The Identification and Characterization of Immunoreactive Fungal Proteins Recognized by Sera from Zimbabweans Sensitized to Fungi

International Archives of Allergy and Immunology

Experimental Immunology – Research Article

Int Arch Allergy Immunol
DOI: 10.1159/000524771

Received: February 18, 2022
Accepted: April 20, 2022
Published online: May 18, 2022

The Identification and Characterization of Immunoreactive Fungal Proteins Recognized by Sera from Zimbabweans Sensitized to Fungi

Lorraine Tsitsi Pfavayi[a, b, c] Richard Burchmore[d] Elopy Nimele Sibanda[e, f, g] Stephen Baker[h, i] Mark Woolhouse[c, j] Takafira Mduluza[g, k] Francisca Mutapi[b, c]

[a]Nuffield Department of Medicine, Centre for Tropical Medicine and Global Health, University of Oxford, Oxford, UK; [b]Institute of Immunology & Infection Research, Ashworth Laboratories, University of Edinburgh, Edinburgh, UK; [c]NIHR Global Health Research Unit Tackling Infections to Benefit Africa (TIBA), Ashworth Laboratories, University of Edinburgh, Edinburgh, UK; [d]College of Medical, Veterinary and Life Sciences, School of Veterinary Medicine, University of Glasgow, Glasgow, UK; [e]Asthma Allergy and Immunology Clinic, Twin Palms Medical Centre, Harare, Zimbabwe; [f] Department of Pathology, National University of Science and Technology (NUST) Medical School, Bulawayo, Zimbabwe; [g]TIBA Zimbabwe, NIHR Global Health Research Unit Tackling Infections to Benefit Africa (TIBA), University of Edinburgh, Edinburgh, UK; [h] Cambridge Institute of Therapeutic Immunology and Infectious Disease, University of Cambridge School of Clinical Medicine, Cambridge Biomedical Campus, Cambridge, UK; [i] Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge Biomedical Campus, Cambridge, UK; [j] Usher Institute of Population Health Sciences and Informatics, Ashworth Laboratories, University of Edinburgh, Edinburgh, UK; [k] Department of Biochemistry, University of Zimbabwe, Harare, Zimbabwe

Keywords
Fungi · Fungal allergens · Zimbabwe · SDS-PAGE

Abstract

Background: Exposure to fungal allergens poses a serious threat to human health, especially to mould-allergic individuals. The prevalence of fungal allergic disease is increasing globally but is poorly studied in Africa. Here, we aimed to identify and characterize fungal proteins that were immunoreactive against serum samples from fungal-sensitized Zimbabweans from Shamva district to inform the development of diagnostics and therapeutics. ***Methods:*** Crude protein extracts of the Ascomycota *Aspergillus fumigatus, Alternaria alternata, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum,* and *Saccharomyces cerevisiae* as well as mucoromycota *Rhizopus nigricans* were individually separated by one-dimensional gel electrophoresis for protein staining and immunoblotting. A pool of eight sera from fungi-sensitive Zimbabwean children aged 3–5 years was used to screen the crude extracts to determine their immunoreactivity. Protein bands recognized by the sera were subjected to mass spectrometry to identify the individual proteins reactive with the sera. ***Results:*** The pooled serum sample reacted with 20 bands, which resolved to 34 distinct proteins, most of which were novel immunogens. The pool was most reactive to *A. alternata*. The proteins identified included peptidases (8/34), hydrolases (6/34), oxidoreductases (5/34), and glucosidases (4/34), while 11/34 were unknown. Eight of the proteins were predicted to be allergens using the Structural Database of Allergenic Proteins (SDAP). ***Conclusions:*** We identified novel immunogens from fungi expanding the number of known fungal allergens. These form a potential basis for diagnostics specific for the Zimbabwean popula-

Edited by: O. Palomares, Madrid.

Karger@karger.com
www.karger.com/iaa

Correspondence to:
Lorraine Tsitsi Pfavayi, lorraine.pfavayi@kellogg.ox.ac.uk

tion. Validation assays will now need to be carried out to further evaluate the cross-reactivity of the identified allergen candidates as well as investigate their potential recognition in a larger cohort of patients. Furthermore, there is now a need to conduct studies relating sensitization to these immunogens and clinical diseases in the population.

Introduction

Allergic diseases associated with IgE-mediated sensitization to fungal allergens are increasing worldwide [1]. With global warming and climate change thought to favour the propagation of fungal spores, allergenicity to fungi has become a serious health concern, triggering or exacerbating respiratory allergic disorders [2, 3].

The prevalence of sensitization to allergens varies across regions worldwide. However, due to a lack of immunological and allergological research facilities in most African countries, there is paucity of allergen prevalence data in this continent [4]. Though documentation is poor, a wide range of aeroallergens has been observed across the continent [5–12] and it has been suggested that there are differences regarding sensitization to allergen molecules in Africa versus Europe [13]. To date, several studies have identified fungal allergens, yet the fungal allergen repertoire has not yet been characterized in an African population.

Fungi are associated with allergic respiratory diseases in humans, such as asthma, rhinitis, allergic bronchopulmonary mycoses, and hypersensitivity pneumonitis [14, 15]. These diseases can result from exposure to different developmental stages of fungi, including spores or vegetative cells [16]. The different developmental stages of fungi produce diverse proteins whose ability to bind to immunoglobulin E (IgE) is heterogeneous [17, 18]. Consequently, commercially available fungal extracts from different suppliers generate discordant results in skin tests and serologic examinations, resulting in pressing diagnostic and therapeutic challenges.

Currently, the management of allergies includes corticosteroids, antihistamines, and in acute cases, non-steroidal anti-inflammatories which may have side effects. To date, the only disease-modifying approach is allergen-specific immunotherapy (AIT) in which allergy-inducing molecules are used for vaccination [19]. AIT is widely used for bee and wasp, house dust mites, pollen, and pet allergies [20]. To date, there is limited evidence supporting the use of AIT in the management of fungi-induced asthma and allergic rhinitis. An exception is *Alternaria alternata* as this is the only standardized allergen extract available [21].

The standard diagnostic test for allergies has been a skin prick test (SPT) using crude extract and/or detection of allergen-specific serum IgE antibodies. However, species-specific IgE reactivity is difficult to confirm due to cross-reactivity between crude allergen extracts from different fungi, representing a significant problem for evaluating IgE tests in clinical practice [22, 23]. Therefore, there is a need to identify specific allergenic fungal proteins that can be used to produce recombinant proteins for therapy and diagnosis [24].

Furthermore, the characterization of the allergenic molecules that an individual is sensitized to can help discriminate between the likelihood of local versus systemic reactions and the persistence of clinical symptoms [25]. For example, some allergens, such as storage proteins in edible nuts (e.g., Ara h 2 and Cor a 9), have been shown to be associated with severe reactions, while other allergens cause sensitization mostly without a clinical reaction.

Most fungi possess multiple and diverse allergens that can be divided into several classes according to their function, e.g., protein hydrolysis (proteases, peptidases), carbohydrate hydrolases (chitinases, glucosidases), and antioxidants (superoxide dismutase, catalases) [26]. These have been described and officially named according to the IUIS nomenclature (http://www.allergen.org), including both major and minor allergens from *Aspergillus fumigatus, Penicillium chrysogenum, Cladosporium herbarum, Rhizopus, Alternaria alternata, and Epicoccum nigrum* [27–36]. However, it has been suggested that many patients display IgE reactivity towards unknown proteins, which may vary between populations [37]. As no studies have been conducted characterizing fungal allergic sensitization in a Zimbabwean population, the present study was conducted to identify allergenic proteins from seven fungal species (*Aspergillus fumigatus, Alternaria alternata, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus nigricans,* and *Saccharomyces cerevisiae*) in Zimbabwe.

Methodology

Study Population

This study was conducted in Shamva district, one of the seven districts in the Mashonaland Central province of Zimbabwe. It was part of a larger study investigating fungal sensitization in pre-

school age children. The area was selected for this current study because the prevalence of fungal sensitization was high, as determined by our previous study [38]. The inhabitants from the study area are of similar ethnicity (Shona) and socioeconomic background (primarily subsistence farmers).

Ethical Approval and Consent

Ethical and institutional approval was obtained from the Medical Research Council of Zimbabwe (MRCZ/A/1964) and the University of Edinburgh. Permission to conduct the study was obtained from the Mashonaland Central Provincial Medical Director. The study aims and procedures were explained to all participants and their parents/guardians in their local language (Shona) before obtaining consent. Written informed consent was obtained from the participants' parents/guardians and recruitment was voluntary with participants free to withdraw from the study at any stage.

Inclusion Criteria

To be included in the study cohort, participants had to meet all of the following criteria: (1) provide a serum sample; (2) ELISA results showing IgE-specific binding to different fungal species; (3) be sensitive to fungi (i.e., IgE positive as determined by SPT). Eight individuals met these criteria (3–5 years old).

Antigen

Freeze-dried crude extracts of *Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus nigricans,* and *Saccharomyces cerevisiae* were obtained from Stallergenes Greer (USA). These extracts were individually reconstituted in Phosphate Buffered Saline and separately run on sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Gel Electrophoresis

One-dimensional gel separation was performed in two parallel samples: one for protein identification and the other for Western blotting. Gel electrophoresis was performed on a 12% polyacrylamide 13-cm gel in a Hoefer SE600 system using sodium dodecyl sulphate (SDS) buffer. The proteins on the gel used for identification were stained with Coomassie blue to visualize them whereas proteins on the gel used for Western blot were transferred onto nitrocellulose membrane as described below.

Immunoblotting

Proteins were transferred from the gel onto a nitrocellulose membrane using semi-dry system (Hoefer) in transfer buffer (Invitrogen) containing 10% methanol at 30 V for 1 h. The membrane was stained with Ponceau S solution to check transfer efficiency and was then blocked at room temperature for 1 h in Tris-buffered saline (TBS) blocking buffer and 0.05% Tween 20. After blocking, the membrane was subjected to 2 separate 10-min washes with TBS, 0.05% Tween, and 0.5% Triton-X 100 (TBS/TT). A pool of serum samples (diluted 1:100 in blocking buffer) was added to the membrane and the membrane was incubated overnight at 4°C and then washed 3 times for 10 min each time in TBS/TT. Horseradish peroxidase-conjugated rabbit anti-human IgE and IgG were diluted 1:1,000 and 1:4,000, respectively, in TBS blocking buffer. The membrane was incubated at room temperature for 1 h and then washed 4 times for 10 min in TBS/TT and 1 time for 10 min in TBS alone. The proteins were visualized using the chemiluminescence product ECL Plus (Amersham), in accordance with manufacturer's instructions. The blots were analysed using Gel Doc from Bio-Rad Image Lab Software.

Image Analysis

The bands on the Coomassie blue-stained gel that matched those on the Western blots were excised and then were analysed by mass spectrometry (MS).

Mass Spectrometry

The excised bands were separately subjected to in-gel trypsin digestion and the resulting peptides solubilized in 20-μL 5% acetonitrile with 0.5% formic acid using the auto-sampler of a nano-flow uHPLC system (Thermo Scientific RSLCnano). Online detection of peptide ions was by electrospray ionization-mass spectrometry MS/MS with an Orbitrap Elite MS (Thermo Scientific). Data were submitted for an MS/MS ion search via the Mascot search engine (http://www.matrixscience.com).

Database Search and Allergen Prediction

For protein identification, the MS/MS data were uploaded on the Mascot search engine (v2.6.2, Matrix Science) and compared against protein sequences in the NCBIprot database using taxonomies *Alternaria alternata, Aspergillus fumigatus, Cladosporiaceae, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus stolonifera (nigricans), and Saccharomyces cerevisiae*. Searches were performed using the following parameters: trypsin as the proteolytic enzyme, allowing for one missed cleavage; for fixed and variable modifications, carbamidomethylation of cysteine and methionine oxidation were used, respectively. The precursor mass tolerance was set at 10 ppm and 0.3 Da for MS/MS matching. Proteins identified with a Mascot score greater than 200 (significant at 95% confidence interval) are reported.

To identify the likely allergen proteins present in the fungal species, the recognized proteins were searched through the structural database of allergenic proteins (SDAP) (http://fermi.utmb.edu/SDAP/index.html), a Web server that provides rapid, cross-referenced access to the sequences, structures, and IgE epitopes of allergenic proteins. The proteins were predicted under the following conditions: (1) sequence similarity >35% between presently obtained proteins and reported allergen proteins and (2) a minimum of 80 amino acid overlap length [39, 40]. Furthermore, predictions for antigenicity were obtained using an online software (http://imed.med.ucm.es/Tools/antigenic.pl) based on the algorithm of Kolaskar and Tongaonkar [41] where the predictions are based on a table that reflects the occurrence of amino acid residues in experimentally known segmental epitopes. Segments are only reported if they are at least eight residues. By these criteria, some of the fungal proteins identified by the sera were classified as likely corresponding to an allergenic protein.

Results

One-Dimensional Gel Electrophoresis Analysis

One-dimensional gel electrophoresis of crude extracts from fungal cells resulted in separation of several proteins

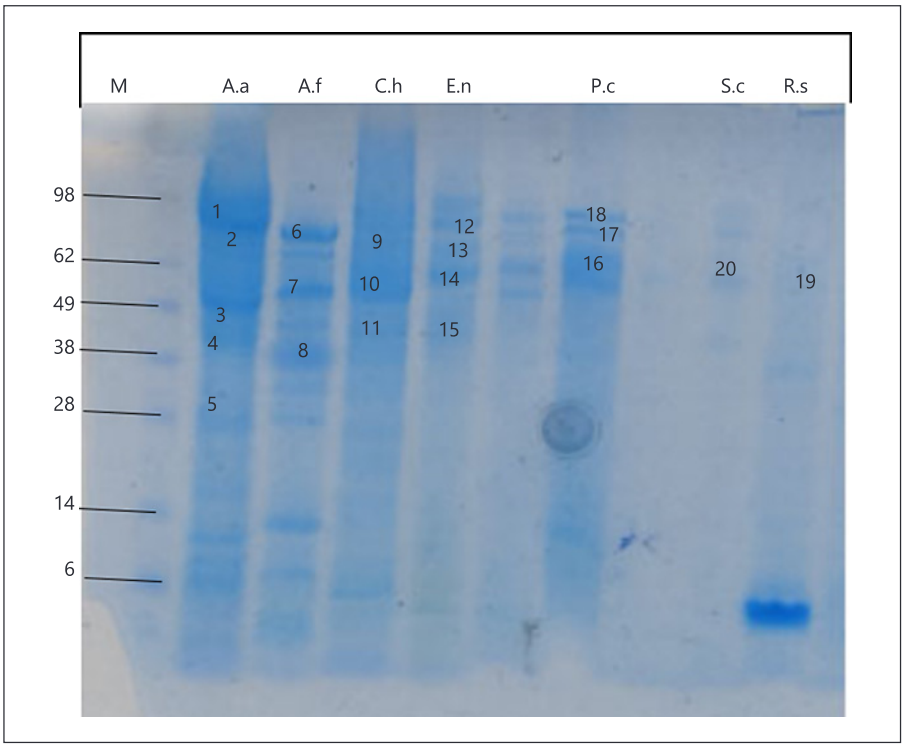

Fig. 1. Coomassie blue-stained one-dimensional gel showing bands matched to the Western blots. Bands on the gel were excised and identified. Molecular weight markers (in kilodaltons) are given on the left. M, marker; *Aa, Alternaria alternata; Af, Aspergillus fumigatus; Ch, Cladosporium herbarum; En, Epicoccum nigrum; Pc, Penicillium chrysogenum; Rs, Rhizopus stolonifer (nigricans)*; *Sc, Saccharomyces cerevisiae.*

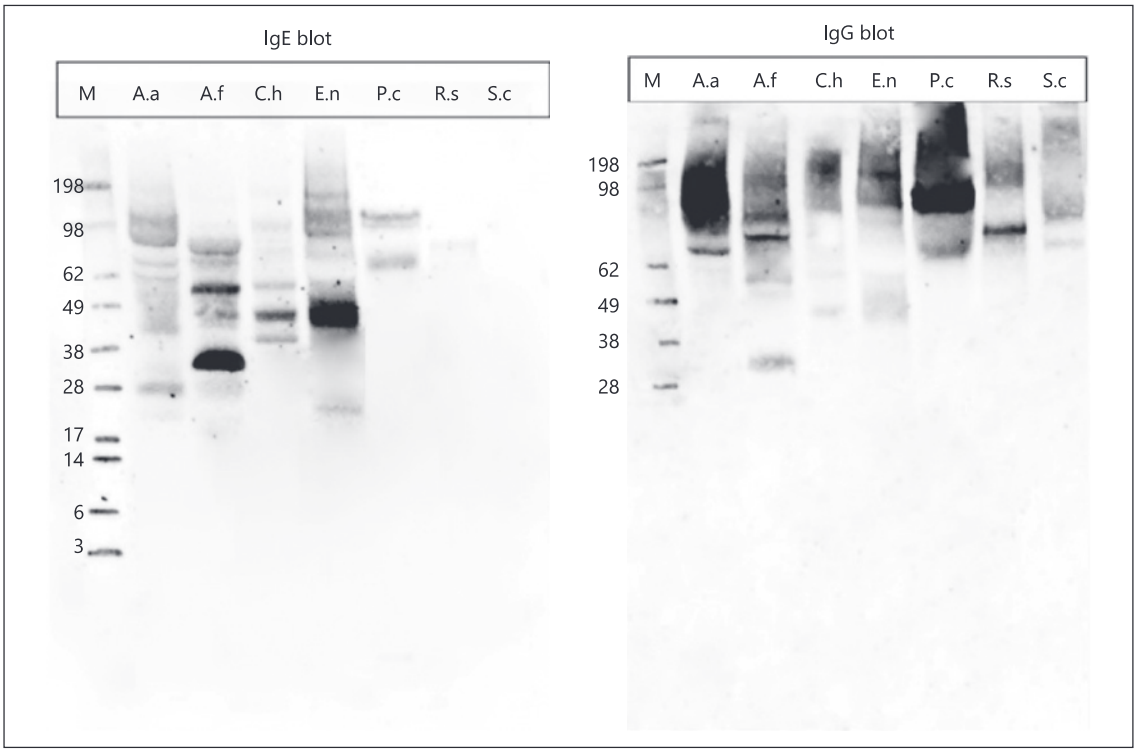

Fig. 2. Western blot analyses of serological reactivity of serum samples. M, marker; *Aa, Alternaria alternata; Af, Aspergillus fumigatus; Ch, Cladosporium herbarum; En, Epicoccum nigrum; Pc, Penicillium chrysogenum; Rs, Rhizopus stolonifer (nigricans)*; *Sc, Saccharomyces cerevisiae.*

Table 1. Fungal proteins identified by MS

Band[a]	Protein name	Species	Accession	Hit score[c]	MW[d]	Molecular function	Reactivity	
							IgE	IgG
1	Glycoside hydrolase	*Alternaria alternata*	XP_018381330.1	2,128	87,237	Hydrolase activity	Yes	No
1	Trehalose	*Alternaria alternata*	XP_018389336.1	2,326	77,106	Alpha, alpha trehalase activity	Yes	No
1	Peptidase s41 family protein	*Alternaria alternata*	OWY41590.1	4,458	83,904	Unknown	Yes	No
2	M6 metalloprotease	*Alternaria alternata*	OWY56860.1	2,911	74,095	Metallopeptidase activity	Yes	No
2	FAS1 domain-containing protein	*Alternaria alternata*	XP_018380929.1	2,217	50,925	Unknown	Yes	No
2	Cyclohexanone 1,2-monooxygenase	*Alternaria alternata*	OWY42352.1	1,663	128,346	Unknown	Yes	No
2	Meiotically up-regulated 157 protein	*Alternaria gaisen*	KAB2110334.1	1,290	57,040	Unknown	Yes	No
3	Vanadium chloroperoxidase	*Alternaria tenuissima*	RYN52497.1	7,156	67,460	Peroxidase activity	Yes	Yes
3	Meiotically up-regulated 157 protein	*Alternaria gaisen*	KAB2110334.1	5,868	57,040	Unknown	Yes	Yes
3	FAS1 domain-containing protein	*Alternaria alternata*	XP_018380929.1	897	50,925	Unknown	Yes	Yes
4	Subtilisin-like serine protease-like protein PR1A	*Alternaria alternata*	XP_018384475.1	2,893	40,384	Serine-type endopeptidase activity	Yes	No
4	Concanavalin A-like lectin/glucanase	*Alternaria alternata*	XP_018390955.1	1,408	46,193	Hydrolase activity	Yes	No
5	GroES-like protein	*Alternaria alternata*	XP_018385554.1	1,675	38,120	Oxidoreductase activity	Yes	No
5	Glycoside hydrolase	*Alternaria alternata*	XP_018382523.1	1,426	44,564	Hydrolase activity	Yes	No
6	Dipeptidyl-peptidase 5	*Aspergillus lentulus*	GFF50131.1	5,526	79,688	Serine-type peptidase activity	Yes	Yes
6	Secreted dipeptidyl peptidase	*Aspergillus fischeri NRRL 181*	XP_001260402.1	5,477	79,675	Serine-type peptidase activity	Yes	Yes
7	Secreted dipeptidyl peptidase	*Aspergillus fischeri NRRL 181*	XP_001260402.1	2,304	79,675	Serine-type peptidase activity	Yes	Yes
7	Catalase B	*Aspergillus minisclerotigenes*	KAB8269428.1	1,472	79,856	Catalase activity	Yes	Yes
7	Dipeptidyl-peptidase 5	*Aspergillus lentulus*	GFF50131.1	1,366	79,688	Serine-type peptidase activity	Yes	Yes
8	Chitinase	*Aspergillus fumigatus*	AAP23218.1	6,271	47,708	Chitinase activity	Yes	Yes
9	GPI-anchored cell wall beta 1,3 endoglucanase EglC	*Aspergillus fumigatus var. RP-2014*	KEY82708.1	2,198	44,923	Unknown	Yes	No
10	Hypothetical protein CNMCM8714_006,228	*Aspergillus fumigatus*	KAF4253478.1	1,319	105,137	Unknown	Yes	No
11	Hypothetical protein CDV57_00,056	*Aspergillus fumigatus*	OXN30505.1	1,063	16,162	Unknown	Yes	No
12	Catalase	*Aspergillus clavatus NRRL 1*	XP_001273665.1	521	80,097	Catalase activity	Yes	No
13	Alpha-glucosidase	*Rachicladosporium antarcticum*	OQO11764.1	625	67,385	Hydrolase activity	Yes	Yes
14	Hypothetical protein B5807_10,540	*Epicoccum nigrum*	OSS44738.1	7,238	112,128	Beta-glucosidase activity	Yes	No
15	S-adenosyl-L-homocysteine hydrolase	*Aspergillus homomorphus CBS 101889*	XP_025550664.1	3,028	49,516	Adenosylhomocysteinase activity	Yes	No
16	Hypothetical protein B5807_10,540	*Epicoccum nigrum*	OSS44738.1	1,485	112,128	Beta-glucosidase activity	Yes	Yes
17	Glycoside hydrolase family 31	*Aspergillus oryzae*	OOO09042.1	2,609	106,688	Beta-glucosidase activity, maltose alpha-glucosidase activity	Yes	Yes
17	Putative dipeptidyl peptidase	*Penicillium chrysogenum*	KZN92610.1	4,136	85,215	Serine-type peptidase activity	Yes	Yes
18	Putative dipeptidyl peptidase	*Penicillium chrysogenum*	KZN92610.1	15,819	85,215	Serine-type peptidase activity	Yes	No
18	Glucose oxidase	*Penicillium chrysogenum*	AFA42947.1	669	66,471	Glucose oxidase activity	Yes	No
19	RecName: full = alpha-(1–6)-linked fucose-specific lectin; AltName: full = RSL	*Rhizopus stolonifer*	P83973.1	7,343	3,199	Unknown	No	Yes
20	Glr1p	*Saccharomyces cerevisiae YJM320*	AJV98761.1	1,540	53,790	Unknown	No	Yes

[a] Band numbers indicated in Figure 1. [b] Accession numbers according to NCBInr database. [c] Mascot score reported after database search, score >200 indicates identity or extensive homology at $p < 0.05$. [d] Theoretical mass retrieved from NCBInr database.

Table 2. Predicted allergen-related proteins in the fungal species investigated

Predicted allergens				Corresponding known allergens				
band[a]	accession No[b]	description	AA[c]	allergen[d]	accession no[b]	AA[c]	bit score[d]	*E* score[e]
1	XP_018381330.1	Glycoside hydrolase (*Alternaria alternata*)	798	Asp n 14	CAB06417	804	500.0	2.8e–142
				Asp n 14	AAD13106	804	498.7	6.5e–142
4	XP_018384475.1	Subtilisin-like serine protease-like protein PR1A (*Alternaria alternata*)		Asp f 13	P28296	403	212.5	2.2e–56
				Asp v 13.0101	ADE74975	403	203.2	1.4e–53
				Pen c 13.0101	AAD25926	397	203.0	1.6e–53
				Pen ch 13	AAF23726	397	200.2	1.1e–52
				Tri r 2.0101	AAD52013	412	197.1	1.0e–51
				Asp fl protease	AAD47202	403	196.8	1.1e–51
				Asp o 13	CAA35594	403	196.8	1.1e–51
				Pen ch 18	AAF71379	494	186.1	2.4e–48
				Cur l 4.0101	ACF19589	506	113.2	2.2e–26
				Asp f 18.0101	Y13338	495	108.9	4.2e–25
				Pen o 18	AAG44478	503	107.2	1.4e–24
5	XP_018385554.1	GroES-like protein (*Alternaria alternata*)	352	Cand a 1	AAA53300	350	307.0	6.3e–85
				Cand a 1	P43067	350	304.3	4.0e–84
6	GFF50131.1	Dipeptidyl-peptidase 5 (*Aspergillus lentulus*)	721	Tri r 4.0101	AAD52012	726	666.6	1.5e–192
7	XP_001260402.1	Secreted dipeptidyl peptidase (*Aspergillus fischeri NRRL 181*)		Tri r 4.0101	AAD52012	726	677.2	9.5e–196
7	KAB8269428.1	Catalase B (*Aspergillus minisclerotigenes*)		Pen c 30.0101	ABB89950	733	930.0	0.0e+00
12	XP_001273665.1	Catalase (*Aspergillus clavatus NRRL 1*)	728	Asp f 15	O60022	152	224.0	1.4e–60
18	KZN92610.1	Putative dipeptidyl peptidase (*Penicillium chrysogenum*)	772	Tri r 4.0101	AAD52012	726	530.8	1.2e–151

[a] Band numbers indicated in Figure 1. [b] Accession numbers according to NCBInr database. [c] Amino acid sequence. [d] Bit score-sequence similarity. [e] *E* score-homology.

that were visible after staining (shown in Fig. 1). Of these, 20 bands reacted with human serum samples from fungal-sensitized individuals as determined by anti-human IgE/IgG immunoblotting (shown in Fig. 2; Table 1).

Immunogenic Protein Identities

The 20 bands identified as serologically reactive by Western blot were excised from the Coomassie blue-stained gel and were subjected to in-gel trypsin digestion. Subsequently, the peptides were analysed by MS/MS and the peptide data obtained were used to search NCBIprot databases. Most of the bands were successfully matched to specific fungal proteins and the identifications of these bands are shown in Table 1. The identity given for each band corresponding to the top hit score (the Mascot output statistic) that had a score >200 (significant at 95% confidence interval), the predicted MW, as well as the associated species are also provided in Table 1. The MS/MS analysis revealed cases in which different bands were derived from the same protein, for example, bands 2 and 3 (FAS1 domain-containing protein and eiotically up-regulated 157 protein) as well as 6 and 7 (secreted dipeptidyl peptidase). The MS/MS analysis also revealed bands that resolved to the same protein but with different accession numbers (e.g., bands 1 and 5, both glycoside hydrolase). The 20 bands recognized by serum samples gave rise to 34 protein identifications. Of the 34 proteins identified, 3 were hypothetical proteins, 11 had no known function,

and the remaining 20 proteins could be grouped by molecular function (Table 1. The identified proteins included enzymes and most of these proteins have not been previously shown to be immunogenic.

Prediction of Allergens Fungi (Alternaria alternata, Aspergillus, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus stolonifera, and Saccharomyces cerevisiae)

Fungal species are known to contain several proteins that act as allergens [42]. Using structural and sequence predictive tools, eight fungal proteins were predicted to be allergens (Table 2. Four of these corresponded with serine proteases from various fungal species, sharing 39.9–58% sequence homology to known fungal allergenic proteases.

The predicted antigenic peptides are shown in online supplementary Figures S1–S4 (for all online suppl. material, see www.karger.com/doi/10.1159/000524771). The remaining proteins were not identified as allergens using these tools.

Discussion

Many allergens have been reported from fungi, including >40 from the fungal species investigated in this study. These include allergens identified functionally as enzymes and regulatory proteins, proteases, enolases, and heat shock proteins, while others currently have unknown biochemical functions and activities [43]. Among the recognized enzymes, the predominant allergens are proteases, ribonuclease, chymotrypsin, catalase, and superoxide dismutase [44, 45].

In this study, the pooled sera reacted mostly with *A. alternata;* we were able to identify 34 immunogenic proteins across the functional spectrum [46]. Among the identified proteins, several were known allergens from other fungal, plant, and insect species, e.g., catalase, chitinase, subtilisin-like serine protease, beta glucosidase, and dipeptidyl-peptidase 5 [47–49]. However, we also identified novel IgE-binding proteins, i.e., allergens. For example, M6 metalloprotease and cyclohexanone 1,2-monooxygenase from *A. alternata* as well as exo-beta-1,3-glucanase and GPI-anchored cell wall beta 1,3 endoglucanase from *A. fumigatus.*

For the majority of detected proteins, there was a disparity between the observed and theoretical molecular weights. These discrepancies may have been due to post-transcriptional modification and/or the structural subunits required for appropriate functioning [50]. Of the 34 proteins recognized by Zimbabwean sera, eight were identified as putative allergens through the allergen-predicting software.

The sera in our study also reacted with Catalase B, which is consistent with other studies that have identified other fungal catalases as allergens including *A. fumigatus* [51], *Aspergillus versicolor* [52], and *Penicillium citrinum* [53]. In the present study, Catalase B exhibited high sequence identity (74.5%) with catalase from *P. citrinum* (Pen c 30.0101) [26]. The high sequence homology observed between these enzymes may represent a conserved allergenicity of the catalase protein. Catalases are ubiquitous iron-containing enzymes that protect cells from oxidative damage through hydrogen peroxide hydrolysis [54, 55]. However, in fungi, catalases have been suggested to play additional roles in conidial germination [56], sporulation [57], and pathogenesis [58], implying that fungal catalases contain some unique epitopes that may be immunogenic [59].

Of all the putative allergens identified here, only one, which was a glycoside hydrolase, corresponded to an occupational (workplace-related) allergen (xylanase [Asp n 14]) from *Aspergillus niger* [60], which is typically associated with baking, farming, and cereal handling [61]. This observation may be associated with para-occupational exposure. Previous studies have shown that occupational allergens can be transported home, presumably on contaminated clothing and skin, with subsequent sensitization of other household residents, including children, leading to severe allergic diseases in atopic patients if not diagnosed and treated [62].

Cross-reactive proteins in fungal allergens complicate the diagnosis and management of fungal allergy and this limitation results in patients having allergic sensitization to many biologically related fungi. In our study, several immunoreactive protein bands of the crude extracts belonged to two main fungi, *Alternaria* and *Aspergillus,* which are likely associated with cross-reactivity amongst the fungal species, as this has been shown between phylogenetically close and even distant species [22]. While molecular diagnostics have improved the ability to identify clinically relevant cross-reactivity, there is still a need to understand the fungal-specific (degree of homology, abundance) and patient-specific factors (immune response, augmentation factors), as well as the epidemiology of cross-reactivity that determines clinical relevance [63].

To further proceed with this work, we will analyse the samples individually for each patient in the study, which

could provide some additional information about the status and frequency of the specific recognition of these candidate allergens. In addition, we will conduct ELISA inhibition studies to further evaluate cross-reactivity of these candidate allergens. Furthermore, research into the potential recognition of these allergens in larger cohort populations will be key in validating these allergens.

In summary, the fact that several immunogenic proteins were novel allergens indicates a need to expand the reference database for the allergen prediction software and highlights potential population differences in genetic variations in the major histocompatibility complex specificity. Therefore, there is need to consider these differences when developing diagnostics and therapeutics for fungal allergy in African populations. The presence of cross-reactivity of allergens amongst related fungal species gives the potential for developing cross species therapeutics and diagnostics.

Conclusion

We identified 34 fungal proteins reactive with serum from a population of Zimbabweans sensitized to fungi. Based on the structural and sequence predictive tools, eight of these were identified as putative allergens. Validation assays will now need to be carried out to further evaluate the cross-reactivity of the identified allergen candidates as well as investigate their potential recognition in a larger cohort of patients. Furthermore, there is need to investigate the role of these immunogens in the aetiology of allergic disease and mechanistic pathways to inform the development of diagnostics and therapeutics appropriate for African populations.

Acknowledgments

We thank all the members of the Parasite Immuno-epidemiology Group at the University of Edinburgh for their valuable comments in shaping the manuscript.

Statement of Ethics

Ethical and institutional approval was obtained from the Medical Research Council of Zimbabwe (MRCZ/A/1964) and the University of Edinburgh. Permission to conduct the study was obtained from the Mashonaland Central Provincial Medical Director. Written informed consent was obtained from the participants' parents/guardians and recruitment was voluntary with participants free to withdraw from the study at any stage.

Conflict of Interest Statement

The authors have declared that no competing interests exist.

Funding Sources

This research was commissioned by the National Institute for Health Research (NIHR) Global Health Research programme (16/136/33) using UK aid from the UK Government. The views expressed in this publication are those of the authors and not necessarily those of NIHR or the Department of Health and Social Care. Lorraine Pfavayi, Francisca Mutapi, and Elopy Sibanda are supported by OAK Foundation.

Author Contributions

Francisca Mutapi, Takafira Mduluza, and Elopy Sibanda conceived the study. Francisca Mutapi, Takafira Mduluza, Elopy Sibanda, and Lorraine Pfavayi conducted the fieldwork; Lorraine Pfavayi curated the field data. Lorraine Pfavayi and Richard Burchmore conducted the laboratory work; Lorraine Pfavayi, Richard Burchmore, and Francisca Mutapi conducted the data analysis. Lorraine Pfavayi and Francisca Mutapi drafted the manuscript; Stephen Baker, Mark Woolhouse, Richard Burchmore, Francisca Mutapi, Takafira Mduluza, Lorraine Pfavayi, and Elopy Sibanda discussed, reviewed, amended, and approved the final version of the manuscript.

Data Availability Statement

All the data that support the findings of this study will be fully available with publication, through the University of Edinburgh Datashare.

References

1 Pawankar R. Allergic diseases and asthma: a global public health concern and a call to action. World Allergy Organ J. 2014 May;7(1):12–3.
2 Garcia-Solache MA, Casadevall A. Global warming will bring new fungal diseases for mammals. mBio. 2010 May 18;1(1):e00061–10.
3 Nnadi NE, Carter DA. Climate change and the emergence of fungal pathogens. PLoS Pathog. 2021;17(4):e1009503.
4 Sibanda EN. Research and clinical aspects of immunology in Africa. Curr Opin Immunol. 2001;13(5):528–32.
5 El-Gamal Y, Awad A, Hossny E, El-Basiony S, Galal E. Cockroach sensitivity in asthmatic Egyptian children. Pediatr Allergy Immunol. 1995;6(4):220–2.
6 Sibanda EN. Inhalant allergies in Zimbabwe: a common problem. Int Arch Allergy Immunol. 2003 Jan;130(1):2–9.

7 Mpairwe H, Muhangi L, Ndibazza J, Tumusiime J, Muwanga M, Rodrigues LC, et al. Skin prick test reactivity to common allergens among women in Entebbe, Uganda. Trans R Soc Trop Med Hyg. 2008 Apr;102(4):367–73.
8 Seedat RY, Claassen J, Claassen AJ, Joubert G. Mite and cockroach sensitisation in patients with allergic rhinitis in the Free State; 2010.
9 Jafta N, Batterman SA, Gqaleni N, Naidoo RN, Robins TG. Characterization of allergens and airborne fungi in low and middle-income homes of primary school children in Durban, South Africa. Am J Ind Med. 2012;55(12):1110–21.
10 Oluwole O, Arinola OG, Falade GA, Ige MO, Falusi GA, Aderemi T, et al. Allergy sensitization and asthma among 13–14 year old school children in Nigeria. Afr Health Sci. 2013;13(1):144–53.
11 Pefura-Yone EW, Kengne AP, Afane-Ze E, Kuaban C. Sensitisation to Blattella germanica among adults with asthma in Yaounde, Cameroon: a cross-sectional study. World Allergy Organ J. 2014 Aug;7:22.
12 Kwizera R, Bongomin F, Olum R, Meya DB, Worodria W, Bwanga F, et al. Fungal asthma among Ugandan adult asthmatics. Med Mycol. 2021;59(9):923–33.
13 Westritschnig K, Sibanda E, Thomas W, Auer H, Aspöck H, Pittner G, et al. Analysis of the sensitization profile towards allergens in central Africa. Clin Exp Allergy. 2003;33(1):22–7.
14 Baxi SN, Portnoy JM, Larenas-Linnemann D, Phipatanakul W; Environmental Allergens Workgroup. Exposure and health effects of fungi on humans. J Allergy Clin Immunol Pract. 2016 May–Jun;4(3):396–404.
15 Pfavayi LT, Sibanda EN, Mutapi F. The pathogenesis of fungal-related diseases and allergies in the African population: the state of the evidence and knowledge gaps. Int Arch Allergy Immunol. 2020;181(4):257–69.
16 Kurup VP, Shen HD, Banerjee B. Respiratory fungal allergy. Microbes Infect. 2000 Jul;2(9):1101–10.
17 Zargari A, Doekes G, van Ieperen-van Dijk AG, Landberg E, Härfast B, Scheynius A. Influence of culture period on the allergenic composition of Pityrosporum orbiculare extracts. Clin Exp Allergy. 1995 Dec;25(12):1235–45.
18 Hemmann S, Menz G, Ismail C, Blaser K, Crameri R. Skin test reactivity to 2 recombinant Aspergillus fumigatus allergens in A fumigatus-sensitized asthmatic subjects allows diagnostic separation of allergic bronchopulmonary aspergillosis from fungal sensitization. J Allergy Clin Immunol. 1999 Sep;104(3 Pt 1):601–7.
19 Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol. 1998 Oct;102(4 Pt 1):558–62.
20 Arshad SH. An update on allergen immunotherapy. Clin Med. 2016;16(6):584–7.
21 Bozek A, Pyrkosz K. Immunotherapy of mold allergy: a review. Hum Vaccin Immunother. 2017 Oct 3;13(10):2397–401.
22 Crameri R, Zeller S, Glaser AG, Vilhelmsson M, Rhyner C. Cross-reactivity among fungal allergens: a clinically relevant phenomenon? Mycoses. 2009 Mar;52(2):99–106.
23 Fukutomi Y, Taniguchi M. Sensitization to fungal allergens: resolved and unresolved issues. Allergol Int. 2015 Oct;64(4):321–31.
24 Corti V, Cattaneo A, Bachi A, Rossi RE, Monasterolo G, Paolucci C, et al. Identification of grass pollen allergens by two-dimensional gel electrophoresis and serological screening. Proteomics. 2005 Feb;5(3):729–36.
25 Canonica GW, Ansotegui IJ, Pawankar R, Schmid-Grendelmeier P, van Hage M, Baena-Cagnani CE, et al. LEN consensus document on molecular-based allergy diagnostics. World Allergy Organ J. 2013 Oct 3;6(1):17.
26 Chiu LL, Lee KL, Lin YF, Chu CY, Su SN, Chow LP. Secretome analysis of novel IgE-binding proteins from Penicillium citrinum. Proteomics Clin Appl. 2008 Jan;2(1):33–45.
27 Baldo BA, Baker RS. Inhalant allergies to fungi: reactions to bakers' yeast (Saccharomyces cerevisiae) and identification of bakers' yeast enolase as an important allergen. Int Arch Allergy Appl Immunol. 1988;86(2):201–8.
28 Sridhara S, Gangal SV, Joshi AP. Immunochemical investigation of allergens from Rhizopus nigricans. Allergy. 1990 Nov;45(8):577–86.
29 Zargari A, Emilson A, Halldén G, Johansson S, Scheynius A. Cell surface expression of two major yeast allergens in the Pityrosporum genus. Clin Exp Allergy. 1997;27(5):584–92.
30 Banerjee B, Greenberger PA, Fink JN, Kurup VP. Immunological characterization of Asp f 2, a major allergen from Aspergillus fumigatus associated with allergic bronchopulmonary aspergillosis. Infect Immun. 1998 Nov;66(11):5175–82.
31 Shankar J, Gupta PD, Sridhara S, Singh BP, Gaur SN, Arora N. Immunobiochemical analysis of cross-reactive glutathione-S-transferase allergen from different fungal sources. Immunol Invest. 2005;34(1):37–51.
32 Simon-Nobbe B, Denk U, Schneider PB, Radauer C, Teige M, Crameri R, et al. NADP-dependent Mannitol dehydrogenase, a major allergen of Cladosporium herbarum. J Biol Chem. 2006;281(24):16354–60.
33 Kukreja N, Singh BP, Arora N, Gaur SN, Sridhara S. Identification of Epicoccum purpurascens allergens by two-dimensional immunoblotting and mass spectrometry. Immunobiology. 2008;213(1):65–73.
34 Rid R, Simon-Nobbe B, Langdon J, Holler C, Wally V, Poll V, et al. Cladosporium herbarum translationally controlled tumor protein (TCTP) is an IgE-binding antigen and is associated with disease severity. Mol Immunol. 2008 Jan;45(2):406–18.
35 Pöll V, Denk U, Shen HD, Panzani RC, Dissertori O, Lackner P, et al. The vacuolar serine protease, a cross-reactive allergen from Cladosporium herbarum. Mol Immunol. 2009 Apr;46(7):1360–73.
36 Luo W, Wilson AM, Miller JD. Characterization of a 52 kDa exoantigen of Penicillium chrysogenum and monoclonal antibodies suitable for its detection. Mycopathologia. 2010 Jan;169(1):15–26.
37 Bordas-Le Floch V, Le Mignon M, Bouley J, Groeme R, Jain K, Baron-Bodo V, et al. Identification of novel short ragweed pollen allergens using combined transcriptomic and immunoproteomic approaches. PLoS One. 2015;10(8):e0136258.
38 Pfavayi LT, Sibanda EN, Baker S, Woolhouse M, Mduluza T, Mutapi F. Fungal allergic sensitisation in young rural Zimbabwean children: gut mycobiome and seroreactivity characteristics. Curr Res Microb Sci. 2021:100082.
39 Ivanciuc O, Schein CH, Braun W. SDAP: database and computational tools for allergenic proteins. Nucleic Acids Res. 2003 Jan 1;31(1):359–62.
40 McClain S. Bioinformatic screening and detection of allergen cross-reactive IgE-binding epitopes. Mol Nutr Food Res. 2017;61(8):1600676.
41 Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 1990 Dec 10;276(1–2):172–4.
42 Twaroch TE, Curin M, Valenta R, Swoboda I. Mold allergens in respiratory allergy: from structure to therapy. Allergy Asthma Immunol Res. 2015 May;7(3):205–20.
43 Kurup VP. Aspergillus antigens: which are important? Med Mycol. 2005 May;43 Suppl 1:S189–96.
44 Hearn VM. Antigenicity of Aspergillus species. J Med Vet Mycol. 1992;30(1):11–25.
45 Singh BP, Banerjee B, Kurup VP. Aspergillus antigens associated with allergic bronchopulmonary aspergillosis. Front Biosci. 2003 Jan 1;8:s102–9.
46 Walker GM, White NA. Introduction to fungal physiology. In: Fungi: biology and applications; 2017. p. 1–35.
47 Palosuo T. Latex allergens. Revue Française d'Allergologie et d'Immunologie Clinique. 1997 Jan;37(8):1184–7.
48 Schiener M, Hilger C, Eberlein B, Pascal M, Kuehn A, Revets D, et al. The high molecular weight dipeptidyl peptidase IV Pol d 3 is a major allergen of Polistes dominula venom. Sci Rep. 2018 Jan 22;8(1):1318.
49 Leoni C, Volpicella M, Dileo M, Gattulli BAR, Ceci LR. Chitinases as food allergens. Molecules. 2019 May 31;24(11):2087.
50 Hasan MK, Cheng Y, Kanwar MK, Chu XY, Ahammed GJ, Qi ZY. Responses of plant proteins to heavy metal stress-a review. Front Plant Sci. 2017;8:1492.

51 Gautam P, Sundaram CS, Madan T, Gade WN, Shah A, Sirdeshmukh R, et al. Identification of novel allergens of Aspergillus fumigatus using immunoproteomics approach. Clin Exp Allergy. 2007 Aug;37(8):1239–49.
52 Benndorf D, Müller A, Bock K, Manuwald O, Herbarth O, von Bergen M. Identification of spore allergens from the indoor mould Aspergillus versicolor. Allergy. 2008 Apr;63(4):454–60.
53 Chiu LL, Lee KL, Lin YF, Chu CY, Su SN, Chow LP. Secretome analysis of novel IgE-binding proteins from Penicillium citrinum. Proteomics Clin Appl. 2008;2(1):33–45.
54 Diamond RD, Clark RA. Damage to Aspergillus fumigatus and Rhizopus oryzae hyphae by oxidative and nonoxidative microbicidal products of human neutrophils in vitro. Infect Immun. 1982 Nov;38(2):487–95.
55 Pradhan A, Herrero-de-Dios C, Belmonte R, Budge S, Lopez Garcia A, Kolmogorova A, et al. Elevated catalase expression in a fungal pathogen is a double-edged sword of iron. PLoS Pathog. 2017 May;13(5):e1006405.
56 Wang N, Yoshida Y, Hasunuma K. Loss of Catalase-1 (Cat-1) results in decreased conidial viability enhanced by exposure to light in Neurospora crassa. Mol Genet Genomics. 2007 Jan;277(1):13–22.
57 Skamnioti P, Henderson C, Zhang Z, Robinson Z, Gurr SJ. A novel role for catalase B in the maintenance of fungal cell-wall integrity during host invasion in the rice blast fungus Magnaporthe grisea. Mol Plant Microbe Interact. 2007 May;20(5):568–80.
58 Shibuya K, Paris S, Ando T, Nakayama H, Hatori T, Latgé JP. Catalases of Aspergillus fumigatus and inflammation in aspergillosis. Nihon Ishinkin Gakkai Zasshi. 2006;47(4):249–55.
59 Ward MDW, Donohue MJ, Chung YJ, Copeland LB, Shoemaker JA, Vesper SJ, et al. Human serum IgE reacts with a *Metarhizium anisopliae* fungal catalase. Int Arch Allergy Immunol. 2009;150(4):343–51.
60 Sander I, Raulf-Heimsoth M, Siethoff C, Lohaus C, Meyer HE, Baur X. Allergy to Aspergillus-derived enzymes in the baking industry: identification of beta-xylosidase from Aspergillus niger as a new allergen (Asp n 14). J Allergy Clin Immunol. 1998 Aug;102(2):256–64.
61 Quirce S, Diaz-Perales A. Diagnosis and management of grain-induced asthma. Allergy Asthma Immunol Res. 2013;5(6):348–56.
62 Vissers M, Doekes G, Heederik D. Exposure to wheat allergen and fungal alpha-amylase in the homes of bakers. Clin Exp Allergy. 2001 Oct;31(10):1577–82.
63 Cox AL, Eigenmann PA, Sicherer SH. Clinical relevance of cross-reactivity in food allergy. J Allergy Clin Immunol Pract. 2021 Jan;9(1):82–99.