

Identification and Cloning of Two Novel Allergens from the Lipophilic Yeast, *Malassezia furfur*

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Two novel allergens, designated Mal f 2 and Mal f 3 according to the WHO/IUIS Allergen Nomenclature Subcommittee recommendation, were isolated from the lipophilic yeast *Malassezia furfur* cell extracts and the genes coding for those were cloned. Mal f 2 and Mal f 3 had apparent molecular weights of 21 kDa and 20 kDa, respectively, on SDS-PAGE under reducing conditions. The identified cDNA clone of Mal f 2 encoded an open reading frame of 177 amino acid residues. Fifty-one percent identity was found between the Mal f 2 and Mal f 3 sequences. Comparison of the Mal f 2 and Mal f 3 sequences with known protein sequences revealed that they had sequence homology with two peroxisomal membrane proteins of *Candida boidinii* and an *Aspergillus fumigatus* allergen, Asp f 3. In RAST, both Mal f 2- and Mal f 3-specific IgE antibodies could be detected in approximately 70 % of sera from *M. furfur* sensitized patients with atopic dermatitis. © 1998 Academic Press

The lipophilic yeast *Malassezia furfur*, formerly denoted as *Pityrosporum orbiculare* or *P. ovale*, is a member of the normal cutaneous flora, which preferentially colonizes the skin of head-neck-face region (1). *M. furfur* is found on normal skin of most of healthy subjects, and is usually non-pathogenic (2, 3), but it can induce infections in the skin and cause skin diseases, such as tinea versicolor, pityrosporum folliculitis and seborrheic dermatitis (4, 5, 6).

The importance of *M. furfur* in the pathogenesis of atopic dermatitis has been shown during the last 10 years

all over the world (7, 8). It is shown that the yeast produces a high incidence of positive skin tests and radioallergosorbent tests (RASTs) in patients with atopic dermatitis (9, 10). A number of IgE binding components were detected by immunoblotting in *M. furfur* extracts using sera from the patients (11, 12). Recently, a first *M. furfur* allergen, Mal f 1, has been identified and cloned (13).

In the present study, two novel allergens which were the most frequent IgE binding were isolated from the *M. furfur* cell extract and cloned. These two allergens, formerly named MF1 and MF2 (14), are now designated Mal f 2 and Mal f 3, respectively, according to the WHO/IUIS Allergen Nomenclature Subcommittee recommendation (15, 16).

MATERIALS AND METHODS

***M. furfur* extract.** *M. furfur* (strain No. 2782, Teikyo Institute for Medical Mycology (TIMM), Tokyo Japan) was grown in liquid Dixon medium composed of Bacto malt extract (Difco) 6%, Bacto Oxgall (Difco) 2%, Tween 40 1% and glycerol monooleate 0.25% at 37°C with shaking. After 3 days, cells were harvested, washed with phosphate buffered saline (PBS) 5 times, disrupted with glass beads (0.5 mm in diameter) using an MSK cell homogenizer (Braun, Melsungen, Germany) and extracted in PBS. After centrifugation (20,000 × g, 30 min) at 4°C, the supernatant was dialyzed extensively against distilled water, passed through 0.45 µm filter and lyophilized.

Isolation of Mal f 2 and Mal f 3. The lyophilized extract was dissolved in 0.05 M Tris-HCl buffer, pH 8.0 and separated by ammonium sulfate precipitation into two fractions, P1 and P2, at 0–50 and 50–90 % saturation, respectively. The fraction P2 was dissolved in 0.05 M Tris-HCl, pH 8.0, dialyzed against the same buffer and applied to a DEAE-cellulose (DE-52, Whatman, Springfield Mill, Kent, England) column equilibrated with the same Tris buffer. The sample was eluted from the column by increasing the NaCl concentration in the Tris buffer in a stepwise fashion. The fraction eluted with Tris-HCl containing 0.1 M NaCl, pH 8.0, was collected, concentrated and applied to a Superdex 75 pg column (Pharmacia Biotech, Uppsala, Sweden). The peak eluted at molecular weight of approximately 40kDa was collected, concentrated, dialyzed against 0.05 M Tris-acetate containing 0.5 M NaCl, pH 8.0, and applied to a Chelating Sepharose Fast Flow column (Pharmacia), which had been che-

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lated with zinc ion and equilibrated with the same Tris-acetate buffer. After elution with the pH 8.0 buffer, the column was eluted by decreasing the pH of the buffer in a stepwise fashion to 7.0, 6.0, 5.0 and 4.0. The fraction eluted from the zinc-chelated column with the pH 8.0 buffer was further separated by copper chelate chromatography. The fraction was applied to the Chelating Sepharose FF column, which had been chelated with copper ion and equilibrated with the same Tris-acetate buffer, pH 8.0. After elution with the pH 8.0 buffer, the column was eluted by decreasing the pH of the buffer to 7.0, 6.0, 5.0 and 4.0. The fraction eluted from the zinc-chelated column with the pH 5.0 buffer and that eluted from the copper-chelated column with the pH 4.0 buffer were further purified by a passage through a Superdex 75 pg column, and named Mal f 3 and Mal f 2, respectively.

SDS-PAGE and immunoblotting. SDS-PAGE was performed with a 12 % polyacrylamide gel (TEFCO, Tokyo, Japan) under either reducing or non-reducing conditions and proteins were stained with Coomassie Brilliant Blue R-250. Electroblothing of SDS-PAGE gels to nitrocellulose paper was carried out in a Model STB-8 Safety Blotting Cell (TEFCO) according to the manufacturer's instructions. After incubation for 1 hr with blocking buffer (PBS containing 3 % bovine serum albumin (BSA) and 0.5 % Tween 20), the nitrocellulose paper was reacted overnight with a pooled allergic serum diluted 1 : 5 in PBS containing 0.2 % BSA and 0.5 % Tween 20, followed by peroxidase-labeled anti human IgE (1 : 5000 diluted, Biosource Int., Camarillo, CA) for 30 min. The IgE reactive bands were detected by the enhanced chemiluminescence system (ECL Western blotting analysis system, Amersham, Buckinghamshire, England) according to the manufacturer's instructions.

Amino acid sequence analysis. The N-terminal amino acid sequence of the native Mal f 2 was determined by Edman degradation with a gas phase protein sequencer (Model 470A, Applied Biosystems) with on-line PTH-derivative analyzer 120A. The N-terminal amino acid of the native Mal f 3 could not be detected, and then the S-pyridylethylated derivative of Cys of Mal f 3 was digested with lysylendopeptidase (Wako Pure Chemical, Osaka, Japan). The generated fragments were extracted and purified by a C18-HPLC to isolate two peptide fragments, the amino acid sequence of which was determined as described above.

PCR amplification of cDNA. Standard molecular cloning techniques were used as described by Sambrook et al (17). The rapid amplification of cDNA ends (RACE) method (18) was employed to produce cDNA fragments coding for Mal f 2 and Mal f 3 using Takara RNA PCR kit (Takara). Degenerated primers for PCR amplification of the Mal f 2 cDNA, MF1F1 and MF1F2, were designed based on the NH₂-terminal amino acid sequences (PGDPTATA and TLMGYI-PWT) of Mal f 2. Degenerated primer for PCR of Mal f 3, MF2F1, was designed based on the internal sequence (TFAQDVNCE) of Mal f 3. Briefly, total RNA from *M. furfur* was reverse-transcribed using oligo(dT)-adaptor primer (Takara), which is composed of oligo(dT) segment and M13 primer M4 segment, as a primer. To produce a cDNA fragment of Mal f 2, the first PCR was carried out by using MF1F1 and M13M4 as primers along with the transcribed cDNA as a template, and then 1 μ l of the amplification reaction product was used as the template in the second PCR, which was carried out using MF1F2 and M13M4. The PCR cycle was performed at 94°C for 1 min, at 55°C for 2 min, and at 72°C for 1.5 min for 40 cycles. To produce a cDNA fragment of Mal f 3, one step PCR was carried out using MF2F1 and M13M4 as primers and the transcribed cDNA as a template under the same conditions described above. The PCR fragment obtained was cloned into pUC118 and sequenced. Nucleotide sequences of the primers used are as follows:

MF1F1 (5'-CC(A/C/G/T)GG(A/C/G/T)GA(C/T)CC(A/C/G/T)AC(A/C/G/T)GC(A/C/G/T)AC(A/C/G/T)GC-3'), MF1F2 (5'-AC(A/C/G/T)(C/T)T(A/C/G/T)ATGGG(A/C/G/T)TA(C/T)AT(A/C/T)CC(A/C/G/T)TGGAC-3'), and MF2F1 (5'-AC(A/C/G/T)TT(C/T)GC(A/C/G/T)CA(A/G)GA(C/T)(A/C/G/T)AA(C/T)TG(C/T)G-3').

Construction of cDNA library and cloning of cDNA. *M. furfur* cells were collected and immediately frozen in liquid nitrogen. Total RNA was extracted according to the procedures described by Oka-yama et al. (19). Poly(A)⁺ RNA was purified using an oligo(dT)-cellulose column. A cDNA library was constructed in the vector λ SHlox (Novagen) and cDNA inserts in the λ SHlox vector were automatically subcloned to obtain as a plasmid form as recommended by the manufacturer. Hybridization was performed under a stringent condition using the PCR cDNA fragment of Mal f 2 or Mal f 3 labeled with [³²P]dCTP as a probe. Ten cDNA clones showing signals with high intensity were selected and a clone having the longest cDNA insert was chosen for further analysis. DNA sequencing was performed by the dideoxy chain termination procedure (20).

Construction of genomic DNA library and screening. Genomic DNA of *M. furfur* was obtained by the following procedure. Cells were pelleted and washed at 3,000 rpm for 15 min and then suspended in PK buffer (0.15 M NaCl, 12.5 mM EDTA, and 0.1 M Tris-HCl pH7.5). The cells were disrupted by Mini-Beadbeater (Biospec Products, Co.). Total genomic DNA was purified from the disrupted cells according to the method of Philippsen et al (21). In order to construct a genomic DNA library of *M. furfur* TIMM2782, the genomic DNA was partially digested with *Pst*I and fragments ranging from 3 to 15 kb were collected by agarose gel electrophoresis. The DNA fragments were inserted into a *Pst*I site of a vector pUC118, and then transformed into *Escherichia coli* HB101. The genomic DNA library was screened by colony hybridization using the 0.6-kb Mal f 2 cDNA labeled with [³²P]dCTP as a probe.

Patient sera. Sera were obtained from patients with atopic dermatitis who gave a positive skin response to the intradermal skin test with the crude *M. furfur* extract (100 μ g/ml). A pooled serum was prepared from 15 sera with high IgE antibody titer to the crude *M. furfur* extract to use in IgE-immunoblot studies.

Measurement of IgE antibodies. Each preparation of *M. furfur* allergens was covalently coupled to cyanogen bromide activated paper disks according to the method of Ceska et al (22). Allergen disks were incubated for 3 hr at room temperature with 50 μ l of serum. When it was needed because of a high IgE antibody concentration, serum was diluted in a horse serum. The disks were then washed and 50 μ l of β -galactosidase conjugated anti human IgE (CAP system, Pharmacia) were added. After an overnight incubation, the disks were washed and a bound enzyme activity was measured according to the method used for the Phadezym RAST system (Pharmacia). The assay was calibrated using a control curve with disks coupled with Japanese cedar pollen extracts and serial dilutions of pooled sera from patients allergic to Japanese cedar pollen that had been substandardized against the Phadebas RAST reference system (Pharmacia) in our laboratory (23). The results were extrapolated from the control curve and expressed as PRU/ml (24).

RESULTS AND DISCUSSION

A large number of proteins and IgE-binding components was detected in the *M. furfur* extract by SDS-PAGE and IgE-immunoblot. Among them, the present study focused on the components of the approximate molecular weights of 20 kDa, which showed strongly stained bands on IgE-immunoblot (Fig. 1A). Two allergenic proteins, named Mal f 2 and Mal f 3, were purified from the crude extract. Although both proteins had very similar physicochemical properties, they could be separated completely by metal chelate chromatography. Under reducing conditions on SDS-PAGE, the apparent molecular weights of Mal f 2 and Mal f 3 were shown to be 21kDa and 20 kDa, respectively, whereas

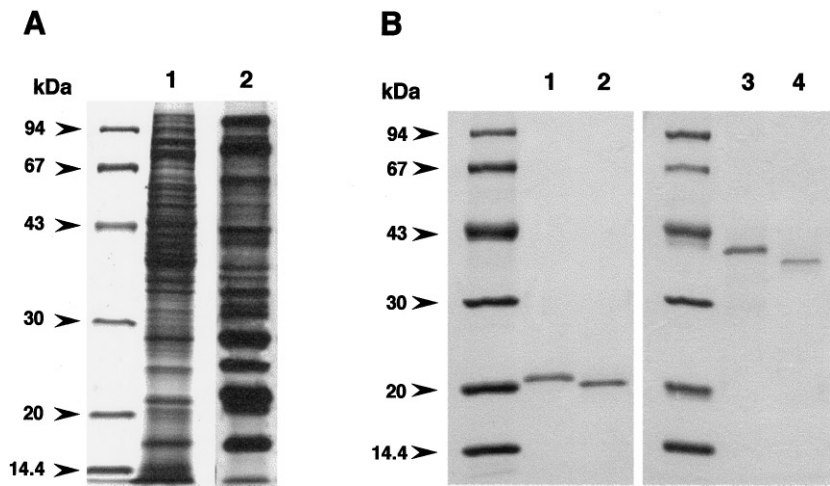


FIG. 1. SDS-PAGE and immunoblot analysis of *M. furfur* allergens. (A) Crude *M. furfur* extract was run under reducing conditions. Lane 1, dye-stained gel; lane 2, immunoblot probed with a pooled allergic serum. (B) Purified Mal f 2 (lanes 1 and 3) and Mal f 3 (lanes 2 and 4) were run under reducing conditions (lanes 1 and 2), or non-reducing conditions (lanes 3 and 4).

under non-reducing conditions the apparent molecular weights of Mal f 2 and Mal f 3 were 42 kDa and 40 kDa, respectively. These results suggest that both allergens were homodimers of single polypeptide chains linked by disulfide bonds (Fig. 1B). The apparent molecular weights of both allergens on Superdex 75 gel filtration chromatography were very similar to those on SDS-PAGE under non-reducing conditions. On isoelectric focusing, Mal f 2 and Mal f 3 were shown to be acidic isoelectric points of 4.7 and 4.8, respectively (data not shown).

The NH₂-terminal amino acid residues of Mal f 2 purified from the *M. furfur* extract were analyzed by the Edman degradation and 29 amino acid residues were determined (Fig. 2). To clone Mal f 2 cDNA, PCR with the nested RACE method was first performed using two PCR primers, MF1F1 and MF1F2, which were designed based on the NH₂-terminal sequence of Mal f 2. A 0.6-kb cDNA fragment was obtained by the two-step PCR, and its nucleotide sequence was determined.

The amino acid sequence deduced from the nucleotide sequence had the same sequence as that of the purified Mal f 2 allergen. Then a cDNA library prepared from *M. furfur* was screened by plaque hybridization using the PCR fragment as a probe. Ten cDNA clones showing signals with high intensity were selected, and then a clone with the longest cDNA (0.6 kb) insert was chosen for further analysis. Nucleotide sequence analysis revealed that the cDNA insert encoded an open reading frame of 177 amino acids, which would generate a 19.2 kDa protein (Fig. 2). To confirm a genomic structure of the *Mal f 2* gene, a genomic library of *M. furfur* was screened using the 0.6-kb Mal f 2 cDNA obtained from the cDNA library as a probe. A clone containing a 4.9-kb *Pst*I DNA fragment was identified, and nucleotide sequence of the only open reading frame of the *Mal f 2* gene was determined based on the cDNA sequence. Comparison of the nucleotide sequence of the genomic DNA with that of the 0.6-kb cDNA revealed that the *Mal f 2* gene composed of three exons separated by 37 bp and 39 bp introns (Fig. 3).

Since the NH₂-terminal amino acid sequence of Mal f 3 could not be obtained, internal sequences of Mal f 3 were determined by analyzing peptide fragments digested with lysylendopeptidase (Fig. 2). Mal f 3 cDNA was cloned by one-step PCR amplification and by screening the *M. furfur* cDNA library using the PCR product as a probe. Ten cDNA clones showing signals with high intensity were selected, and then a clone with the longest cDNA (0.6 kb) insert was chosen for further analysis. Nucleotide sequencing revealed that its deduced amino acid sequence included the sequences identified by the amino acid sequence of proteolytic fragments of the mature protein, and that the cDNA obtained lacked an NH₂-terminal region (Fig. 2).

The predicted amino acid sequence of Mal f 3 have

Mal f 2	<u>MPGDPTATAK</u>	<u>GNEIPDTLMG</u>	<u>YIPWTPELDS</u>	GEVCGIPTTF	40
Mal f 3	-----EI	GSTIPNATFA	YVPYSPELED	HKVCGMPTSF	
		* * *	* * *	* * *	
Mal f 2	KTRDEWKGGK	VVIVSIPGAY	TPICHQQHIP	PLVKRVDELK	80
Mal f 3	QSHERWKGKK	<u>VVIVAVPGAF</u>	<u>TPTCTANHVP</u>	<u>PYVEKIQELK</u>	
	*****	*****	*****	*****	
Mal f 2	AKGVDVAVYVI	ASNDPFVMAA	WGNFNNAKDK	VVFATDIDLA	120
Mal f 3	SKGVDEVVVI	SANDPFVLSA	WGITEHAKDN	<u>LTFAODVNCE</u>	
	*****	*****	*****	*****	
Mal f 2	FSKALGATID	LSAKHFGERT	ARYALIIDDN	KIVDFASDEG	160
Mal f 3	<u>FSKHFNATLD</u>	LSSKGMGLRT	ARYALIANDL	<u>KVEYFGYDEG</u>	
	***	*****	*****	*****	
Mal f 2	DTGKLQNASI	DTILTQV			177
Mal f 3	<u>EP</u> ---KQSSA	ATVLTKL			
	*	* * *			

FIG. 2. Amino acid sequences of Mal f 2 and Mal f 3. Identical residues are indicated by asterisks. Underlined regions represent sequences established by Edman degradation.

be needed to determine the degree of cross-reactivity between Mal f 2 and Mal f 3.

In the present study, we have reported the isolation and genomic cloning of the two allergens from the lipophilic yeast, *M. furfur* and their allergenic characterization. The sequence data of Mal f 1 and Mal f 2 will facilitate the mapping of T and B cell epitopes of the allergens, and will help to clarify the mechanism of the human immune response to *M. furfur*, which is usually known as a normal cutaneous flora.

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