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Identification of spore specific allergens from *Penicillium chrysogenum*

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Abstract

Background: The common indoor mould *Penicillium chrysogenum* is reported to be associated with respiratory diseases and allergic asthma. Beside the already known specific allergens stemming from mixtures of hyphae and spores the question was which allergens are specifically abundant in the airborne spores.
Methods: Serum samples of 50 patients with allergic symptoms were screened for a positive reaction with spore proteins using 1D immunoblotting. The sera of 10 patients with different immunopositive patterns in the 1D blots were selected for 2D westernblotting and IgE reactive proteins were identified by mass spectrometry.
Results: 29 proteins specifically interacting with IgE from sera were indentified. 5 of them showed a frequency higher than 50% and were identified as Glyceraldehydes-3-phosphate dehydrogenase, Catalase A, delta-1-pyrroline-5-carboxylate dehydrogenase, glucose-6-phosphate isomerase and transaldolase. The vacuolar serine protease, known as the major allergen Pen ch 18, was identified in 3 cases.
Conclusions: Beside the confirmation of known allergens like Pen ch 18, new spore specific IgE binding potential allergens were identified. The differentiation into hyphae and spore specific allergens might be helpful for a more specified diagnosis and to differentiate the source of exposure leading to sensitization.

Keywords: Fungi allergens; Penicillium chrysogenum; SDS Page.

1. Introduction

Factors affecting indoor air quality are of increasing concern because, in industrialized societies, people spend up to 80% of their lifetime indoors [1]. There they are exposed to various factors including a large variety of chemical substances and allergens from different sources. Beside the house dust allergens, consisting mostly of house dust mite allergens, moulds are a dominant source of allergenic substances [1]. Among them there are species which occur predominantly in the outdoor environment but enter households by ventilation like species of *Cladosporium* and *Alternaria*. These typical outdoor fungi often grow on rotting plant material with a seasonal peak in summer [2].

In temperate areas construction and leakage problems or poor ventilation can result in increased dampness indoors promoting mould growth on organic material. The fungal profile in houses with dampness and mould problems is shifting to species of *Penicillium* and *Aspergillus*, such as *Penicillium chrysogenum*, *P.expansum* and *Aspergillus versicolor* with higher spore concentration in winter [3, 4].

In recent years the health risk of living in damp and mouldy environment was analysed in numerous studies. Mould exposure was identified as risk factor for various respiratory diseases, allergic symptoms and non specific symptoms like headache, eye and skin irritation [1, 5, 6]. Especial-

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ly, exposure with *Penicillium* spores is associated with asthma attacks and wheezing [7, 8].

The extraction of proteins was performed in earlier studies from complete fungal mycelium, consisting mostly out of hyphae and containing only a minor fraction of spores. The spores of *P. chrysogenum* are relative small with 2.6 µm as the average aero-dynamic diameter [9] resulting in a higher tendency to become airborne. This is in particular true in comparison to fungal hyphae, which are first most often attached to the substrate and second are much larger and therefore more prone to end up in the house dust rather than becoming airborne. Since the most direct contact of patients occurs via the airborne spores rather than by mycelium we investigated the IgE reactive proteins out of extracts from spores from *P. chrysogenum*.

From *P. chrysogenum* there are so far seven allergens described in the Allergome database [10]. The list includes the following allergens: Pen ch 13 (Subtilisin like serin protease; Uniprot:Q9URR2), Pen ch 18 (Vacuolar serine protease, Uniprot:Q9P8G3), Pen ch20 (N-Acetyl Glucosamidase; Uniprot:Q02352), Pen ch 31 (Calreticulin; Uniprot:Q2TL59), Pen ch 33 (an uncharacterized 16 kDa protein; Uniprot: B0L0W9), Pen ch 35 (Transaldolase) and Pen ch MnSOD (Mangan Superoxide Dismutase; Uniprot: B6H9W9). The best characterized allergen, the serine protease from the subtilisin-type [11] is described as Pen ch 13 [12]. The induction of airway inflammation is associated with changes in junctional structure alterations resulting in increased permeability [13]. Another serine protease, the vacuolar serine protease (Pen ch 18) [14] is described as allergen from *P. chrysogenum* [15] and also from several other fungi [16-18]. Among the different serine proteases there occurs a relative high amount of cross-reactivity of IgE antibodies [19]. The Pen ch 20 allergen, the N-acetyl glucosamidase [20] is involved in the modification of sugars, relevant for building the chitin layer of the fungal cell wall. Calreticulin (Pen ch 31) is a chaperone and as such of relative high abundance. A rather meager characterization is available for Pen ch 33, which was identified once and for which only the open reading frame without even an supposed function is known. In a recent study transaldolases, listed as Pen ch 35 were described as a family of cross-reacting allergens in *C. cladosporioides* and *P. chrysogenum* [21].

Mold spores can be associated with Type I – IV allergies, but the type I is the most prevalent [22]. Allergic rhinitis, allergic asthma and conjunctivitis are all symptoms of Type I allergies, which are caused by the interaction between IgE antibodies and the allergens [23]. The classical way to find new allergens is to blot protein extracts, probe with sera from affected patients and detect the interaction between IgEs and potential allergens by an IgE specific antibody. This works especially well, when the proteins from a crude mixture are separated to near homogeneity into spots by 2D-gel electrophoresis [24-26].

By this approach sera from 50 patients with symptoms of atopic diseases were screened by 1D gel IgE-immunoblotting and the patients with the strongest signals were selected for 2D-gel IgE immunoblotting. Among the identified IgE-binding proteins were well known allergens from *P. chrysogenum* and some so far not described putative allergens. The identified proteins were analysed in terms of their frequencies in order to obtain a semi-quantitative ranking of their importance and to allow a comparison between the spore specific allergen spectra from the one described for extracts obtained from hyphae or mixtures of hyphae and spores.

2. Material and Methods

Serum samples

Serum samples of 50 patients were randomly selected from a population of patients with allergy diagnoses. The samples were taken from a blood repository, more specifically from 33 women and 17 men, which age ranged between 12 and 73 years, with a mean of 46.2 years. These patients were diagnosed for different allergic diseases at the Medical Department for Dermatology and Allergology at the University of Leipzig. (urticaria/angioedema n=16, adverse drug reactions n=14, contact allergy n=5, anaphylaxis n=3, atopic eczema n=3, mastocytosis n=2, food allergy n=2, others n=5).

Cultivation of P. chrysogenum and preparation of spore extract

P. chrysogenum was cultivated for 21 days at 20°C in plastic flasks for cell culture containing dicloran-glycerol agar with chloramphenicol (DG 18, Oxoid, Wesel, Germany). The DG 18 agar reduces the growth mycelium and boosts the development of the spores [27]. The cultivated strain belongs to the Centre of Environmental Research (UFZ), Leipzig, Germany. The produced spores were collected from surfaces of the cultures and suspended in a solution of 3 mM chloramphenicol and 1 mM phenylmethylsulfonylfluoride (PMSF) in 50 mM Tris/HCl (pH 7.5). This suspension was centrifuged at 15,000 g at 4°C for 30 min. The pellet was resuspended in a solution of 3 mM chloramphenicol in 20 mM Tris/HCl (pH 7.5). The spores in this suspension were disintegrated using a bead mill (Retsch GmbH, Haan, Germany). The disintegration was conducted at 4°C for 1.5 h, adding 1 to 1.5 mg glass beads (d=0.75 to 1 mm) per 1 ml of suspension. The suspension resulted from the disintegration process was twice centrifuged at 16,000 g for 20 min at 4°C and the supernatant was frozen at -20°C. The protein concentration in this supernatant was determined using the DC Bio-Rad Protein Assay, (Bio-Rad Deutschland, Munich) which derives from the method of Lowry [28].

1D SDS-PAGE, 2D electrophoresis and Immunoblotting

A discontinuous SDS-Page with two different acrylamide concentrations was used for more precise bands. A 12% resolving gel (12%, 0.05% APS- 0.1% SDS, 0.075% TEMED,

0.375 Tris-HCl) and a 4% stacking gel which contains in contrast to the resolving gel 4% Acrylamide-Bisacrylamide and 0.125 M Tris-Cl (pH8.8) were produced.

A solution of 20% ice-cold TCA was used to precipitate 50 µg proteins after incubation for 4 h and the pellets were washed twice with 100% ice-cold acetone. The obtained pellet was dissolved in 10 µL sample buffer (Bromophenol Blue 0.1% (w/v), 5% 2-mercaptoethanol, 10% Glycerol, 2% SDS, 125 mM Tris-Cl), heated for 5 min at 60°C and applied to the gel.

For 2D-gel electrophoresis, 500 µg protein were purified by phenol extraction with subsequent solvent precipitation and washing steps as described earlier [29]. The resulting protein pellets were dried at ambient temperature and dissolved in 135 µl of DeStreak rehydration solution with 0.5% IPG (immobilized pH gradient) pH 3–10 non-linear (NL) buffer (v/v) (GE Healthcare, Uppsala, Sweden). The electrophoresis was performed as described earlier [30]. Gels were stained by Coomassie Brilliant Blue G-250 (CBB) staining (Roth, Kassel, Germany).

A second gel, which was handled parallel with the same procedure, was used for immuno-Blotting. The proteins were transferred by electroblotting to a nitrocellulose membrane with pores of 0.2 µm, (Schleicher & Schuell, Dassel, Germany) using a Western Blot chamber as described previously [31]. In brief the chamber XCell II Blot Module (Invitrogen, Karlsruhe, Germany) and 10 mM CAPS buffer (10 mM CAPS- *N*-cyclohexyl-3-aminopropanesulfonic acid, 10% (v/v) Methanol (pH 11) were used. A current of 1.5 mA/cm² was applied for 90 min at ambient temperature. The blotting efficiency was checked by reversible PonceauS staining (0.2% PonceauS in 3% TCA).

For immunological detection, the membranes were washed with distillate water and blocked with 3% BSA in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) overnight, and incubated with or without the serum samples that were diluted 1:40 in antibody buffer (TBS with 0.05% Tween 20 and 1% BSA) for 120 min at ambient temperature. Afterwards, the membranes were washed twice with TTBS (0.05% Tween 20 in TBS) for 10 minutes each and incubated with goat anti-human IgE secondary antibody conjugated with alkaline phosphatase (Sigma-Aldrich, Germany) in a dilution of 1:2000. After incubation for 2 h, the membranes were washed twice with TTBS and once with TBS. The chemiluminescence emitted was detected with the AP Conjugate Kit (Bio-Rad, Geisenhofen, Germany).

Protein identification by mass spectrometry

Protein spots that react positively with the serum samples were cut from polyacrylamide gels and digested overnight with trypsin (Sigma-Aldrich, Geisenhofen, Germany) as described previously [32]. The resulting peptides were eluted, concentrated by vacuum centrifugation and thereafter separated by RP nano-LC 1100 series (Agilent Technologies, Paolo Alto, USA), with an analytical column Zorbax 300SB-C18, 3.5 µm, 150 x 0.075 mm, linear gradient with 0-60% acetonitrile (ACN) in 35 min, using solvent A (97% water/3% ACN/0.1% formic acid) and solvent B (97% ACN/3% water/0.1% formic acid). The peptides were identified by on-line MS/MS (LC/MSD TRAP XCT mass spectrometer, Agilent Technologies), The peptide identifications were achieved by comparisons with the NCBInr database, 20090310, all fungal entries, using Mascot in-house version 2.2.1 (Matrix Science, London, UK) with subsequent parameters: trpysin digestion, up to one missed cleavage, fixed modifications: carbamidomethyl (C) and with the following variable modifications: methionine oxidation and carbamidomethylation of cysteines, peptide tol.: ± 1.2 Da, MS/MS tol.: ± 0.6 Da and peptide charge: +1, +2 and +3. The amino acid sequences were compared for similarity with known proteins using the Allergome database with the algorithm Basic Local Alignment Search Tool protein (BLASTP) under the standard parameters BLOSUM62.

3. Results

All 50 sera were tested for a reaction with *P. chrysogenum* and additionally with *A. versicolor* in 1D blots. A set of 10 sera which were tested also in 2D gels, are presented in figure 1. For control we also incubated a blot without serum and solely with the secondary antibody. The result (lower right panel in Figure 1) indicate very weak bands for A at ~26 kDa and 32 kDa and for lane B at ~28 kDa and ~35 kDa. Since we used standardized conditions in terms of the amount of protein and serum and also for western blotting we are convinced that the background is very low and does not influence the signal intensity of the samples in a significant way. The sera showed a variety of IgE positive bands, for *A.versicolor* as well as for *P.chrysogenum.* Serum 12, 23, 29, 34, 36 and 47 are examples for a strong immune reactivity to both investigated spore extracts in terms of detected bands and staining intensity. It is remarkable that most sera exhibited different patterns in their reactivity. A dominant band for *P.chrysogenum* with an apparent molecular weight of 35 kDa was found in nearly all sera. A second band with a molecular weight approximately 55 kDa was also frequently detected.

From the 50 sera screened by 1D blots, 10 strongly reacting sera, which showed a variety of positive bands, were selected for detecting and identification of proteins in 2D blots.

After incubating 2D blots of extract from *P.chrysogenum* spores with patient sera, IgE-reacting spots were detected and in order to align the spots on the membrane with spots in the gel, the membranes were stained by Ponceau S and the resulting spot patterns on membranes and gels were aligned by the 2D-analysis software DECODON. Three representative examples are shown in Figure 2. The signals show detection of several groups of spots that resemble a pearl-chain appearance. There are spots at different molecular weight, also several at the apparent molecular weight of 35 kDa as

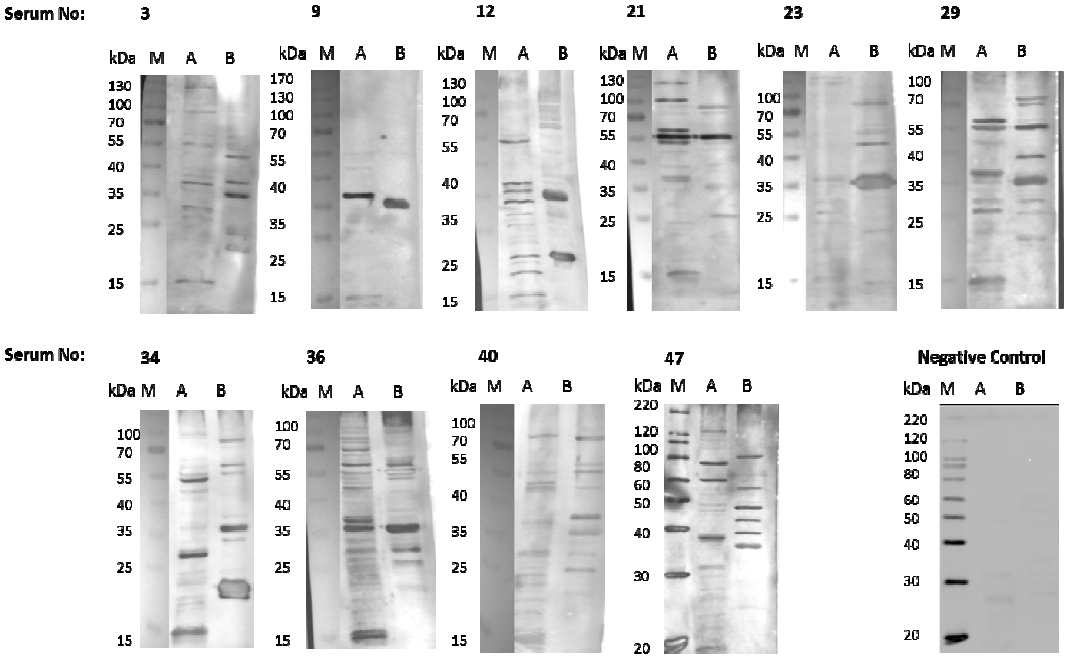

Figure 1. 1-D Immunoblot of selected sera, Immunological reaction against *A.versicolor (A)* and *P.chrysogenum (B),* M – unstained protein molecular weight marker, negative control without serum.

already seen in 1D blot analysis and at different pI. The horizontal pearl chain points to pI effective modifications or isoforms of one protein.

For identification of the immunoreactive proteins, a master gel was prepared and stained with Coomassie, shown in figure 3. All proteins spots, which reacted with one or more of the analyzed sera, were marked and the gels were aligned to the mastergel by DECODON. Coomassie stained spots corresponding to immune reactive spots were cut and proteins contained within were identified by mass spectrometry. The list of all detected allergens with decreasing frequency is given in table 1. All spots yielded unambiguous identifications and no spot revealed second hits, underlining the quality of the achieved resolution on the gel.

The immunoreactive spots were ranked according to their frequency within the tested 10 patients and the highest frequency (6/10) was found for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A second group with a frequency of 5/10 included Catalase, 1-pyrroline-5-carboxylate dehydrogenase, Glucose-6-phosphate isomerise and Transaldolase. Pc21g01220 from the oxidoreductase family and fumarate hydratase were identified 4 times, a protein from the aldehyd-dehydrogenase family and hydralase 3 times in 10 sera. Pen ch 18, a vacuolar serine protease which is known as allergen from *P.chrysogenum,* was found also 3 times. We identified 18 more proteins which showed an immune reaction with human sera (Table 1).

4. Discussion

The advancement of this study compared to other quests to identify allergens of *P. chrysogenum* lies in the focus on extracts from spores. This allows identifying spore specific IgE reactive proteins. Among them only one already known allergen, Pen ch 18, a vacuolar serine protease was detected in a minority of cases. Shen et al. [33] described Pen ch 18 as allergen with an association to asthma. Within the selected patients for this study, no asthmatic diseases were included. Nevertheless in three sera also Pen n 18 was identified. Other known allergens from *P. chrysogenum* like Pen ch 13, an alkaline serine protease and Pen ch 20, N-Acetyl glucosamidase were not found in this study underlining the difference between whole cell lysates and spore specific extraction [12, 20].

The major allergens are usually defined as those allergens which can be found in 50% of people who suffer from the corresponding allergy [34]. Since the patients analyzed in this study did not mainly suffer from fungal allergies this characterization cannot be used without problems. Additionally, the 1D blots were previously screened for strong bands and hence cannot be used as a reference point for overall frequency. Therefore, an arbitrary minimum limit of 50% should include most major allergens. However, the term major allergen is avoided in favor of high-frequency allergens. As high-frequency allergen the proteins GAPDH, catalase A, delta-1-pyrroline-5-carboxylate dehydrogenase, glucose-6-phosphate isomerase and transaldolase were identified.

GAPDH is an enzyme, which appears to be a main allergen in *P. chrysogenum* and *A. versicolor* [35]. Also the proteins stemming from *P. expansum* had shown a certain amount of cross-reactivity (unpublished data).

Interestingly, wheat glyceraldehyde-3-phosphate dehydrogenase has been identified as an allergen as well [36]. For the

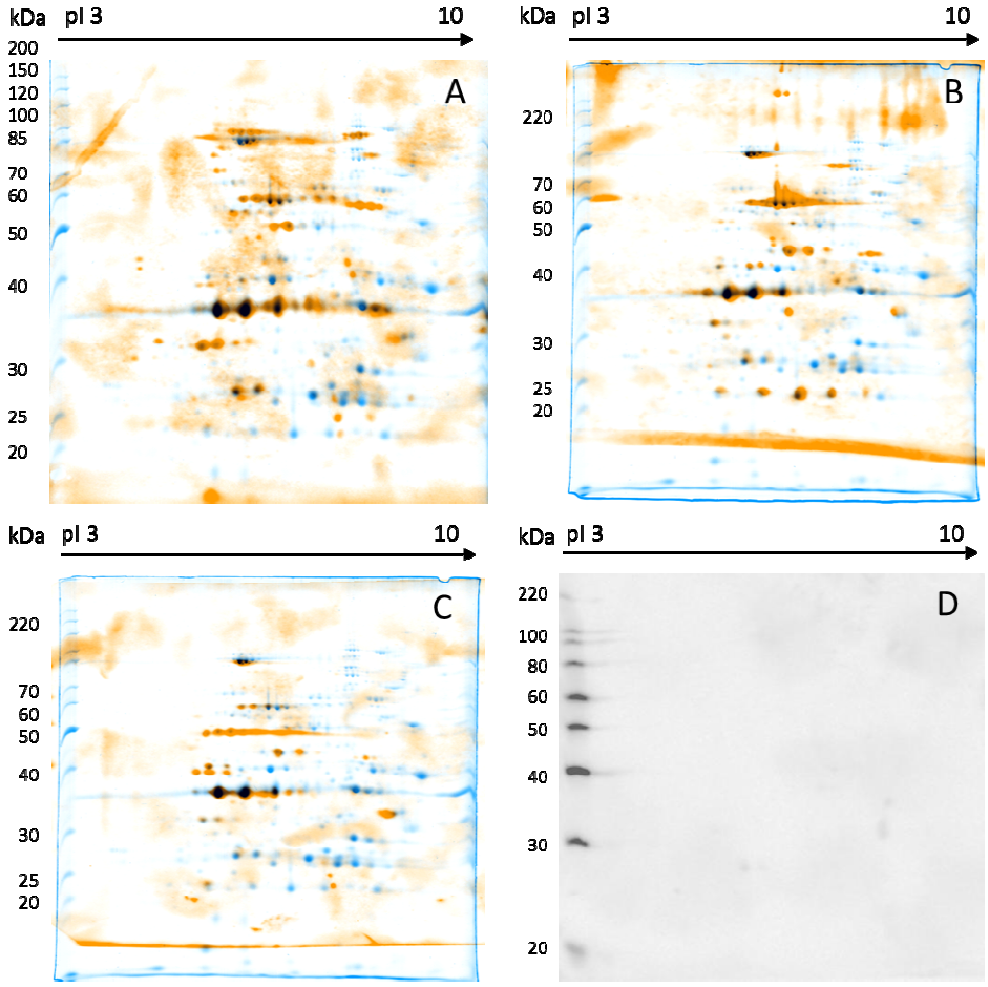

Figure 2. Examples of layered 2 D gels Coomassie-stained gel (blue) and chemiluminescence detection (orange) A-C selected sera (A-serum 23, B-serum 29, C-serum 47, D-negative control without serum).

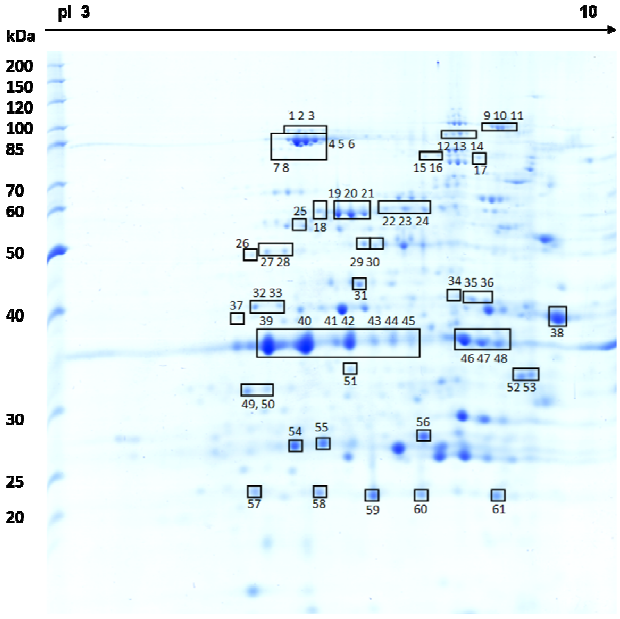

Figure 3. Positive reacting spots in Coomassie stained master gel. First Lane: Unstained Protein Marker. Remaining figure: Two dimensionally separated proteins of *P. chrysogenum.*

alignment, the nucleotide sequence of *Hordeum vulgare*, was taken since the exact sequence from the *Triticum aestivum* enzyme was not revealed in the respective paper [36]. A comparison between the enzyme of *Hordeum vulgare* and *P. chrysogenum* showed an identity of 71% and 84% positives. However, the length of both enzymes is similar. This points towards the epitope being in the 200 amino acid sequence which does not correspond to the *Alternaria* variant of the enzyme. This theory still has to be confirmed by epitope analysis.

The identity between the GAPDH of *P. chrysogenum* and humans is 70%, as determined by BlastP (84% positives and 0% gaps), thus only hardly allowing an allergic reaction according to the statistical estimation that proteins with more than 62% identity to human proteins are only rarely allergenic [37]. It will be assumed that a development of IgE reactivity to highly conserved allergenic molecules leads to cross-reactivity with divergent mould species [38]. Autoimmune responses caused by the production of IgE antibodies against fungal allergens have been described before in various papers [39-41]. The human variant of the analyzed enzymes is able to cause a strong reaction in skin prick tests. It has been sug-

Table 1. Name and molecular function of identified proteins.

Uniprot ass No.	Protein Molecular Function	Species	Protein scores	Mol W (Da)	Frequencyncy	Spot
Pc21g14560	Glyceraldehyde-3-phosphate dehydrogenase	P.chrysogenum	1353, 1361, 1283, 1155, 1012, 1044, 1251	36149	6	39, 40, 41, 42, 43, 44, 45
Pc20g06360	Catalase	P.chrysogenum	1692, 1081, 624, 711	84305	5	4, 5, 6, 7, 8
Pc12g08900	1-pyrroline-5-carboxylate dehydrogenase activity	P.chrysogenum	1433	62610	5	18
Pc22g19730	Glucose-6-phosphate isomerase	P.chrysogenum	1357, 1403, 522	61256	5	19, 20, 21
Pc21g16950	Transaldolase	P.chrysogenum	1239, 1094, 1166	35589	5	46, 47, 48
Pc21g01220	oxidoreductase activity	P.chrysogenum	535,202	35392	4	54, 56
Pc22g23810	fumarate hydratase activity	P.chrysogenum	1007	58125	3	29
Pc06g01800	oxidoreductase activity	P.chrysogenum	763, 649	37416	3	32,33
Pc06g00180	aldehyddehydrogenase family	P.chrysogenum	840	54046	3	34
Pen ch 18	vacuolar serine protease	P.chrysogenum	612, 569	38489	3	49,50
Pc13g08730	hydrolase activity,	P.chrysogenum	804, 664	34666	3	52,53
Pc16g11860	Catalase	P.chrysogenum	839, 917, 808	79912	2	12, 13, 14
Pc22g11240	heat shock protein familie	P.chrysogenum	1285	69692	2	25
Pc22g05800	Saccharopine Reductase	P.chrysogenum	865	49177	2	30
Pc20g04720	Dihydrolipoyl dehydrogenase	P.chrysogenum	1171	54589	2	31
Pc21g17460	adenosine kinase activity	P.chrysogenum	953	43589	2	35,36
Pc13g07960	oxidoreductase activity	P.chrysogenum	799	36973	2	37
Pc20g11580	Superoxide dismutase	P.chrysogenum	428	24668	2	57
Pc22g25220	FMN binding oxidoreductase activity	P.chrysogenum	575, 342, 242	21307	2	59, 60, 61
Pc22g18630	methionine biosynthetic process	P.chrysogenum	1544, 1136, 1641	87540	1	1,2,3
Pc18g03470	metallopeptidase activity	P.chrysogenum	2005, 2183, 2105	98916	1	9, 10, 11
Pc22g15910	Proteolysis	P.chrysogenum	752, 696	67986	1	15,16
Pc22g16760	dipeptidyl-peptidase activity	P.chrysogenum	1078	78783	1	17
Pc18g00980	Not identified	P.chrysogenum	723, 585, 678	57162	1	22, 23,24
Pc13g12450	ATP binding heat shock protein 70 family	P.chrysogenum	305	74784	1	26
Pc18g00650	phosphotransferase activity	P.chrysogenum	550, 688	48009	1	27, 28
Pc13g09680	aspartic-type endopeptidase activity	P.chrysogenum	953	43589	1	38
Pc13g15120	oxidoreductase activity	P.chrysogenum	329	31448	1	51
Pc22g08970	Not identified	P.chrysogenum	324	21844	1	58

gested that auto antigens against manganese superoxide dismutase play a role in atopic dermatitis [42].

Catalase is an enzyme that is abundant in tetramers. It catalyzes the reaction of hydrogen peroxide to oxygen and water. Since hydrogen peroxide is a side product of challenge for cells exposed to oxygen. Therefore, almost all aerobic organisms have this enzyme. Its catalytic activity is attained through its heme groups. Each tetramer unit binds to one heme group [43].

Catalase A is another allergen found in all three species. It is the third most reactive allergen found in *A. versicolor* [35] and the fourth most abundant allergen in *P. chrysogenum*. The analyzed sequence contains the information for one of the subunits. In previous studies, it has been shown that humans react to catalase of *Metarhizium anisopliae*, a mold which infects insects. The positive reaction is attributed to cross-reactivity with different fungi [44]. This theory coincides with the observation that catalase A is a major allergen in many indoor fungi. However, an alignment of catalase of *Metarhizium anisopliae* and catalase A of *P. chrysogenum* only shows 43% identity, 58% positives and 4% gaps. Catalase has been identified as an allergen in *Penicillium citrinum* [16].

50% of the chosen sera were from patients with urticaria, whereas the overall frequency was merely 20%. This might lead to the conclusion that patients with urticaria show a stronger reaction to indoor molds than the average patient with atopic disease. Nevertheless, 4 out of the 10 sera showed only a very slight reaction with the indoor mold proteins.

Urticaria is an allergic disease that can be caused either by contact, after inhalation or by digestion of allergens. After digestion the remaining peptides of the allergen are released into the blood stream. After skin contact, a localized reaction occurs which produces hives. The mast-cell activation in-

creases the vascular permeability and causes vasodilation of blood vessels [45]. A correlation between urticaria and dermatophysis has been found [46].

Beside the protein Pen ch 18 as a major allergen from *P.chrysogenum* related to asthma we identified other proteins with a high frequency. It can be assumed, that these patients were exposed to *P. chrysogenum* resulting in a sensitization. The Allergome database has introduced a classification system that illustrates the hierarchy in allergen characterisation ranging from first indications of allergenicity over IgE and several *in vivo* tests up to tests in epidemiological studies [10].The identification of immune-reactive proteins from spores of *P. chrysogenum* will facilitate further clinical studies with the aim to provide an individual and source and allergen specific allergy profile that in turn will support prevention and treatment.

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