

Two-dimensional electrophoresis of *Malassezia* allergens for atopic dermatitis and isolation of Mal f 4 homologs with mitochondrial malate dehydrogenase

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The yeast *Malassezia furfur* is a natural inhabitant of the human skin microflora that induces an allergic reaction in atopic dermatitis. To identify allergens of *M. furfur*, we separated a crude preparation of *M. furfur* antigens as discrete spots by 2-D PAGE and detected IgE-binding proteins using sera of atopic dermatitis patients. We identified the known allergens, Mal f 2 and Mal f 3, and determined N-terminal amino acid sequences of six new IgE-binding proteins including Mal f 4. The cDNA and genomic DNA encoding Mal f 4 were cloned and sequenced. The gene was mitochondrial malate dehydrogenase and encoded Mal f 4 composed of 315 amino acids and a signal sequence of 27 amino acids. We purified Mal f 4, which had a molecular mass of 35 kDa from a membrane fraction of a lysate of cultured cells. Thirty of 36 *M. furfur*-allergic atopic dermatitis patients (83.3%) had elevated serum levels of IgE to purified Mal f 4, indicating that Mal f 4 is a major allergen. There was a significant correlation of the Phadebas RAST unit values of Mal f 4 and the crude antigen, but not between Mal f 4 and the known allergen Mal f 2.

Keywords: *Malassezia furfur*; malate dehydrogenase; allergen; atopic dermatitis; 2-D PAGE.

Atopic dermatitis is a chronic inflammatory skin disease that is associated with high levels of total and allergen-specific serum IgE and immediate-type reactions to a wide variety of allergens. The lipophilic yeast *Malassezia furfur* (previously known as *Pityrosporum orbiculare* or *Pityrosporum ovale*), is a natural inhabitant of the microflora of human skin [1,2]. Usually nonpathogenic, this yeast can act as a pathogen causing pityriasis versicolor and seborrheic dermatitis [3,4]. Furthermore, a role in the pathogenesis of atopic dermatitis has been suggested by the high prevalence (40–65%) of type I skin reactions of such patients to *M. furfur* extracts [4,5], and the presence of specific anti-(*M. furfur*) IgE in the sera of many atopic dermatitis patients [6–9]. These studies have used 1-D SDS/PAGE and immunoblotting, and identified a variety of IgE-binding components with molecular masses ranging from 10 to 100 kDa. This variability has caused difficulties in defining the allergens by molecular mass, because some bands are too close to differentiate, particularly between 30 and 67 kDa [9,10]. Recently, use of a monoclonal antibody has enabled identification of Mal f 1 (*m* = 37 kDa) [11,12]. Additionally, two allergens, Mal f 2 (21 kDa) and Mal f 3 (20 kDa) have been identified by chromatographic separation [13].

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Abbreviations: RAST, radio allergosorbent tests; PRU, Phadebas RAST unit; IPG, immobilized pH gradient gel.

Note: The nucleotide sequences of genomic DNA of the *M. furfur* Mal f 4 gene have been deposited in the GenBank/EMBL/DDJB databases under accession numbers AF084828.

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Here we further analyze *M. furfur* allergenic proteins by immunoblotting bands separated by 2-D PAGE, using sera from atopic dermatitis patients. We identified a new 35 kDa allergen and designated it Mal f 4 according to the WHO/IUIS Allergen Nomenclature Subcommittee recommendation [14,15]. We also show the sequence of the gene encoding Mal f 4 and describe the homology of Mal f 4 protein with mitochondrial malate dehydrogenase.

MATERIALS AND METHODS

Preparation of *M. furfur* antigens

M. furfur crude extract and the fraction P2 were prepared as described [13]. Briefly, *M. furfur* TIMM 2782 was cultivated at 37 °C for 3 days in Dixon medium [6% malt extract broth (Difco), 2% Oxgall (Difco), 1% Tween 40, 0.25% glycerol monooleate]. Cells were harvested by centrifugation, washed with phosphate-buffered saline (NaCl/P_i), and disrupted using a homogenizer with glass beads. The cell lysate was separated by centrifugation and the supernatant was dialyzed against distilled water, then lyophilized to yield the crude extract used for the skin test. The crude extract was dissolved in 0.05 M Tris/HCl buffer (pH 8.0) and precipitated with 50–90% ammonium sulfate. The precipitate was dialyzed against the Tris/HCl buffer yielding fraction P2. Protein content was determined using a bischinchoninic acid protein assay reagent kit (Pierce).

Measurement of IgE levels

Each preparation of *M. furfur* antigens was covalently coupled to cyanogen bromide-activated paper disks as described by Ceska *et al.* [16]. Allergen disks were incubated for 3 h at room temperature with 50 µL of serum. The disks were washed, then

50 µL of β-galactosidase conjugated anti-(human IgE) (CAP system, Pharmacia) was added. After an overnight incubation, the disks were washed and bound enzyme activity was measured according to the method used in the Phadezym radio allergosorbent tests (RAST) system (Pharmacia). The assay was calibrated using a control curve with disks coupled to Japanese cedar pollen extracts and serial dilutions of pooled sera from patients allergic to Japanese cedar pollen that had been standardized to the Phadebas RAST unit (PRU) reference system (Pharmacia) in our laboratory [13]. The results were extrapolated from the control curve and expressed as PRU·mL⁻¹.

Serum samples

Sera were obtained from atopic dermatitis patients who reacted positively to an intradermal skin test using the *M. furfur* crude extract (100 µg·mL⁻¹). A pooled serum containing high levels of IgE from 15 atopic dermatitis patients were used for IgE-immunoblotting studies. Sera from three healthy adults with no history of atopy and a negative response to the skin test were used as a control.

SDS/PAGE

One dimensional SDS/PAGE was performed on 12.5% vertical slab gels and molecular mass of resolved bands was determined by comparison with molecular mass markers (BioRad). SDS, glycerol and 2-mercaptoethanol were added to samples, which were then boiled, and applied to lanes. The gels were stained using a silver staining kit (Pharmacia) or 0.25% Coomassie brilliant blue R-250 to detect proteins.

2-D PAGE

Antigens were dissolved in a sample buffer composed of 8 M urea, 2% 2-mercaptoethanol, 0.8% Pharmalyte (2-D 3-10; Pharmacia), 0.5% Nonidet P-40, and 0.01% bromophenol blue. After centrifugation, the supernatant was applied to a sample cup placed on an immobilized pH gradient gel (IPG) strip (Immobiline DryStrip pH 4–7 or pH 3–10; Pharmacia), which was rehydrated in a solution composed of 8 M urea, 10 mM dithiothreitol, 0.5% Nonidet P-40, and 2 mM acetic acid. Isoelectric focusing was performed using Multiphor II (Pharmacia) according to the manufacturer's instructions. After IEF, the IPG strip was equilibrated in two stages with gentle shaking in a 50 mM Tris/HCl buffer (pH 6.8) containing 6 M urea, 1% SDS, 16 mM dithiothreitol, 30% glycerol. Iodoacetamide, 243 mM, and bromophenol blue were added to the second stage in place of 16 mM dithiothreitol. The strip was electrotransferred to ExcelGel SDS Homogeneous 12.5 (Pharmacia) at

the maximal setting (600 V, 20 mA, 30 W) for about 20 min at 15 °C. Electrophoresis proceeded for about 1 h at the maximal setting (600 V, 50 mA, 30 W). The gel was fixed and stained as described above for protein detection.

Immunoblotting and sequencing of N-terminal amino acid residues

Two dimensional gels were removed from their backing and proteins were electrotransferred to a polyvinylidene difluoride membrane (Millipore). The membranes were either immunoblotted as described below or stained with Coomassie brilliant blue R-250 for protein visualization. Membranes were incubated with NaCl/P_i containing 3% BSA overnight, then reacted overnight with the pooled serum diluted 1 : 5 in NaCl/P_i containing 0.2% bovine serum albumin and 0.5% Tween 20, followed by 1 : 5000 diluted peroxidase-labeled anti-(human IgE) (Tago, Burlingame, CA) for 30 min. IgE-binding proteins were detected using the enhanced chemiluminescence ECL Western blotting system (Amersham) according to the manufacturer's instructions. Protein spots were then excised from the membrane for N-terminal sequence analysis by Edman degradation using an HP G1000 A protein sequencer (Hewlett Packard). The sequences determined were searched for homology with known proteins using the SWISSPROT database.

Purification of Mal f 4

M. furfur TIMM 2782 was cultured in YNB medium (0.67% yeast nitrogen base (Difco), 2% glucose, 0.5% Casitone (Difco), 1% Tween 60, 0.1% glycerol monooleate, 5% MEM vitamin solution (Gibco, Gaithersburg, MD, USA) at 30 °C for 4 days. Cells (1 × 10¹⁰) obtained from 0.9-L cultures were collected and digested with *Trichoderma* lysing enzymes and β-glucuronidase (Sigma) in 50 mM potassium phosphate buffer (pH 7.5) containing 2 M NaCl, 10 mM EDTA, and 10 mM 2-mercaptoethanol to remove cell walls. The cells were then suspended in NaCl/P_i containing 1 mM phenylmethanesulfonyl fluoride and 2 mM EDTA and separated by centrifugation at 10 000 g for 30 min. The precipitate collected as a membrane fraction was suspended in the same buffer, frozen at –30 °C, then thawed at room temperature before use. Membrane-bound proteins were solubilized by sonication, then separated by centrifugation at 100 000 g for 1 h. The supernatant (166 mg of protein) was precipitated with 90% ammonium sulfate, then dissolved in 50 mM sodium phosphate buffer (pH 7.4). After dialysis the solution was applied to a Blue Sepharose column (HiTrap Blue 5 mL, Pharmacia) equilibrated with the same buffer. The column was eluted using a linear 0–2 M KCl gradient

Table 1. N-terminal sequences of IgE-binding proteins identified by 2-D PAGE. Spot numbers correspond to those in Fig. 2. x, not identified; – no homologous proteins in Swiss-Prot database; ND, not determined as blocked.

Spot no.	Allergen name	N-terminal amino acid sequence			Homology
		1	11	21	
MF1	Mal f 2 ^a	PGDPTATAKGNEI	PDTLMGYI	PWTPELD	Peroxisomal membrane protein 20 of <i>Candida boidinii</i>
MF2	Mal f 3 ^a	ND			
MF3		ND			
MF4		KYTLPLPYDYGAL	EPAISGEIMETHYEKH		Manganese superoxide dismutase
MF5		xxxxxEPYDVIVIGGGPGGYV	AxxKxxE		
MF6	Mal f 4	RKVAVLGASGGIGQPLSLMLKLNPKVTELR			Dihydroipoamide dehydrogenase
MF7 ^b		GNNGLSEVVYKPDxQxTxExFxxVI			
MF8		ND			
MF10		VDQxyFGLx			–
MF11		ND			
MF13		SNVFFDITKNGSPLTIKFKLFDDV			Cyclophilin

^a [13]; ^b MF7 was detected at the front (probably 9–10 kDa) and at a pI of 6.0 in a 2-D PAGE gel.

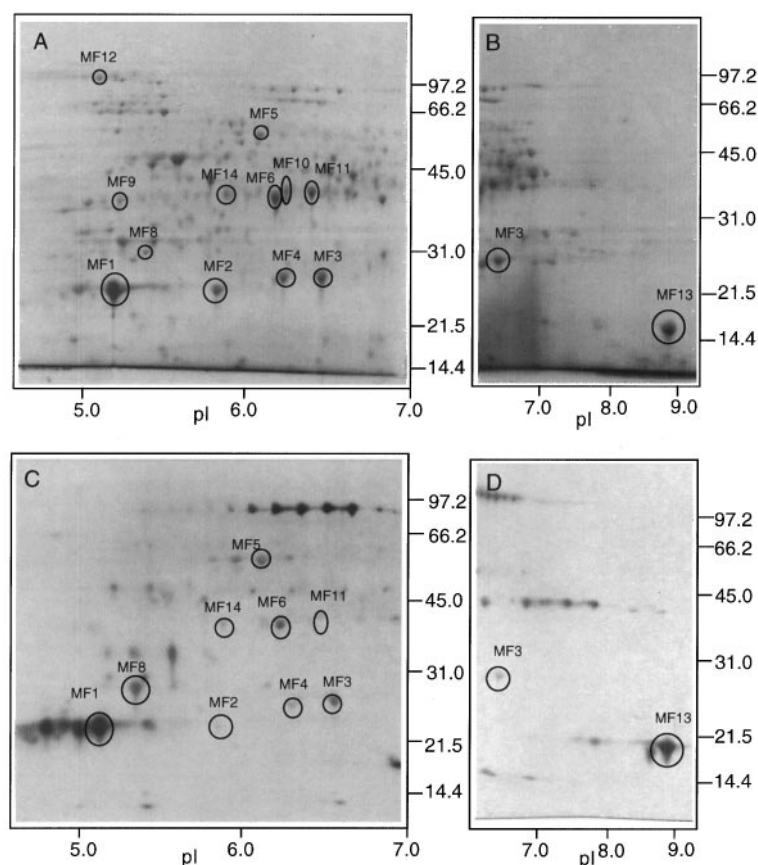


Fig. 1. Separation of crude *M. furfur* antigens by 2-D PAGE and identification of IgE-binding proteins. Crude *M. furfur* antigen P2 fraction was lyophilized and dissolved in sample buffer (100 µg protein per 90 µL), centrifuged, and the supernatant was resolved by 2-D PAGE (see Materials and methods). After electrophoresis using an IPG strip pH 4–7 (A, C) or pH 3–10 (B, D), proteins were stained by Coomassie brilliant blue R-250 (A, B), and IgE-binding proteins were detected by immunoblotting using pooled sera from atopic dermatitis patients (C, D) or from healthy individuals. The serum from healthy individuals gave no positive spots (data not shown). Spots MF9, MF10, and MF12 did not bind to IgE from the pooled serum used for the experiment shown in this figure, but bound to that from another sample of pooled serum. MF7 was not observed because of a low molecular mass. Molecular mass standards (kDa) are indicated at the right of the gels.

in the same buffer. Fractions containing Mal f 4 that eluted at about 0.5 M KCl (3.8 mg of protein), were desalted and applied to a hydroxyapatite column (EconoPac CHT-II 1 mL, BioRad) equilibrated with 10 mM sodium phosphate buffer (pH 6.8). The column was eluted using a linear gradient of 0–0.6 M sodium phosphate buffer (pH 6.8). Fractions that eluted at about 0.1 M sodium phosphate buffer (830 µg of protein), were concentrated by ultrafiltration, and applied to a Blue Sepharose column (HiTrap Blue 1 mL, Pharmacia) equilibrated with 50 mM sodium phosphate buffer (pH 7.4). The column was eluted stepwise with 5 mM and 12 mM NADH in the same buffer. The fraction that eluted with 12 mM NADH (98 µg of protein) was mixed with an equal volume of 4 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.0) and applied to a Phenyl Superose column (0.1 mL, Pharmacia) equilibrated with 2 M ammonium sulfate in the same buffer. The column was eluted using a gradient to the same buffer without ammonium sulfate. Fractions that eluted at about 1 M ammonium sulfate (43 µg of protein) were dialyzed against 20 mM sodium borate buffer (pH 8.5) containing 0.15 M NaCl, then used to determine IgE.

The activity of malate dehydrogenase of Mal f 4 protein was determined by measuring the change in optical density at 340 nm caused by oxaloacetate-dependent NADH oxidation in an assay mixture composed of 94 mM potassium phosphate buffer (pH 7.5), 0.23 mM oxaloacetate, 0.22 mM NADH, and a protein sample. Pig heart mitochondrial malate dehydrogenase (Boehringer Mannheim) was used as a positive control.

PCR amplification of cDNA

Standard molecular cloning proceeded as described by Sambrook *et al.* [17]. *M. furfur* TIMM 2782 was cultured in YNB medium at 30 °C for 3 days. Cells were collected, frozen in

liquid nitrogen, then total RNA was extracted as described by Okayama *et al.* [18]. A cDNA fragment was generated by the rapid amplification of cDNA ends (RACE) [19] using an RNA PCR kit (Takara, Shiga, Japan). Degenerate primers for PCR amplification of the Mal f 4 cDNA, MF6F1 and MF6F2, were designed based on the N-terminal amino acid sequences (KVAVLGASG and LSLLMKLNP; see Table 1) of Mal f 4 protein isolated by 2-D PAGE. Briefly, total RNA from *M. furfur* cells was reverse-transcribed using an oligo(dT)-adapter primer (Takara) consisting of oligo(dT) and M13 M4 segments and avian myeloblastosis virus reverse transcriptase. To produce a cDNA fragment of Mal f 4, the first PCR was performed using MF6F1 and M13M4 as primers along with the transcribed cDNA as a template. Thereafter, 4 µL of the amplification reaction product was used as the template for the second PCR, which used MF6F2 and M13 M4 as primers to yield a cDNA fragment of about 1.0 kb in length. PCR amplification proceeded as follows: 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 1.5 min for 40 cycles. The amplified PCR fragment was cloned into pUC118 and sequenced by dideoxy chain termination [20]. Nucleotide sequences of the primers were as follows: MF6F1, 5'-AA(A/G)GT(A/C/G/T)GC(A/C/G/T)GT(A/C/G/T)(C/T)T(A/C/G/T)GG(A/C/G/T)GC(A/C/G/T)(A/T)(G/C)(A/C/G/T)GG-3' and MF6F2, 5'-(C/T)T(A/C/G/T)(A/T)(G/C)(A/C/G/T)(C/T)T(A/C/G/T)(C/T)T(A/C/G/T)ATGAA(A/G)(C/T)T(A/C/G/T)AA(C/T)CC-3'.

Construction of cDNA library and cDNA cloning

Poly(A)⁺ RNA was purified using an oligo(dT)-cellulose column. A cDNA library was constructed in the vector λSHlox (Takara) and a Mal f 4 cDNA was screened by plaque hybridization using the 1.0-kb PCR fragment labeled with

A

-240	GAATTCATAGATTCTGAGGCCGCGCGTCGAGTCACGTTCCCTGAGTTATGTAATTCCGCCAT	-181
-180	CGAGTCCCAGGGTGGGGACCTGGGTCCGGGCGGGGGGAAGGGTGGGTTCGGAAGGTGGA	-121
-120	GGGCGTTT ¹ TAGTGCCAGTGCAGACCAGTTCGGGCGACGTCACAAAGTCCGGCGCTTGCC	-61
-60	GGCGCTTAAGTGACAGACCGTTGCGCGAGCGTCTCTCTCCATCATCTTTATCGATC	-1
1	ATGTTTGCCTGCGCGCCCGTCGTAACAGSCVTRAPAPASARVYF	60
61	TCGCAGATCGCGTACGAAACCGCAAGGTGCGCTGCTCGGTGCTCGGCGGCTATTGGC	120
121	SGQTAAAGNRKVAIVLGASGGTIG	180
181	CAGCCGctagtgtgcgcttcgcctaaacacaa ⁴¹ CGTCTCGTCTCGTGAAGCTCAACCCCA	240
241	Q ⁴¹ P ⁴¹ <u>LSLSLMLKLNLP</u>	300
301	AGVTTACCGAGTCTCCGCTGTGACAGATCCGCTCGCTCGGGTGTGCTGATGATCTCT	360
361	K ⁴¹ T ⁴¹ <u>ETELRL</u> LYDIRLAFGLVAAADL	420
421	CGCACATCAACACCCCGCGGTGATCTCGGCTACGCCAGGACGACCTCGAGGGTGGCG	480
481	SHINTPAAVTSGLYADLESA	540
541	TCGACGGTCCGGAGATTGTGCTGATCCCCCGCGATGCCCGCGAAGCCGGCATGACC	600
601	V ⁴¹ D ⁴¹ G ⁴¹ A ⁴¹ E ⁴¹ V ⁴¹ L ⁴¹ I ⁴¹ P ⁴¹ A ⁴¹ G ⁴¹ M ⁴¹ P ⁴¹ R ⁴¹ K ⁴¹ P ⁴¹ G ⁴¹ M ⁴¹ T	660
661	GTGACGACCTGTTCAACGctacgtgaatcagctataac ¹¹² CTGCAAGCTCGATTTCGCT	720
721	112 ⁴¹ R ⁴¹ D ⁴¹ L ⁴¹ D ⁴¹ L ⁴¹ F ⁴¹ N ⁴¹ <u>SNASIVIR</u>	780
781	GACCTTCCGCAAGGTCTGGCGTAAAGTTCGCCCAAGGCTTACCTCGGCTCATCTCGAAC	840
841	125 ⁴¹ D ⁴¹ LA ⁴¹ K ⁴¹ V ⁴¹ V ⁴¹ A ⁴¹ K ⁴¹ V ⁴¹ A ⁴¹ P ⁴¹ K ⁴¹ A ⁴¹ I ⁴¹ T ⁴¹ G ⁴¹ V ⁴¹ T ⁴¹ S ⁴¹ N ⁴¹	900
901	CCGCTCAACTCGACGGTGGCGATCTGCTCGCTGAGGTGTTCAAGAAGCGGTGTGTACGAC	960
961	145 ⁴¹ P ⁴¹ V ⁴¹ N ⁴¹ S ⁴¹ T ⁴¹ V ⁴¹ P ⁴¹ I ⁴¹ V ⁴¹ A ⁴¹ E ⁴¹ V ⁴¹ F ⁴¹ K ⁴¹ A ⁴¹ CG ⁴¹ V ⁴¹ Y ⁴¹ D ⁴¹	1020
1021	CCCAAGCGCCTCTCGGTGTGACCACTCGACACACCGCGCGGCACCTCTCTGTGC	1080
1081	165 ⁴¹ P ⁴¹ K ⁴¹ R ⁴¹ L ⁴¹ F ⁴¹ G ⁴¹ V ⁴¹ T ⁴¹ L ⁴¹ D ⁴¹ T ⁴¹ T ⁴¹ R ⁴¹ A ⁴¹ A ⁴¹ T ⁴¹ F ⁴¹ L ⁴¹ S ⁴¹	1140
1141	GGCATGTGCTGCGACCCGACGACCAACGCTCCCGCATCTGGTGGCCATCTCGGT	1200
1201	185 ⁴¹ G ⁴¹ I ⁴¹ A ⁴¹ G ⁴¹ S ⁴¹ D ⁴¹ P ⁴¹ Q ⁴¹ T ⁴¹ T ⁴¹ N ⁴¹ V ⁴¹ P ⁴¹ I ⁴¹ G ⁴¹ H ⁴¹ S ⁴¹ G ⁴¹	1260
1261	GTGACATTTGTGCCCTGATCTCGCAGCGCGCCAGGGTGACAAGGTGCAGGCTGGCGAC	1320
1321	205 ⁴¹ V ⁴¹ T ⁴¹ I ⁴¹ T ⁴¹ V ⁴¹ L ⁴¹ I ⁴¹ S ⁴¹ Q ⁴¹ A ⁴¹ A ⁴¹ D ⁴¹ K ⁴¹ V ⁴¹ Q ⁴¹ A ⁴¹ G ⁴¹ E ⁴¹	1380
1381	225 ⁴¹ CAGTCAACAAGCTTGTGCACCGCATCTCAGTTCTCGTGGTGACGAGTCTGTCAGAGCCAAG	1440
1441	225 ⁴¹ A ⁴¹ Y ⁴¹ D ⁴¹ K ⁴¹ L ⁴¹ V ⁴¹ H ⁴¹ R ⁴¹ I ⁴¹ Q ⁴¹ F ⁴¹ G ⁴¹ E ⁴¹ V ⁴¹ V ⁴¹ K ⁴¹ A ⁴¹ K ⁴¹	1500
1501	241 ⁴¹ GACGTGCGCGCTCGCGACAGCTCTCGATGCTTACGCGCGCGCTCTTCACGAGAGGGC	1560
1561	245 ⁴¹ D ⁴¹ G ⁴¹ A ⁴¹ G ⁴¹ S ⁴¹ A ⁴¹ T ⁴¹ L ⁴¹ S ⁴¹ M ⁴¹ A ⁴¹ Y ⁴¹ A ⁴¹ A ⁴¹ V ⁴¹ F ⁴¹ T ⁴¹ E ⁴¹ G ⁴¹	1620
1621	265 ⁴¹ CTCGTCAAGGTC ⁴¹ TCGACGGT ⁴¹ GAGGCG ⁴¹ GAGCA ⁴¹ GATGACCT ⁴¹ TCGT ⁴¹ CGAG ⁴¹ AGCCCT ⁴¹ CT	1680
1681	265 ⁴¹ L ⁴¹ L ⁴¹ K ⁴¹ G ⁴¹ G ⁴¹ L ⁴¹ D ⁴¹ G ⁴¹ E ⁴¹ A ⁴¹ V ⁴¹ T ⁴¹ Q ⁴¹ C ⁴¹ T ⁴¹ TCG ⁴¹ V ⁴¹ F ⁴¹ E ⁴¹ S ⁴¹ P ⁴¹ L ⁴¹ A ⁴¹	1740
1741	901 ⁴¹ TTCAGGACAGGTGCATCTTCTCGCTCGCCGCTCGATCTCGGCGCGAGGGTGTGAAG	960
961	285 ⁴¹ F ⁴¹ K ⁴¹ D ⁴¹ Q ⁴¹ V ⁴¹ D ⁴¹ F ⁴¹ F ⁴¹ A ⁴¹ S ⁴¹ P ⁴¹ V ⁴¹ E ⁴¹ F ⁴¹ G ⁴¹ P ⁴¹ E ⁴¹ G ⁴¹	1020
1021	961 ⁴¹ AAGATCCCTGCTCTGCCGAAGCTCACCGCCGAGGAGCAGAAGCTGCTCGACGCTCGCTG	1080
1081	305 ⁴¹ N ⁴¹ I ⁴¹ P ⁴¹ A ⁴¹ L ⁴¹ F ⁴¹ K ⁴¹ L ⁴¹ T ⁴¹ G ⁴¹ E ⁴¹ V ⁴¹ Q ⁴¹ K ⁴¹ L ⁴¹ D ⁴¹ A ⁴¹ C ⁴¹ L ⁴¹	1140
1141	1021 ⁴¹ CCGCAAGTGCACAAGTctacgtatatgaattggtggtcgacacacATCATCAAGAGGG	1200
1201	325 ⁴¹ P ⁴¹ D ⁴¹ L ⁴¹ A ⁴¹ <u>NI</u> K ⁴¹ K ⁴¹ G ⁴¹	1260
1261	1081 ⁴¹ CGTTGCTGTGGCGCGCGAGAAACCCGTAAATTCGCGCAAGCAATCTTTTACGAGCTTGCGC	1320
1321	335 ⁴¹ V ⁴¹ W ⁴¹ A ⁴¹ A ⁴¹ E ⁴¹ N ⁴¹ P ⁴¹ *	1380
1381	1141 ⁴¹ GAAAGAAAGAAATGACGTTTCTATAGAAGCTAGATCTGTCCCTTTCCACCTATTTTGTG	1440
1441		

B

	10	20
Mal f 4	MFARAALRNSVRAPASARYFSQTAAAN	
<i>S. cerevisiae</i>	*LS*V-----AK*A**S*V*NP	
mouse	*LSAL*-*-PVGA*LR*S**TS*ON*	

Fig. 2. Nucleotide sequence of the cDNA and genomic DNA of *Mal f 4* and its deduced amino acid sequence (A) and homology of the signal sequence encoded by the *Mal f 4* gene with those of *S. cerevisiae* and mouse mitochondrial malate dehydrogenase (B). (A) Primers, above the nucleotides shown as single-headed arrows, MF6F3 (sense), MF6R4 (antisense), MF6F5 (sense), and MF6R6 (antisense), were used for cassette-ligation mediated PCR to isolate the genomic *Mal f 4* DNA. The nucleotide sequences of three introns are shown by small letters and consensus intron sequences are double-underlined. Sequenced N-terminal amino acid residues are shown in bold. The cDNA obtained started from the nucleotide (box) at position 232. (B) Conserved amino acids are shown by asterisks and gaps are shown as dashes.

[³²P]dCTP by random priming as a probe. Ten cDNA clones showing high intensity signals were selected. The cDNA inserts in the λSHlox vector were automatically subcloned for use as a plasmid as recommended by the manufacturer. The longest cDNA insert (0.9 kb) was subcloned into pUC118, sequenced and further analyzed.

PCR amplification of genomic DNA

Genomic DNA of *M. furfur* cells was obtained as follows. Cells cultured in YNB medium were washed and suspended in PK

Mal f 4	1		RKVAVLGASGGIGQPLSLMLK	-----NPKVTE-	28
mMDH	1		Y* ⁺ T* ⁺ ***G* ⁺ *****I*	-----H* ⁺ *D-	28
mouse MDH	1		A* ⁺ *****N	-----S* ⁺ *SR-	28
pMDH	1		V* ⁺ *I* ⁺ *****V* ⁺ *****	-----S* ⁺ *S-	28
cmDH	1		PHSVTPSPTEQDSI* ⁺ I* ⁺ A* ⁺ *****S* ⁺ * ⁺ I* ⁺ AQLQYQLKES	RS* ⁺ HI	50
Mal f 4	29		LRLYDIRLAP--GVAADLSHINTPATVSGYAQDD--	LEGAVDGAIEVL	72
mMDH	29		*****LKG* ⁺ K--* ⁺ T* ⁺ *****P* ⁺ NS* ⁺ VK* ⁺ PEEPED* ⁺ NN* ⁺ LKDTDM	75	
mouse MDH	29		*****AHT--* ⁺ T* ⁺ *****E* ⁺ R* ⁺ NK* ⁺ LGPE--G* ⁺ PDCLC* ⁺ CDV* ⁺ V	75	
pMDH	29		--A* ⁺ *****A* ⁺ E--* ⁺ TGK*****NSSCV* ⁺ D* ⁺ S--	* ⁺ NCLSN* ⁺ QV* ⁺ T	78
cmDH	51		H*A* ⁺ ***VNOEAIN* ⁺ T* ⁺ *****D* ⁺ ISV* ⁺ SHSPAG--G* ⁺ NCLHN* ⁺ S* ⁺ V	92	
Mal f 4	73		IPAGMRPRKGTRDDLPNSNASIVRDLAKVVAKVA--	PKAYIGWISNPVN	120
mMDH	76		*****V*****AI*****AA* ⁺ ES* ⁺ --N* ⁺ A* ⁺ L*****	123	
mouse MDH	74		***V*****T* ⁺ T* ⁺ *AT* ⁺ AAT* ⁺ QHC--E* ⁺ MVCI* ⁺ A*****	121	
pMDH	73		***V*****KM* ⁺ G* ⁺ KS* ⁺ VDSI* ⁺ G* ⁺ F* ⁺ --N* ⁺ R* ⁺ L*****	120	
cmDH	99		***V*****V* ⁺ G* ⁺ ISQ* ⁺ GTA* ⁺ ECCDLS* ⁺ VFVL*****	148	
Mal f 4	121		STVPI-VAEVFKKAGVYDPK--	RLFGV* ⁺ TLDTTRAATFLSGI----	159
mMDH	124		*****Q* ⁺ L* ⁺ NK* ⁺ N* ⁺ --K* ⁺ *****SI* ⁺ * ⁺ I* ⁺ EV----	162	
mouse MDH	122		*I* ⁺ * ⁺ T* ⁺ *****N* ⁺ N--KI*****V* ⁺ R* ⁺ IAEVL----	160	
pMDH	121		*L* ⁺ * ⁺ AV* ⁺ T* ⁺ *M* ⁺ KFK* ⁺ G--NVM* ⁺ * ⁺ LV* ⁺ E* ⁺ V* ⁺ LYMLLNK	165	
cmDH	149		*L* ⁺ VM* ⁺ SNIL* ⁺ NHPQSNISGIER* ⁺ IM* ⁺ * ⁺ K* ⁺ V* ⁺ IS* ⁺ * ⁺ RE* ⁺ ----NI	194	
Mal f 4	160		----AGSDPQT* ⁺ TNPVIGHSGSV* ⁺ IVPLISQAAGDKVKVAGEQYDKLVH	204	
mMDH	163		----ENT* ⁺ TOER* ⁺ N*****I* ⁺ * ⁺ *****TNH--LMSDDKRRIE* ⁺ I	205	
mouse MDH	161		----K* ⁺ L* ⁺ AR* ⁺ V*****A* ⁺ K* ⁺ * ⁺ *****C* ⁺ TP-KVDFPD* ⁺ LAT* ⁺ TG	204	
pMDH	166		KIGQEGEQLDTHMRK* ⁺ T*****E* ⁺ * ⁺ I* ⁺ TKDSL-V* ⁺ LDK* ⁺ * ⁺ EHFI* ⁺	213	
cmDH	195		ESGLTPRVNSMP-D*****E* ⁺ * ⁺ I* ⁺ * ⁺ SNFLNRS-ED* ⁺ LKY* ⁺ I	242	
Mal f 4	205		RIQFGGDEVFKAKDGAGSATLSMAYAA--	AVFTEGLKGLDGE-AV* ⁺ TG	249
mMDH	206		*****N*****H* ⁺ G--* ⁺ K* ⁺ ANAV* ⁺ S* ⁺ FK* ⁺ LD* ⁺ IE	251	
mouse MDH	205		* ⁺ EA* ⁺ T*****A*****G--	* ⁺ R* ⁺ VFS* ⁺ VDAM* ⁺ RG* ⁺ VE	250
pMDH	214		V*****I*****Q*****FRG--	* ⁺ K* ⁺ A* ⁺ EV* ⁺ RSFH* ⁺ LP* ⁺ VE	259
cmDH	243		V* ⁺ Y*****K*****H* ⁺ G* ⁺ YKCVQ* ⁺ VSL* ⁺ G* ⁺ NIEQHGT* ⁺ Y	292	
Mal f 4	250		C-TFVESPLFK-----	DQ-VDFASF* ⁺ PVEFG* ⁺ PEGVKNIPA--LP	283
mMDH	252		P-S* ⁺ *D* ⁺ *****SGEIG*****TL* ⁺ * ⁺ D* ⁺ IEK* ⁺ HP--IG	286	
mouse MDH	251		--S* ⁺ *Q* ⁺ K-----	ETECTY* ⁺ ST* ⁺ LLL* ⁺ K* ⁺ LEKNLIG--IG	282
pMDH	260		SLSA* ⁺ YL* ⁺ GI* ⁺ NGKGAQQLVG--NSIEY* ⁺ SL* ⁺ IVLRN* ⁺ GS* ⁺ V* ⁺ DS* ⁺ V	308	
cmDH	293		V--PLKDANN* ⁺ PL* ⁺ AGGADLLPLVDGA* ⁺ Y* ⁺ * ⁺ TLITTK* ⁺ * ⁺ SVYD* ⁺ VDIVN	340	
Mal f 4	284		KLTAEE-QKLLDACLPDLAKNIKKGVAAEAENP		315
mMDH	287		E* ⁺ SS* ⁺ --EEM* ⁺ QK* ⁺ KET* ⁺ * ⁺ E* ⁺ FN* ⁺ VSK		317
mouse MDH	283		I* ⁺ FP--EKMIAEIAE* ⁺ E* ⁺ KAS*****EDFVKNM		314
pMDH	309		* ⁺ SPR* ⁺ -Q* ⁺ VNTAVKE* ⁺ R* ⁺ * ⁺ E* ⁺ KSFLDSSKL		342
cmDH	41		RMNDM* ⁺ RNM* ⁺ P* ⁺ VSO* ⁺ * ⁺ D* ⁺ LEF* ⁺ SRASS		376

Fig. 3. Homology of the mature protein encoded by *Mal f 4* gene with mature malate dehydrogenase of *S. cerevisiae* and mouse mitochondrial malate dehydrogenases. Conserved amino acids are shown by asterisks. Gaps are shown as dashes. mMDH, pMDH, and cMDH are mitochondrial, peroxisomal, and cytoplasmic malate dehydrogenases of *S. cerevisiae*, respectively. The cDNA shown in Fig. 2A encoded a protein starting from the amino acid residue at position 43.

buffer (0.15 M NaCl, 12.5 mM EDTA, 0.1 M Tris/HCl; pH 7.5). The cells were disrupted using a Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA). Total genomic DNA was purified from the disrupted cells as described by Philippsen *et al.* [21]. *Mal f 4* genomic DNA was obtained by cassette-ligation mediated PCR [22] using an *in-vitro* cloning kit (Takara). Genomic DNA digested separately with the restriction enzymes, *Eco*RI, *Eco*T22I, *Hind*III, *Mun*I, *Nhe*I, *Pst*I, *Spe*I, and *Xba*I, was ligated to cassettes (Takara) and used as the template for PCR as described below. Specific primers (see Fig. 2A), MF6F3 (sense, 5'-GCCCCGGCATGACCCGTGACG-3'), MF6R4 (antisense, 5'-CTTGGCGAGGTCACGGAC-3'), MF6F5 (sense, 5'-GTCAAGGCCAAGACGGTGC-3'), and MF6R6 (antisense, 5'-GTGAAGACAGCGGCGGCGTA-3'), and cassette primers C1 and C2 (Takara) were used for PCR according to the manufacturer's protocol. To obtain a fragment containing the N-terminal of *Mal f 4* gene, we performed first PCR using MF6R6 and C1 as primers and second PCR using MF6R4 and C2 as primers. A fragment containing the C-terminal was obtained by a first PCR using MF6F3 and C1 as primers and a second PCR using MF6F5 and C2 as primers. The central fragment of the *Mal f 4* gene was obtained by PCR using MF6F3 and MF6R6 as primers. PCR proceeded as follows: 25 cycles of 94 °C for 0.5 min, 55 °C for 1.5 min and 72 °C for 2 min. The three PCR fragments were cloned into pUC118 and the DNA fragments of two or three clones from the respective DNA fragments were sequenced.

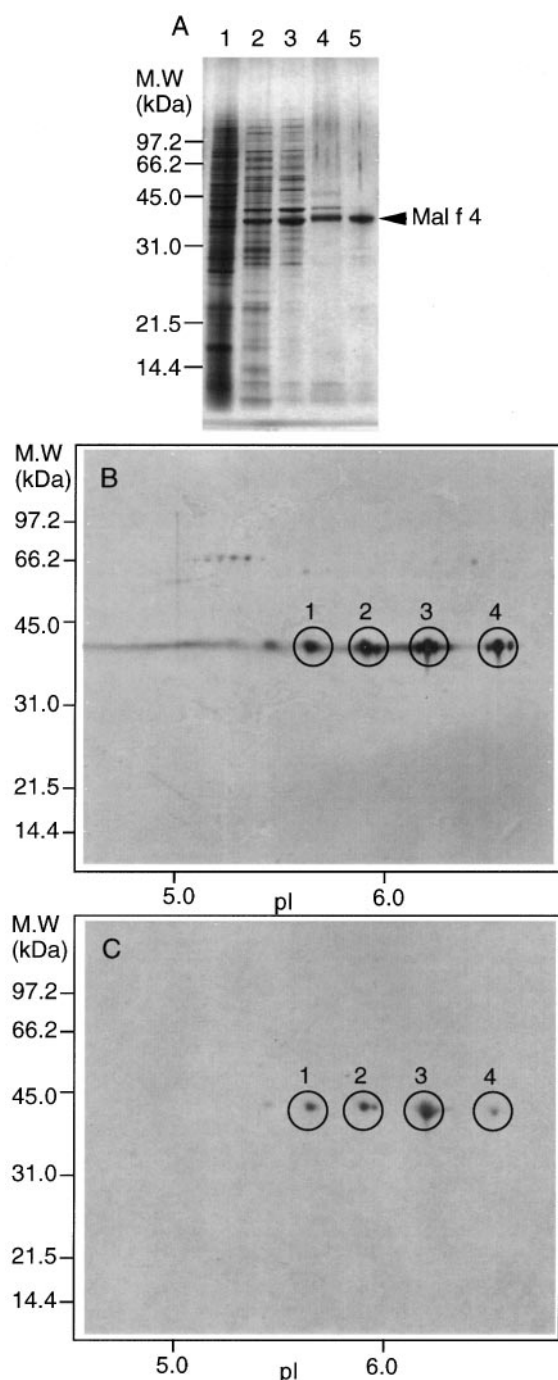


Fig. 4. SDS/PAGE of purification procedure of Mal f 4 (A), and analysis of the purified Mal f 4 preparation by 2-D PAGE (B) and IgE-immunoblotting (C). (A) Purification and recovery of proteins are described in Materials and methods. Samples were taken after purification by precipitation with ammonium sulfate (lane 1, 900 ng protein), sequential chromatography using Blue Sepharose (lane 2, 350 ng), hydroxyapatite (lane 3, 160 ng), Blue Sepharose (lane 4, 76 ng), and Phenyl Superose (lane 5, 80 ng). Proteins were detected by silver staining (A, B). Purified Mal f 4 (B, 860 ng for silver staining; C, 4.3 μ g for immunoblotting) was applied to 2-D PAGE. Spots 1 (pI 5.6), 2 (pI 5.8) identical to MF14 (Fig. 1), 3 (pI 6.2) identical to MF6 (Fig. 1 and Table 1), and 4 (pI 6.5) were identified as IgE-binding proteins. Molecular mass standards (kDa) are indicated on the left of the gels.

RESULTS

Analysis of IgE-binding components by 2-D PAGE and N-terminal amino acid sequencing

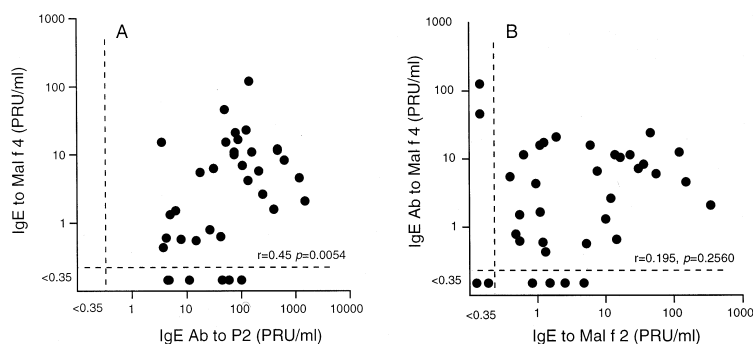
M. furfur proteins in the P2 fraction were separated by 2-D PAGE in pH gradients of 4–7 or 3–10 and tested for IgE-binding (Fig. 1). We detected a number of spots reactive to IgE from atopic dermatitis patients. The IgE-binding proteins were heterogeneous in terms of molecular mass and isoelectric point (pI). Most of these proteins located between pI 5–7 and molecular mass of 30–90 kDa. The IgE-binding spots MF1 and MF2 (Fig. 1) were identified as Mal f 2 and Mal f 3 by comparing their N-terminal amino acid sequences and 2-D PAGE pattern with the purified sample [13]. Mal f 2 was the most abundant protein and was the most densely stained by immunoblotting. We determined the N-terminal amino acid sequences of the six novel IgE-binding proteins shown in Table 1, which includes proteins that are homologous to manganese superoxide dismutase, dihydrolipoamide dehydrogenase, and cyclophilin. A new allergen designated Mal f 4 (spot MF6 in Fig. 1) had 30 N-terminal amino acid residues with significant homology to malate dehydrogenases from *Saccharomyces cerevisiae* and mammals [23,24]. Among the 30 amino acid residues, 24 and 22 of them shared identity with the N-terminal amino acid residues of mature mitochondrial and peroxisomal malate dehydrogenases of *S. cerevisiae*, respectively.

Cloning and analysis of the *Mal f 4* gene

Based on the N-terminal amino acid sequence of Mal f 4, we prepared two degenerate primers corresponding to positions 2–10 and 16–24 of the N-terminal amino acid residues and performed PCR by nested RACE [19] using these primers. The cDNA fragment obtained by PCR was about 1.0 kb in length, and it coded for the amino acid residues (KVTELK, positions 25–30 of the N-terminal amino acid sequence) of Mal f 4. The PCR fragment was used as a probe to clone the cDNA of the *Mal f 4* gene from a cDNA library of *M. furfur* by plaque hybridization. Ten cDNA clones were selected, and the longest cDNA fragment (0.9 kb) was sequenced. An open reading frame of 819 bp encoding a protein of 273 amino acid residues (Figs 2A, 3) was revealed. This protein lacked the N-terminal amino acid sequence (Table 1) of the native Mal f 4 protein. Nevertheless, the protein encoded by the cDNA had high homology with *S. cerevisiae* malate dehydrogenase (Fig. 3), indicating that the cloned cDNA was the objective *Mal f 4* gene.

We confirmed the genomic structure of *Mal f 4* gene as follows. We performed cassette-ligation mediated PCR [22] using cassettes and four specific oligonucleotides (Fig. 2A) as the primers and the genomic DNA obtained by digestion with eight restriction enzymes and ligation to cassettes as the template. We obtained a 5'-upstream region containing nucleotides coding for the N-terminus, a 3'-downstream region containing nucleotides coding for the C-terminus, and a central region of the genomic DNA. Their nucleotide sequences were compared with the cDNA encoding the protein of 273 amino acids and with the N-terminal amino acid sequence of Mal f 4 protein. The results revealed that the *Mal f 4* gene is composed of four exons separated by 26-bp, 22-bp, and 31-bp introns, and that it encodes a protein consisting of 342 amino acids (Fig. 2A). The protein deduced from the cloned gene contained a 30 amino acid sequence identical to that of the Mal f 4 isolated by 2-D PAGE, indicating that the gene coded for

Fig. 5. Correlation of IgE levels in sera of atopic dermatitis patients reactive to Mal f 4, Mal f 2, and crude antigen fraction P2. (A) Mal f 4 vs. fraction P2 (B) Mal f 4 vs. Mal f 2. The coefficient of correlation and statistical significance was calculated based upon PRU values below 0.35 = 0.34. ($n = 36$).



Mal f 4. These studies revealed that Mal f 4 protein is composed of 315 amino acids. The calculated molecular mass of the protein (32 928) was close to that shown by 2-D PAGE (Fig. 1) and the 35 kDa estimated by SDS/PAGE (Fig. 4A). Furthermore, the calculated pI of 5.65 was close to the pI of 6.2 estimated by 2-D PAGE.

The predicted amino acid sequence had higher homology with the sequence of mitochondrial malate dehydrogenase than of cytoplasmic or peroxisomal malate dehydrogenases from *S. cerevisiae* [21]. The predicted sequence had 57%, 46%, 41% and 52% identity with mitochondrial, peroxisomal, and cytoplasmic malate dehydrogenases from *S. cerevisiae* and mouse mitochondrial malate dehydrogenases, respectively (Fig. 3). The N-terminal sequence of mature cytoplasmic malate dehydrogenase of *S. cerevisiae* showed little similarity to Mal f 4 (Fig. 3). The signal sequence deduced from the *Mal f 4* gene was composed of 27 amino acids, of which many had basic and hydroxylated side chains and none of which had acidic chains. This sequence shared similarity with the mitochondrial targeting sequences of mitochondrial malate dehydrogenase of *S. cerevisiae* and mammals (Fig. 2B) [24]. Mal f 4 lacked a peroxisomal targeting amino acid sequence similar to the sequence (SKL) of *S. cerevisiae* peroxisomal malate dehydrogenase at the C-terminal (Fig. 3). These results suggested that Mal f 4 is a *M. furfur* mitochondrial malate dehydrogenase.

Purification of Mal f 4 protein and measurement of IgE titers to the purified Mal f 4 in the sera of atopic dermatitis patients

To characterize Mal f 4 protein as an allergen, we purified it from a membrane fraction of *M. furfur* cells by chromatography using mainly Blue Sepharose, based on the fact that malate dehydrogenase has affinity to this resin [21]. The resulting preparation migrated as a single band at 35 kDa in SDS/PAGE (Fig. 4A). However, the preparation consisted not only of Mal f 4 as a major spot at pI 6.2 (spot 3), but also three other spots with pI values of 5.6, 5.8, and 6.5 in 2-D PAGE (Fig. 4B). Their 30 N-terminal amino acid residues were identical to those of Mal f 4. The purified Mal f 4 separated by 2-D PAGE was tested for IgE-binding by immunoblotting, and all four spots bound to IgE from atopic dermatitis patients (Fig. 4C). The staining density correlated to the amount of Mal f 4 in the preparation. Spot 2 was identical to spot MF14 shown in Fig. 1.

To analyze the incidence of sensitization to Mal f 4, we selected 36 atopic dermatitis patients sensitized to *M. furfur* and three healthy individuals with no history of atopy as a control, and determined levels of serum IgE to the purified Mal f 4. We also compared the IgE to Mal f 4 in the sera from the atopic dermatitis patients with those to the crude antigen fraction P2 and to Mal f 2 allergen. As shown in Fig. 5, 30 of 36 patients (83.3%) had IgE reactive to Mal f 4 allergen (> 0.35

PRU·mL⁻¹), whereas control individuals did not. The percentage was almost as high as that of Mal f 2 (32/36, 88.8%). There was a significant correlation between the PRU values of Mal f 4 and fraction P2 ($r = 0.45$, $P = 0.0054$) (Fig. 5A), whereas there was no such correlation between PRU values of Mal f 2 and Mal f 4 (Fig. 5B) and between Mal f 3 and Mal f 4 (data not shown). The geometric mean values of patient IgE reactive to Mal f 4, Mal f 2, and the P2 fraction were 10.5, 25.7, and 183.8 PRU·mL⁻¹, respectively.

DISCUSSION

We analyzed *M. furfur* allergens reactive to IgE of atopic dermatitis patients by 2-D PAGE and identified known Mal f 2 and Mal f 3, as well as several new allergens including Mal f 4. SDS/PAGE and IgE-immunoblotting have revealed a number of 10–100 kDa IgE-binding components in *M. furfur* cell extracts [6–9]. 2-D PAGE followed by immunoblotting separated complex *M. furfur* components and revealed distinct IgE-binding proteins. Separation by 2-D PAGE, isolated proteins that were sufficiently pure to sequence their N-terminal amino acids. These proteins included Mal f 2 and six novel allergens. Four allergens showed homology with malate dehydrogenase, manganese superoxide dismutase, dihydrolipoamide dehydrogenase, and cyclophilin. Manganese superoxide dismutase from *Aspergillus fumigatus*, and cyclophilin from *Psilocybe cubensis* are fungal allergens [25,26]. However, we could not determine the N-terminal amino acid sequences of several major IgE-binding proteins because the N-terminals were blocked or because of low levels of protein. Mal f 1 [12] was not identified in the crude antigen fraction P2. This may be a result of differences not only in the sera, but in the antigen, because the culture conditions include incubation periods that influence the allergenic composition [27].

The four IgE-binding spots with different pI values separated by 2-D PAGE from a chromatographically pure Mal f 4 sample, all had the same N-terminal amino acid sequences. We could not determine the chemical cause of the difference in pI. It may have been a result of derivatization during sample preparation for 2-D PAGE, which uses 8 M urea and therefore causes carbamylation [28]. A purification step such as freeze-thawing could also be responsible [29], because the purified Mal f 4 lost activity of malate dehydrogenase (data not shown). However, the spot 2 of the four spots was detected as an IgE-binding protein, MF14 in 2-D PAGE (Fig. 1), suggesting that it is an isoallergen. We isolated part of a gene that was over 90% identical to the *Mal f 4* gene, by PCR cloning *M. furfur* cDNA (data not shown), although the cDNA of the entire gene remains to be cloned. Such highly homologous genes may encode the IgE-binding spots as isoallergens.

M. furfur allergens including Mal f 1 that has no homology with known proteins, as well as Mal f 2 and Mal f 3, which are

homologous to peroxisomal membrane proteins such as *Candida boidinii* PMP20 and *A. fumigatus* Asp f 3, have recently been isolated and their genes cloned [12,13]. Mal f 4 had no amino acid sequence homology with these known *M. furfur* allergens. Although Mal f 4 and Mal f 2 showed significant correlations with the fraction P2 in IgE levels, there was no significant correlation between the two allergens. The geometric mean value of IgE reactive to Mal f 4 from 36 patients was one-seventeenth of that to the crude antigen, while three patients had IgE levels to Mal f 4 that were as high as those to the crude antigen (Fig. 5), indicating that Mal f 4 was the major *M. furfur* allergen for the three atopic dermatitis patients. Proteins homologous to malate dehydrogenase from *Schistosoma manni* and latex are IgE-binding components [29,30], but no reports indicate fungal malate dehydrogenase as an allergen. Cross-reactivity between Mal f 4 and malate dehydrogenase homologs from environmental allergens including latex and various fungi such as *C. albicans*, the cell extract of which contains IgE-binding components cross-reactive to *M. furfur* antigens [10,31], remains to be determined.

The fungal allergen extracts currently used for *in-vivo* and *in-vitro* diagnosis of sensitization are prepared from raw materials without purification, and are not well defined. Therefore, extracts must be improved to ensure dependable diagnosis. Allergen Mal f 4 as well as Mal f 2 will be useful for this purpose. Furthermore, the *M. furfur* allergens identified here will help clarify the pathogenesis of atopic dermatitis.

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