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Identification of the major allergen of *Malassezia globosa* relevant for atopic dermatitis

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ABSTRACT

Background: *Malassezia globosa* constitutes a part of the normal flora of human skin, but may induce IgE production in atopic dermatitis (AD). However, information on *M. globosa* allergens is scant.

Objective: To identify the major *M. globosa* allergens by using proteomic analysis.

Methods: Immunoglobulin E (IgE) immunoblotting and cross-inhibition tests for *M. globosa* allergens were performed using sera from AD patients and control subjects. These allergens were identified and characterized using the proteomics approach involving a combination of two-dimensional (2D) electrophoresis, mass spectrometry, and bioinformatics tools. We cloned the cDNA of this allergen using sequences obtained by 5'- and 3'-rapid amplification of cDNA ends polymerase chain reaction.

Results: The sera of the AD patients had IgE-reactive 40–45-kDa protein components. By 2D immunoblotting, we detected a 42-kDa protein spot with an isoelectric point (pI) of 4.8; the protein was highly reactive to IgE and was designated MGp42. Full-length MGp42 cDNA contained a 1908-bp open reading frame encoding 635 amino acid residues (calculated molecular mass, 69.7 kDa; pI, 6.02). The N-terminal MGp42 sequence started from the 250th residue (Asp-250) of the deduced amino acid sequence and consisted of 386 amino acid residues; these results are consistent with those of 2D immunoblotting. MGp42 showed sequence similarity to members of the heat shock protein 70 (hsp70) family. Immunoblot inhibition tests revealed no IgE cross-reactivity between MGp42 and human HSP70.

Conclusions: MGp42 may be a cleavage product of intact HSP70. This novel *M. globosa* allergen could be useful for the diagnosis of AD.

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1. Introduction

The lipophilic yeasts of the genus *Malassezia* are natural inhabitants of the human skin, preferentially inhabiting seborrheic skin areas such as the head and neck; however, these organisms are associated with several skin diseases such as pityriasis versicolor, pityrosporum folliculitis, and seborrheic dermatitis [1–3]. They are also considered to be one of the factors that exacerbate atopic dermatitis (AD) located primarily in the head and neck area. This consideration is based on the finding that over 90% of AD patients exhibited specific serum immunoglobulin E (IgE) antibodies against *Malassezia* yeasts [4–8]. In particular, AD patients with head and neck dermatitis have high levels of *Malassezia*-specific IgE [7,9]. In addition, treatment with antifungal agents such as ketoconazole results in clinical improvement of head and neck AD in association with decreased *Malassezia* colonization [5,10,11]. The genus *Malassezia* consists of 13 species,

including *Malassezia furfur*, *Malassezia sympodialis*, *Malassezia globosa*, *Malassezia restricta*, *Malassezia slooffiae*, *Malassezia obtusa*, and *Malassezia pachydermatis* [12–17]. Of these, *M. globosa* and *M. restricta* may play an important role in the pathogenesis of AD [12]. Although a number of *Malassezia* allergens have been identified and studied [18–21], data on allergens of *M. globosa* is unavailable. The identification and characterization of clinically relevant *Malassezia* allergens remain incomplete; this limits our understanding of the roles of these allergens in the immunopathogenic mechanisms involved in AD.

The identification and characterization of allergens require extensive effort and a large amount of raw material. Newly developed proteomics approaches involving the combined application of separation techniques, mass spectrometry (MS), and bioinformatics tools have been proposed for the identification and characterization of proteins in complex biological mixtures under various experimental conditions [22,23]. In this study, we propose the use of a proteomics approach combining two-dimensional (2D) western blotting and matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) for the identification of *M. globosa* allergens. In addition, we cloned and

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sequenced the gene encoding the major allergen of *M. globosa* by using 5'/3'-RNA ligase-mediated rapid amplification of cDNA ends polymerase chain reaction (5'/3'-RLM-RACE PCR). Comparison of this sequence with known protein sequences revealed that the *M. globosa* allergen is highly similar to members of the heat shock protein 70 (hsp70) family. This study suggests that the major allergen of *M. globosa* is a cleavage product of a protein belonging to the hsp70 family.

2. Materials and methods

2.1. Yeast strains, culture conditions, and extract preparation

M. globosa CBS7966 was cultured on modified Leeming and Notman agar (LNA; 20 g glucose, 50 g malt extract, 1 g polypeptone, and 20 g bile salts) (Oxoid, Hampshire, United Kingdom) containing 1% Tween-40, 0.2% glycerol, and 50 µg/mL chloramphenicol (Sankyo, Tokyo, Japan) at 32 °C [24]. After cultivation for 5 days, the yeasts were harvested, washed 5 times with Dulbecco's phosphate-buffered saline (PBS), suspended in an equal volume of PBS, and then disrupted using a French pressure cell (Amicon) at 14,500 psi. The crude lysate was centrifuged at 100,000 × *g* for 30 min, and the supernatant was collected. The protein concentration of the crude extract was estimated using a Protein Assay Rapid Kit (Wako Pure Chemical Industries Ltd., Osaka, Japan) according to the manufacturer's instructions.

2.2. Patient sera

Sera from 28 AD patients, including 12 males and 16 females, whose ages ranged from 20 to 64 years (mean age, 35.4 years) were used in this study. Allergic response was confirmed on the basis of the patient's clinical history and diagnosis, and characterized using the AlaSTAT system (Diagnostic Product Corporation) for measuring IgE reactivity. The diagnoses were made according to the criteria of Hanifin and Rajka [25]. The initial inclusion criterion for this study was an *M. furfur*-specific IgE titer of >3 IU/mL, as determined using the AlaSTAT system. The severity of AD was minor in 6 cases, moderate in 18 cases, and severe in 4 cases. Sera from 12 healthy adult subjects (mean age, 26.8 years) were used as controls. This study was approved by the ethics committee of Meiji Pharmaceutical University, and informed consent was obtained from the patients or their relatives.

2.3. Immunoblotting

Crude extracts (100 µg) were subjected to sodium dodecyl sulphate (SDS)–polyacrylamide (with a linear 10–20% gradient) slab-gel electrophoresis and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane [26]. The blotted membranes were incubated with blocking buffer (PBS containing 5% skim milk powder) for 30 min at room temperature, cut into 2-mm wide strips, and used for immunoblotting. IgE-binding components were detected using an AlaBlot kit, according to the manufacturer's instructions. In brief, the membrane strips were incubated overnight at 4 °C with each patient's serum samples diluted at a ratio of 1:10 in PBS. After incubation, the strips were washed 3 times with washing buffer and subsequently incubated with anti-human IgE alkaline phosphatase conjugate for 1 h. After incubation, the IgE-binding components were visualized using a substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

2.4. Immunoblot inhibition analysis

Immunological cross-reactivity was examined by performing immunoblot inhibition experiments [21]. To inhibit specific IgE

antibodies, the pooled sera from the AD patients were diluted to a ratio of 1:10 in PBS and incubated overnight at 4 °C with 0.1, 1, and 10 µg/mL of recombinant human HSP70 (StressMarq Biosciences Inc.) as an inhibitor. Unabsorbed sera and sera absorbed with 100 µg/mL of crude extract obtained from *M. globosa* were run in parallel as controls. The membrane strips were incubated overnight at 4 °C with the mixtures and subsequently developed as described above.

2.5. Sodium metaperiodate treatment

Carbohydrates of antigenic components present on the blotted membrane strips were disrupted by incubation with 50 mM sodium metaperiodate for 20 h at 4 °C in the dark [27]. After the membrane strips were washed, they were processed for antibody reactivity, as described above.

2.6. 2D electrophoresis

Crude extracts were subjected to duplicate 2D electrophoresis: one sample was used for immunodetection, and the other, for proteomic analysis. Isoelectric focusing was performed using an Ettan IPGphor II system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with 7-cm strips of immobilized pH gradient (IPG; pH 3–10). We used a programmed voltage gradient of 300 V for 30 min, wherein the voltage was linearly increased to 1000 V within 30 min and maintained at 5000 V for 1.2 h to generate a total of approximately 2 kVh. After isoelectric focusing, the IPG strips were washed for 15 min first with equilibration buffer (0.05 M Tris, 6 M urea, 30% glycerol, and 1% SDS) containing 32 mM dithiothreitol and then with equilibration buffer containing 216 mM iodoacetamide. The proteins were then separated according to their molecular weight on a 10–20% gradient slab gel under standard SDS-PAGE conditions. The gels were either immunoblotted as described below or stained with Coomassie brilliant blue (Daiichi Pure Chemicals, Japan). For specific IgE immunodetection, the proteins on the 2D gel were blotted onto a PVDF membrane and stained with Cypro Ruby to enable visualization. The membrane was then blocked with skimmed milk and incubated overnight with patient serum diluted at a ratio of 1:5 in Tris-buffered saline (TBS). After the membrane was washed, it was incubated with anti-human IgE peroxidase conjugate for 1 h. IgE-binding proteins were detected using the ECL Plus western blotting system (Amersham), according to the manufacturer's instructions.

2.7. Sequencing of internal amino acid residues

Protein spots corresponding to the IgE-reactive component were excised from the 2D gel for *de novo* sequence analysis [28]. Excised spots were trypsinized in gel, desalted, and analyzed by orthogonal matrix-assisted laser desorption/ionization quadrupole time-of-flight (oMALDI Qq-ToF) MS, which was performed on a QSTAR Pulsar i instrument (Applied Biosystems, Foster City, CA, USA). Data were screened against the SWISS-PROT database by using the MASCOT search program. Ions of interest were obtained after isolation of the appropriate derivatized precursor ions using timed-ion selection and were used for post-source decay (PSD) analysis. The fragment ions were refocused onto the final detector by incrementally increasing the voltage applied to the reflectron. The individual segments were subsequently stitched together with a software developed by PerSeptive Biosystems, and the PSD mass spectra were searched using the MS-Tag program.

2.8. N-terminal amino acid sequencing

After 2D electrophoresis, the blotted proteins were visualized by Coomassie blue staining, and the protein spots containing the

Table 1

List of primers used in PCR amplifications and nucleotide sequencing.

Primers	Nucleotide sequence
GeneRacer RNA Oligo	5'-CGACUGGAGCACGAGGACACUGACAU-GGACUGAAGGAGUAGAAA-3'
GeneRacer Oligo dT Primer	5'-GCTGTCAACGATACGCTACGTAACGGCAT-GACAGTG(T) ₂₄ -3'
5' RACE GSP	5'-AGCTCCTTGCCRTCGAAGAAGTCAC-3'
3' RACE GSP	5'-ATCGTGTGGTGGCTGGCTCCACTC-3'
GeneRacer 5' Primer	5'-CGACTGGAGCACGAGGACACTGA-3'
GeneRacer 5' Nested Primer	5'-GGACACTGACATGGACTGAAGGAGTA-3'
GeneRacer 3' Primer	5'-GCTGTCAACGATACGCTACGTAACG-3'
GeneRacer 3' Nested Primer	5'-CGCTACGTAACGGCATGACAGTG-3'
3' RACE seqFw1	5'-GAGGTGACTTTCGATGTGGACG-3'

GSP: Gene-specific primer.

presumed allergens were cut out and subjected to N-terminal sequence analysis in a Procise 494 protein sequencer (Applied Biosystems, Foster City, CA, USA).

2.9. Total RNA isolation, cDNA cloning, and sequencing

Total RNA was extracted from yeast cells by using the hot phenol extraction method [29]. The complete cDNA sequence of the major allergen of *M. globosa* was amplified using degenerate primers (Table 1) designed on the basis of the internal sequence of

the cDNA, using the Gene Racer full-length RLM-RACE kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The amplified products were analyzed using electrophoresis, subcloned into the pCR4Blunt-TOPO vector, and transformed into *Escherichia coli* (strain TOP10). After transformation, plasmids from positive clones were subjected to sequence analysis by using an ABI 377 sequencer (Applied Biosystems) and a dye-terminator cycle sequencing FS ready reaction kit.

3. Results

3.1. Reactivity of serum IgE from AD patients to *M. globosa* crude extract

To identify IgE-binding proteins, we analyzed a crude extract of *M. globosa* by using SDS-PAGE and western blotting. Serum samples from 28 AD patients were tested on immunoblots of *M. globosa* crude extracts for IgE binding. All the patient serum samples exhibited IgE binding to protein bands with apparent molecular masses of 4.4–219 kDa (Fig. 1A, lanes 14–41). Allergens with molecular masses of 40–45 kDa were detected in the sera of all patients (Fig. 1B). Further, 6.5-, 9.5-, 26-, and 83-kDa allergens were identified at frequencies in the range of 30–61% (Fig. 1A, arrow; and Fig. 1B), whereas 19-, 28-, 34-, 48-, 62-, and 70-kDa allergens were identified at lower frequencies, i.e., in the range of 18–22%. The remaining IgE-binding components with various

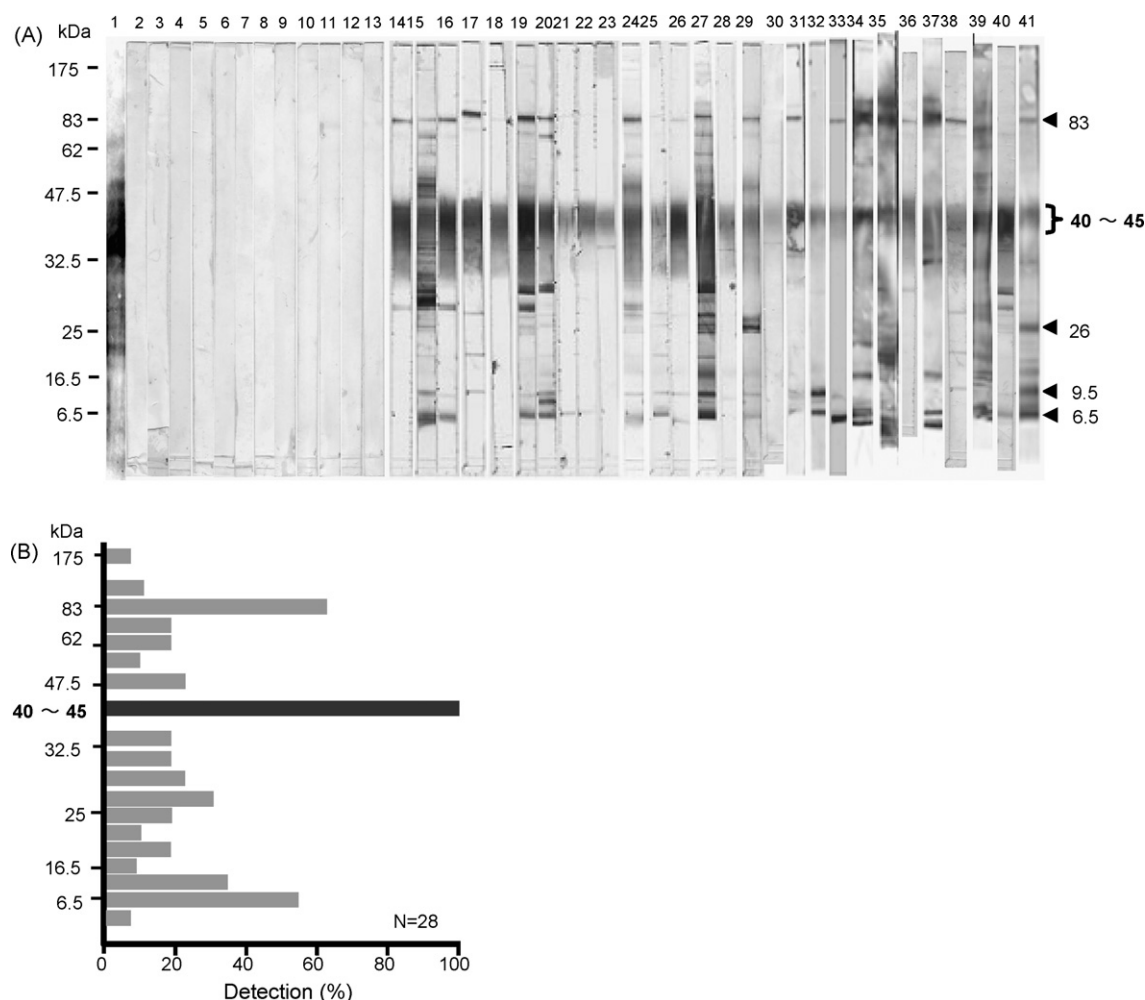


Fig. 1. SDS-PAGE and immunoblot analysis of *M. globosa* allergens. (A) IgE-binding proteins in a crude extract of *M. globosa* were detected by immunoblotting with sera from AD patients. Lane 1, Cypro Ruby staining; lanes 2–13, immunoblots with serum samples from non-allergic individuals; and lanes 14–41, immunoblots with serum samples from AD patients. Lanes 14–41 show IgE binding. (B) The detection frequency of *M. globosa* allergens.

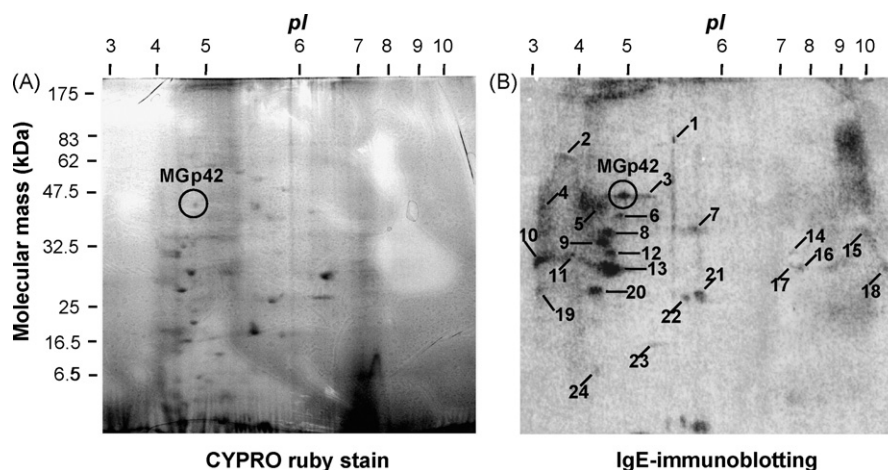


Fig. 2. Immunoblot analysis and 2D electrophoresis of *M. globosa* allergens. (A) A 2D membrane stained with Cypro Ruby. (B) Representative immunoblots probed individually with 8 serum samples from AD patients. The immunoreactive protein spot corresponding to the major allergen from *M. globosa*, designated MGp42 has been circled. The detection frequency of each spot when using the sera of 8 AD patients was as follows: MGp42 spot (8/8), spot 1 (5/8), spot 2 (2/8), spot 3 (1/8), spot 4 (2/8), spot 5 (1/8), spot 6 (2/8), spot 7 (2/8), spot 8 (3/8), spot 9 (2/8), spot 10 (2/8), spot 11 (3/8), spot 12 (2/8), spot 13 (3/8), spot 14 (2/8), spot 15 (2/8), spot 16 (2/8), spot 17 (1/8), spot 18 (1/8), spot 19 (2/8), spot 20 (3/8), spot 21 (3/8), spot 22 (2/8), spot 23 (3/8), and spot 24 (5/8).

molecular masses, such as the 17-, 55-, and 126-kDa components, were detected at frequencies lower than 11%. No IgE binding was observed when the sera of healthy donors were used (Fig. 1A, lanes 2–13). For our analysis, we selected the 40–45-kDa protein because it is a novel IgE-binding protein identified in the sera of AD patients at a considerably high frequency.

3.2. 2D immunoblotting

To better characterize the *M. globosa* allergens detected in the sera from AD patients, crude protein extracts were subjected to 2D-gel electrophoresis and subsequently, to immunoblotting. Fig. 2A shows the 2D-gel profile of the crude extract of *M. globosa*, in which >80 distinct protein spots were detected on Cypro Ruby staining. To identify the spots corresponding to allergens, the IgE-binding spots on the 2D gel were visualized by immunoblotting with 8 individual serum samples from AD patients; at least 20 different reactive spots with molecular masses of 6–90 kDa and isoelectric point (pI) values ranging from 3 to 10 were observed (Fig. 2B). A highly reactive protein spot with a molecular mass of 42 kDa and a pI of 4.8, designated MGp42, was detected in each of the 8 serum samples from the AD patients, whereas several spots, such as spots

1 and 24, were detected in 63% (5 of 8) of the serum samples from the AD patients. Other spots were detected at lower frequencies (in 1–3 of 8 serum samples). No positive spots were detected in sera from non-allergic individuals (data not shown).

3.3. Determination of internal and N-terminal amino acid sequences of the *M. globosa* allergen

To identify the IgE-reactive spot, it was excised and digested in-gel with trypsin; the resultant peptide mixtures were analyzed by MALDI-TOF-MS. The MS profile of the peptides (molecular mass, 42-kDa; pI, 4.8; MGp42) showed multiple peaks in the range of 1000–2700 Da (Fig. 3). Of these, 3 peptides showing prominent peaks were selected for comparison with established databases, and the protein with the highest correlation to the spot was found to be a member of the hsp family. To further characterize the internal sequence of the spot, the signals at 1183.63, 1330.63, and 1445.76 Da were selected for PSD analysis. The amino acid sequences of the fragments are shown in Table 2A.

After western blotting, analysis of the N-terminal amino acid sequence of MGp42 was performed, and it yielded the sequence DLTTN (Table 1B).

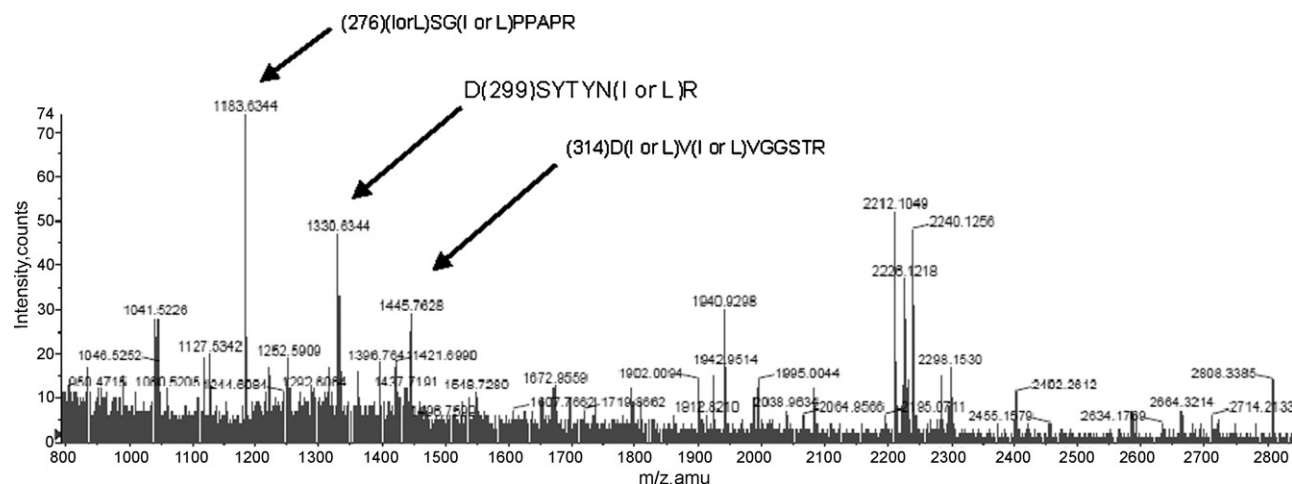


Fig. 3. MALDI-TOF-MS profiles of a tryptic digest of the spot corresponding to the major allergen from *M. globosa*. The spot was digested *in situ* with trypsin. The peptides were analyzed by MALDI-TOF-MS, and prominent mass peaks were selected for database searches.

Table 2Determination of amino acid sequences of the major allergen of *M. globosa* Cf.(A) Internal amino acid sequences of the major allergen of *M. globosa* (MGp42) determined by MALDI–TOF–MS and PSD analysis.^a

Peak <i>m/z</i>	Amino acid sequence	Similarity
<i>m/z</i> 1183	(276) (IorL)SC(IorL)PPAPR	Heat shock protein 70 of <i>Guanica lacunosa</i>
<i>m/z</i> 1330	D(299) SYTYN(IorL)R	None
<i>m/z</i> 1445	(314) D(IorL)V(IorL)VGGSTR	ACR038Wp of <i>Ashbya</i> <i>gossypii</i> ATCC10895

(B) The N-terminal amino acid sequence of the major allergen of *M. globosa* (MGp42) as determined by Edman degradation and mass spectrometry.^b

N-terminal amino acid sequence	DLTTN
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^a The sequences of the identified peptides were compared with the peptide sequences of annotated coding regions by using the MASCOT search. The underlined parts indicate sequences similar to those of known peptides. Full-length MGp42 cDNA contained 1908 bp of open reading frame encoding 635 amino acid residues (Fig. 4) with a calculated molecular mass of 69.7 kDa and a *pI* of 4.92.

^b Cf. The MGp42 protein consisted of 386 amino acid residues (Fig. 4) with a calculated molecular mass of 42.1 kDa and a *pI* of 4.75; these results are consistent with those of 2D immunoblotting.

3.4. Sequence analysis of full-length cDNA

To obtain full-length MGp42 cDNA, we cloned and sequenced 5′- and 3′-RACE clones. Fig. 4 shows the nucleotide sequence of the full-length cDNA and the deduced amino acid sequence. The full-

length cDNA contained 1908 bp of open reading frame (ORF) encoding 635 amino acid residues with a calculated molecular mass of 69.7 kDa and a *pI* of 4.92. The N-terminal sequence of the mature protein, MGp42, started from the 250th residue (Asp-250) of the deduced amino acid sequence. This protein consisted of 386 amino acid residues and had a calculated molecular mass of 42.1 kDa and a *pI* of 4.75, which is consistent with the results of 2D immunoblotting.

3.5. Sequence similarities and comparisons between HSPs

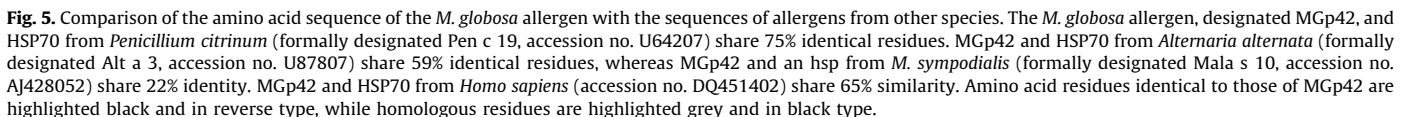
We used the basic local alignment search tool (BLAST) and found that MGp42 showed high similarity to proteins from the hsp 70 family (Fig. 5). For instance, MGp42 and HSP70 from *Penicillium citrinum* (formally designated Pen c 19, accession no. U64207) share 75% identical residues. MGp42 and HSP70 from *Alternaria alternata* (formally designated Alt a 3, accession no. U87807) share 59% identity, whereas MGp42 and the HSP from *M. sympodialis* (formally designated Mala s 10, accession no. AJ428052) share only 22% sequence identity. Further, MGp42 shares 65% identity with the HSP70 of *Homo sapiens* (accession no. DQ451402).

3.6. Evaluation of cross-reactivity of MGp42 with human HSP70

The IgE immunoblot inhibition studies using pooled sera from AD patients revealed that human HSP70 did not inhibit the binding of IgE to the 42-kDa band, corresponding to MGp42, even at high

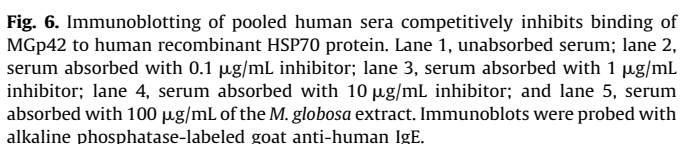
AGACTACAGTATGACCAAAGCTATCGGTATTGATTGGGAACCTACTACTCCTGTGTGGCCGCTGTGCAAAATGACCGCGTCGAGGTCATCGCCAACGATCAAGGTAACCGTACTACTCCA	121
M T K A I G I D L G T T Y S C V A V W Q N D R V E V I A N D Q G N R T T P	37
TCATATGTTGCATTACCGCACTGAGCGTTTGTATCGGTGACGCTGCCAAGAACCAAGTGGCTATGAACCCATCAACACTGTGTTTGAACGCCAAGCGTCTGATCGGTGCAAGTTTGTAC	241
S Y V A F T D T E R L I G D A A K N Q V A M N P H N T V F D A K R L I G R K F D	77
GACTCTGAGGTGCAAGCCGACATGAAGCACTGGCCCTTCTACTGTCTGCTGATGGTAAGCCAGTGATCCAAATCGAATACAAAGGTTGAGAAGACCTTCACTCTGAAGAAGTC	361
D S E V Q A D M K H W P F T V I A R D G K P V I Q I E Y K G E K K T F T P E E V	117
TCGGCTATGGTCTCACCAAGATGCGTGAATTCGCGAGGCGCTACTTGGGCAGCAACCTGAAGGACGCGCTTATCACCGTCCCTGCATCTTCAACGATTCGCAACGCTCAAGCTACCAAG	481
S A M V L T K M R E I A E A Y L G S N V K D A V I T V P A Y F N D S Q R Q A T K	157
GACGCGCGTGTGATCGTGGATTGAACGTGCTCCGTATCATCAACGAGCCGACTGCCGCCGCTATTGCTTACGCTCTCGACAAAAGTCCGAGCAGAGAAGAGCTTTTGATCTTCGAC	601
D A G V I A G L N V L R I I N E P T A A A I A Y G L D K K S E Q E K N V L I F D	197
TTGGTGGTGGTACTTTTCATGTGTCCCTCTTGACCATTTGAAGAGGGCATCTTCGAAGTGAAGGCTACTGTGTTGACACTCACTTGGGTGGTGAAGACTTTGACAACCGTCTGGTGAAC	721
L G G G T F D V S L L T I E E G I F E V K A T A G D T H L G G E D F D N R L V N	237
TTCTCTGTCGAAGAGTTCAAGCGCAAGTTCAAGAGGACCTCACTACCAACGCTCGTCTCCGTCGCTCTCGTACCGCTGCCGAGCGCGCTAAGCGTACGCTCTCATCTCGGCACAG	841
F F V Q E F K R K F K K D L T T N A R A L R R L R T A A E R A K R T L S S S A Q	277
ACCAGCATCGAAATCGACTCGCTGTTCGAGGGTGTGCACTTCTACACATCATCACCGCGCTCGTTTCGATGAGTTGTGCGGTGACTTGTTCGAAGCCACTGTCAGCGCTGTCGAGAAG	961
T S I E I D S L F E G V D F Y T S I T R A R F D E L C G D L F Q A T L Q P V E K	317
GTCTCCGCGACTCCAAGATCGACAAGAGCCAAAGTGAACGATATCGTGTGGTGGTGGCTCCACTCGTATTTCCCGCTGTGCAAGAAGTGTCTAGTGACTTCTTCAACGGCAAGGAGCTC	1081
V L R D S K I D K S Q V N D I V L V G G S T R I P R V Q K L L S D F F N G K E L	357
AACAAGTCGATCAACCCCGCAGAGGCTGTTGCCTACGGTGCCGTGTGCAAGCTGCTATTTTACCGGTGACACCTCTGAGAAGACGCAAGACTTGTCTTGTGGACGTTGCTCTCTA	1201
N K S I N P D E A V A Y G A A V Q A A I L T G D T S E K T Q D L L L L D V A P L	397
TCATGGGTATCGAGACCGCTGGTGGTGTCTTCACTCCGCTCATCAAGCGTAACACCCCGTACCAACCAAGAAGTCTGAGGTATTTCTCGACCTATGCCGACAACGAGCGTGGTGTGTTG	1321
S M G I E T A G G V F T P L I K R N T T V P T K K S E V F S T Y A D N Q P G V L	437
ATTCAAGTGTGTAAGTGTGAGCGTGCAGCGCAACGACAACCTGCTCGCAAGTTTGAAGTGTCCGGTATTTCCCGCTGCGCGCGTGGTGTGCCTCAAAATCGAGGTGACTTTTCGAT	1441
I Q V F E G E R A R T K D N N L L G K F E L S G I P P A P R G V P Q I E V T F D	477
GTGGACCGCAACCGCATCTTGAACGTGTCTGCTGTGACAAGACACCGCAAGTCCGAGAAGTACCACTACCAACGCAAGGTCGTCTATCGAAGGAGGACATTGAGCGTATGGTA	1561
V D A N A I N V S A A I D K T T T G K S E K I T I T N D K G R L S K T L E R M V	517
TCTGATCCGAGAGTACAAGCTGAGGACGAGGCTGTCTGTGAGCGTATCCAAGCTAAGAAGCGTTTGAATCTTACACTTACACTTGCCTCAAGTCAAGGAGGCTGTGTC	1681
S D A E K Y K A E D E A A A E R I Q A K N G L E S Y T Y N L R N S L N E E G L S	557
AGCAAGCTCGAGGCTCTGACAAGGACAACCTCAAGAGGCCATTGATGAGACCATTTGAATGGCTCGACAACGCAAGAGGCTCCATTGACGAATACAGGAGAAGCAAAAGAGTTG	1801
S K L E A S D K D N L K K A I D E T I E W L D N S Q E G S I D E Y K E K Q K E L	597
GAAGAGATTGCCAACCAATTCTCCAAAGGCATACGGTGTGTGCGGTGCACCAACACCAACCAAGATGTCCATCTGCCGATGGCGGTCTCAAGTCAAGGAGATCGACTAAGCG	1921
E E I A N P I L Q K A Y G A A G G A P P P P Q D A P S A D G G P Q V E E I D *	635
CATCTTGCATTGGACATAGCTTCTTAGATATCCCTAGTAATTGATCCATCTGTTACGAAAAAATAAAAAAAAAA	1996

Fig. 4. The nucleotide sequence and deduced amino acid sequence of the gene encoding the major allergen from *M. globosa*. The grey boxed region represents the N-terminal amino acid sequence established by Edman degradation. The internal amino acid sequences determined using MALDI–TOF–MS and PSD analysis are indicated in the box.



4. Discussion

Many studies have attempted to find a differential pattern of *Malassezia* colonization on skin affected by AD [30]. However, the results of culture-based methods have differed among studies [30–32]. This may be due to the difficulties in culturing *Malassezia* species. Recently, a molecular-based (nested PCR) analysis has revealed that *M. globosa* and *M. restricta* are detected in approximately 90% AD patients, while other *Malassezia* species are detected in less than 40% AD patients [12]. By using a quantitative real-time PCR method, Sugita et al. [33] have shown that *M. globosa* and *M. restricta* account for 80% of all *Malassezia* colonization on skin affected by AD. Further, it has been reported that AD patients have specific serum IgE antibodies against *Malassezia* [7,8]. Treatment of AD patients with antifungal agents decreases *Malassezia* colonization and the severity of eczematous lesions, suggesting a major role for *Malassezia* species in AD [5,34]. *Malassezia* extracts have been shown to contain 13 different



IgE-binding components (Mala f 2–4, Mala s 1, and Mala s 5–13) [18–21]. However, none of the IgE-binding components from *M. globosa* have been identified and studied. In the present study, we used the proteomics approach to identify and characterize an allergen from *M. globosa*.

Immunoblot analysis showed that IgE-reactive protein components with molecular masses of 40–45 kDa were present in all the serum samples obtained from the AD patients. On 2D immunoblots, we observed an IgE-binding protein with a molecular mass of 42 kDa and a pI of 4.8; this protein was designated MGp42. The IgE reactivity of MGp42 was retained even when its carbohydrate moieties were disrupted by sodium metaperiodate treatment (data not shown), suggesting that the carbohydrates in this compound do not function as antigenic determinants. Proteomics analysis involving the MALDI-TOF-MS PSD method revealed that MGp42 showed high sequence similarity to HSP70. On the basis of the internal amino acid sequences of MGp42, the full-length cDNA of MGp42 was cloned and sequenced by 5'/3'-RLM-RACE PCR analysis. The full-length cDNA contained a 1908-bp ORF encoding 635 amino acid residues with a calculated molecular mass of 69.7 kDa and a pI of 6.02. Recently, the genomes of *M. globosa* and *M. restricta* were sequenced using the whole-genome shotgun method [35]. The ORF sequence of full-length MGp42 cDNA matched the gene sequence of *M. globosa* (MGL_0058). The N-terminal sequence of the mature MGp42 protein started from the 250th residue, i.e., Asp-250, of the deduced amino acid sequence. It consisted of 386 amino acid residues, and its molecular mass and pI were consistent with the results of 2D immunoblotting. These results suggest that MGp42 may be a cleavage product of intact HSP70.

We found that the amino acid sequences of MGp42 from *M. globosa* and Mala s 10 (HSP from *M. sympodialis*) show low homology to each other (22%) even though they belong to the same genus. The diversity of HSP70 isoforms is probably attributable to the presence of multiple HSP70 gene copies [36,37]. For instance, the hsp70 family of *Saccharomyces cerevisiae* contains at least 10 members, including cytosolic HSP70 [38]. Cytosolic HSP70 is further divided into 2 groups: Ssa and Ssb. The essential Ssa proteins are most closely related to the major cytosolic hsp70s of mammalian cells, while Ssb proteins differ from the members of the Ssa group by 37% [39]. Alternatively, the less-conserved COOH-terminal domain of HSP70 might account for isoform diversity [40].

Proteins of the hsp70 family appear to be phylogenetically highly conserved across human, fungi, and bacterial species, thus facilitating immunological cross-reactions between pathogens and human HSP70 [41]. Autoreactivity to human proteins has been postulated as a decisive pathogenetic factor for patients with AD [42,43]. AD patients reacting to human manganese superoxide dismutase (MnSOD) are sensitized against the MnSOD of *M. sympodialis* (Mala s 11) [44]. Since MnSODs of fungal and human origin are highly similar in terms of their structure, sensitization is most likely induced by exposure to fungal MnSOD of *M. sympodialis* present in the environment. This results in molecular mimicry due to secondary autoreactivity of fungal MnSOD with its human counterpart and may contribute to the perpetuation of inflammatory skin reactions [45]. Similarly, thioredoxins (Trxs) of *M. sympodialis* (Mala s 13) and *Aspergillus fumigatus* (Asp f 28–29) have been identified as novel IgE-binding proteins [46]. Mala s 13 shares 45% sequence identity with human Trx. Trxs of 3 different origins (*M. sympodialis*, *A. fumigatus*, and human) represent cross-reactive structures recognized by serum IgE from individuals sensitized to Trx of *M. sympodialis* [46,47]. In the present study, however, immunoblot inhibition analysis revealed no IgE cross-reactivity between MGp42 and recombinant human HSP70. This finding is in agreement with the result that an IgE-reactive component with a

molecular mass of approximately 70 kDa was detected at low frequencies (18%) (Fig. 1). Thus, it seems possible that the epitopes of MGp42 recognized by serum IgE of AD patients are masked by steric hindrance in intact HSP70 and exposed because of conformation changes during HSP70 cleavage. We cannot rule out the possibility that a cleavage product of human HSP70 cross-reacts with MGp42. Further studies involving epitope mapping and structural analysis of MGp42 are required to clarify the molecular basis of interaction between MGp42 and IgE antibodies. In addition, the IgE cross-reactivities between MGp42 and homologues of other pathogens must be explored in future studies.

Several research groups have attempted to produce recombinant *Malassezia* allergens (rMala s1, rMala s5–11) for diagnostic purposes [19,45,46,48]. Using *M. sympodialis* extract and 3 recombinant allergens (rMala s 1, rMala s 5, and rMala s 6), Johansson et al. [49] obtained positive reactions with specific IgE tests, skin prick tests (SPTs), and/or atopy patch tests (APTs) in 67% of the AD patients examined in their study.

No studies have investigated the clinical application of *M. globosa* MGp42 for the diagnosis of AD. Recombinant MGp42 allergens are required for preparing a reliable diagnostic tool for AD. Further studies are required to assess the clinical relevance of positive results of SPTs and APTs and of specific IgE levels to the recombinant MGp42 protein, and to evaluate the practical application of these findings in the clinical management of patients with AD.

Proteomics is a powerful tool for the identification of allergenic proteins from *Malassezia* species and will greatly help further studies addressing the mechanism underlying allergy sensitization.

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