

Human Serum IgE Reacts with a *Metarhizium anisopliae* Fungal Catalase

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Key Words

Allergen · Catalase · Fungus · IgE · Mass spectrometry

Abstract

Background: Previous studies have demonstrated that *Metarhizium anisopliae* extract can induce responses characteristic of human allergic asthma in a mouse model. The study objectives were (1) to identify and characterize the *M. anisopliae* mycelia extract (MYC) proteins that are recognized by mouse serum IgE, (2) to determine if human serum IgE reacts with these proteins, and (3) to determine if these IgE-reactive proteins are found in other fungi. **Methods:** Asthmatic human serum IgE, *M. anisopliae* crude antigen (MACA) immunized mouse serum IgE, and anti-catalase antibodies were used to probe one- and two-dimensional gel electrophoresis blots of MYC. **Results:** Mass spectrometry analysis identified catalase as a mouse IgE-reactive protein. This identification was confirmed by assaying catalase activity in the extract and extract immunoblots probed with anti-catalase antibody. Six adult asthmatic sera contained IgE, but not IgG, that was reactive with mycelia extract proteins. A similar protein profile was seen when blots were probed with either mouse anti-MACA IgE or anti-bovine liver catalase antibodies. Furthermore, these mouse anti-MACA and anti-catalase antibodies were cross-reactive with other mold extracts (skin prick testing mix) and *Aspergillus niger* cata-

lase. **Conclusions:** Some human asthmatics have developed IgE that reacts with an *M. anisopliae* catalase, most likely due to cross-reactivity (minimal IgG development). The cross-reactivity among fungal catalases suggests that IgE-reactive catalase might be useful for exposure assessment. Additionally, the similarity of protein profiles visualized with both human and mouse serum IgE suggests that allergy hazard identification can be facilitated using a mouse model.

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Introduction

Molds/fungi are ubiquitous in the environment and have been implicated in the etiology of respiratory hypersensitivity diseases such as allergic rhinitis, allergic asthma, allergic bronchopulmonary mycoses and hypersensitivity pneumonitis [1, 2]. Asthma is of particular concern because over the past 25–30 years [3, 4] the incidence of asthma has dramatically increased in industrialized

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countries, especially among children. The reasons for the worldwide increase in asthma incidence are unclear, but one contributing factor could be childhood exposure to molds [5, 6]. The Institute of Medicine's report on dampness and health found that there was sufficient scientific evidence linking molds and damp environments with asthma symptom exacerbation but not with asthma induction [7]. Therefore, the report recommended more research to clarify this relationship.

The percentage of adult asthmatics that are allergic is estimated to range from 50 [8] to >90% [9]. Although estimates suggest that 3–10% of people worldwide have fungal allergies [10], Mari et al. [11] found that 19.1% of their allergy study population had positive mold skin prick tests.

The entomopathogenic fungus, *Metarhizium anisopliae*, is a soil-borne fungus found worldwide. Due to the pathogenicity of *M. anisopliae* to a broad range of insect hosts, it has been used since the late 1800s for pest control. Currently, it is used as a biopesticide to control insects such as grasshoppers, termites, and thrips. Additionally, *M. anisopliae* has been licensed in the US for indoor cockroach control. Although there is no evidence that *M. anisopliae* is either infectious or toxic to mammalian species, previous studies in our laboratory [12, 13] have demonstrated that respiratory exposure to *M. anisopliae* extract can induce physiological and immune responses characteristic of human allergic asthma in BALB/c mice. To date, there are only anecdotal information and limited clinical data suggesting that some individuals exposed occupationally to *M. anisopliae* have become sensitized [14]. However, *M. anisopliae* has been investigated as a mosquito control to combat malaria [15, 16] by impregnating mosquito netting with viable conidia. These conidia are the environmentally stable portion of the fungal life cycle and a viable reproductive unit. Infection of the host insect induces proteases and chitinases to comprise the exoskeleton and subsequent mycelium development and sporulation leading to host death. Furthermore, Ravichandra [17] reported at the 2007 International Conference on Bioengineering and Nanotechnology that his laboratory has used *M. anisopliae* in an energy-efficient method to produce biodiesel. These studies indicate a potential increase in opportunities for both indoor residential and occupational exposures to what is ecologically an outdoor organism.

Fungal allergen identification/characterization can provide insight about allergenic proteins in general (what makes a protein allergenic, enzyme class) as well as lead to better tools for assessing fungal allergen environmen-

tal levels, biomarkers of exposure (e.g. serum antibodies), and potentially allergy-preventive therapies. Although allergens have been identified in 23 fungal genera [18], the diagnosis of mold-induced allergic disease has been hampered by the lack of standardized reagents [19].

The study objectives were (1) to identify and characterize the *M. anisopliae* mycelia extract proteins (MYC) that are recognized by mouse serum IgE; (2) to determine if human serum IgE reacts with these proteins, and (3) to determine if these IgE-reactive proteins are found in other fungi.

Materials and Methods

Experimental Approach

To identify MYC IgE-reactive proteins that might account for the respiratory allergy responses previously observed in mice, immunoblots of MYC were probed with mouse anti-*M. anisopliae* serum. One protein was identified as a catalase by mass spectrometry (MS) analysis and protein database interrogation. The subsequent demonstration of extract catalase activity and the anti-catalase antibody reactivity with MYC immunoblots provided confirmation. Six asthmatic adult sera were used to probe MYC immunoblots to identify IgE reactivity. To provide insight into the prevalence of IgE-reactive catalase among molds, two commercially available mold mix extracts were immunoblotted and probed with anti-catalase antibody and mouse anti-MACA serum (IgE-reactive protein detection).

Fungal Antigen Preparation and Immune Serum Production

M. anisopliae strain 1080 was obtained from the Entomopathogenic Fungus Collection of the Agricultural Research Service of the United States Department of Agriculture in Ithaca, N.Y., USA. In previous studies, we have exposed mice to MACA consisting of equal protein concentrations of mycelia, conidia and inducible enzyme extracts [12]. In order to identify and characterize those proteins that were IgE reactive, we began with the MYC.

Seven-week-old, female BALB/c mice (Charles River, Raleigh, N.C., USA) were housed in groups in polycarbonate cages with hardwood chip bedding in an environmentally controlled animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Mice were maintained on a standard diet (Purina rodent lab chow, St. Louis, Mo., USA) and water ad libitum. Additionally, mice were acclimated for 1 week before exposure to MACA. Anti-MACA-IP antibodies were raised using a traditional immunization protocol to produce a relatively large serum pool with a relatively high IgE titer. This protocol consists of three intraperitoneal (IP) injections (25 µg MACA in alum and 2–15 µg MACA in HBSS) over a 9-week period. A second source of anti-MACA IgE (anti-MACA-IA) was raised using our standard experimental protocol; this exposure route is via the airways exclusively, consisting of four intratracheal aspiration exposures (20 µg MACA each) over a 4-week period [20]. The mice were exsanguinated 5 or 3 days after the final exposure, respectively, and the sera pooled for each

method. The anti-MACA serum was the source of primary antibodies (IgE) against MYC in immunoblot analysis.

Gel Electrophoresis and Immunoblotting

SDS-PAGE (separation by molecular weight): MYC, *Aspergillus niger* catalase (EMD Biosciences, San Diego, Calif., USA), bovine liver catalase (EMD Biosciences) and two commercial mold mixes (mold mix 1 and 2; Greer Laboratories, Lenoir, N.C., USA) were electrophoresed using 10 × 10 cm, 1.5-mm-thick NuPage 4–12% Bis-Tris gels (Invitrogen, Carlsbad, Calif., USA) and immunoblotted as described below. Mold mix 1, *Alternaria alternata*, *A. niger*, *Bipolaris sorokiniana*, *Cladosporium sphaerospermum*, and *Penicillium notatum* (now *P. chrysogenum* [21]), and mold mix 2, *Aureobasidium pullulans*, *Dreschlera spicifera*, *Fusarium moniliforme* (now *F. verticillioides* [22]), *Mucor plumbeus*, and *Rhizopus stolonifer*, are used for human skin prick testing.

Two-dimensional gels: for the first dimension [separation by isoelectric focus point (pI)], MYC was mixed with isoelectric focusing sample buffer (Invitrogen) and applied to Novex isoelectric focusing gels, pH 3–7 (Invitrogen). Subsequently, the gels were fixed in 12% trichloroacetic acid containing 3.5% sulfosalicylic acid.

For the second dimension, two isoelectric focusing sample lanes were excised and electrophoresed in tandem gels (SDS-PAGE above). Subsequently, one gel was stained with colloidal Coomassie blue (Invitrogen) for protein visualization and the other immunoblotted (see below). Alignment of the stained gel and the IgE immunoblot allowed identification and collection of the IgE-binding protein spots for mass spectrometric analysis.

Immunoblotting: proteins were transferred from SDS-PAGE gels onto 0.2-μm nitrocellulose membranes and non-specific binding blocked with casein blocker in phosphate-buffered saline (PBS; Pierce, Rockford, Ill., USA) or 5% non-fat dried milk. Blots were incubated with serum or purified antibodies followed by a secondary antibody for detection and amplification of the visualizing signal. All washes and incubations were performed with either PBS or Tris-buffered saline (TBS; Pierce) each containing 0.05% (v/v) Tween-20 (PBST; TBST; Sigma, St. Louis, Mo., USA). To visualize the IgE- and/or anti-catalase-reactive proteins, blots were either incubated with Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, Mass., USA) and exposed to Hyperfilm (Amersham, Piscataway, N.J., USA) or incubated with Supersignal West Dura (Pierce), and the image captured by a FluorChem CCD imaging system (Alpha Innotech, San Leandro, Calif., USA).

Antibody systems used for immunoblots were as follows:

(1) *Detection of Mouse IgE Reactivity.* Mouse anti-MACA was followed by biotin-labeled anti-mouse IgE (BD Biosciences Pharmingen, San Diego, Calif., USA) and streptavidin-horseradish peroxidase (HRP) conjugate (Zymed, South San Francisco, Calif., USA).

(2) *Detection of Anti-Catalase Reactivity.* (2a) Rabbit polyclonal anti-CatB was a gift from Dr. Takasuka [23]. The anti-serum raised against purified glutathione S-transferase-CatB (Ser-31 to Pro-580 fragment of *Aspergillus fumigatus* catalase CatB) fusion product did not react with glutathione S-transferase nor *Escherichia coli* (expression host) protein. The antibodies used for visualization were HRP-labeled goat anti-rabbit IgG (Zymed) or (2b) biotin-labeled anti-bovine liver catalase antibody (Abcam, Cambridge, Mass., USA) and streptavidin-HRP conjugate (Zymed).

(3) *Detection of Human IgE Reactivity.* Sera from adult, mold skin prick test-positive, asthmatic patients (living within a 30 mile radius of Chapel Hill, N.C., USA) were followed by biotin-labeled mouse anti-human IgE (Southern Biotechnology, Birmingham, Ala., USA) and streptavidin-HRP conjugate (Zymed). The sera were collected under a research protocol reviewed by the University of North Carolina Biomedical Institutional Review Board and approved by the US Environmental Protection Agency [24]. All study subjects gave informed consent before participation.

(4) *Detection of Human IgG Reactivity.* Sera from adult asthmatics (described above) were followed by HRP-labeled mouse anti-human IgG (all subclasses; Zymed).

Dot Blots

Dot blots were performed to detect both human serum IgE and IgG reactivity with non-denatured proteins. MACA (0.5 μg) was applied to the nitrocellulose membrane pre-wetted with TBS and mounted along with three pieces of dot blot SF filter paper (Bio-Rad Laboratories, Hercules, Calif., USA) in the Bio-Dot® micro-filtration apparatus. After the wells had completely drained by gravity filtration, the membrane was removed from the apparatus and allowed to dry overnight. Subsequently, the membrane was rehydrated with TBST for 5 min and non-specific binding sites blocked by incubation for 1 h in 5% non-fat dried milk in TBST with agitation at room temperature (RT). The membrane was then washed for 5 min with TBST. Both anti-human IgE and anti-human IgG antibodies (as described above) and streptavidin-HRP (Zymed) were diluted in 1% bovine serum albumin (Sigma) in TBST.

To detect IgE reactivity, the dot blots were incubated overnight at 4°C with human asthmatic sera. Subsequently the membrane was washed once for 15 min, and 4 times for 5 min with TBST. The dot blots were then incubated for 1 h at RT first with anti-human IgE antibody, then washed as before, followed by a 1-hour incubation at RT with streptavidin-HRP.

To detect IgG reactivity, the dot blots were incubated for 2 h at RT with human asthmatic sera, then washed as previously described. Subsequently, HRP-labeled mouse anti-human IgG antibody was added and the membrane incubated for 1 h at RT.

A chemiluminescence signal was generated using Supersignal West Dura (Pierce) for 5 min following membrane washing as previously described. The chemiluminescence signal was quantified by densitometry analysis following a 1-min exposure using the FluorChem CCD imaging system (Alpha Innotech).

AlphaEase® FC Imaging System software (Alpha Innotech) was used to automatically calculate the integrated density value for the defined area (in pixels) by summing each pixel value and subtracting the background value (10 lowest pixel values within the area) and the AVG (integrated density value/area). A dot blot control blank (no MACA protein bound) was also produced. The values reported are the densitometry ratios: Ratio = AVG/blank AVG, where blank is the detection system without MACA protein. Due to non-specific binding of the detection system, the ratio value for the IgE blots was adjusted by subtracting the densitometry ratio value for control blots that consisted of MACA blot and the detection system, but without primary antibodies.

Mass Spectrometry

The IgE-reactive protein spots were dehydrated and rehydrated 4 times in a 2:1 solution of acetonitrile/25 mM ammonium bi-

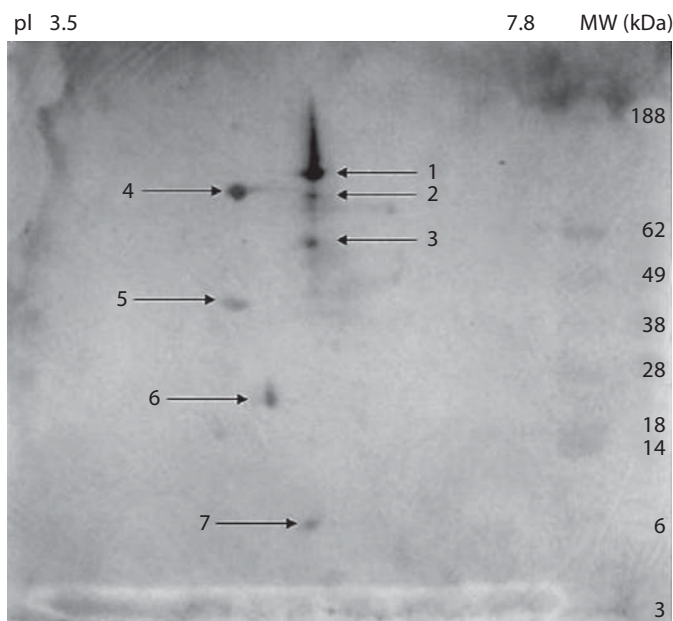


Fig. 1. Two-dimensional electrophoresis and immunoblot analysis of mycelia protein from *M. anisopliae*: mouse anti-MACA-IP IgE immunoblot of MYC.

carbonate buffer and 25 mM ammonium bicarbonate solution, respectively, for Coomassie stain removal. Subsequently, the gel pieces were shrunk (acetonitrile), dried by vacuum centrifugation, and incubated in 10 mM dithiothreitol:50 mM ammonium bicarbonate for 45 min at 57°C. Following the incubation in alkylation solution (55 mM iodoacetamide:25 mM ammonium bicarbonate) for 45 min at 25°C in the dark, the gel pieces were washed [25 mM ammonium bicarbonate; acetonitrile:25 mM ammonium bicarbonate (2:1), and 25 mM ammonium bicarbonate], dehydrated in acetonitrile and dried by vacuum centrifugation. The gel pieces were then digested with 8.0 ng/μl trypsin (Sigma) in 25 mM ammonium bicarbonate, pH 8.0, at 37°C overnight. The resulting peptides were extracted with 60% acetonitrile in 5% trifluoroacetic acid (v/v) by sonication (10 min), lyophilized, and stored at -80°C.

The tryptic peptides were rehydrated in water, desalted, and concentrated (ZipTip₁₈; Millipore, Bedford, Mass., USA). An aliquot of the peptide sample was mixed with α-cyano-4-hydroxycinnamic acid (Bruker, Billerica, Mass., USA) solution (20 mg/ml) in 50% acetonitrile and 0.5% trifluoroacetic acid and air-dried at RT.

The peptides were analyzed with a Biflex III matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS (Bruker). Angiotensin I, *m/z* 1045.5, and ACTH 18-39 clip, *m/z* 2465.1 (Peptide Calibration Standard; Bruker) were used for calibration. The TOF was operated using positive polarity in the reflectron mode. The MALDI ions were generated using a nitrogen laser with a 200-ns delay extraction time, and a 20-kV acceleration voltage. Each spectrum was an average of 100 shots.

Additionally, the peptides were sequenced with an Applied Biosystems QSTAR quadrupole TOF MS (Framingham, Mass., USA) equipped with nanoESI interface (Proxeon, Odense, Denmark). The QSTAR was operated in the product ion scan mode in which predetermined precursor ions were chosen for MS/MS analysis. For each peptide analyzed by MS/MS, the following parameters were adjusted: ion spray voltage 900–1,200 V, collision energy 20–35 V, curtain gas 6, pulse frequency 7 kHz, and pulse duration 18 μs. Furthermore, the instrument was calibrated daily.

The peptide amino acid sequence was determined by BioAnalysisTM (version 1.1) software (Applied Biosystems) from the resulting product ions (y-ion series). The resulting sequence was submitted to EMBL PeptideSearch (version 3.0) with the following restrictions: a mass tolerance set at 0.05-Da for the precursor and for product ions and no limitations on protein molecular weights, pI, or species.

MascotTM (<http://www.matrixscience.com>) was used to match the in silico-derived tryptic peptide masses to experimental tryptic peptide masses to within a mass tolerance of ± 1 Da for fungal proteins and allowing one missed cleavage. Cysteine alkylation and methionine oxidation were considered modifications. The comparisons were evaluated by the number of matched tryptic peptides, the percentage protein sequence coverage, and the experimental molecular weight and pI of the MYC protein.

Catalase Assay

MYC catalase activity was assessed using a catalase assay kit (Calbiochem, Carlsbad, Calif., USA) following the manufacturer's instructions. Briefly, following a 1-min incubation of MYC and a known H₂O₂ concentration, the reaction was quenched. HRP catalyzed the oxidative coupling of 4-aminophenazone and 3,5-dichloro-2-hydroxybenzenesulfonic acid in the presence of the remaining H₂O₂. The resulting quinone imine dye was measured at 520 nm and the MYC catalase concentration calculated.

Results

Detection and Identification of MYC IgE-Reactive Proteins

A 2D gel of MYC which was blotted and probed with mouse anti-MACA-IP IgE (fig. 1) demonstrated seven defined IgE-reactive protein spots. Based on Coomassie blue gel staining there was sufficient protein at spot 2 for MS analysis (data not shown).

Interrogation of the MascotTM database with the observed spot 2 mass values found the highest correlation with *Claviceps purpurea* catalase. The relative sequence coverage was 17%. However, the *C. purpurea* catalase molecular weight (78,785 Da) and pI (5.5) correlated well with MYC spot 2 values (data not shown).

MS/MS spectra were acquired from three doubly charged precursor ions at *m/z* 450.8, 654.3 and 717.4 representing the single charged peptides of 901.6, 1,307.6,

Fig. 2. MS analysis: the full amino acid sequence for *M. anisopliae* catalase. The underlined and bold text identifies the peptides that were sequenced by nanoESI-MS/MS.

A) gi|88766403|gb|ABD49721.1| Catalase [*Metarhizium anisopliae*]
 MVQARLV AISLALASLASSQCPFADPGRLAAR TEGRPDSREHLKGYEVDGDVFLTSDVGGPI
 EDQNSLKAGER**GPTLLEDIFIR**QKITHFDHERVPERAVHARGAGAHGTFTSYGDFSNITAAS
 FLGAKDKQTPVVRVSTVAGSRGSADTAR **DVHGFAVR**LYTDEGNFDIVGNVVPVFFIQDAIQ
 FPDLVH SVKPRSDNEIPQAATAHDTAWDFFSQETSTLHTLFWAMAGYGIPRSFRHMDGHGVH
 TFRVLNENGDTKLKWHWKTQKGKASMVWD EAQHVAGKNADFHRQDLFDAIESGNFPEWEL
 NIQMINEQALAFGFDVLDPTKIPEELAPLRPLGILRLDANPANYFAETEIMYQPGHIVR **GVD**
FTDDPLLQGRIFSYLDTQLNRHGGPNFEQLPINRPVPIHNNNRDGAQNLHKNNTAPYIPNS
 MNKGFPKQANRTQGGKFFTAPGRQASGALERRRSSTFQDHWSPRLFFNSITPIEQQLIDAI
 RFETSGLTREVQQNVLVQLNRISHDIARVVG KTLGLEAPAPDDTYHDNTTQGLSIFGQELPTI
 ATLRVGVLVSTESSDSLQANALKEQF SASKVTVVTVGESRVDGIDQTYTSAEAVGFDGIIVTD
 GAELFDPKNKSTLFPPGRPLQIVVDGYNWGPVGLGKARDVLGAAGASEGAGVFIKDGPA
 DIVEEFKKGLATFKFTDRFAQDGG

Table 1. Blastp results and significance values shown for the three peptides sequenced

Sequences producing significant alignments	E value
<i>Peptide 1: GPTLLEDIFIR mass: 1,307.6 Da (position 75–85)</i>	
gb ABD49721.1 catalase (<i>Metarhizium anisopliae</i>)	0.040
gb AAB48485.1 catalase (<i>Aspergillus fumigatus</i>)	0.040
gb ABB89950.1 catalase (<i>Penicillium citrinum</i>)	0.040
<i>Peptide 2: DVHGFAVR mass: 901.6 Da (position 155–162)</i>	
gb ABD49721.1 catalase (<i>Metarhizium anisopliae</i>)	50
gb AAR17474.1 catalase (<i>Cochliobolus heterostrophus</i>)	50
emb CAA04716.1 catalase (<i>Claviceps purpurea</i>)	50
<i>Peptide 3: GVDFTDDPLLQGR mass: 1,433.8 Da (position 373–385)</i>	
gb ABD49721.1 catalase (<i>Metarhizium anisopliae</i>)	0.001
gb ABB89950.1 catalase (<i>Penicillium citrinum</i>)	0.001
ref XP_001596889.1 catalase ¹ (<i>Sclerotinia sclerotiorum</i>)	0.001

¹ Hypothetical protein similar to catalase.

and 1,433.8 Da (spectra not shown), respectively. The amino acid sequence for these peptides was determined from manually sequencing the γ -series product ions within each spectrum. For all peptides sequenced, the derived amino acid code aligned perfectly with the predicted sequence determined for catalase from *M. anisopliae* (fig. 2). The derived amino acid sequences for these peptides were peptide 1: GPTLLEDIFIR, peptide 2: DVHGFAVR, and peptide 3: GVDFTDDPLLQGR. Peptides 1 and 3 had significant homology and specificity to catalase from *M. anisopliae* as well as to catalases from sev-

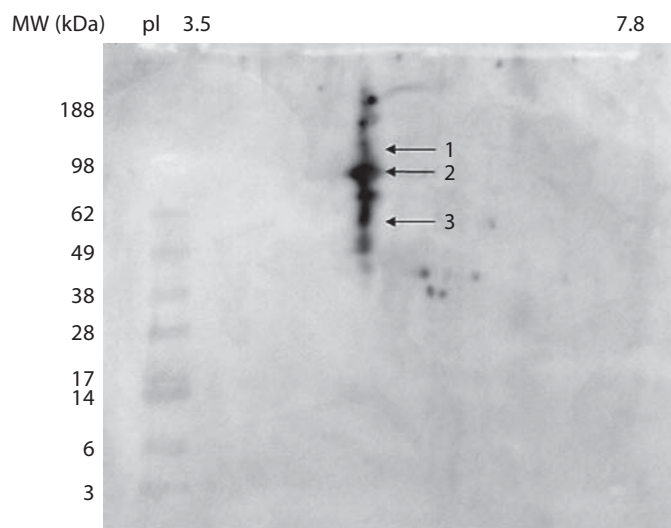


Fig. 3. Polyclonal anti-CatB immunoblot of the MYC 2D gel. The protein spots labeled 1–3 correlate to spots 1–3 on the IgE immunoblot (fig. 1).

eral other fungal species (table 1). Peptide 2 was the shortest peptide, thus it had the lowest significance value. Nevertheless, *M. anisopliae* was found in the group that had the lowest E value in the Blastp search.

Confirmation of Catalase Identification

MYC was shown to contain 6,000–15,000 U/ml of catalase activity. Further evidence was provided by anti-*A. fumigatus* catalase B antibodies recognition of MYC IgE-reactive proteins 1, 2, and 3 (fig. 3).

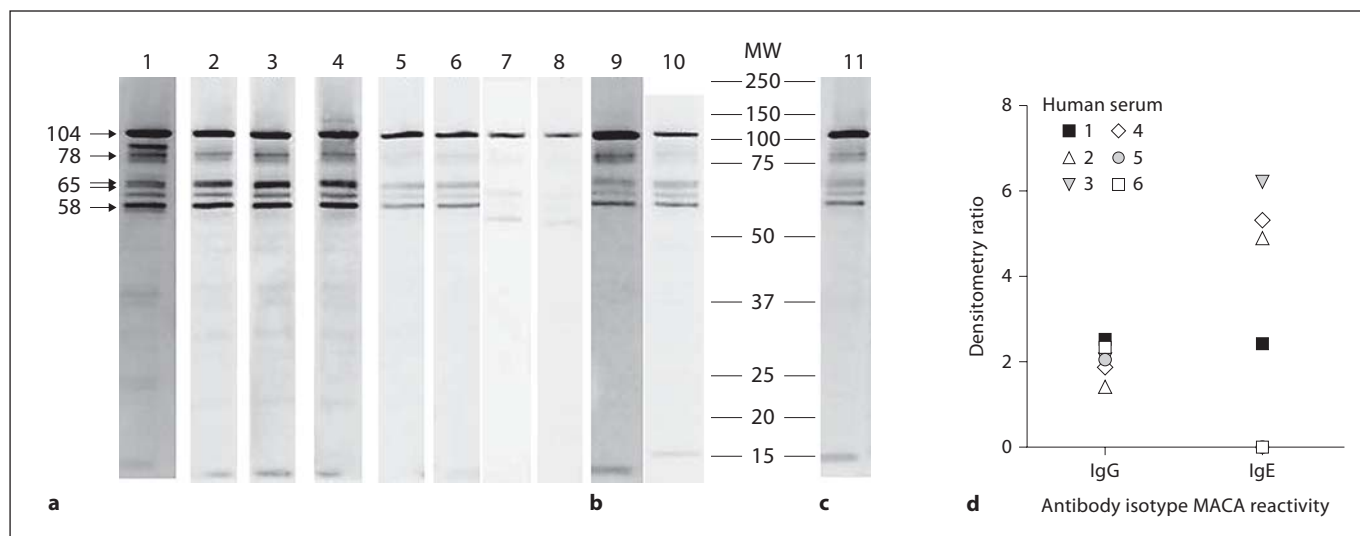


Fig. 4. Western blots of MYC were probed with human asthmatic serum (blots 1–6; **a**) or anti-human IgE only (control blots 7, 8; **a**); mouse anti-MACA-IP (blot 9; **b**), and mouse anti-MACA-IA (blot 10; **b**). **c** Biotin-labeled anti-catalase antibody (blot 11). **d** Densitometry values for MACA dot blot reactivity with human asthmatic serum IgG and IgE.

Human IgE-Reactivity with MYC

When the sera from 6 adult asthmatics [20] were used to probe MYC immunoblots, a number of IgE-binding protein bands were detected (fig. 4a, blots 1–6). Five of these proteins, 104, 78, 65 (doublet), and 58 kDa, appear to be immunodominant in their ability to react with human IgE. Control blots (anti-human IgE antibody only) did demonstrate some non-specific reactivity with the 104-kDa protein (fig. 4a, blots 7–8). However, the chemiluminescence signal was stronger when the human sera were present, therefore this non-specific binding does not account for all of the protein-antibody reactivity observed. When MYC blots were probed with either mouse anti-MACA-IP IgE (raised using an IP injection protocol) or mouse anti-MACA-IA IgE (raised using an exclusively airway exposure protocol) similar protein profiles of reactivity were detected (fig. 4b). This suggests that IgE antibodies induced by airway exposures, in this case at least, react with the same proteins as antibodies produced by a systemic route and more aggressive protocol (IP plus adjuvant). Additionally, a similar protein profile of reactivity was detected when MYC blots were probed with anti-bovine liver catalase (fig. 4c).

In general, the detection of an IgG response to an antigenic agent is indicative of exposure to that agent. Therefore, resolving gel blots of MYC extract were probed with human sera for IgG reactivity. There was no visible

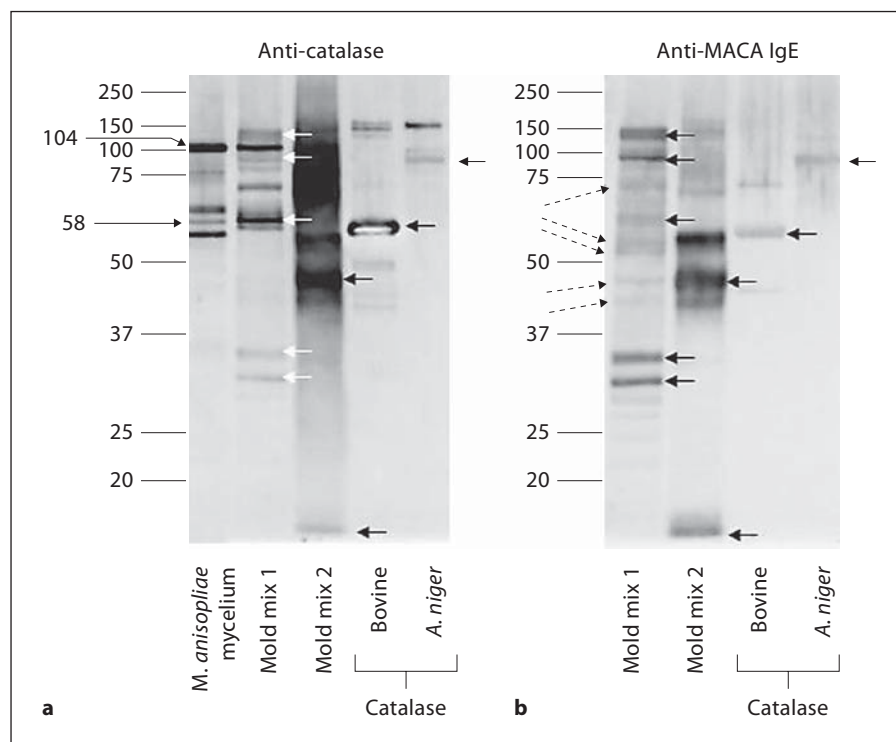
binding (data not shown). Since the resolving gel blot was of denatured proteins (linear epitopes), a dot blot (non-denatured proteins) was also performed to determine if structural epitopes would be detected. Densitometry values for IgG reactivity with the MACA proteins were minimal at best (fig. 4d). However, when dot blots were probed for IgE reactivity, there was a variable but generally positive densitometry value. These data suggest that the IgE reactivity identified is most likely a result of cross-reactivity rather than direct exposure to *M. anisopliae*.

Determination of Cross-Reactivity among Mold Catalases

To assess the possibility that catalase might be a common fungal IgE-reactive protein, immunoblots of two mold mix extracts and two purified catalases (*A. niger* and bovine liver) were probed with anti-bovine liver catalase (fig. 5a) and with mouse anti-MACA-IP IgE (fig. 5b).

The anti-catalase antibody reacted with proteins in all samples but at varying molecular weights. Two protein bands (104 and 58 kDa) were detected in both MYC and mold mix 1. Additionally, both mold mix 1 (which includes *A. niger*) and purified *A. niger* catalase have a protein band detected by anti-catalase (fig. 5a). However, the dominant protein detected in purified bovine liver catalase has a lower molecular weight.

Fig. 5. a Western blot of MYC: commercial mold mix extracts (mold mix 1 and 2; used in human skin prick testing) and purified catalases (*A. niger* and bovine liver) were probed with biotin-labeled anti-bovine liver catalase antibody and streptavidin-HRP conjugate. **b** Western blot of commercial mold mix extracts and purified catalases was probed with polyclonal mouse anti-MACA followed by rat anti-mouse IgE. Solid arrows (**a**, **b**) indicate protein bands that are detected by both anti-catalase antibodies and IgE antibodies raised against *M. anisopliae* extract. Dashed-line arrows (**b**) indicate anti-MACA IgE-binding protein bands in mold mix 1 that may not be catalases.



Mouse anti-MACA-IP IgE antibodies bound to proteins in both mold mixes and both purified catalases, indicating cross-reactivity between the extracts.

Some protein bands were detected by both anti-catalase and anti-MACA-IP antibodies (fig. 5). However, anti-MACA-IP antibodies also bound to several other proteins in mold mix 1 that were not bound by the anti-catalase antibodies, suggesting that non-catalase cross-reactive proteins may also be present in this extract (fig. 5b).

Discussion

In this study, we have demonstrated that both human and mouse serum IgE were reactive with *M. anisopliae* protein(s) that have been identified as catalase. Additionally, cross-reactivity among the mold species tested in these studies was demonstrated.

Previous studies in our laboratory have indicated that mice sensitized and challenged by respiratory exposure to *M. anisopliae*, a biopesticide, developed responses characteristic of human allergic asthma [12, 13]. Occupational exposures to fungi have been associated with hypersensitivity pneumonitis (e.g. *Aspergillus* species with

tobacco worker's lung, *Penicillium casei* with cheese washer's lung, and *P. chrysogenum* with woodworker's lung) and allergy/asthma (e.g. *Saccharomyces cerevisiae* with baker's asthma).

None of the adults that provided serum samples were involved in biopesticide applications. Yet all six serum samples reacted with proteins in the *M. anisopliae* extract, including proteins at the same molecular weight as those reacting with both anti-catalase and mouse anti-MACA IgE (fig. 4). Although exposure to *M. anisopliae*, a soil fungus, is a possible explanation for the IgE reactivity with *M. anisopliae* mycelial catalase, a more likely scenario would be exposure to other more common indoor fungi which produce cross-reactive protein(s).

In our study, catalase was identified by MS as one of the MYC IgE-reactive proteins. Other studies have identified other fungal catalases as allergens including *A. fumigatus* [25], *A. versicolor* [26], and *P. citrinum* [27]. Our data demonstrate that *A. niger* also contains IgE-reactive catalase. In fact, purified *A. niger* catalase, bovine liver catalase, and both commercial 'mold mixes' cross-reacted with IgE antibodies raised against *M. anisopliae* extract (anti-MACA-IP).

Other cases of cross-reactive fungal proteins are known. For example, baker's asthma patients were found

to produce IgE that also reacted with common indoor fungi like *Cladosporium herbarum*, *A. alternata*, *A. fumigatus*, and *Candida albicans* [28]. This cross-reactivity appears to originate from conserved genes, i.e. orthologues, in fungal evolution.

Bowyer et al. [29] used comparative genomics to assess the occurrence of fungal orthologues associated with proteins that are often allergens. Some orthologue classes were ubiquitous in fungi, others were less well conserved and still others occurred in only a limited set of species. It appears that fungal catalases may originate from one of the more conserved classes of fungal genes resulting in some with immunologically related and some with distinct epitopes.

For example, the polyclonal anti-CatB antibody was also reactive with several MYC proteins (fig. 3) that were not detected with anti-MACA IgE probes. Therefore, not all fungal catalases can induce allergy nor do all catalase epitopes induce IgE reactivity. This dichotomy in IgE induction by a class of ubiquitous enzymes, catalases, may help explain why not all fungi induce allergic responses or at least allergic responses of equal severity.

Catalases (hydroperoxidases) are biologically ubiquitous metalloenzymes that protect cells from oxidative damage through hydrogen peroxide hydrolysis. However, in fungi, catalases also play additional roles in conidial germination (*Neurospora crassa*) [30], sporulation (*Magnaporthe grisea*) [31], pathogenesis (*A. fumigatus*) [32] and other functions. These presumed functional differences between fungal and mammalian catalases suggest sequence differences that would allow mammalian immune systems to recognize some fungal catalases as foreign, i.e. the fungal catalases contain some unique epitopes that may be immunogenic. The type of immune response developed may be allergenic in a genetically predisposed population.

The ability of an apparently ubiquitous protein to induce allergic responses is not unique to fungal proteins/enzymes. Tropomyosin, a highly conserved ubiquitous protein, is integral in muscle contraction regulation and is a common food allergen. Although it is generally accepted that the tropomyosin food allergen source is crustaceans, a clinical study by Purohit et al. [33] concluded that tropomyosin may be the relevant cross-reacting allergen responsible for cockroach-, mite-, shrimp-, and crab-induced clinical symptoms. Additionally, it was found that a third of the Orthodox Jews in a study group with no previous exposure to shrimp- but with cockroach- and/or dust mite-specific IgE showed IgE reactivity for *Pen a 1* (shrimp tropomyosin) [34].

There are precedents that indicate that both mouse and human adaptive immune responses can develop against the same protein. For example, Tamura et al. [35] showed that mice can develop IgE against Sugi basic protein, the major human allergen in Japanese cedar pollen extract. Therefore, one might expect that the *M. anisopliae* immunodominant human allergens might also be present among the mouse IgE-reactive *M. anisopliae* proteins. In this study, IgE-reactive proteins detected by both human sera and mouse anti-MACA serum had the same molecular weight and similar reaction intensity suggesting immunodominance of these proteins. Therefore, these data suggest that a mouse model of allergic asthma can be important in allergy risk assessment associated with fungal or other microbial exposures, and may also be useful in identifying proteins that may be allergens.

The identification and characterization of mold allergens will lead to a better understanding of the relationship between fungal exposure and adverse health outcomes. This knowledge will aid in improving tools/methods for assessing environmental levels of fungal allergens and/or biomarkers of exposure. This study demonstrates that some human asthmatics have developed IgE that reacts with a *M. anisopliae* catalase. The fact that the IgE reactivity identified in this study most likely resulted from catalase cross-reactivity rather than exposure does not negate the potential to induce allergic responses if and when increased occupational and residential exposures to this fungus occur.

Furthermore, the data also suggest that allergy hazard identification can be facilitated using a mouse model. The identification of fungal catalases as allergens [25–27] as well as the cross-reactivity among the fungal catalases reported here suggests that IgE-reactive catalase might be useful for exposure assessment. Whether or not indoor environmental levels of IgE-reactive catalase are sufficiently elevated warrants further investigation.

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