

Proteome of Conidial Surface Associated Proteins of *Aspergillus fumigatus* Reflecting Potential Vaccine Candidates and Allergens

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Aspergillus fumigatus is a mold causing most of the invasive fungal lung infections in the immunocompromised host. In addition, the species is the causative agent of certain allergic diseases. Both in invasive and in allergic diseases, the conidial surface mediates the first contact with the human immune system. Thus, conidial surface proteins may be reasonable vaccine candidates as well as important allergens. To broaden the list of those antigens, intact viable *Aspergillus* conidia were extracted with mild alkaline buffer at pH 8.5 in the presence of a 1,3- β -glucanase. The proteome of this fraction was separated by two-dimensional gel electrophoresis (2-DE) and analyzed by liquid chromatography coupled with tandem mass spectrometry. Altogether 26 different *A. fumigatus* proteins were identified, twelve of which contain a signal for secretion. Among these were the known major conidial surface protein rodlet A, one acid protease PEP2, one lipase, a putative disulfide isomerase and a putative fructose-1,6-biphosphatase. The known allergen Asp f 3 was identified among the proteins without a signal for secretion. On the basis of the recently annotated *A. fumigatus* genome (*Nature* **2005**, *438*, 1151–1156), proteome analysis is now a powerful tool to confirm expression of hypothetical proteins and, thereby to identify additional vaccine candidates and possible new allergens of this important fungal pathogen.

Keywords: *A. fumigatus* • proteome • conidia • conidial surface • vaccine • allergens

Introduction

Among the molds, *A. fumigatus* is the most common species that causes invasive mycoses of heavily immunocompromised patients.^{1–4} Patients at highest risk for acquiring the disease are those with a significant reduction in the number or function of granulocytes such as leukemia patients undergoing chemotherapy or patients on long-term treatment with immunosuppressive drugs, especially cortisone (e.g., organ transplant patients).^{5,6} In both groups, the fatality rate of invasive aspergillosis is high and most of the patients die despite antimycotic treatment.⁷ Thus, in addition to antimycotic therapy, prophylaxis of the disease is highly desirable and may be achieved by vaccination with protection-inducing fungal antigens, a principle that has been suggested and discussed more recently.^{8,9} It has been shown that an acquired immunity is

inducible in animals and this is mediated primarily by a cellular immune response involving macrophages and CD4⁺ T-cells.^{10,11}

Following the classical models of immunology, such a protective T-cell mediated immunoreaction should primarily involve macrophages that have ingested the infectious agent and then present its peptide antigens via MHCII-class molecules to T-cells with a specific receptor (for review, see ref 12). Macrophages then are activated via lymphocytic IL2 and IFN- γ secretion and thus, may kill the ingested parasite efficiently. Consequently, if such a model is valid for a protective immune response against invasive aspergillosis, only the very first part of the infection, which starts with the ingestion of inhaled *Aspergillus* conidia by resident alveolar macrophages, should be attributed to those immune mechanisms. All other growing stages of *Aspergillus* as young hyphae and mycelia, are too large to be ingested and thus may be only a target for an unspecific attack by granulocytes or, as proposed recently a specific target for cytotoxic lymphocytes that do not follow the known classical immune mechanisms.¹³ At any rate, in immunocompetent humans, the first line of defense is the resident alveolar macrophage. Conidia are not killed immediately after ingestion but when they start to swell and become metabolically active.¹⁴ This mechanism per se is not

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the result of a specific immune response. However, if such a response is present, and conidial antigens were presented to the macrophage surface, killing of conidia via T-cell dependent activation of these macrophages would likely be far more efficient. In the situation of immunosuppression, where the natural unspecific defense functions of macrophages are impaired, or where the second unspecific line of defense, the granulocytes are impaired in number or function, specific activation of macrophages may substantially enhance the residual defense against aspergillosis. In this connection, proteins associated with the conidial surface and thus, readily accessible to the enzymes of the fungiphorus vacuole seem to be the first candidates that may supply peptide antigens for MHCII-presenting on the macrophage surface. They may therefore be ideal candidates for a future recombinant vaccine prophylactically administered to high-risk patients.

Conidial antigens could also be decisive for the pathogenesis of *Aspergillus*-related allergic diseases since *Aspergillus* asthma, the extrinsic allergic alveolitis (EAA) and the allergic bronchopulmonary aspergillosis (ABPA) (for reviews see refs 3,15,16) start with the inhalation of the conidia. In the case of *Aspergillus* asthma and EAA, an immediate reaction or a reaction with several hours delay respectively, is typical.³ In contrast, in ABPA patients the fungus colonizes the lungs^{17,18} and allergic reactions are not exclusively directed against conidial antigens but most likely also against antigens of the mycelial phase. For the pathogenesis of allergic disease, especially for *Aspergillus* asthma and EAA, antigens of the conidial surface may be a clue to the pathogenesis and thus be of outstanding interest.

Using 2D-gel electrophoresis and mass spectrometry, we have therefore analyzed the proteins released from intact viable conidia on the basis of the recently annotated *Aspergillus* genome.

Materials and Methods

Strains and Culture Conditions. In all experiments, *A. fumigatus* D141 (NRRL 6585; U.S. Department of Agriculture, Peoria, IL) as a clinical isolate derived from an aspergilloma that had developed in a 45-year-old human with tuberculosis¹⁹ was used. The fungus was grown on regular Sabouraud-agar plates at 37 °C for 3 days prior to extraction of conidial surface or cellular proteins.

Extraction of Conidial Surface Proteins. Conidia of 45 densely sporulated plates were suspended in 300 mL of 10 mM sodium phosphate buffer pH 6.0 + 0.9% NaCl + 1 mM phenylmethylsulfonylfluoride (PMSF) (Serva, Heidelberg, Germany) + 1 μ M pepstatin (Peptide Institute, Osaka, Japan). Large aggregates of conidia, conidiophores, and hyphae were then removed by rigorous shaking on a vortex mixer and a brief centrifugation step, followed by passage of the supernatant through woven fabric with a mesh opening of approximately 5 μ m (Sefar, Wasserburg, Germany). The flow through, which was examined by microscopy and contained conidia only but no other visible fungal elements, was then transferred to a bottle top filter (0.2 μ m (Corning, NY)) and conidia were washed twice with the suspension buffer. After the second washing, the buffer was drained completely, conidia were weighed and about 6 g were suspended in a 50 mL polystyrene tube containing 40 mL 0.1 M Tris-HCl-buffer pH 8.5 + 50 μ L of a recombinant 1,3- β -glucanase (20 U/ μ L) (Quantazyme, MP Biomedicals, Eschwege, Germany) also containing 1 mM 1,10 phenanthroline (Sigma, Taufkirchen, Germany) + 1 μ M chy-

mostatin (Sigma) + 1 μ M leupeptin (Sigma) + 1 μ M E-64 (Sigma) + 1.0 μ M pepstatin. The sample was then incubated for 1 h at 21 °C with constant gentle movements (vertical turning wheel: 10 rpm) allowing the proteins from the conidial surface to be released into the buffer. Subsequently, the tube was centrifuged (5000 $\times$ g; 10 min), and the supernatant aspirated with a 30 mL syringe and passed through a 0.2 μ m syringe filter (Corning, NY) to remove any residual conidia. Pelleted conidia were not discarded but frozen until needed for cellular protein extraction (see protocol below). After addition of 100 μ L 0.15% deoxycholic acid solution (Sigma) per mL supernatant and incubation for 15 min at room temperature, proteins were precipitated with 50 μ L trichloroacetic acid (Sigma) per mL sample and incubation on ice for 30 min. Precipitated proteins were pelleted by centrifugation (15 000 $\times$ g; 15 min; 4 °C). Then, 14 mL of 10 mM Na-phosphate buffer pH 7.4 were pipetted on the drained pellet, which was dissolved under continuous addition of 1 M NaOH to keep the pH in the neutral range. This protein solution was incubated for 1 h with vigorous shaking at room temperature. Residual nondissolved particles were removed by another centrifugation step (15 000 $\times$ g; 30 min; 4 °C). Thereafter, the protein solution was dialyzed against H₂O and reduced to a volume of approximately 500 μ L using Vivaspinn concentrator tubes (10 000 MWCO PES) 20 and 2 mL according to the protocols of the manufacturer (Vivascience, Hanover, Germany). Finally, the protein concentration of the sample was determined following the Bradford method²⁰ using Bioquant protein reagent solution (Merck, Darmstadt, Germany). Samples were stored in aliquots at -80 °C until their use for 2-DE.

Extraction of Conidial Cellular Proteins. For extraction of cellular proteins, conidia from which the surface proteins had been extracted (see above) were thawed, weighed and 5 g (wet weight) were suspended in 50 mM Tris-HCl pH 7.0 + 1 mM PMSF + 1 μ M pepstatin to a final volume of 10 mL. This suspension was then equally distributed between 10 PP Micro tubes (2 mL) (Sarstedt, Nümbrecht, Germany) and 0.4 volumes of glass beads ($\varnothing$ 0.5 mm) were added to each tube. The tubes were then placed into a Fast Prep FP 120 cell disrupter (Bio101, Savant). Disruption of conidia was achieved at 6.5 m/s for 20 s followed by a brief cooling step on ice. This disruption cycle was repeated 25 times. Subsequently, samples were centrifuged (10 000 $\times$ g; 10 min). The supernatants containing the proteins were pooled into a fresh tube. Conidial debris was removed from these samples via an ultracentrifugation step (100 000 $\times$ g; 60 min). Thereafter, the protein solution was dialyzed against H₂O and reduced to a volume of approximately 500 μ L using Vivaspinn concentrator tubes as described above for the extraction of conidial surface proteins. Finally, the protein concentration of the sample was determined with the Bradford method²⁰ followed by its storage at -80 °C until use in 2-DE.

2D-Gel Electrophoresis and Staining Procedures. The first dimension was accomplished with an IPGphor isoelectric focusing unit (Amersham Pharmacia Biotech, Freiburg, Germany) and 18 cm pH 4–5 L or pH 3–10 NL Immobililine DryStrip (Amersham Pharmacia order No.: 17-6001-84 (pH 4–5 L) and 17-1235-01 (pH 3–10 NL)). Sample aliquots generated as described above, containing 650 μ g protein were freeze-dried, then redissolved in standard rehydration buffer and thereafter focused for a total of 100 000 Vhrs according to the manual and instructions of the manufacturer.

For the second dimension, SDS-polyacrylamide gels 19 $\times$ 23 $\times$ 0.15 cm with an acrylamide gradient of 15–18% were

prepared using a Hoefer DALT Gradient Maker and a Hoefer DALT Multiple Gel Caster (both Amersham Pharmacia). The IPG strips from the first dimension were loaded onto the slab gels together with a molecular mass size marker and run in a Hoefer DALT Electrophoresis Tank at 100 V overnight following the instructions of the manufacturer.

After electrophoresis gels were stained with colloidal Coomassie blue using Roti-Blue CBBG-250 solution (Roth, Karlsruhe, Germany) using the protocol provided by the supplier.

In Gel Digestion of Proteins and Extraction of Peptides.

Coomassie blue stained spots were manually excised from the gels and washed with distilled water for 15 min. The destaining procedure comprised three 5 min washing steps alternately with 50% acetonitrile (ACN) and 100 mM ammonium bicarbonate. Destained gel slices were then dehydrated with ACN for 15 min and completely dried in a vacuum centrifuge. Thereafter, the slices were rehydrated for digestion with 40 μ L trypsin (Promega, Mannheim, Germany) (10 ng/ μ L in 100 mM ammonium bicarbonate) for 45 min on ice. Excess trypsin solution was removed and the volume was replaced with the digestion solution but without trypsin. After overnight digestion at 37 °C, peptides were extracted with repeated cycles of different concentrations of ACN and trifluoroacetic acid (TFA). At the end of the extraction, solutions were pooled and completely dried in a vacuum centrifuge.

Protein Sequence Analysis by LC–MS/MS. Extracted peptides were dissolved in 0.1% formic acid (FA) and one microliter of sample was introduced using a CapLC autosampler (Waters, Manchester, UK) onto a μ -precolumn Cartridge (C18 pepMap, 300 μ m $\times$ 5 mm; 5 μ m particle size, LC Packings Idstein, Germany) and further separated through a C18 pepMap100 nano Series (75 μ m $\times$ 15 cm; 3 μ m particle size) analytical column (LC Packings Idstein, Germany). The mobile phase consisted of solution A (5% ACN in 0.1% FA) and solution B (95% ACN in 0.1% FA). The total sample running time was set to 60 min. Peptide sequencing was performed on a Q-TOF Ultima Global (Waters) mass spectrometer equipped with a nanoflow ESI Z-spray source in the positive ion mode. Data were acquired using MassLynx (v 4.0) software (Waters) on a Windows NT PC, and were further processed on a ProteinLynx-Global-Server (PLGS) v. 2.1, (Waters, Manchester, UK).²¹ The raw data files were deconvoluted and deisotoped using the Max Ent lite algorithm (Waters). A PLGS module was used to generate Mascot-searchable *.pkl files. The *.pkl processed data were searched against a NCBI database via a Mascot search engine using a peptide mass tolerance of 100 ppm. The search criteria were set with one missed cleavage by trypsin allowed and protein modifications set to methionine oxidation and carbamidomethylcysteine when appropriate. Scanning of identified proteins for signal sequences was done with the program SignalP 3.0 Server²² available at <http://www.cbs.dtu.dk/services/SignalP/>.

Genomic data for *A. fumigatus* were provided by The Institute for Genomic Research (www.tigr.org/tdb/e2k1/afu1) and The Wellcome Trust Sanger Institute (www.sanger.ac.uk/Projects/A_fumigatus); genomic data for *A. nidulans* were provided by The Broad Institute (<http://www.broad.mit.edu/annotation/fungi/aspergillus/>); and genomic data for *A. oryzae* was provided by The National Institute of Advanced Industrial Science and Technology (<http://oryzae.cbrc.jp/> and www.bio.nite.go.jp/dogan/Top). The analysis of these data was enabled by an international collaboration involving more than

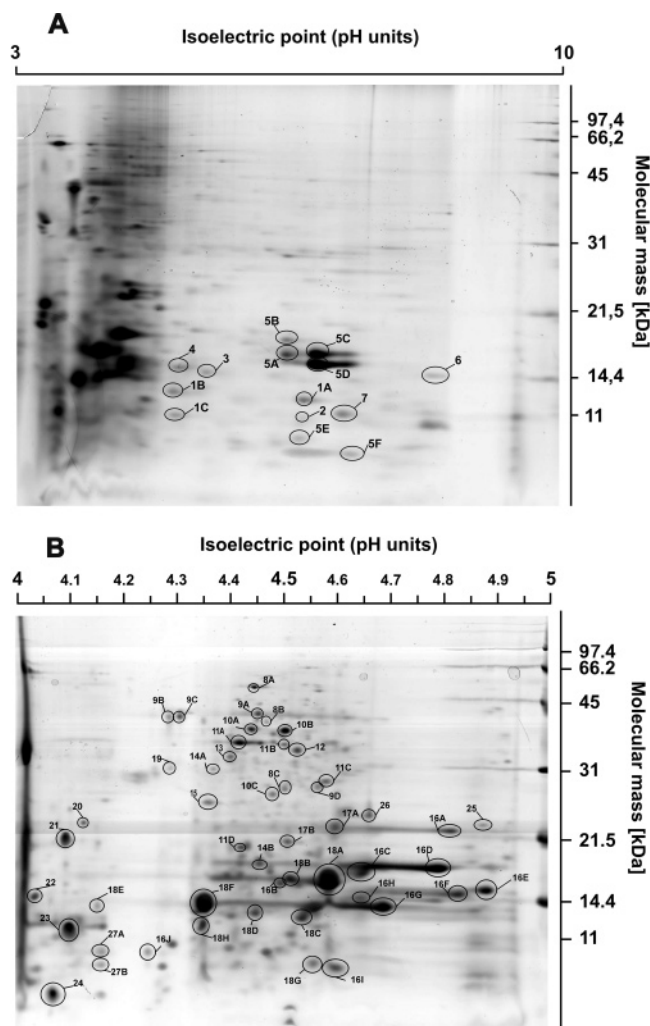


Figure 1. 2-DE (pH 3–10 NL (1A) and pH 4–5 L (1B); 15–18% acrylamide gradient) of *A. fumigatus* conidial surface proteins extracted with mild alkaline buffer (pH 8.5) in the presence of a 1,3- β -glucanase. About 650 μ g of proteins were loaded on pH-gradient strips and then separated horizontally according to their isoelectric point followed by vertical acrylamide gel separation according to their molecular mass. After Coomassie staining, encircled spots were excised, in-gel trypsin digested and subjected to liquid chromatography coupled with tandem mass spectrometry. The results of spot identification are provided in Tables 1 and 2. Note that most proteins focused in the acid pH-range between pH 4 and 5 (1B).

50 institutions from 10 countries and coordinated from Manchester, UK (www.cadre.man.ac.uk and www.aspergillus.man.ac.uk).

Results

A. fumigatus conidial surface extraction according to Materials and Methods from 45 densely grown regular Sabouraud agar plates resulted in an average total of 850 μ g protein. Approximately 650 μ g were applied to a pH 3–10 nonlinear pH gradient strip followed by electrofocusing and second-dimension SDS-PAGE including Coomassie staining (Figure 1A). Since the bulk of proteins focused between pH 4 and 5, a corresponding linear immobilized pH gradient strip was used for another 2-DE in order to provide additional spot separation (Figure 1B).

Table 1. Results of Spot Identification of Conidial Surface Proteins (2-DE pH 3–10 NL; Figure 1a)

spot no.	match ^a	sequence coverage ^b (%)	identification (<i>A. fumigatus</i> Af293)	accession no.	score ^c	theoretical pI/mass (kDa)	observed range of pI/mass (kDa)	signal peptide
1A	3	10	allergen Asp F3	EAL85811	52	5.4/18.4	6.5/12.5	no
1B	2	10	"	"	51	"	4.7/13	"
1C	4	19	"	"	83	"	4.8/10	"
2	6	8	hypothetical protein Afu1 g11480	EAL90475	65	6.6/20.5	6.6/11	yes
3	5	32	hypothetical protein Afu2 g08190	EAL93116	156	5.4/13.6	5.2/15	no
4	10	26	acyl CoA binding protein family	EAL93401	118	5.4/17.3	4.8/17	no
5A	13	75	CipC protein	EAL91668	325	5.8/15	6.3/15	no
5B	6	40	"	"	211	"	6.3/16	"
5C	8	40	"	"	225	"	6.5/15	"
5D	41	67	"	"	346	"	6.5/14.5	"
5E	20	46	"	"	231	"	6.4/8	"
5F	20	46	"	"	231	"	7/7	"
6	8	31	Single-strandbinding protein family domain protein	EAL91809	260	10.4/20.5	8.5/14.4	yes
7	5	51	hypothetical protein Afu1 g09890	EAL90318	320	5.9/9.2	7/11	no

^a Match = Number of peptides matched from protein in MS/MS query. ^b Sequence coverage = percentage of amino acid sequence of protein covered in MS/MS analysis. ^c MASCOT scores > 24 indicate identity or extensive homology ($p < 0.05$).

Fifty-one spots were excised from the Coomassie stained 3–10 NL and 4–5 L gels, respectively. All spots were trypsin digested and peptides were extracted as described in Materials and Methods. The Extracted peptides were analyzed by liquid chromatography coupled with tandem mass spectrometry to obtain de novo sequencing data as described. The search against a NCBI database resulted in the identification of all proteins except one as being attributable to *A. fumigatus* expression products that were postulated via the annotation of the recently sequenced fungal genome.²³ Sequence coverage to respective *A. fumigatus* proteins ranged between 4 and 59% (Tables 1 and 2). Typical MS/MS spectra generated by Q-TOF (ESI–MS/MS) from three of these proteins are shown in Figure 3. The peptides from one protein spot did not significantly match with any *A. fumigatus* protein but with alcohol dehydrogenase 1 from *Saccharomyces pastorianus* (Spot No. 12 in Figure 1B and Table 2). Several spots were attributable to the same *Aspergillus* proteins, which resulted in an overall identification of 26 different *A. fumigatus* proteins as part of the conidial surface proteome. Of these 26 proteins, 12 contained a signal peptide while 14 did not (Tables 1 and 2). The three most dominant protein spots in the pH 4.0–5.0 gel (Spot Nos. 16, 18, and 23) represented a conserved hypothetical protein (Acc. No. EAL90699), the spore coat hydrophobin Hyp1 (Acc. No. EAL91643) and another hypothetical protein (Afu6 g14470; Acc. No. EAL89283). Both contained a signal peptide. Conidial surface spots listed in Tables 1 and 2 were identified not only in the gel depicted in Figure 1, parts A and B, but were confirmed with additional gels run with similar protein preparations and under similar conditions.

The extraction of cellular conidial proteins (see Methods and Materials) derived from 5 g of conidia, the surface proteins of which had already been extracted, resulted in an overall yield of approximately 2250 μ g protein in 1500 μ L H₂O. This cellular protein fraction was also subjected to 2-DE under similar conditions to those used for the conidial surface protein fraction (Figure 2, parts A and B). The spot pattern obtained with the pH 4.0–5.0 gel as well as with the 3.0–10.0 gel was

quite different compared to the corresponding spot pattern obtained for the protein surface fraction (Figure 1, parts A and B).

Discussion

On the basis of the recently sequenced and annotated *A. fumigatus* genome,²³ 26 proteins of the conidial cell surface were identified by proteome analysis. Among those proteins, the most prominent is the spore coat hydrophobin Hyp1 (No. 18 in Figure 1B) a fungal hydrophobin that is also known as the rodlet A protein in *A. fumigatus*.^{24–26} The rodlet A protein constitutes a major part of the outer conidial cell wall layer and disruption mutants of the corresponding gene did not display a rodlet layer.²⁶ The identification of this hydrophobin migrating in prominent spots in the 2-DE underlines that the experimental conditions for extracting conidial surface proteins were suitable. These conditions consisted of a mild alkali treatment of conidia at pH 8.5 combined with a 1,3- β -glucanase digestion. The results were confirmed in several independent experiments. The addition of glucanase to the extraction buffer did not substantially change the protein spot pattern, but enhanced protein extraction efficacy (not shown). Thus, most extracted proteins are not covalently linked to the conidial surface, but rather attached to it noncovalently, likely on a hydrophobic interaction basis.

At this point, it is important to emphasize that we do not claim to have extracted all noncovalently bound conidial surface proteins but only those that are released under conditions of mild alkali treatment. Changing pH to acidic values did not lead to liberation of proteins in substantial amounts (not shown). Strong alkaline conditions were avoided since these might have resulted in loss of protein integrity as well as unwanted liberation of cellular nonsurface proteins. For comparative purposes, the latter were extracted after mechanical destruction of those conidia that had undergone previous surface protein extraction. A completely different pattern was observed in 2-DE (Figure 2, parts A and B). This difference in pattern should serve as additional evidence that the conidial

Table 2. Results of Spot Identification of Conidial Surface Proteins (2-DE pH 4–5 L; Figure 1B)

spot no.	match ^a	sequence coverage ^b (%)	identification (<i>A. fumigatus</i> Af293)	accession no.	score ^c	theoretical pI/mass (kDa)	observed range of pI/mass (kDa)	signal peptide
8A	21	43	disulfide isomerase, putative	EAL87706	738	4.6/56.2	4.4–4.5/55	yes
8B	13	31	“	“	303	“	4.4–4.5/33	“
8C	20	36	“	“	726	“	4.5–4.6/26	“
9A	10	31	aspartic endopeptidase Pep2	EAL92441	357	4.8/43.3	4.4–4.5/45	yes
9B	6	20	“	“	304	“	4.3/43	“
9C	6	20	“	“	312	“	4.3–4.4/43	“
9D	6	24	“	“	226	“	4.5–4.6/26	“
10A	9	25	hypothetical protein Afu8 g01980	EAL84970	225	4.5/45.9	4.4–4.5/33	no
10B	11	26	“	“	391	“	4.4/33	“
10C	4	9	“	“	218	“	4.4–4.5/25	“
11A	11	44	eukaryotic translation elongation factor 1 beta subunit (EF-1-beta), putative	EAL90446	467	4.5/30.1	4.4–4.5/32	no
11B	11	25	“	“	199	“	4.5/32	“
11C	8	36	“	“	268	“	4.5–4.6/27	“
11D	4	19	“	“	160	“	4.4–4.5/17	“
12	8	25	alcohol dehydrogenase 1 (<i>Saccharomyces pastorianus</i>)	AAP51050	266	6.4/36.8	4.5–4.6/31	no
13	6	18	extracellular lipase, putative	EAL86100	180	5.6/31.4	4.4/31	yes
14A	6	30	WW domain protein	EAL85931	162	4.4/28.4	4.3–4.4/29	no
14B	1	4	“	“	42	“	4.4–4.5/ 14.4–21.5	“
15	4	40	phosphoglycerate mutase family protein, putative	EAL86798	186	4.7/20.8	4.3–4–4/24	yes
16A	10	51	conserved hypothetical protein	EAL90699	278	4.9/28.2	4.85/21.5	yes
16B	5	22	“	“	126	“	4.5/15	“
16C	10	33	“	“	471	“	4.6–4.7/16	“
16D	7	33	“	“	371	“	4.8–4.9/16	“
16E	10	26	“	“	413	“	4.9–5.0/15	“
16F	9	33	“	“	409	“	4.8–4.9/14.4	“
16G	19	39	“	“	426	“	4.8–4.9/13.5	“
16H	9	33	“	“	297	“	4.6–4.7/14.4	“
16I	4	12	“	“	58	“	4.6–4.7/5–11	“
16J	5	24	“	“	45	“	4.2–4.3/10	“
17A	5	21	hypothetical protein Afu6 g04690	EAL85571	151	5.1/22.9	4.6/22	yes
17B	5	26	“	“	133	“	4.5/21.5	“
18A	10	50	spore coat hydrophobin Hyp1	EAL91643	366	4.9/16.2	4.6/15	yes
18B	4	38	“	“	169	“	4.4–4.5/ 14.4–21.5	“
18C	4	43	“	“	236	“	4.5–4.6/12	“
18D	5	50	“	“	285	“	4.4–4.5/12	“
18E	2	18	“	“	51	“	4.2/13	“
18F	14	74	“	“	495	“	4.3–4.4/14.4	“
18G	2	18	“	“	47	“	4.6/5–11	“
18H	5	59	“	“	226	“	4.3–4.4/11	“
19	3	8	conserved hypothetical protein, putative	EAL85104	38	5.2/38.2	4.3/31	yes
20	4	10	p21 protein	EAL91226	68	4.4/22.2	4.1/22	no
21	2	9	hypothetical protein Afu1 g10260	EAL90354	116	5.7/27.5	4.1/21.5	no
22	3	53	60s acidic ribosomal protein superfamily	EAL88394	83	4.1/11.1	4.0/14.4	no
23	2	4	hypothetical protein Afu6 g14470	EAL89283	66	4.4/26.0	4.1/11	yes
24	1	18	conserved hypothetical protein	EAL87941	167	4.4/11.8	4.0–4.1/5–10	no
25	9	51	tropomyosin 1	EAL87049	302	4.9/17.9	4.9/22	no
26	5	40	conserved hypothetical protein	EAL86162	152	4.9/22.0	4.7/21.5–31	yes
27A	1	18	conserved hypothetical protein	EAL87941	74	4.4/11.8	4.1–4.2/8–11	no
27B	2	29	“	“	107	“	4.1–4.2/8–11	“

^a Match = Number of peptides matched from protein in MS/MS query. ^b Sequence coverage = percentage of amino acid sequence of protein covered in MS/MS analysis. ^c MASCOT scores > 24 indicate identity or extensive homology ($p < 0.05$).

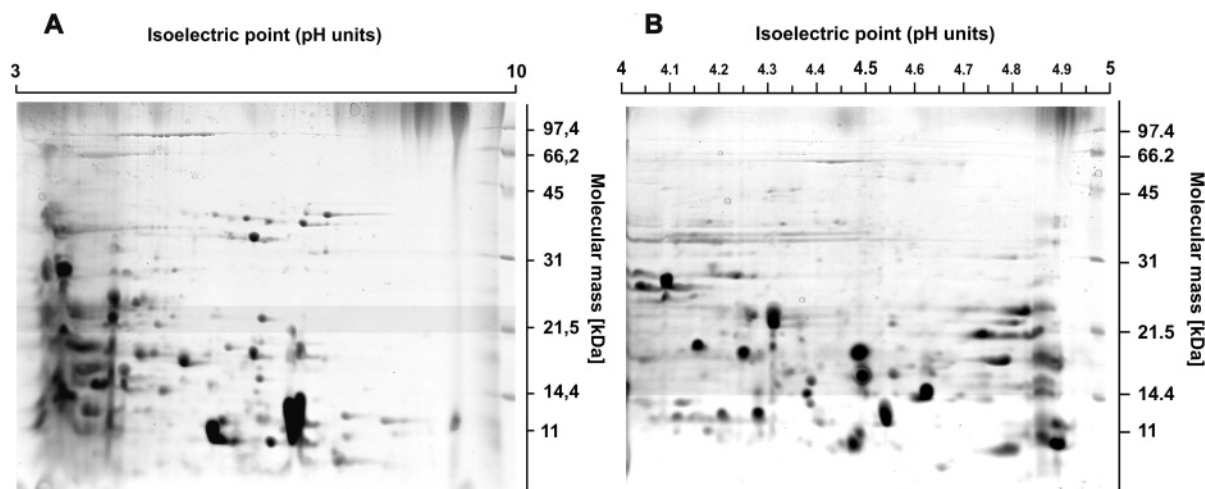


Figure 2. 2-DE (pH 3–10 NL (2A) and pH 4–5 L (2B); 15–18% acrylamide gradient) of *A. fumigatus* cellular proteins obtained by mechanical destruction of conidia, the surface proteins of which had been previously extracted to obtain the protein fraction used for the gels displayed in Figure 1, parts A and B. Note that the protein patterns compared to the surface protein patterns depicted in Figures 1A and B are different.

surface protein fraction that was analyzed (displayed in Figure 1, parts A and B) was not substantially contaminated by cellular protein components. The 26 proteins that were identified in this study as being a part of the *Aspergillus* conidial surface proteome may be divided into those with a signal for secretion and those without.

Proteins that Contain a Signal Peptide. Altogether 12 from 26 *A. fumigatus* proteins displayed a signal sequence for secretion. Among these, hydrophobin Hyp1 (No. 18 in Figure 1B) is a known major conidial surface protein. This and several other proteins did not occur as a single spot, but rather as several spots with various IPs and molecular masses. The most likely explanation for this behavior either lies in variable glycosylation of the respective proteins or, in their partial hydrolysis due to proteolytic activity present on the conidial surface. Strong proteolytic activity of the conidial surface associated with the degradation of complement (C3) bound to the conidial surface,²⁷ a feature that may influence pathogenicity, has been described.²⁸ As we were conscious about such activity, various proteinase inhibitors were included in the extraction buffers, but proteolytic action may well have taken place before extraction while the conidial surface had developed.

Compatible with the putative proteolytic activity of the conidial surface, one of the proteins was identified as the endoprotease PEP2 (No. 9 in Figure 1B). An orthologue of this enzyme, is known to have a vacuolar location in *Saccharomyces cerevisiae*, and it is also known to be secreted when overexpressed in yeast.^{29,30} In accordance with this finding, PEP2 was previously isolated from an *A. fumigatus* cell wall fraction.³⁰ Its presence on the conidial surface may well have some impact on *Aspergillus* related allergic diseases such as asthma since proteolytic activity is a central biochemical property that endows molecules with intrinsic allergenicity.³¹ Thus, if not an allergen itself, as is the case for its relative the secreted aspartic protease PEP1 (Asp f 10),³² PEP2 on the conidial surface may well play a pivotal role in the processing of allergens. Indeed, in this context, it has been demonstrated, that fungal proteases are specifically capable of conferring potential to otherwise innocuous allergens applied to the respiratory tract by acting as an essential adjuvant factor.³³

Among the proteins isolated from the conidial surface is an extracellular lipase (No. 13 in Figure 1B). In contrast to other fungi, lipases in *A. fumigatus* are not very thoroughly characterized. However, the presence of a lipase as part of the conidial surface may be of importance for the interaction with the host cell at an early stage when the conidia are inhaled. Lipases could induce cell damage as well as facilitate adherence. As a parallel example, a lipase from the plant pathogenic fungus *Alternaria brassicicola* was detected by SDS-PAGE and immunoblotting in the water washings of ungerminated spores. Anti-lipase antibodies were added to a conidial suspension of *A. brassicicola* prior to inoculation. As a result, blackspot lesions were reduced by 90% on intact cauliflower leaves, but not on leaves from which surface wax had been removed. Thus, spore surface-bound lipase is thought to interact closely with epicuticular leaf waxes for adhesion and/or penetration of the fungal propagules during the early stages of host-parasite interactions.³⁴ In *A. fumigatus*, the role of this lipase for virulence, and thus possibly also as a vaccine candidate remains to be determined by further investigations involving also gene knock-out experiments.

Another potential vaccine candidate and allergen that was isolated from the conidial surface represents a putative disulfide isomerase (No. 8 in Figure 1B). Disulfide isomerases have been described in other *Aspergilli* as well as in *A. fumigatus*.^{35,36} Nigam et al. suggested that they possibly play an important role in the protein folding mechanisms of *A. fumigatus* antigens/allergens, since they isolated such an enzyme via the screening of a cDNA expression library with sera of patients suffering from ABPA.³⁶ Most likely, the disulfide isomerase that was isolated from the conidial surface and identified as such on the basis of the annotated *A. fumigatus* genome is encoded by the same gene as the isomerase sequenced by Nigam et al., despite substantial sequence variation toward the middle and end of the protein. This assumption is justified because the first 100 amino acids of the N-terminus including the usually variable signal of both proteins are completely identical and because the genome project so far has revealed only a single gene to encode this sequence (own blast search).

One of the proteins identified having a signal for secretion putatively belongs to the phosphoglycerate mutase family (No.

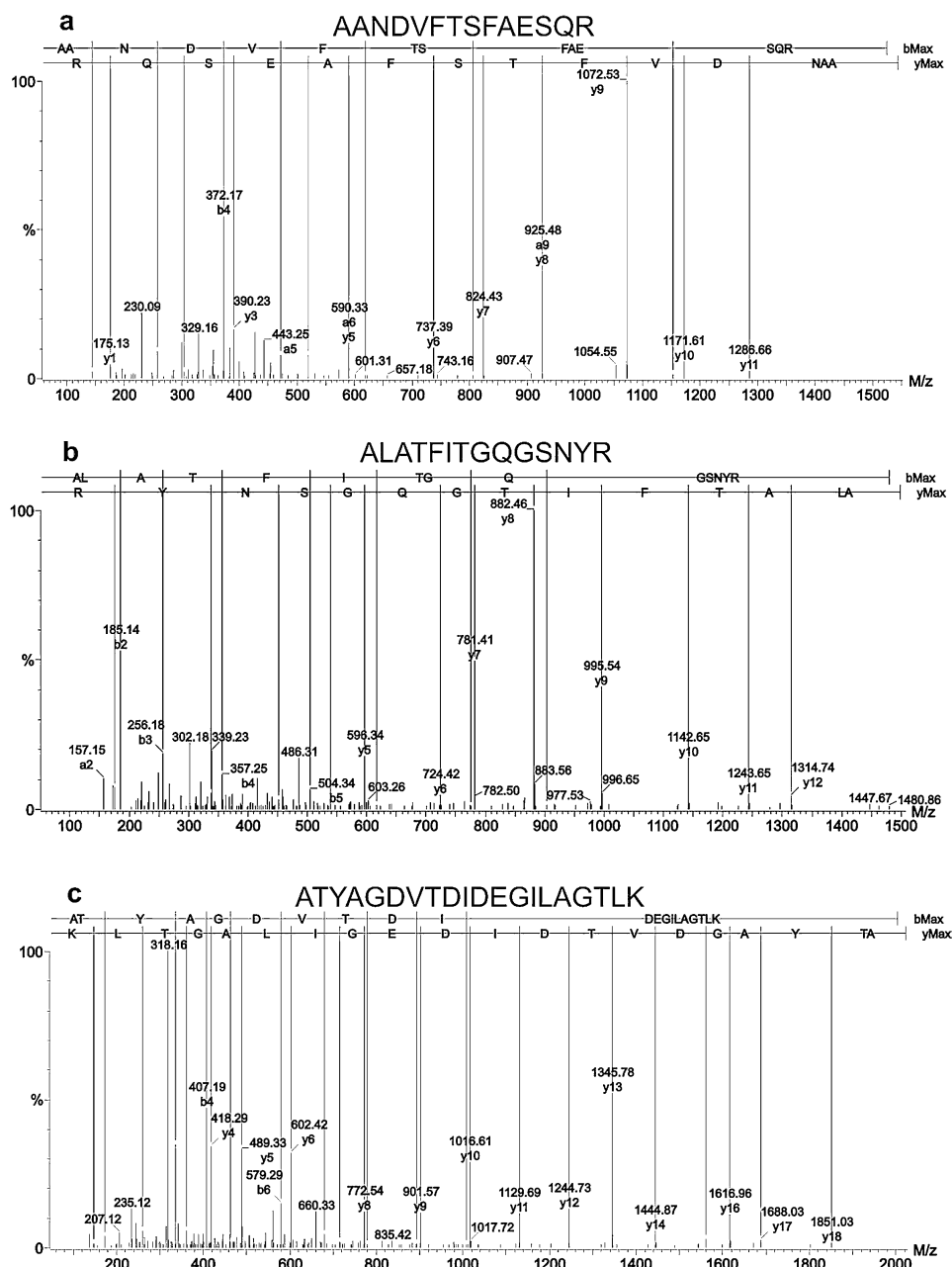


Figure 3. MS/MS spectrum generated by Q-TOF (ESI–MS/MS) for doubly charged precursor ions of (a) m/z 771.87, identified as protein disulfide isomerase (Spot No. 8, Table 2), (b) m/z 749.92, identified as extracellular lipase (Spot No. 13, Table 2) and (c) m/z 1011.94, identified as hydrophobin (Spot No. 18, Table 2). The amino acid positions of the identified sequences in the respective proteins are as follows: 166–179 (disulfide isomerase; Acc. No. EAL87706), 212–225 (extracellular lipase; EAL86100), and 68–87 (hydrophobin; Acc. No. EAL91643).

15 in Figure 1B), and exhibit a conserved GpmB region (NCBI conserved domain search),³⁷ typical for enzymes with a fructose-2,6-biphosphatase activity. This protein may well be involved in carbohydrate metabolism of the cell wall and thus, possibly in germination of the activated conidium in the host.

Seven other putatively secreted proteins with still unknown function were identified in the alkaline wash of *A. fumigatus* conidia (Nos. 2, 6, 16, 17, 19, 23, and 26 in Figure 1, parts A and B). Two of these proteins (Nos. 16 and 23) are present in fairly high amounts. Those two proteins are therefore of special interest and will be the subject of future investigations in terms of recombinant expression, functional analysis, and vaccination trials.

Proteins without a Signal Peptide. Fourteen from 26 *A. fumigatus* proteins derived from conidial surface extraction did not reveal a signal for cellular secretion. One might argue that these proteins may be contaminants from intracellular material. However, conidia were treated in a way that should not have harmed their integrity. Furthermore, corresponding spots in the 2-DE gels of the conidial surface protein fraction (Figure 1, parts A and B) were not present or visibly prominent in the gels representing the intracellular protein fraction (Figure 2, parts A and B) neither were observed comparing the gels by spot match analysis (not shown). In addition, it is now widely accepted that nonclassical secretory pathways exist, which do not require the presence of a signal peptide.^{38–40} Accordingly,

many proteins previously thought to have an intracellular location, only are also present in fungal cell walls.⁴¹

Among those proteins for example is the elongation factor 1 (No. 11 in Figure 1B), that has been shown to have an additional surface location in *Candida albicans*.⁴¹ Interestingly, the same subunit of elongation factor 1 (beta) that has been identified in this study as being a part of the conidial surface of *Aspergillus*, has been identified as a novel allergen in another fungus, *Penicillium citrinum*.⁴² In the tape *Echinococcus granulosus* the orthologue elongation factor 1 subunit was presumed to play a key role in the allergic disorders and in the complex host-parasite relationship of patients with cystic echinococcosis.⁴³

Another, already known allergen of *A. fumigatus*, the Asp f 3 was also identified (No. 1 in Figure 1A). This allergen (also known as AHP1), is produced by the fungus⁴⁴ and is thought to be a peroxisomal membrane protein. In addition to its allergenic potency and presence on the conidial surface, it is also expressed by hyphae during invasive disease as indicated by a regularly occurring corresponding antibody response.⁴⁵

An intriguing protein found to be conidial surface associated is CipC (No. 5 in Figure 1A). The name CipC derives from concanamycin-induced protein because its orthologue in *A. nidulans* was shown by 2DE-analysis of an intracellular protein fraction to be upregulated in response to the antibiotic concanamycin A, produced by *Streptomyces* species.⁴⁶ To our knowledge, nothing is known about the exact function of CipC. However, the presence of the protein on the conidial surface infers direct rather than indirect tasks in competitive interactions between bacteria and filamentous fungi.

Another four proteins identified on the conidial surface are associated with specific functions: tropomyosin (No. 25 in Figure 1B), a protein of the acyl CoA-binding protein family (No. 4 in Figure 1A), a p21 protein that is a putative co-chaperon of Hsp90 (No. 20 in Figure 1B) and a protein belonging to the 60s acidic ribosomal protein superfamily (No. 22 in Figure 1B). With the exception of the ribosomal protein spot, the other protein spots were rather weak. A Ribosomal protein associated with a fungal cell surface has already been described.⁴¹

No specific function was attributable to 7 of the 14 proteins without a signal peptide (Nos. 3, 7, 10, 14, 21, 24 and 27 in Figures 1, parts A and B), and one protein (No. 12 in Figure 1B) could not be identified as an *A. fumigatus* derived protein at all, but rather as a protein that has significant similarities to alcohol dehydrogenase 1 from *Saccharomyces pastorianus*. As the corresponding orthologue from *A. fumigatus* did not show a significant peptide match, the identity of this protein spot remains unclear. However, deduced from spot intensity, 3 of the 6 proteins identified as *Aspergillus* derived (Nos. 10, 21, and 24 in Figure 1B) are present on the conidial surface in fairly high amounts and, as mentioned for the proteins with signal peptides but without attributable function, should be the subject of further investigations.

In addition to their potential use as vaccine candidates against invasive aspergillosis, conidial surface proteins are of interest because of their possibly crucial role in triggering certain *Aspergillus*-related allergic diseases. The tool of proteomics together with the recently annotated genome of *A. fumigatus* offers novel strategies to complete the list of allergens and vaccine candidates as well as molecules decisive for the pathogenesis of invasive aspergillosis.

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Supporting Information Available: Peptides identified within the protein sequences are available as supplemental data at <http://pubs.acs.org>. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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