



Allergenic evaluation of *Malassezia furfur* crude extracts

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Received 6 December 2001; accepted in revised form 22 June 2002

Abstract

Crude extracts of the lipophilic yeast *Malassezia furfur* were obtained from 2, 6, 10 and 28 day old cultures. The *in vitro* cultivation periods corresponded, respectively, to the lag phase, middle of the log phase, end of log phase and the decline phase of the growth curve, which was based on viable cell counts obtained with a fluorescent viability test. Biochemical analyses showed that the protein and carbohydrate contents were greater in day 10 extracts. Seventy patients with different allergic manifestations and 30 healthy volunteers were skin prick tested using the extracts. Of these, thirteen (18.57%) patients gave positive responses. SDS PAGE gradient electrophoretic profiles of the preparations indicated that the 28 day extracts contained the greatest number of protein bands with molecular weights ranging mostly between 30 and 94 kDa. Immunoblots incubated with individual patient sera showed that four IgE binding *M. furfur* allergens of approximately 88, 61, 52 and 39 kDa were present in the 28 day extracts. The components identified could be used for detecting IgE mediated responses to *M. furfur* among individuals affected with different allergic conditions.

Key words: *Malassezia furfur*, allergens, prick test, immunoblotting.

Introduction

The lipophilic yeast *Malassezia furfur* is a common saprophyte of the normal human skin [1]. This organism has complex lipid requirements for growth and has been isolated from the upper trunk, head and face from sebum-rich areas of the body [2].

Two distinct classes of *M. furfur* allergens are known to induce type I hypersensitivity reactions. One corresponds to cell wall or cytoplasmatic protein and the other to polysaccharides or glycoproteins, probably mannans or mannoproteins. Their relative frequencies may vary according to the length of the *in vitro* growth period of the yeast [3]. Several studies have demonstrated the involvement of fungi in allergic conditions [4–7] and *M. furfur* specific IgE antibodies have been detected in both healthy individuals and patients with skin allergies mainly atopic dermatitis [2, 8, 9–16]. Crude preparations of fungi are often used in skin prick and intradermic tests. However, the

characterization and standardization of the allergens is fundamental to understanding the allergenic profile of *M. furfur*.

The aim of the present study was to obtain crude extracts of *M. furfur* derived from different growth phases of the yeast. Then to analyze their protein and carbohydrate contents, evaluate their *in vivo* allergenic potential and characterize the allergens by immunoblotting. Sera would be used from patients with various allergic manifestations, including not only the commonly investigated atopic dermatitis but also disorders frequently associated with *M. furfur*, such as rhinitis, asthma, bronchitis and urticaria.

Materials and methods

Identification of *Malassezia furfur* strains

We identified the *M. furfur* isolates according to the method of Guillot et al. [17]. Briefly, *M. furfur* yeast suspensions were adjusted at about 10⁵ cells/mL and

* Published in 2002.

were mixed with Sabouraud dextrose agar, and the mixtures were plated. Four holes were made in the agar with a 3-mm diameter punch and filled with 5 μ L each of Tween 20, 40, 60 and 80, respectively. The agar plates were cultivated at 32 °C for 1 week. *Malassezia furfur* exhibited similar growth with Tween 20, 40, 60 and 80. In addition, we utilized two complementary tests: inoculation of the strains in Sabouraud dextrose agar without addition of oil (no growth) and assimilation tests with Cremophor El (Sigma) according Mayser et al. [18]. The strains grew in this medium. The strains exhibited the typical morphology and the biochemical tests were compatible with the *M. furfur* type species.

Growth curve

The growth curve of *M. furfur* was determined on the basis of viable cell counts, using the fluorescent viability test for fungal cells described by Calich et al. [19]. A 1 mL inoculum (10^6 cells) of the yeast was transferred to a 500 mL flask containing 100 mL of Sabouraud dextrose broth supplemented with 1% oxgall and 1% olive oil. Incubation was carried out at 25 °C for 32 days, at 70 rpm. Enumeration of total cells and viable cells was performed after the first 24 h of culture and every 24 h thereafter. All the experiments were performed in triplicate. At appropriate times, 1 mL samples of the cell suspension were drawn from the cultures, mixed with an equal volume of a solution containing fluorescein diacetate 0.005% (w/v) (Sigma) and ethidium bromide 0.005% (w/v) (Sigma) and incubated for 15 min at 25 °C. Next, a drop of the suspension was placed on a slide, covered with a coverslip and examined with a fluorescence microscope (Zeiss, Germany). One hundred cells per slide were counted in four separate fields and the percentage of living cells (green fluorescence) was evaluated. Dead cells presented a red fluorescence. Total cell counts were determined with a hemocytometer [20].

Malassezia furfur extracts

After establishing the growth curve, four different growth periods were selected for obtaining *M. furfur* allergenic crude extracts. A 2 mL inoculum (10^6 viable cells/mL) was transferred to a 1,000 mL flask containing 200 mL of Sabouraud dextrose broth supplemented with 1% oxgall and 1% olive oil and cultured at 25 °C, at 70 rpm. At the selected growth periods (namely: 2, 6, 10 and 28 days) the cells were harvested, washed three times with distilled water by

centrifugation ($800 \times g$) and heat dried at 40 °C. Dry cells were mixed with Coca extractor liquid (0.033 M sodium bicarbonate, 0.085 M sodium cloreto, pH 9.1) [21] to give a 5% final suspension (w/v) and kept at 4 °C for one week. The crude extracts were sterilized using 0.22 mm pore size filters (Millipore SA 67 Molsheim, France), lyophilised and stored at –20 °C.

Biochemical analyses of the extracts

Protein concentrations and the carbohydrate contents of the crude extracts were determined using the method of Lowry [22] and the anthrone method [23], respectively.

Prick test

Seventy patients with different allergic manifestations (rhinitis: 38; asthma: 5; bronchitis: 4; atopic dermatitis: 5; urticaria: 8; rhinitis and asthma: 6; rhinitis and urticaria: 2; rhinitis and atopic dermatitis: 2) were selected; thirty healthy volunteers served as normal controls. The *M. furfur* extracts (concentration: 5mg/mL) were applied to each subject. Histamine (Pharmacia Diagnostics AB) diluted 1:1000 was used as our positive control and Coca extractor liquid as our negative control. A whealing response was checked 15 min after application. Tests were considered positive when the erythema observed was equal to or greater than that induced by the histamine solution.

SDS page

The *M. furfur* crude extracts of 2, 6, 10 and 28 day old cultures were submitted to SDS-PAGE electrophoresis as described by Nichols [24] using a 2.5–15% concentration gradient gel. The extracts were diluted 1:5 in sample buffer (0.062 M Tris-HCl, pH6.8; - 20% SDS; 10% glycerol; 5% 2-mercaptoethanol and 10% Bromophenol Blue) and boiled for 5 min. The protein content of the extracts applied to the slots was 50 μ g. SDS-PAGE runs were performed at a constant current of 70 mV for approximately 2 h. MW standards were run in parallel using low MW proteins. After electrophoresis, the separated yeast components were detected by staining with Coomassie blue at room temperature, for 24 h.

Western blotting

For this assay, *M. furfur* extracts of 28-day-old cultures were used as antigen and tested with sera from 10 skin prick positive patients. A pool of sera from 12 normal individual was used as the negative control. After electrophoresis, the separated yeast components and the standards were transferred electrophoretically to a nitrocellulose (NC) membrane (pore size 0.45 mm, BioRad) in a Mini-Trans Blot cell (BioRad). The transfer was run overnight at 30V and 4 °C. Protein binding sites still available on the NC membrane after complete transfer were blocked by incubation for 2 h at 37 °C with 0.5% Tween 20 in 0.05 M phosphate buffered saline containing 5% non-fat milk (PBS-M). The NC membranes with blotted yeast components were cut into approximately 5 mm-wide strips and incubated overnight at room temperature with individual sera diluted 1:4 in PBS-M, on a rocking shaker. The strips were then washed three times with 0.5% Tween 20 in 0.05 M PBS (PBS-T) and incubated for 2 h with an anti-human IgE alkaline phosphatase conjugate 1:50 (Sigma). After washing, the IgE-binding compounds were visualized with an alkaline phosphatase conjugate substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitroblue-tetrazolium (Sigma). The molecular weights of the resolved bands were determined by comparison of their relative mobilities with those of the low MW standards.

Results

Malassezia furfur growth curve and characteristics of the extracts

Data from the *M. furfur* growth used in this study are presented in Table 1 and Figure 1. A 48 h lag phase was observed. During the logarithmic phase, the highest numbers of viable cells were observed from the 6th to 10th day of culture, after which the beginning of the decline phase was detected. The cellular viability decreased (5.2×10^7 viable cells/mL) on the 32nd day of incubation. Protein and carbohydrate contents were measured in the extracts obtained from the different phases of *M. furfur*'s growth (2, 6, 10 and 28 days). They were selected on the basis of the growth curve data. The highest protein and carbohydrate concentrations were detected in the 10 day extracts (end of the log phase). The protein and carbohydrate contents of the 6 day extracts were found to be significantly

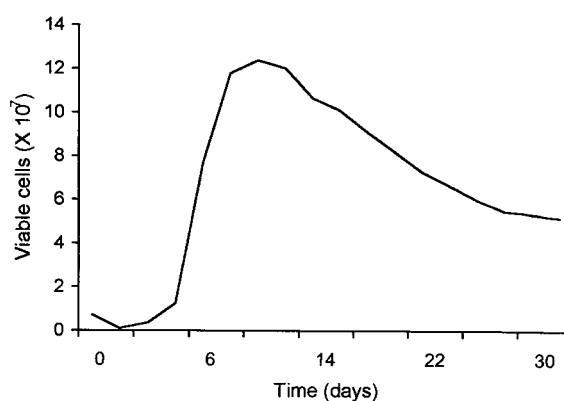


Figure 1. *Malassezia furfur* growth curve, obtained with a fluorescent method using fluorescein diacetate and ethidium bromide.

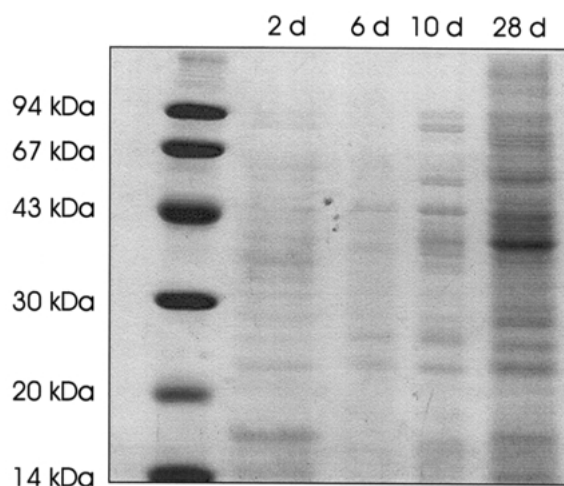


Figure 2. SDS-PAGE of *Malassezia furfur* extracts of 2, 6, 10 and 28d (days).

higher than those derived from 2 day and 28 day cultures (Table 2). Figure 2 shows the electrophoretic profiles of the crude extracts of *M. furfur* prepared from 2, 6, 10 and 28 day old cultures. The 28 day extracts presented the greatest number of IgE binding components with molecular weights ranging from 30 to 94 kDa.

Prick tests

Seventy patients with various allergic conditions and thirty healthy individuals were skin prick tested with the *M. furfur* preparations obtained at different growth phases of the yeast. Table 3 shows that 13 (18.57%) of the patients reacted positively after 15 min of extract application. No positive skin tests were observed among the healthy controls. In addition, the highest

Table 1. Viability values (%) of *Malassezia furfur* estimated* by the FD-EB fluorescent method

Cultivation time (days)	Total cells ($\times 10^7/\text{ml}$)	Viable cells ($\times 10^7/\text{ml}$)	Viability (%)
Inoculum	1.60	0.73	46
2	0.89	0.38	43
4	3.15	1.26	40
6	14.55	7.71	53
10	37.55	12.39	33
28	68.75	5.50	8

*Mean of three determinations. FD – Fluorescein Diacetate. EB – Ethidium Bromide.

Table 2. Protein and carbohydrate concentrations* of *Malassezia furfur* extracts

Cultivation time (days)	Protein concentration $\mu\text{g/ml}$	Carbohydrate concentration $\mu\text{g/ml}$
2	855	33
6	1441	660
10	1620	937
28	406	274

*Mean of three determinations.

frequency of skin prick responses were observed with 2 and 28 day *M. furfur* extracts (Table 4).

Western blotting

Immunoblots revealed that the *M. furfur* extracts of the 28 day old cultures contained four protein components that were able to bind IgE with approximate molecular weights of 88, 61, 52 and 39 kDa (Figure 3). Sera of patients A, D, I and J reacted with a protein band of about 52 kDa. These subjects had been respectively, diagnosed with asthma, rhinitis, urticaria and atopic dermatitis. Another band of 61 kDa was recognized by

Table 3. Prick test responses to *Malassezia furfur* extracts* in 70 patients with allergic manifestations and 30 healthy controls

Groups	Prick test			
	Positives		Negatives	
	No. total	%	No. total	%
Patients	13	18.6	57	81.4
Controls	0	0	30	100

*Extracts of 02, 06, 10 and 28 days obtained with Coca extractor liquid.

Table 4. Skin reactivity of allergic patients to prick testing with different *Malassezia furfur* extracts

Patients	Symptoms	Extracts			
		02 days	06 days	10 days	28 days
1	Rhinitis	+	+	+	+
2	Rhinitis	+	–	+	+
3	Rhinitis	+	+	+	+
4	Rhinitis	+	+	+	+
5	Asthma	+	–	–	+
6	Asthma	+	+	+	+
7	Bronchitis	+	+	–	+
8	Atopic dermatitis	+	–	–	+
9	Atopic dermatitis	+	+	+	+
10	Urticaria	+	+	+	+
11	Urticaria	+	–	–	+
12	Rhinitis and asthma	+	–	–	+
13	Rhinitis and asthma	+	–	+	+
Total		100%	53.8%	61.3%	100%

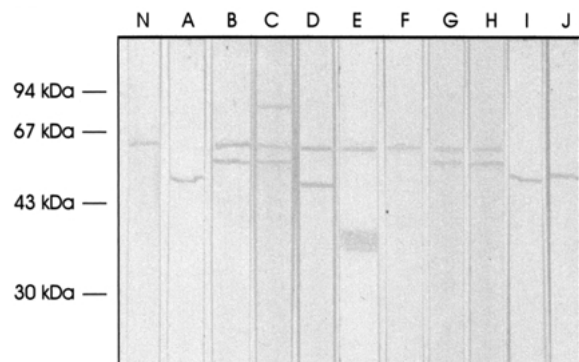


Figure 3. IgE binding components in *Malassezia furfur* extract of 28 days old cultures. Sera of patients with different allergies (A-J), normal individuals sera (N).

sera of patients B (asthma), C (rhinitis), G (bronchitis) and H (urticaria). Antigenic components of 88 and 39 kDa were recognized in sera from two subjects with rhinitis; the former band by patient C and the latter by patient E (Figure 3).

IgE from sera of patients B, C, D, E, F, G and H as well as the control pool of serum N bound to a 66 kDa component.

Discussion

The characterization and standardization of allergens is fundamental to understanding the allergenic profile

of *M. furfur*. The determination of a growth curve is of fundamental importance for research work on allergens. By establishing the time course of fungal growth, it is possible to standardize the time of antigen production, the minimal time required for significant antigen yields and the growth phase at which maximum antigen expression occurs [25]. Several procedures, such as total cell counts, turbidimetric methods and estimation of weights of heat-dried cells, are employed to determine growth curves, however, they have the drawback of not differentiating viable from non-viable microorganisms.

Ran et al. [26] used total cell counts to determine the growth kinetics of *M. furfur* and found that the logarithmic phase reached a maximum after four days of cultivation. In our study, based on viable cell counts, maximum values during the log phase were observed on day 10 of culture (Figure 1). Several studies have demonstrated that growth curves obtained by enumeration of viable cells are quite reproducible [25, 26]. The fluorescent cell viability method presently used, in addition to good reproducibility, permits very accurate evaluations as it makes a sharp distinction between viable (green fluorescence) and non viable (red fluorescence) fungal cells.

Based on our *M. furfur* growth data, we selected the lag phase (2 days of culture), middle and end of the log phase (days 6 and 10 of culture, respectively) and decline phase (day 28 of culture) to make crude allergenic preparations of this yeast. The second day of the lag phase was chosen as the sampling time because the number of cells at the beginning of this growth period was too small to ensure good extractions. Day 10 corresponded to the peak of cell division during the exponential period. As there was practically no stationary phase under our culture conditions (cultures were found to be already in the decline phase after day 10), this growth period was not included in our study.

According to Mendes [21], when fungal cells are suspended in the Coca extractor liquid at 4 °C for one week, extraction of antigenic material is slow and promotes a greater yield of cell wall components. Additionally, this type of process does not favor significant hydrolysis of the extracts and therefore preserves their allergenic potential.

The development of the various components of the *M. furfur* extracts may depend on the length of the *in vitro* culture period. There is experimental data suggesting that proteins, defined by immunoblotting, would be mainly produced during the first days of

culture but would be readily degraded by proteolytic enzymes upon prolonged cultivation [3].

Generally speaking, mannans constitute one of the main components of yeast cell walls, corresponding to 35–45% of heat dried cell weight. These molecules are extensively branched and span the whole cell wall, prevailing in the outermost fibrillar layer. They bind covalently to proteins forming the mannoproteins that can be extracted by mild procedures [29].

In this study, the biochemical analyses of the *M. furfur* crude preparations showed that both their protein and carbohydrate contents paralleled the yeast growth curve; i.e., as viable cell counts increased, so did the protein and carbohydrate concentrations.

By and large, there is no standardization of the concentrations of allergen used in skin prick tests, which can vary from 0.002 to 5 mg/mL [30–34]. We chose to apply extracts at 5 mg/mL. These were tested in patients diagnosed with different allergic conditions and selected according to symptoms that would be associated with the presence of fungi after excluding allergies to foods, drugs or inhalants.

The percentage of positive skin prick tests to *M. furfur* extracts among the patients analyzed was 18.57% (13 out of 70) (Table 3). A similar result was obtained by Broberg et al. [32] when prick testing individuals presenting with atopic dermatitis, asthma and rhinoconjunctivitis with extracts of the same fungal species.

The yeast extracts obtained by us after 2, 6, 10 and 28 days of *in vitro* culture contained 16, 8, 13 and 28 protein fractions, respectively (Figure 2). These values are in accordance with the results from other workers, such as those reported for *Alternaria* spp (40 protein fractions) [35], *Cladosporium herbarum* isolates (10 and 15 fractions) [5], *Aspergillus* spp (approximately 10 fractions) [6] and *Penicillium notatum* (15 fractions) [7].

We chose day 28 extracts for evaluation of IgE binding proteins by immunoblotting because their SDS-PAGE electrophoretic profiles revealed the highest number of protein bands as compared to the other ones (Figure 2) and because all the patients were skin prick positive to it (Table 4). Furthermore, in three cases, the whealing response to this extract was greater than that induced by crude preparations from 2 day old culture (data not shown).

According to Doekes et al. [3], *M. furfur* proteins with molecular weights within the range of 10 to 100 kDa can be detected by immunoblotting. In our study,

four protein bands of approximately 88, 61, 52 and 39 kDa were resolved by the same technique (Figure 4).

Broberg et al. [32] found allergens of 10, 23, 26, and 34 kDa using sera of patients diagnosed with atopic dermatitis and head and neck dermatitis. By contrast, our data revealed that, expanding the range of allergic manifestations associated with *M. furfur* so as to include less studied conditions such as asthma, rhinitis, bronchitis and urticaria, IgE binding components of 88, 61, 52 and 39 kDa were recognized with patient sera (Figure 3).

To conclude, the results presented herein indicate that, after establishing the *M. furfur* growth curve by an accurate fluorescent cell viability method, cells obtained on the decline phase of the yeast (28 days of culture) contained a range of components that could be used for detecting IgE mediated responses to *M. furfur* among individuals affected with different allergic conditions.

Acknowledgment

We thank all patients and the control group who took part in this study. Our study was supported by a grant of the FAPESP (Fundação de Amparo à Pesquisa de São Paulo).

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