

Characterization of a 52 kDa Exoantigen of *Penicillium chrysogenum* and Monoclonal Antibodies Suitable for its Detection

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Abstract The indoor clade of *Penicillium chrysogenum*, the so-called Fleming clade, is the most common species of *Penicillium* on moldy building materials. In a previous study, we identified a 52 kDa human antigen characteristic of the indoor clade of *P. chrysogenum* not present in a taxonomically diverse selection of fungi. Further investigations revealed that it is a modestly glycosylated mature protein with a pI 5.3. The protein is apparently identical to a glucoamylase previously reported from an aluminum-tolerant *P. chrysogenum* mutant. Based on sequence similarity, molecular weight, and pI, it is distinct from a number of other glucoamylases from domesticated strains of *Aspergillus oryzae* and *A. niger* used to produce industrial enzymes. Surprisingly, it had not been reported as an allergen. The monoclonal antibodies developed have the potential for use in assays of *P. chrysogenum* antigens in spores and spore/mycelial fragments in dust.

Keywords 52 kDa antigen · Glucoamylase · *Penicillium chrysogenum* clade 4 · Monoclonal antibodies

Introduction

In a series of studies of fungi associated with damp building materials, we are interested in identifying antigenic proteins in humans that are produced by strains found in the built environment and producing antibodies for their detection [1–3]. This follows a recommendation from the US National Academy of Sciences that a reliable way to assess disease associated with fungi in the environment would be to develop “standardized methods for assessing exposure to fungal allergens” [4]. While qualitatively similar, fungal proteins that accumulate in laboratory media are typically quantitatively different in distribution than those that occur in nature [2]. Wijands et al. [5, 6] raised rabbit polyclonal antibodies by immunizing cell wall extracts of *Alternaria alternata* and *Aspergillus fumigatus*. For each fungus, a number of apparently characteristic proteins were produced under all growth conditions tested. However, none of the proteins corresponded to the human allergens from these well-studied fungi. Another strategy, i.e., using spores from several fungi as the immunogen also has produced mixed results because of cross-reactivity [7, 8], and no specific human antigen or allergen was being measured.

This prompted our interest in using sera from atopic individuals to screen fungal proteins. The use of human sera to identify, rather than raise antibodies to mycelial cultures or to spores, means that the proteins identified were present in nature. Unlike the

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situation for the fungi ubiquitous in outdoor air, exposures to the fungi that grow on damp building materials is a recent phenomenon (past 20 years), and, are highly variable. Progress has required access to large numbers of sera from atopic patients collected over a large geographic area (as described in [9]). With the *Stachybotrys chartarum sensu lato* antigen, we found approximately 10% of the pre-screened sera we studied responded to the ultimate unique antigenic protein. The protein was shown to be an exoallergen produced on the preferred natural substrate of the fungus and on paper faced gypsum wallboard [2].

Penicillium chrysogenum is the most common species of fungi found on damp building materials [10]. It comprises four molecular species (clades) of which only one (clade 4, the Fleming strain is included in this clade) is common in the built environment in North America [11, 12]. Since this fungus is more common, we found that ~50% of the individuals in the patient sera reacted to protein extracts of clade 4 permitting the identification of a 52 kDa protein. This protein had apparently not been reported as a human allergen and many strains from the indoor clade did not produce allergens. It appears that previous studies by others were focused on the remaining three clades. This protein was present in high concentrations in spores and extracts from moldy paper faced gypsum wallboard [1].

The purposes of these studies were to characterize this protein and to produce production of antibodies suitable for its detection.

Materials and Methods

Spores were produced on rice cultures following the method developed by Murad et al. [13]. Uncle Ben'sTM converted long grain rice (50 g) was placed in a 500 mL wide-mouth Erlenmeyer flasks with 30 mL of ultrapure water. The flasks were autoclaved for 30 min. The rice cultures were inoculated with macerated *Penicillium chrysogenum*-MEA slant (DOAM 234056) and then incubated at 25°C in the dark for 3 weeks, then air dried in an oven at 30°C. The spores were carefully removed from the rice grains with a spatula and stored in vials at -20°C.

Spore extracts were produced as follows. One milligram of harvested spores was weighed with a

Mettler 163 analytical balance (± 0.02 mg) and then placed in a vial with a polystyrene bead. The vial with the spores was then milled for 30 min with a Spex-Certiprep mixer mill (model 5100, Metuchen, NJ). The crushed spore fragments were then dissolved in 0.1 mL of phosphate buffered saline-0.05% (v/v) Tween 20 (PBST, pH 7.4).

Penicillium chrysogenum CBS 277.067 was obtained from Centraalbureau voor Schimmelfcultuur, the Netherlands. *P. chrysogenum* DAOM 234056 cultures were maintained on 2% malt extract agar (Difco, Lawrence, KS) slants at 5°C and used for the production of the target protein as follows. A slant was macerated in 400 mL (5% v/v of total media) sterile ultrapure water. Then, a 5% (v/v) aliquot of *P. chrysogenum*-MEA was used to inoculate 2.8 L Fernbach flasks containing 560 mL of Enniatin (ENN) medium or 2.0 L Erlenmeyer flasks containing 400 mL. The ENN medium was comprised of the following: 50 g maltose, 8 g peptone, 5 g yeast extract, 0.75 g KH_2PO_4 2 g, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.067 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ per L ultrapure water. The cultures were put on a rotary shaker (3.81-cm throw) at 220 rpm at 25°C for 48 h in the dark. All cultures were filtered through cheese cloth to separate the culture filtrate from the mycelium. The culture filtrate was pooled for protein extraction. Protein was precipitated overnight by adding cold acetone to final concentration of 75% (v/v) at -20°C overnight. Protein pellets were collected after centrifugation at $17,400 \times g$ for 15 min at -8°C and then dissolved in a small volume of 50 mM Tris-HCl buffer (pH 7.5). The protein extract was further fractionated at 4°C by stepwise precipitation using $(\text{NH}_4)_2\text{SO}_4$ at saturations ranging from 50 to 80%.

The ammonium sulfate-precipitated protein was dissolved in a small volume of deionized water and subjected to buffer exchange by diafiltration against 50 mM Tris-HCl buffer (pH 7.5) using Centriplus YM-10 filters (Millipore, Billerica, MA). The buffer-equilibrated protein preparation was filtered through a 0.22- μm membrane and loaded to an anion exchanger column (16 mm $\times$ 60 mm) containing Q-Sepharose FF resin (Amersham Bioscience, Montreal, Quebec). Proteins bound to the column were eluted at a flow rate of 2.0 mL min^{-1} in a linear gradient increasing from 0 to 0.1 M NaCl in 50 mM Tris-HCl (pH 7.5) using the AKTA PrimePlus protein chromatography system (GE Healthcare). UV absorption of the

fractions (2 mL) was monitored at 280 nm and those with the target antigen as detected by SDS–PAGE and immunoblotting were pooled and concentrated.

After anion exchange chromatography, the target antigen was further purified by gel filtration on a Sephacryl S-200 column (16 mm× 800 mm) pre-equilibrated in 150 mM NaCl–50 mM Tris–HCl (pH 7.5). The sample was loaded onto the column and proteins were eluted with the equilibrating buffer at a flow rate of 0.1 mL min^{−1}. The fractions containing the target antigen were pooled, concentrated, and stored at −20°C.

Protein concentration was determined at room temperature on a 96-well microplate reader set at 595 nm using the Bradford protein assay kit (Bio-Rad, Mississauga, ON) according to the manufacturer's instructions. A calibration curve was generated with bovine serum albumin (BSA, Sigma, St. Louis, MO) as the reference.

Antibody Purification

The enhanced goat polyclonal antiserum [1] and mouse monoclonal antibody ascites were purified using affinity chromatography before use in characterization experiments. Briefly, an antibody sample was equilibrated with 20 mM sodium phosphate buffer (pH 7.0) and then applied to a 1-mL HiTrap protein G column (GE Healthcare) pre-treated in the same buffer. The bound IgG was eluted with 0.1 M glycine–HCl (pH 2.7) and immediately neutralized with 1 M Tris–HCl (pH 9.0). The homogeneity of the purified antibodies was verified by SDS–PAGE. Their activities against 52 kDa antigen were tested by Western blotting.

SDS–PAGE and Immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 10% (v/v) separation gels. Gels were run at a constant voltage of 170 V for 120 min in a Hoefer-260 Mini gel electrophoresis unit (Amersham Biosciences). A low molecular mass markers (Amersham Biosciences) was used as protein reference in SDS electrophoresis, including rabbit phosphorylase b (97 kDa), bovine albumin (66 kDa), chicken ovalbumin (45 kDa), bovine carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine α -lactalbumin

(14.4 kDa). Proteins in the gel were visualized with the GelCode Blue stain reagent (Pierce) or PlusOne silver staining kit (Amersham Biosciences) according to manufacturer's instruction.

For Western blotting, proteins separated by SDS–PAGE were transferred to Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences) in Towbin buffer using a Hoefer miniVE electrotransfer unit (Amersham Biosciences) at the constant current 400 mA for 30 min. Then, the membrane was blocked with 1% bovine serum albumin (BSA, Sigma) in 0.5 M Tris-buffered saline (TBS, pH 7.5) with 0.05% Tween 20 (TBST). Selected patient sera (for more information regarding the atopic patient sera see [1]), or goat anti-52 kDa protein polyclonal antibody (pAb), or mouse anti-52 kDa protein monoclonal antibodies (mAbs) were variously used as the primary antibody. Immunoblots were visualized with alkaline phosphatase (AP)-conjugated goat anti-human IgE, IgG, IgA, IgM, or rabbit anti-goat IgG, or rabbit anti-mouse IgG (all from Sigma) as the secondary antibody in combination with the chromogen *p*-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP, Sigma).

Glycoprotein Assays

Carbohydrate moieties covalently linked to a protein backbone were detected in SDS gels by using the GelCode glycoprotein staining kit (Pierce, Rockford, IL). In brief, the protein gel was fixed in 50% methanol (v/v in dH₂O) for 30 min after electrophoresis, and washed twice with 3% acetic acid (v/v in dH₂O) for 10 min. Then, following the manufacturer's protocol the gel was treated with oxidizing reagent (periodic acid) and reducing reagent, respectively. Glycoprotein is visualized as a magenta band in the gel. Horseradish peroxidase and soybean trypsin inhibitor were included in this assay as the positive and negative control, respectively.

The removal of carbohydrates from glycoproteins to determine the deglycosylated molecular weight was achieved using the trifluoromethanesulfonic acid (TFMS) method [14]. Briefly, the lyophilized protein sample (200 μ g) in a vial was treated with 40 μ L of the pre-cooled TFMS in the presence of 10% (v/v) anisole (Sigma, St. Louis, MO) at 4°C for 4 h, with occasional swirling. The reaction mixture was then

neutralized with approximately 80 µL of 60% pyridine solution pre-cooled in the methanol–dry ice bath (ca. -15°C), followed by desalting using Microcon YM-10 filter (Millipore).

N-Terminal Amino Acid Sequence Analysis

The purified 52 kDa protein (ca. 1 µg per lane) was run on a 10% SDS–polyacrylamide gel and then transferred onto a PVDF membrane as described earlier. The protein blots visualized by staining with the GelCode Blue dye were excised, destained, and subjected to Edman degradation for N-terminal amino acid sequencing at the Advanced Protein Technology Centre, the Hospital for Sick Children, Toronto, ON, Canada.

Determination of Protein Isoelectric Point

The isoelectric point (pI) of the 52 kDa *P. chrysogenum* antigen protein was determined using 7-cm ReadyStrip immobilized pH gradient (IPG) strips with a linear pH gradient ranging from 5 to 8 (Bio-Rad, Hercules, CA). The lyophilized 52 kDa protein was dissolved in the buffer solution containing 8 M urea, 2% CHAPS, 50 mM dithiothreitol, 0.2% Bio-Lyte 3–10 ampholytes, and trace bromophenol blue. The protein sample (150 µL) was loaded onto a gel strip by means of passive rehydration overnight at room temperature (ca. 20°C). Isoelectric focusing was performed on a PROTEAN IEF Cell (Bio-Rad) at 4,000 V for 10,000 V-h as recommended by the strip manufacturer. Proteins could then be visualized with silver stain, and pI values were calculated according to the percentage of protein migrating distance from the acidic end of the strip relative to the IPG gel length.

Monoclonal Antibodies

Monoclonal antibodies were prepared by Immuno-Precise Antibodies (Victoria, BC, Canada) as follows. Four female BALB/c mice were immunized by intraperitoneal injections with 25 µg of purified *P. chrysogenum* DAOM 234 056 52 kDa protein per mouse, in complete Freund's adjuvant. Four subsequent boosts were administered as indicated earlier, spaced at 3-weekly intervals, with incomplete Freund's adjuvant. When the serum titer had increased

>10,000-fold from a pre-immune serum sample, as determined by ELISA, it was tested for response to the pure 52 kDa protein by Western blotting. The two highest responders with a clear Western blot response were each boosted intravenously with 10 µg of the 52 kDa protein in 100 µL of sterile PBS pH 7.4. After 3 days, the donor mice were killed, and the spleen cells were harvested and pooled.

Fusion of the splenocytes with SP2/0 BALB/c parental myeloma cells was performed according to Kohler and Milstein [15], except that one-step selection and cloning of the hybridomas was performed in ClonacellTM-HY medium (Stemcell Technologies, Inc., Vancouver, BC). This method involves the use of an enriched, semi-solid medium, optimized for the growth of single cells. This in turn permits the identification and isolation of essentially every antigen-specific hybridoma in the population. The resulting clones were picked 11 days post-fusion and re-suspended in wells of 96-well tissue culture plates in culture medium for 4 days.

The supernatants from 1,000 clones (1:800 dilution) were screened by ELISA for antibody activity. This was done on 96-well Immuno MaxiSorp microplates (Nunc) coated with 100 µL of the 52 kDa target antigen (1 µg/mL) in carbonate buffer, pH 9.6 (Sigma) and detected using both goat anti-mouse IgG and anti-mouse IgM conjugates (Sigma) as secondary antibodies, respectively. The same clones were also screened by a dot blot technique. Briefly, BioTrace pure nitrocellulose blotting membranes (GelmanScience P/N S80209) were cut to same dimensions as 96-well plate. This was incubated face down with 120 µg 52 kDa denatured antigen (boiled in 2× sample buffer for 20 min) in 15 mL TBS for 1 h at room temperature and washed. Blocking was done with 5% milk powder in TBS for 1 h at room temperature and washed. Blots were allowed to dry on paper towels face up for 10 min prior to primary antibody dotting. Mouse anti-52 kDa protein immune serum and mouse pre-immune serum were diluted 1:400 in SP2/0 tissue culture supernatant and dotted as controls on the membrane with a multi-channel pipette in the template of the 96-well plate. Blots were incubated dry (with dotted on primary) for 1 h at room temperature. Blots were washed for 30 min after primary antibody incubation with five changes of TBST. The goat anti-mouse IgG/IgM (H+L) with alkaline phosphatase conjugated (Pierce)

secondary antibody (15 mL) was prepared at a 1:5,000 dilution in TBST. This was added to the blots incubated with shaking in Petri dishes for 1 h at room temperature and washed. The reaction with substrate NBT/BCIP was stopped after 5 min using cold tap water.

Based on the results from the screening, the cell culture supernatants from the IgG isotype clones were screened by ELISA in our laboratories. The 100 µL of 52 kDa protein (1 µg/mL) was coated to each well of a microplate. The plate was incubated on a microplate shaker overnight at 4°C and then washed three times for 5 min with 150 µL PBST between incubations. The coated plate was blocked with 100 µL of 1% bovine serum albumin (BSA) in PBST buffer for 1 h, washed as mentioned earlier and incubated for 1 h with 100 µL of the cell culture supernatant (100-time dilution for initial experiments). The plate was then incubated for 1 h with 100 µL of horse radish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma) that was diluted 1:2,000 in 1% BSA–PBST. The color was developed by adding 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) substrate, shaken for 10 min, and the color development was stopped by adding 50 µL of 0.5 M H₂SO₄. The plate was read using a Max340PC microplate spectrophotometer at 450 nm (Molecular Devices, CA). The experiments were repeated using 35 µg/mL (100 µL/well) of crude protein extracted from spores of *P. chrysogenum* DAOM 234056. The tissue culture supernatants were then screened with Western blotting to verify the dot blot results and to confirm a response to the protein in spores. These assays identified the top performing cell culture supernatants. Further testing was done to determine their ELISA response to 52 kDa protein as mentioned earlier by diluting the primary antibody (i.e., the culture supernatants) stepwise over two orders of magnitude. This was tested against 0.33 and 1 µg/mL of the 52 kDa protein samples.

Final selection of monoclonal antibody producing hybridoma cells was based on a combination of the ELISA data and the Western blot cross-reactivity experiments. Cell culture supernatants 2F9, 4H2, 7H7, and 8H1 were tested for possible cross-reactivity on Western blot with 1.5 µg (per lane) of the purified target protein, 35 µg crude protein extract from culture filtrate of *P. chrysogenum* DAOM 234056 as well as extracts of other selected fungi

representing the diverse related and unrelated species as described previously [1].

Monoclonal Performance

The performance of two purified IgG₁ mAbs, 2F9 and 7H7, was assessed by indirect ELISA. Pure 52 kDa protein was diluted in 50 mM sodium carbonate–bicarbonate buffer, pH 9.6 (Sigma). Aliquots of 100 µL (0.05 and 0.015 µg protein/well) of the protein preparation were coated to individual wells of a microplate and incubated overnight at 4°C. The unbound antigen was removed by washing three times with PBST and 200 µL of blocking buffer containing 1% BSA in PBST was dispensed to each well. After 1 h incubation, the plates were washed and 100 µL primary antibody in serial twofold dilutions (starting with 20 nM) with 1% BSA–PBST was added. The plate was then incubated for 1 h and 100 µL aliquots of HRP-conjugated anti-mouse IgG diluted (1:10,000) in 1% BSA–PBST were added to each well. After incubation for 45 min, the colour reaction was developed at 37°C for 10 min with 100 µL of TMB. The absorbance at 450 nm was recorded immediately after terminating the reaction with 50 µL of 0.5 M H₂SO₄.

The affinities of two mAbs, 2F9 and 7H7, directed at 52 kDa protein were determined using the method of Friguet et al. [16]. The antibody dissociation constant (K_D) was calculated with the aid of a Scatchard plot using the equation $v/a = (1 - v)/K_D$.

The monoclonal antibodies (2F9, 4H2, 7H7, 8H1) were tested for cross-reactivity to spore extracts from a wide array of related and unrelated fungi by indirect ELISA as earlier. The fungi tested included the following: *Aspergillus flavus* 12-7-4, *A. fumigatus* DAOM XXX**, *A. niger* 5869-6, *A. sydowii* DAOM 235363, *A. versicolor* DAOM 235361, *Chaetomium globosum* DAOM XXX, *Cladosporium halotolerans* DAOM XXX, *C. sphaerospermum* DAOM XXX, *Epicoccum nigrum* 51-2, *Paecilomyces variotii* DAOM 235357, *Penicillium brevicompactum* DAOM 234049, *P. corylophilum* 5585.1, *P. expansum* DAOM 235358, *P. crustosum* DAOM 215343, *Stachybotrys chartarum* DAOM 235364 and *P. chrysogenum* DAOM 234056. *Paracel Laboratories, Ottawa, ON number; **National Mycological Herbarium, Ottawa. [XXX means the culture is deposited and the number is pending.] Note that

C. halotolerans and *C. sphaerospermum* are the new indoor species of the former morphospecies previously known as *C. sphaerospermum*.

Results

Purification of the 52 kDa Antigenic Protein

The crude protein extract obtained from the acetone precipitation was subjected to stepwise fractionation by adding $(\text{NH}_4)_2\text{SO}_4$ to saturation of 50, 60, 65, 70 and 80%. The fraction from 50% $(\text{NH}_4)_2\text{SO}_4$ precipitation contained some insoluble precipitates and little protein, while most proteins were found in fractions extracted by 65 and 80% $(\text{NH}_4)_2\text{SO}_4$. Protein profiles and the isotypes with reactivity against the IgG of the patient serum QC2397 are shown in Fig. 1. Of these IgG-binding isotypes, a protein band with about 52 kDa molecular mass was recognized as the target from 65 to 80% ammonium sulfate-treated fractions.

Because of proteins that eluted near the target protein, the purity from subsequent purification steps was best if the 70% fraction was applied to a Q-Sepharose column for protein separation by anion exchange. At pH 7.5, the bound proteins were retrieved by a linear gradient of NaCl concentration increasing from 0 to 0.1 M. The 52 kDa protein was

largely found from the fractions eluted with NaCl between 0.04 and 0.07 M (data not shown).

Gel electrophoresis analysis demonstrated that most unwanted proteins were removed by the anion exchange step (Fig. 3). The partially purified protein gave a yield of 3.8% of the total protein extracted by acetone from 5.6 L culture filtrate (Table 1). The rest of unwanted proteins present in the anion exchanged sample were further removed by gel filtration chromatography (Fig. 2). The homogeneity of the 52 kDa protein was verified on SDS gel (Fig. 3). The final yield for the purified 52 kDa protein accounted for about 0.6% of the crude protein (Table 1).

The 52 kDa antigen was recognized from the *P. chrysogenum* protein extracts by IgG in human serum QC2397. Apart from this IgG-binding capacity, tests were made for whether the protein contained antigenic epitopes for other antibody components in QC2397. The purified 52 kDa protein did not produce positive reaction with IgE, IgM, or IgA, except for IgG (Fig. 4).

Protein Characterization

The first 21 amino acid residues from the N-terminus of the 52 kDa antigen were ADSXPAALQAILNNIGA DGSA. An amino acid residue at the position 4 was unidentified (denoted as X, probably in a modified form). This peptide sequence was compared with other known fungal protein sequences accessible at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). It showed that all the amino acid residues except the one unidentified in the N-terminus exactly matched to a sequence between positions 29 and 49 of a glucoamylase which was deduced previously from the cDNA library of a *P. chrysogenum* mutant strain by Sugimoto et al. [17]. A Western blot of an extract of a culture of

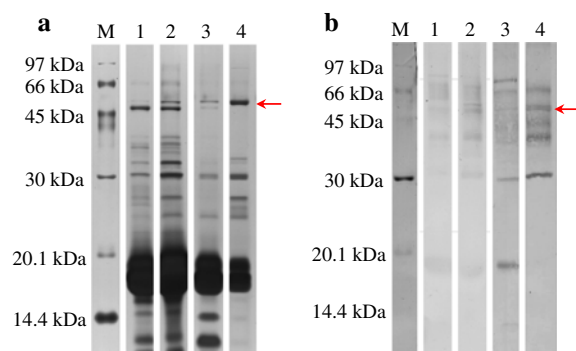


Fig. 1 Detection of 52 kDa antigen from ammonium sulfate-precipitated fractions. **a** protein profiles on the silver-stained SDS gel. **b** Antigenic proteins recognized by IgG of patient QC2397 on PVDF membrane. *M* molecular mass markers; lanes 1–4 for protein extracts obtained at 60, 65, 70, and 80% of ammonium sulfate saturation, respectively. The target protein is marked with an arrow

Table 1 Target protein yields from purification steps

Treatment	Volume (mL)	Protein (mg)	Yield (%)
Acetone precipitation	123	199.87	100
Ammonium sulfate fractionation	15	68.97	34.51
Ion exchange	8	7.67	3.84
Gel filtration	6	1.25	0.63

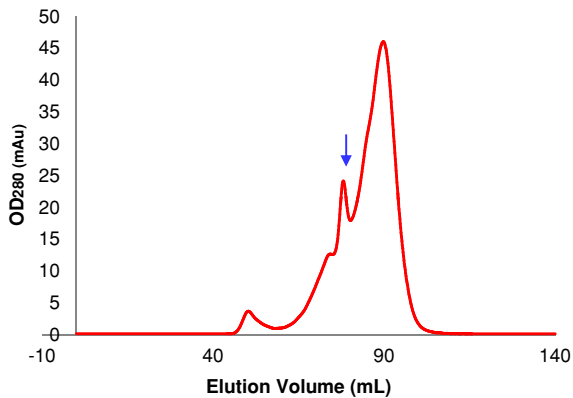


Fig. 2 Gel filtration chromatography for purification of 52 kDa antigen. The target protein peak is marked with an arrow

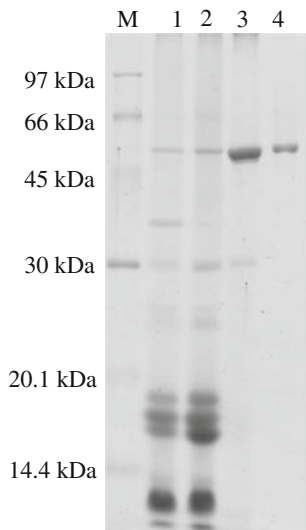


Fig. 3 Purification of 52 kDa antigen. Samples collected from relevant extraction and purification steps were run on a 10% SDS gel. Protein bands on the gel were then stained with GelCode Blue dye. *M* molecular mass markers; *lane 1* protein extracts by 75% (v/v) acetone; *lane 2* protein extracts by 70% ammonium sulfate; *lane 3* the sample collected after anion exchange; *lane 4* the 52 kDa antigen purified by gel filtration

the mutant studied by Sugimoto (CBS 277.067) prepared as mentioned earlier shows that the protein is apparently the same as that purified here (Fig. 5).

The purified 52 kDa protein, shown as a single band on SDS gel, was subjected to isoelectric focusing electrophoresis (IEF) to determine its pI. After IEF on a linear IPG gel strip with pH 5–8, the 52 kDa protein exhibited one major band with pI 5.3

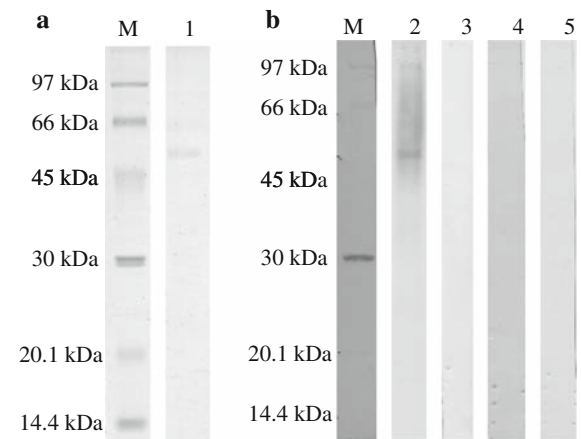
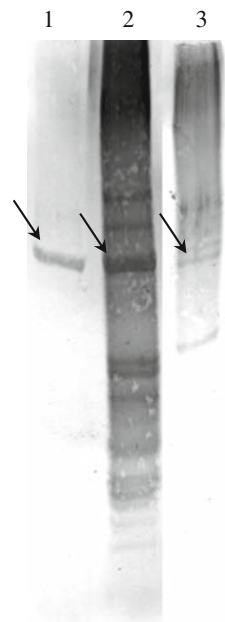


Fig. 4 The immunoglobulin-binding characteristic of 52 kDa antigen with human serum. **a** GelCode Blue-stained SDS gel. *M* molecular mass markers, *lane 1* the purified 52 kDa antigen. **b** Specific antibody recognition. The 52 kDa antigen on PVDF membrane was detected with patient serum QC2397 as the primary antibody and one of the following rabbit anti-human immunoglobulins as the secondary antibody; *lanes 2–5* for IgG, IgE, IgM, and IgA, respectively

Fig. 5 Western blot with a comparison of 52 kDa antigen (*lane 1*) with the proteins produced by *P. chrysogenum* DAOM 234056 (*lane 2*) and CBS 277.067 (*lane 3*). The arrows indicate the 52 kDa protein in each sample. Each sample was tested against purified goat anti-*P. chrysogenum* polyclonal antibodies (1:2,000 dilution)



and two minor isoforms with pIs of 5.2 and 5.4, respectively (Fig. 6).

To determine whether the purified antigen is a glycoprotein, a staining kit was employed after SDS electrophoresis, which detects carbohydrate moieties covalently linked to proteins. The 52 kDa protein band produced a reaction identical to horseradish



Fig. 6 The measured isoelectric points of 52 kDa antigen. On this silver-stained IPG gel strip (pH 5–8), three protein isoforms are displayed, with close pI values: A (5.2), B (5.3), and C (5.4)

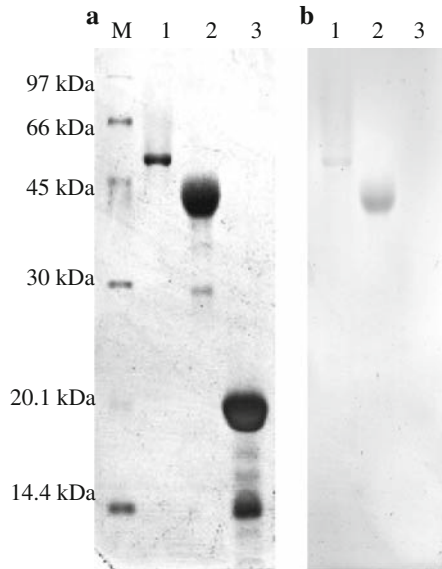


Fig. 7 Glycoprotein staining analysis. Proteins on SDS gel were visualized by silver staining (*panel A*) and periodic acid-Schiff base (PAS) staining (*panel B*). *M* molecular mass markers; *lane 1* 52 kDa antigen; *lane 2* horseradish peroxidase (HRP, as the positive control for glycoprotein); *lane 3* soybean trypsin inhibitor (negative control)

peroxidase (a positive control for glycoproteins), both of which turned magenta after staining, while soybean trypsin inhibitor (as a negative control) was not stained by the treatment (Fig. 7). This suggested that the 52 kDa antigen was a glycoprotein. The influence of protein deglycosylation on its IgG-binding activity was investigated with the TFMS-treated 52 kDa antigen. After deglycosylation, the molecular weight decreased from 52 kDa to approximately 50 kDa and the resultant protein lost its IgG-binding reactivity (Fig. 8).

Production and Testing of Monoclonal Antibodies

From the 1,000 clones screened, 44 were found to be potentially useful based either on the ELISA and dot blot results performed by the laboratory that produced

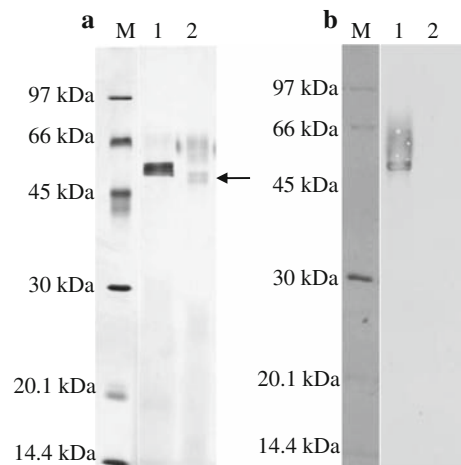


Fig. 8 IgG-binding reactivity of the 52 kDa antigen before and after deglycosylation. **a** The silver-stained SDS gel. **b** Western blotting analysis on PVDF membrane detected with monoclonal antibody (2F9) against 52 kDa protein. *M* molecular mass markers, *lanes 1 and 2* for 52 kDa antigen and TFMS-treated 52 kDa antigen (arrow), respectively

the mAbs. Of these, 21 were of the IgG isotype and the remainder were IgM. Most (21/44) had a good ELISA response during the screening (data not shown). A number had good responses on Western blot but low response by ELISA. Eight of these antibodies had good to very good responses to the 52 kDa protein in pure form and in extracts shown in Western blot using the cell supernatant from the monoclonal antibody clone screenings (data not shown). The responses of six mAbs that emerged from this process were further tested by diluting both the primary antibody and 52 kDa protein (33 vs. 100 ng/well, data not shown). From these tests, two mAbs with strong ELISA response and no apparent cross-reactivity with other fungal culture filtrates in Western blot were ultimately identified and used for ascites production, 7H7 and 2F9.

The responses of monoclonal IgG₁ (2F9 and 7H7) antibodies toward 52 kDa antigen were individually determined by indirect ELISA. Both produced specific binding with 52 kDa protein (Fig. 9). Low non-specific backgrounds (<0.05 OD units) were observed in control wells where the antigen was absent. The mAb 2F9 exhibited higher binding activity to the 52 kDa antigen. Assays using 2F9 the OD value reached maximum dilutions as low as 1.25 nM with 5 ng of 52 kDa protein per well. For 7H7, higher antibody and antigen concentrations

Fig. 9 Titration of antibodies against 52 kDa antigen using indirect ELISA. The 52 kDa antigen in doubling dilutions was coated to a 96-well microplate. Antibody was added at various concentrations as shown in the plot legends (see text). Plots **a** and **b** are for monoclonal antibodies 2F9 and 7H7, respectively

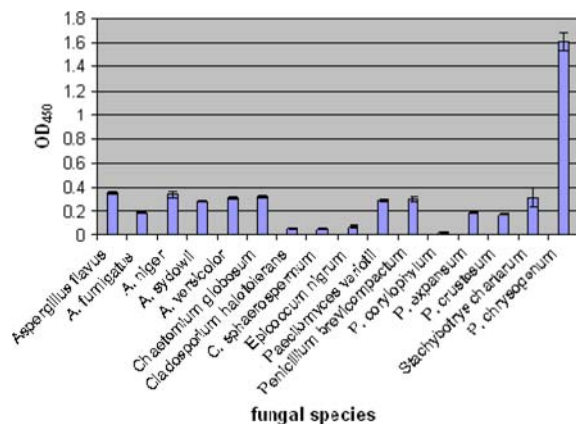
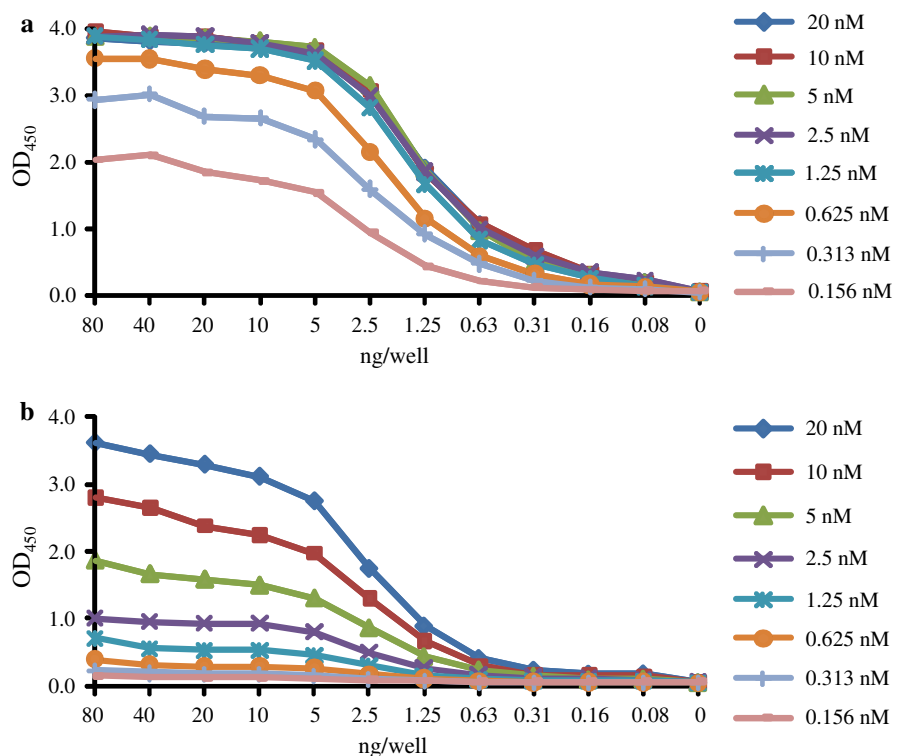


Fig. 10 Cross-reactivity of monoclonal antibody 2F9 to spore extracts to closely related, and taxonomically diverse species found in the built environment

were necessary to obtain maximum OD. The 2F9 monoclonal had a stronger affinity to 52 kDa protein than 7H7. The K_D values were 0.10 nM for 2F9 and 0.31 nM for 7H7. Linear equations for 2F9 and 7H7 were $y/a = -9.316v + 9.085$, $R^2 = 0.919$, and $y/a = -3.213v + 3.035$, $R^2 = 0.922$, respectively.

Cross-reactivity against various spore extracts by indirect ELISA are shown for the monoclonal with the best K_D value, 2F9 (Fig. 10). Cross-reactivity was

not observed and the data for 7H7 was similar (not shown).

Discussion

In spite of the progress made in defining important allergenic proteins from common airborne fungi over the past 15 years, fungal allergen extracts have largely remained uncharacterized. The identified allergens have a diversity of biologic functions, e.g., serine proteases, enolases, and heat shock proteins (see www.allergen.org). In the current list, only three published allergens have been recognized from *P. chrysogenum*: *Pen ch 13* (34 kDa, alkaline serine proteinase) [18], *Pen ch 18* (32 kDa, vacuolar serine protease) [19], and *Pen ch 20* (68 kDa, *N*-acetyl glucosaminidase) [20]. In addition, there are two allergens listed which at the time of writing remain unpublished, calreticulin and a 16 kDa protein of unknown function. All of these IgE reactive proteins have different molecular weights from the glucoamylase purified in our study, which has a molecular weight of 52 kDa as estimated by SDS-PAGE.

Alonso et al. [21] suggested that there was a 52 kDa antigen that developed in rabbits in response to poorly described *P. chrysogenum* mycelial extracts that they suggested were IgE-specific “presumably related to their atopic condition”. This was never pursued. While the few human sera we tested were not IgE reactive, response to this fungus in the US and Canada or Europe is still not common [1]. Without continued exposure, the IgE antibody serum titer declines quickly, whereas IgG antibodies remain in the serum for much longer [22].

In moldy buildings, the bulk of the fungal exposure is to spore and hyphal fragments [23, 24]. This is because these fragments are much smaller and are more efficiently inspired. Exposure to spores and fragments can be seen from direct methods (i.e., immunodetection) such as those described by Green et al. [25]. Since antigenic proteins in conidia are usually expressed in very low absolute amounts in spores, we developed culture conditions and purification methods to recover adequate amounts to isolate the target protein [1]. This allowed us to produce highly pure protein for characterization and antibody production.

Based on the N-terminal amino acid sequence, the 52 kDa protein is glucoamylase apparently similar to the one reported from the aluminum-tolerant *P. chrysogenum* mutant by Sugimoto et al. [17]. The entire glucoamylase sequence they deduced from cDNA library consists of 488 amino acid residues. The first 21-amino acid sequence identified from our purified protein has high homology (95%) to a segment beginning from position 29–49 at the N-terminus of the deduced amino acid sequence. The first 28 amino acid segment of the deduced glucoamylase sequence contains a long stretch of hydrophobic amino acids, likely indicating the presence of a signal peptide. The N-terminus signal peptide is usually cleaved from the precursor and does not appear in a mature protein. The 52 kDa protein purified in our study is the mature form of a protein which is secreted by *P. chrysogenum* to culture fluid and present in high concentration in spores. In addition, two potential *N*-glycosylation sites are found in the deduced sequence: Asn¹¹⁴–Pro–Ser and Asn¹⁹²–Gln–Thr. This agrees with the positive glycoprotein staining result observed for our 52 kDa protein. No other post-translational processing site is found within the remaining 460-amino acid

sequence. This suggests that *P. chrysogenum* glucoamylase is composed of a single polypeptide, with a calculated molecular mass of 49,224 Da for the backbone. Considering the carbohydrate contents, the final molecular mass of the mature enzyme is close to 52 kDa as determined by SDS–PAGE in our study.

The glucoamylase isolated from *P. chrysogenum* (DAOM 234 056) has a 60% sequence identity with a 53 kDa amylase by from *Aspergillus oryzae* [17]. *A. oryzae* amylase is an important occupational allergen that causes Baker’s asthma. The pI of the *A. oryzae* amylase allergen Asp o 21 is 4.0 [26], which is more acidic than that of the *P. chrysogenum* amylase discussed here (pI 5.3). Both of these enzymes are mature proteins. *A. oryzae* is a domesticated fungus that essentially does not occur outside enzyme production facilities and is closely related to *A. parasiticus* and less so to some uncommon related species [27].

In a case report, Quirce et al. [28] reported that sera from 3/4 individuals who were allergic to an *A. niger*-derived glucoamylase from occupational exposure reacted to proteins with molecular weights of 33.5, 47, 54, 68, and 96 kDa with one individual responding to the three lower molecular weight proteins only. At least five glucoamylases are present in commercial preparations of *A. niger*, of which the dominant form was reported to be 58 kDa with a pI of 3.6 [29]. This has a 62% sequence similarity to *P. chrysogenum* glucoamylase. In previous work testing building material *A. niger* samples, no reaction in the 50 kDa region was observed when probed with the human sera or with the enhanced *P. chrysogenum* goat polyclonal antiserum. [1]. Nor was this the case for the monoclonal antibodies discussed here (for 2F9, Fig. 10). Antibodies raised against one commercial preparation of a 53 kDa *A. niger* glucoamylase were responsive to Asp o 21 [30].

There are two enolases that were reported to have sequence similarities to the *P. chrysogenum* glucoamylase [17]. These are 47 kDa enolases from *A. fumigatus* (Asp f 22) and from *P. citrinum* (Pen c 22) which cross-react with each other and with similar proteins from *A. fumigatus* and from the unrelated common fungus *A. alternata* [31]. Mycelial extracts of *A. fumigatus* demonstrated proteins close to the 52 kDa protein reported here. Based on Western blotting of the 47 kDa protein with the

enhanced polyclonal antibody, the 47 kDa protein appears to be present in cellular extracts of *A. fumigatus* [1]. However, cross-reactivity tests with ELISA tests were not observed, nor was the protein seen in extracts of *P. chrysogenum* or *A. flavus* [1] or in spore extracts of these species (Fig. 10). This appears to be an intracellular protein.

Of the mAbs produced to the 52 kDa protein approximately half were of the IgG isotype and the remainder were IgM. Two mAbs, 2F9 and 7H7, free of cross-reactivity to culture filtrates of a diverse taxonomic range of fungi on immunoblot [32], produced specific binding with 52 kDa protein. The K_D value was 0.10 nM for 2F9 and 0.31 nM for 7H7, comparable to the values for antibodies shown useful for assays of *S. chartarum* in dust [3].

When indirect ELISA using mAb 2F9 was tested against spore extracts, somewhat better K_D values were observed (Fig 10). The species used are common in the built environment [10] represent closely related species within the terverticilliate *Penicillia* (*P. brevicompactum*, *P. crustosum*, *P. expansum*) as well as other similar fungi (*A. flavus*, *A. sydowii*, *A. versicolor*, *Paecilomyces variotii*) and taxonomically distant species (*Chaetomium globosum*, *C. halotolerans*, *C. sphaerospermum*, *S. chartarum*). This confirms earlier data showing that an enhanced polyclonal antibody to *P. chrysogenum* showed no material cross-reactivity to spores or mycelial extracts of other fungi either on ELISA or immunoblot [1, 32].

As noted by Wilson et al. [1], a major difficulty in performing this type of research is that the problem of mold growth in the built environment on any scale is a relatively new phenomenon. Although roughly one-third of the sera pre-selected for response to *Penicillium* responded to the indoor clade of *P. chrysogenum*, it remains to determine the extent of IgE response in the population. The antibodies produced will be useful for exposure assessments similar to those reviewed by Earle et al. [33] and Tsay et al. [34]. Further development depends on increasing the sensitivity by producing a capture ELISA and testing their performance in house dust using the methods described in [3]. This would appear to be important because a report from the German Environmental Survey for Children found that sensitization to *P. chrysogenum* was among the most common of those tested [35].

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