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# Characterization of two related exoantigens from the biodeteriogenic fungus *Aspergillus versicolor*

Yinan Liang, Wendy Zhao [1], Jianping Xu [2], J. David Miller*

*Department of Chemistry, Carleton University, Steacie Building, Ottawa, Ontario K1S 5B6, Canada*

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ABSTRACT

*Aspergillus versicolor* is one of the most common fungi in damp buildings in U. K., various European and Scandinavian countries as well as the United States and Canada. It is a proxy for species that occur at similar material water activities. Based on studies from Finland, Norway and Germany, it is among the common species resulting in an IgE reaction. Using pre-screened human sera with antibodies to various fungi, two related proteins were discovered with molecular weights 43 and 41 kDa based on SDS electrophoresis. Both proteins were excreted on the surfaces of spores and into culture media. The 41 kDa protein has a pI of 4.5. Based on a partial sequence, it is a serine protease. There are a number of *Aspergillus* proteases with overlapping sequences but these have different molecular weights and pI values. Polyclonal and monoclonal antibodies were developed that were specific compared to a diverse taxonomic array of related and unrelated fungi that commonly occur in the built environment. Initially this was done to ensure the specificity of the target protein. The measurement of other allergens and antigens associated with the built environment has a number of uses. Most importantly, these can be used to assess reliably biodeterioration and contribute to improved exposure assessments for population health studies.



## 1. Introduction

The common approach for detecting fungi in environmental samples depends on assessment of culturable propagules. This method is not inherently quantitative because there is large variation in propagule half-life, choice of medium and interference competition in culture affect recovery of viable propagules (Hung et al., 2005). There are two other tools that have received considerable attention for exposure assessment, quantitative PCR (QPCR) and measurements of exoantigens. In the few published studies, that have examined QPCR for fungi, limited or no correlation has been found with the various existing physical and mycological techniques (Dillon et al., 2007; Pietarinen et al., 2008; Pitkäranta et al., 2008). In the buildings context, various expert panels indicated that best approach for exposure assessment to fungi was to measure relevant allergens and antigens of the fungi found in damp buildings (NAS, 2000; Health Canada, 2004; NAS, 2004).

Xu et al. (2007) reported a potential marker protein antigenic in humans from the biodeteriogen *Stachybotrys chartarum*. Antibodies to this protein were used to develop an assay in dust (Xu et al., 2008) that can be applied to different analytical platforms (Smith et al., 2009) and the protein has been sequenced and expressed (Shi et al., 2011). A similar approach was used with the indoor clade of *Penicillium chrysogenum* (Wilson et al., 2009; Luo et al., 2010).

*Aspergillus versicolor* is common in soil, hay, and various foods (Samson et al., 2004). This fungus has long been known to be associated with damp, stored grain (Ominski et al., 1994). Its toxin, sterigmatocystin, was reported in grain from the early 1970s (Scott et al., 1972). It is a biodeteriogen of paper, artworks, photographic film, polymers and freshly cut wood (Mikluscak and Dawson-Andoh, 2004; Zhao et al., 2005; Abrusci et al., 2006; Michaelsen et al., 2009). In a large data compilation, *A. versicolor* was "common" on insulation, textiles, ceiling tiles, paper-faced gypsum wallboard, and manufactured wood from the US and Canada (Miller et al., 2008). It has been reported as a dominant mold in many problem buildings (e.g. Hodgeson et al., 1998; Jarvis and Morey, 2001). *A. versicolor* was also very common on moldy building material samples in Denmark, Belgium, Finland and the U.K. (Beguin and Nolard, 1994; Gravesen et al., 1999; Salonen et al., 2007; Flannigan and Miller, 2011) as well as carpet/floor dust in the Europe, US and Canada (e.g. Beguin and Nolard, 1996; Miller

* Corresponding author. Tel.: +1 613 520 2600; fax: +1 613 520 3749.
*E-mail address:* david_miller@carleton.ca (J.D. Miller).
[1] Present address: Food Research Division, Health Canada, Ottawa, Ontario K1A 0K9.
[2] Present address: Biovision, Mountain View, CA 94043, USA.

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and Day, 1997; Engelhardt et al., 2002; Horner et al., 2004). Its toxin (sterigmatocystin) has been detected on water-damaged building materials (Nielsen et al., 1999; Tuomi et al., 2000; Bloom et al., 2009) and in dusts collected from carpets and floors (Engelhardt et al., 2002; Bloom et al., 2009).

At the same time, there is widespread evidence of human exposure to proteins from this fungus from buildings exposure. Median blood IgG response to *A. versicolor* in sera from 214 children in Finland was highest of 21 fungi tested (Korppi et al., 2003). Rydjord et al. (2007) evaluated the frequency of mold exposure in an adult healthy population in Norway had *A. versicolor* IgG antibodies in a panel of 1100 sera collected in blood donor clinics. In the entire collection of sera, 12.5% had specific IgG antibodies to *A. versicolor*. In a study of child health in Germany, IgE was analyzed in ~1800 sera against commonly occurring fungi including *P. chrysogenum*, *A. versicolor*, *Wallemia sebi*, *Eurotium* species and *Alternaria alternata*. Approximately 10% of the children were sensitive to at least one of the fungi and 2% of the children responded to *A. versicolor* (Kolossa-Gehring et al., 2007).

Even though allergens of *Aspergillus* species were among the first to be recognized as important aeroallergens, few species-specific allergens have been characterized from this genus (Horner et al., 1994; Simon-Nobbe et al., 2008). At present, the International Union of Immunologic Societies' Subcommittee for Allergen Nomenclature lists allergens from two facultative pathogens (*Aspergillus fumigatus, Aspergillus flavus*) and two fungi used in industrial fermentations (*Aspergillus niger, Aspergillus oryzae*). Of the currently accepted major and minor allergens/antigens from *Aspergillus* species (www.allergen.org; www.allergenome.org), the proteins include alkaline serine proteases, a peroxisomal protein, a metalloprotease, Mn-superoxide dismutase, ribosomal protein P2, aspartic protease, peptidyl-prolyl isomerase, heat shock protein P90, enolase, L3 ribosomal protein, cyclophilin, thioredoxin, beta-xylosidase, 3-phytase B, amylase A as well as a variety of other related uncharacterized proteins including various cellulases and other degradative enzymes.

Culture conditions have a large effect on the production of proteins that might be used to produce antibodies for measuring fungal biomass. Proteins that might be present in spores, spore fragments and mycelia in nature are not necessarily those first or easily accumulated in culture. For this reason we have used collections of pre-screened human sera from a clinical testing lab with a large catchment area. Unlike allergy to fungi for which there is near universal exposure such as those common in outdoor air, exposures to damp building fungi are much less common and sometimes transient. For example, with the *S. chartarum sensu latto* antigen, we found approximately 10% of the pre-screened sera we studied had an IgG response to the protein. For the most common *Penicillium* species isolated from the built environment, *P. chrysogenum*, approximately 25% had a strong IgG response to the selected antigen. In addition, we have found that the important antigens in our hands are accumulated on the surfaces of spores and are water soluble. This means that they are removed if the spores are harvested or rinsed with buffers (Xu et al., 2007; Wilson et al., 2009).

This report concerns the identification and characterization of antigenic proteins in humans excreted by *A. versicolor* strains isolated from the built environment. This antigen accumulates on the surfaces of spores and, by virtue of presence to antibodies in humans is excreted in high abundance and/or immunogenicity when the fungus grows in nature.

## 2. Materials and methods

The overall research strategy is illustrated in Fig. 1. Briefly, (1) cultures of *A. versicolor* from around the North American continent were screened with diverse human sera to identify common antigens; (2) goats were immunized with unwashed spores and boosted with the target protein to ensure that the protein was present on spores and not an artifact of isolation thus yielding an enhanced polyclonal antibody, (3) the protein was produced and characterized and (4) monoclonal antibodies were produced and characterized for their specificity.

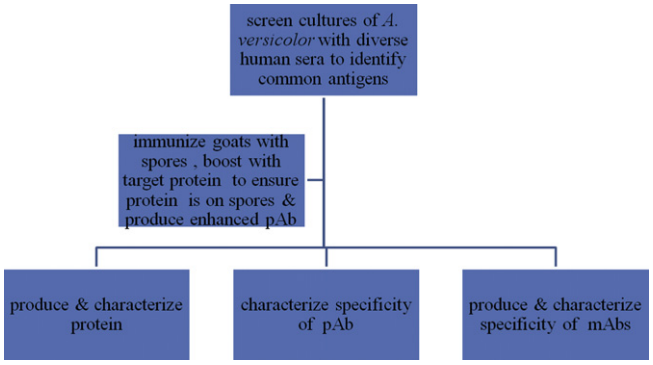


**Fig. 1.** Flow chart describing experimental approach.

### 2.1. Isolates and cultures

Isolates of *A. versicolor* from visibly moldy building materials or indoor air samples, selected for a geographic representation across the US and Canada, were obtained from Paracel Laboratories (Ottawa, Ontario). Strains from single spore isolates representing 24 genotypes were derived from mass cultures and transferred to 2% malt extract agar slants (Difco). Each strain was transferred to 2% malt extract agar slants and stored at 5 °C. Liquid cultures for culture filtrate and mycelium as well as spores were prepared according to Xu et al. (2007) and Wilson et al. (2009). Briefly, a slant was macerated in sterile water from which a 2.5% aliquot was used to inoculate 2.8-L Fernbach flasks each containing 560 ml of a medium developed for the accumulation of proteins and peptides (Traber et al., 1989) and demonstrated to be useful in this context (Xu et al., 2007). The liquid cultures were put on a rotary shaker (3.81 cm throw) at 220 rpm at 25 °C for 48 h in the dark. Time course experiments were performed, and for *A. versicolor,* the growth rate is such that after 48 h, the majority of the cells are well into stationary phase with consequent proteases accumulation (data not shown). At the end of the incubation period, all cultures were filtered through cheese cloth using a Buchner funnel. The filtrate from each flask was collected and stored at 5 °C and processed within 24 h. The mycelium was rinsed twice with water, wrapped in aluminum foil, frozen and then dried under vacuum.

Spores were produced on rice cultures adapted from the method of Murad et al. (1993). Rice (50 g; Uncle Ben's™) was placed in 500 ml wide-mouth Erlenmeyer flasks, 30 ml of water was added and the mixture was autoclaved for 30 min. Cultures were inoculated and incubated at 25 °C in the dark for 3 weeks, and dried at 30 °C in an oven. Spores were harvested by physical removal from the rice grains and stored in vials at 4 °C.

The fungi at one or more stages of the process included *Acremonium strictum* 50-7*, *A. alternata* 27-1, *Aspergillus calidousus* IBT 2826, *Aspergillus ochraceous* 12-7-3, *A. flavus* 12-7-4, *A. fumigatus* 31, *A. ochraceous* 12-7-3, *A. niger* 5869-6, *Aspergillus glabrum* 5053-15, *Aspergillus sydowi* DAOM 235363, *A. versicolor* DAOM** 235361 (Winnipeg), *A. versicolor* 1-2b, 1-3b (Hawaii), 2-2a, 2-2b, 2-3a, 2-3b (Winnipeg), 38-2a (Ottawa), 40-1a, 40-3b (Ottawa), 41 (Montreal), 41-1a, 42-1a (Saskatoon) 42-1b, 42-2a, 43-3a, 43-2b (Ottawa), 44-3

(Vancouver), 138-1 (Hawaii), 152, 159 (San Diego), *Cladosporium cladosporioides* 4240450, *Cladosporium halotolerans* DAOM240238, *Cladosporium sphaerospermum* DAOM 240243, *Epicoccum nigrum* 240452, *Eurotium amstelodami* IBT 28246***, *Eurotium herbariorum* CBS 47-F3****, *Empetrum rubrum* CBS 47-F4, *Chaetomium globosum* DAOM 240348, *Memnoniella echinata* DAOM 235365, *Paecilomyces varioti* DAOM 235357, *Penicillium brevicompactum DAOM 234049*, *Penicillium corylophilum* 12-7-3, *P. chrysogenum* DAOM 234056, *Penicillium crustosum* DAOM 245045, *Penicillium expansum* DAOM 235358, *Penicillium decumbens* 212029, *Penicillium spinulosum* 12-7-1, *Penicillium thomii* 6322-4, *Scopulariopsis brevicaulis* DAOM 240454, *S. chartarum* (DAOM 235364), *Stachybotrys chlorohalonata* (*****ATCC 201863), and *W. sebi* 12-7-2. *Paracel Laboratory Ltd; **National Mycological Herbarium, Ottawa; *** Technical University of Denmark, ****Centraalbureau voor Schimmelcultures, Utrecht; *****American Type Culture Collection, Manassas, VA.

### 2.2. Identification of target proteins

Sera from ~40 patients were taken from a clinical laboratory that serves the United States and Canada for routine and non-routine allergen testing (IBT Reference Laboratory, Lenexa, KS) that were reactive to various fungi. Reactivity was determined using the Pharmacia ImmunoCAP® reagents during diagnostic testing (Halsey et al., 2000).

SDS-PAGE electrophoresis and immunoblots were carried out following Wilson et al. (2009).

Initial screenings for a target antigen using the 21 *A. versicolor* genotypes noted above were performed as follows. Approximately 1.0 g of freeze-dried mycelium was mixed with 10 ml of extraction buffer pH 7.5 (see below) and 40 μl fungal protease inhibitor cocktail (Roche, 1,697,498) prepared according to the manufacturer's instructions. The resulting suspension was vortexed and kept on ice for ≥10 min until the mycelium was hydrated. The material was homogenized on ice with a Polytron (Kinematica, Switzerland) 5 × 3 min at 1–2 min intervals to limit warming. The homogenate was centrifuged at 13,000 × *g* for 10 min at 4 °C. Following this, 4 × volume of cold acetone was added to the supernatant and kept at −20 °C overnight. After centrifugation at 13,000 × *g* for 10 min at 4 °C, the resulting pellets were freeze-dried and dissolved in 1 ml TBS buffer. The protein concentration of the extract was determined and stored at −20 °C. The sera were screened by ELISA and immunoblotting for maximum response to the mycelial protein extracts as previously described (Wilson et al., 2009). The protein concentration of the final homogenates used was 5 mg $mL^{-1}$ with the human sera diluted 1:1000. Based on the repeated observation of antibodies in ~20% of the human sera tested reacting to most strains of the fungus tested, a number of potential candidate proteins were identified (Zhao, 2006). Of greatest interest were two proteins around 43 kDa. Both were equally reactive to the sera used at a dilution of 1:1000 (Zhao, 2006; Liang, 2008).

To ensure that the target protein, as isolated, was present in spores and not an artifact of isolation, and to develop a polyclonal antibody, a procedure described by Xu et al. (2007) was used. Briefly, spores of *A. versicolor* DAOM 235361 were used to raise polyclonal antibodies in goats. A test bleed was taken at one month to ensure reaction with target protein. The remaining extract was subjected to SDS electrophoresis. Bands of a 43-kDa protein (hereafter Avs 43), the protein visualized by immunoblot as described above with strongly positive human serum sample in one lane and Coomassie blue (CBB) and silver in the remaining lanes, were cut from several gels. The gel pieces (1 mg protein) were ground with a mortar and pestle in sterile phosphate buffered saline (PBS). The protein suspension (~500 μg) was emulsified with Freund's incomplete adjuvant emulsion and used as the antigen solution to boost the goat previously immunized with spores. The antibodies that resulted had an attenuated response to spore-borne proteins other than the target protein. During this period, a 41 kDa protein was also observed (hereafter Avs 41; Zhao, 2006).

For purification of the identified target protein, the crude protein was isolated from fungal cell culture filtrate with 75% acetone precipitation described as above and further fractionated with ammonium sulphate (AS) precipitation from 55% to 65% of AS saturation (Zhao, 2006). The protein pellet was resuspended in Tris buffer and desalted using a 10 kDa ultra-15 centrifuge tube (Millipore Corp., Billerica, MA). Anion exchange chromatography was then performed using an ÄKTAprime™ plus fraction collector (GE Healthcare, San Francisco, CA) with 1.5 ml of Q-Sepharaose fast flow resin while separation was achieved with stepwise NaCl concentrations (0.14 M, 0.16 M, 0.18 M, 0.2 M and 0.25 M) in Tris buffer. Further purification was accomplished by gel filtration, using Sephacryl-S-200-HR resin (Sigma–Aldrich, Oakville, ON) in a 96 × 1.6 cm column. Molecular weight calibration was accomplished using the Sigma, MW-GF-200; 12.4–200 kDa molecular weight marker kit. Peaks were subsequently concentrated and visualized by both SDS-PAGE and immunoblotting using polyclonal (Pab) or monoclonal (Mab) antibodies.

More than 10 mg Avs 43 (>98% pure; Zhao, 2006) was produced for mAb production and approximately 5 mg of Avs 41 protein was obtained to a similar level of purity.

### 2.3. Protein characterization

To determine the glycosylation of purified protein, the materials were first separated on SDS-PAGE along with positive and negative controls and stained with GelCode glycoprotein staining kit (Pierce, Rockford, IL). The isoelectric point (pI) was then measured on a 2-D gel. Samples were applied to an immobilized pH gradient (IPG) strip (Bio-Rad, Hercules, CA) with a pH range 3–6 (7 cm) by the active rehydration method for ~12 h. The IPG gel was focused for 40,000 V hour on a Protean IEF system (Bio-Rad). After equilibration with SDS-PAGE loading buffer for second-dimension gel, the IPG strip was loaded onto the SDS-PAGE gel as described above for running the second-dimension electrophoresis.

To obtain the N-terminal amino acid sequence, the purified proteins were separated on 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. Both Avs 41 and Avs 43 were identified by CBB staining. The protein bands on the membrane were excised and automatic Edman degradation sequencing was performed using an ABI 492 Procise cLC sequencer (Torød, Norway).

### 2.4. Monoclonal antibodies

Monoclonal antibodies were prepared and screened by Immunoprecise Laboratories (Victoria, BC) after Luo et al. (2010). Four female BALB/c mice were immunized by intra-peritoneal injections with 25 μg of the 43 kDa protein per mouse, in FCA. Four subsequent boosts were administered as above, spaced at 3 week intervals, with FIA. When the serum titer had risen more than 10-fold from a pre-immune serum sample, as determined by ELISA, the sera were tested for response to the pure 41 and 43 kDa proteins on immunoblot. All the mice had a good response on immunoblot, and two mice demonstrated antibodies to both Avs 41 and Avs 43. All four mice were used to prepare mAabs. Each was boosted intravenously with 10 μg of Avs 43 in 100 μl of sterile PBS pH 7.4. Three days later the donor mice were sacrificed and the spleen cells were harvested and pooled followed by the splenocytes with SP2/0

BALB/c parental myeloma cells. The supernatants from 1000 clones (1:800 dilution) were screened by ELISA for antibody activity and by dot blot (also at Immunoprecise; Luo et al., 2010).

Based on the results from the above screening, the cell culture supernatants from the IgM isotype clones were screened by ELISA at Carleton University. The target protein (1 μg) 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 phosphate buffered saline-0.05% (v/v) Tween-20 (PBST, pH 7.4) between incubations. The coated plate was blocked with 100 μl of 2% bovine serum albumin (BSA) in PBST buffer for 1 h, washed as above and incubated for 1 h with 100 μl of the cell culture supernatant. The plate was then incubated for 1 h with 100 μl of horse radish peroxidase (HRP) conjugated anti-mouse IgM (Sigma) that was diluted 1:2000 in 1% BSA/PBST. The colour was developed by adding 100 μl of 3,3′, 5,5′-tetramethylbenzidine (TMB, Sigma) substrate, shaken for 10 min, and the colour development was stopped by adding 50 μl of 0.5 M $H_2SO_4$. The plate was read using a Max 340 PC microplate spectrophotometer at 450 nm (Molecular Devices, CA). The tissue culture supernatants were then screened by immunoblot to confirm 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 performed to determine their ELISA response to 41 kDa protein as above by diluting the primary antibody (i.e. the culture supernatants) stepwise over a two orders of magnitude. This was repeated except with addition of 0.33 μg per well instead of 1 μg. From this process, 12 antibodies emerged, 10 of which were the IgM isotype with the remaining two being IgG.

The two IgG monoclonals had low response on immunoblot and by ELISA. Final selection of mAab producing hybridoma cells was based on a combination of the ELISA, dot blot and the dot blot cross-reactivity experiments. The five different cell supernatants (1:500 and 1:1000) were tested on immunoblot against pure Av protein (0.8 μg per lane). Three cell supernatants (6E2, 2H12 and 8A10) were tested for cross reactivity to extracts of other selected fungi representing the diverse related and unrelated species as described previously (Liang, 2008). From this process, five mAbs (2H12, 5G5, 5C9, 6E2 and 8A10) were chosen for further work and mouse ascites were produced.

### 2.5. Antibody purification

Affinity purification of IgG antibodies from the enhanced anti-*A. versicolor* pAb was done after Wilson et al. (2009).

Mouse monoclonal IgM antibodies were purified using gel filtration chromatography. A HiPrep 16/60 Sephacryl-S-300 HR gel filtration column calibrated using a high molecular weight calibration kit (GE Healthcare) was used. The column was conditioned using 200 ml of 0.15 M NaCl Tris buffer at a flow rate 0.5 ml $min^{-1}$. The ascites (2 ml) of various mAbs were filtered through 0.45 μm filters (Millipore Corp.) prior to use. The ascites were resuspended in 0.15 M NaCl Tris buffer as above and then concentrated to 0.5 ml by 50 kDa cut off Amicon Ultra-15 centrifuge tubes. The concentrated ascites were applied to the column, eluted at 0.5 ml $min^{-1}$ and collected in 1 ml aliquots with a fraction collector. Each eluted peak was concentrated separately and the response for each Mab was examined by immunoblot. Efforts to purify the antibodies using the IgM purification kit (Pierce) or HiTrap IgM purification HP (GE Healthcare) after consultation with the manufacturers were unsuccessful (Liang, 2008).

IgM titer was quantified by the mouse IgM Isotyping TiterZyme® Enzyme Immunometric Assay (EIA) kit (Assay Design, MJS BioLynx Inc., Brockville, ON) according to the manufacturer's instructions. The plate was read on a microplate plate reader at 450 nm (Max 340 PC, Molecular Devices, CA).

### 2.6. ELISA

All ELISA experiments were performed on 96-well MaxiSorp microplates (Nunc-Immuno, VWR, Ottawa, ON) with each experiment repeated three times in duplicate. Both protein standards and spore samples were directly prepared in coating buffer, 50 mM carbonate–bicarbonate pH 9.6 (Sigma), in a polystyrene microtitre plate. Two different ELISA protocols were used as follows.

For protocol 1, each well was coated with the diluted *A. versicolor* samples or the blank overnight at 4 °C. The standard curve for purified Av protein (100–0.195 ng per well) and disrupted Av spores (40–78 ng per well) were diluted by 50% across the plate, sequentially. After coating, the solution was removed the next day and blocked 1 h at room temperature with 150 μl 2% BSA/PBST. The blocking solution was removed and the plate was washed three times with 150 μl PBST (PBS buffer with 0.1% Tween-20). The purified pA from protein G column (1000× dilution) was diluted with 1% BSA/PBST and added to the plate. After 1 h incubation on the shaker, the plate was washed, the secondary antibody anti-goat IgG HRP (Jackson Laboratory, West Grove, PA) was diluted 20,000× with 1% BSA/PBST and added to each well. Tetramethylbenzidine (TMB) (100 μl) was used as a substrate after incubation for 1 h and the reaction was stopped by adding 50 μl 0.5 M $H_2SO_4$ after 10 min, with the absorbance recorded at 450 nm. The second protocol involved the following changes. Coating was accomplished by adding diluted *A. versicolor* samples to the microtitre plate and shaking for 4 h at room temperature. The coating solution was then removed, 200 μl 1% skimmed milk/TBS (Blotto solution) was added and left to block overnight at 4 °C. The blocking solution was removed next day and the plate was washed by TTBS (TBS solution with 0.05% Tween-20) using a plate washer (three times each with 250 μl TTBS). The purified mAbs (1:600 dilution) as well as the secondary antibody, anti-mouse IgM HRP, were diluted in Blotto solution and added into the plate, similarly to ELISA protocol 1.

### 2.7. Testing of spore extracts and antibody performance

Briefly, 1 mg of harvested spores, milled and the crushed spore fragments dissolved in 0.1 ml PBST for 2 h while shaking at 4 °C (Zhao, 2006; Wilson et al., 2009) were tested. Purified enhanced pAb and mAb 6E2 were used to examine cross reactivity to a wide taxonomic diversity of fungi.

## 3. Results

### 3.1. Identification of target proteins

Of the collection used, 8 of 40 human sera demonstrated high ELISA and immunoblot response to the 24 strains of *A. versicolor* (Table 1; data for two negative strains not listed). The strains were representative of the geographic range considered (Montreal to San Diego to Saskatoon, i.e. within a triangle comprising c. 150,000 $km^2$). One of the high responding sera (1506) was used to screen 13 strains representative of the geographic range covered in North America by immunoblot for response to the target proteins (Table 2). From this work, three proteins were consistently observed in crude mycelial extracts on immunoblot with the various positive human sera. The first was at ~68 kDa, and there were two proteins somewhat above 40 kDa (arrows, Fig. 2). Once potential target proteins were determined, all further work was done using *A. versicolor* DAOM 235361, a single spore isolate from an air sample from a crawl space in Winnipeg, Manitoba. After

**Table 1**
*A. versicolor* proteins detected by immunoblot with various human pAbs.

| Protein MW (kDa) | Serum sample number | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1303 | 1304 | 1307 | 1310 | 1501 | 1502 | 1503 | 1504 | 1505 | 1506 | 1287 | 1288 | 1289 | 1290 | 1293 | 1294 | 1297 |
| 68 | ++ | ++ | + | + | +++ | + | + | ++ | ++ | ++ | + | + | | + | + | ++ | + |
| 43 | + | ++ | + | ++ | | ++ | | +++ | ++ | +++ | + | + | + | | ++ | + | ++ |
| 30 | + | + | + | +++ | + | ++ | | +++ | +++ | + | + | + | | | ++ | + | + |

Note: the symbol indicates presence of the proteins noted as measured by SDS-PAGE with + being present, +++ being highly expressed and ++ intermediate.

further preliminary work, the higher molecular weight protein was not pursued (Zhao, 2006).

A goat was successfully immunized with *A. versicolor* spores, with the sera reacting with many proteins in spore extracts, including the ~40 kDa target proteins. This reaction was demonstrated by the presence of antibodies to spore proteins as well as culture filtrate extracts of increasing purity (Fig. 3). After the boost with 1 mg of the target protein, there was a ~four-fold increase in antibody titer to the target protein (Fig. 3B, arrow lanes 1–3; serum dilution 1:2000). Further, there was a reduction in intensity of several non-target bands on immunoblot. This included response to a number of protein bands above Avs 43 target band (shown by an arrow). In addition, detection of bands below 14 kDa and above 45 kDa in spore extracts was decreased (Fig. 3A, lane 1,2; Fig. 3B, lane 1,2 indicated by arrows). The antibodies that resulted had an attenuated response to spore-borne proteins other than the target protein(s). As with the various human sera, the goats developed antibodies to two proteins at 41 and 43 kDa.

Typically, 1 g crude protein was obtained from 8 L culture filtrate. The resulting extract was fractionated and purified through a series of steps (Table 3). The proteins were enriched in 75% acetone followed by ammonium sulphate fractionation. The majority of the target protein was found in the fraction between 55 and 65% ammonium sulphate. Anion exchange chromatography removed most of the extraneous proteins with the majority of target protein was found between 0.18 and 0.20 M NaCl eluate. The target antigen fraction was further purified by gel filtration chromatography, which also removed the remaining soluble pigments (Fig. 4). Depending on fermentation, two proteins eluted in varying amounts in the most immunologically active fractions that came off the gel filtration column. These had apparent molecular weights of 43 kDa and 41 kDa by gel filtration. While both proteins accumulated in the mycelium and culture filtrate, the larger protein was present in greater concentration in the mycelium than in the filtrate (data not shown). The gel filtration step resulted in a ≈20-fold purification over the AS precipitation (Table 1) for Avs 43 while yields of Avs 41 were lower.

### 3.2. Protein characterization

By 2-D gel electrophoresis, pI value for Avs 41 was pH 4.5, an acidic protein (Fig. 5). A glycoprotein stain was used to detect the existence of sugar moiety on Avs 41. The positive control HRP and

**Table 2**
Antigenic proteins found in various *A. versicolor* strains on immunoblot with human pAb 1506.

| Protein MW (kDa) | Serum sample number | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| 68 | | ++ | ++ | + | | | | + | | | |
| 43 | + | +++ | +++ | +++ | + | +++ | ++ | +++ | ++ | ++ | ++ |
| 30 | | + | + | + | + | | | | | | |

1. 1-2b Hawaii; 2. 235361, (2-2a), 3. 2-2b; 4. 2-3b; 5 Winnipeg; 40-1a, 40-3b Ottawa; 6. 41-1a Montreal; 7. 42-1, 42-1b Saskatoon; 8. 43-2b Ottawa; 9. 138-1 Hawaii 10. 152 San Diego; 11. 159 San Diego; symbols as in Table 1.

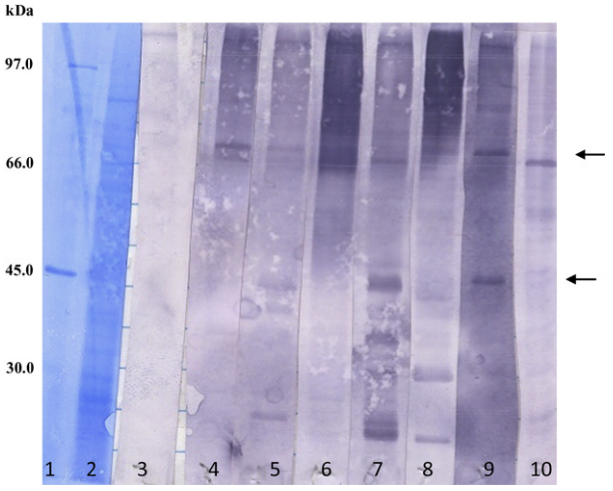


**Fig. 2.** Immunoblot of mycelial extract of *A. versicolor* DAOM 235361 stained with different patient sera (protein 5 mg ml$^{-1}$; serum dilution, 1:1000). Lanes: 1. molecular weight marker, 2. CBB stain, 3. Patient 1500, 4. 1501, 5. 1502, 6. 1503, 7. 1504, 8. 1505, 9. 1506, 10. 1294; common bands are indicated by arrows.

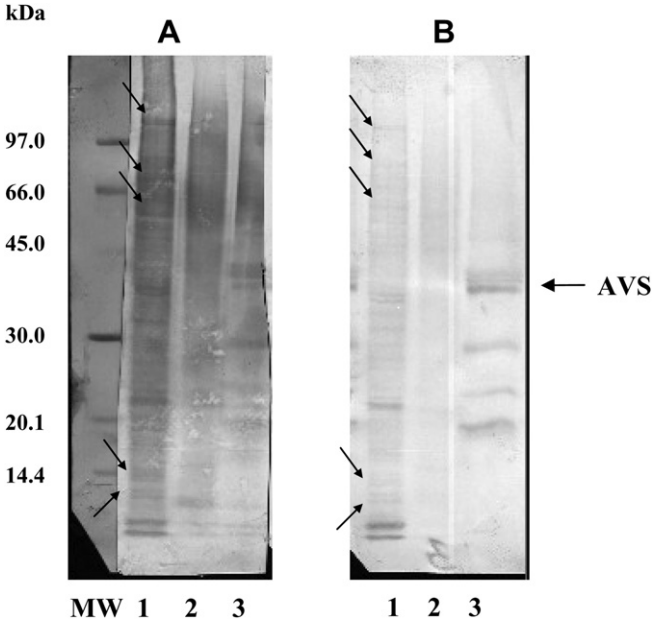


**Fig. 3.** Comparison of the polycolonal antibody through development stages. A. MW marker, B. After immunization with spores, C. After boost with Avs 43 cut from gels; 1. extract of *A. versicolor* spores; 2. AVS acetone precipitation of culture filtrate; 3. Avs preparation from ion exchange column (serum dilution 1:2000). After the boost with the 43 kDa protein, the antibody titer to the target protein increased Avs is indicated by arrow on right of Fig. 3B; some proteins attenuated after boosting with pure Avs 43 are indicated by arrows on left of Figs 3A and 3B.

**Table 3**
Purification of Avs 41 protein.

| Purification step | Protein (mg) | Recovery (%) |
|---|---|---|
| 75% acetone | 250 | 100 |
| AS precipitation | 80 | 32 |
| Ion exchange | 1.2 | 0.48 |
| Gel filtration | 0.2 | 0.08 |

the negative control was soybean trypsin inhibitor. Avs 41 kDa was determined to be non-glycosylated (data not shown).

The N-terminal sequence is shown on Table 4. A search of the protein database of NCBI/BLAST showed that Edman sequence of Avs 41 shared 69%, 62% and 62% identity with homologous proteins from three different *Aspergillus* species, *A. fumigatus* (34 kDa), *A. flavus* (34 kDa) and *A. oryzae* (34 kDa). All three homologous proteins were predicted to be alkaline serine proteases. Based on the complete protein sequence, the N-terminal sequence for the mature protein starts at amino acid 122.

### 3.3. Monoclonal antibodies

After gel filtration, each of the five mAbs were of much improved purity based on reduced non-specific responses in both immunoblot and ELISA. For example, IgM titer in the 6E2 ascites was ca. 1 mg mL IgM antibody in 25 mg $mL^{-1}$ total protein in ascites (4%) and the purified mAb 6E2 after gel filtration column contained approximately 56% IgM antibody (1.26 mg $mL^{-1}$ IgM antibody in 2.25 mg $mL^{-1}$ solution). The purity of IgM after gel filtration increased 14× compared to the ascites. Recovery of 6E2 from gel filtration column was 59%.

### 3.4. Antibody performance

As demonstrated in Fig. 6, the Pab was specific for spore extracts of *A. versicolor* compared to similar extracts of the spores from other species.

When Avs 41 was tested by ELISA, two monoclonal antibodies, 6E2 and 5C9 had the highest response, which were consistent over the operating range for the indirect ELISA used (Fig. 7 for 6E2). By indirect ELISA, response to spore extracts of five *A. vesicolor* strains (Winnipeg, Ottawa, Vancouver and Hawaii) were tested with different mAbs. For example, 6E2 produced a repeatable response with extracts of spore fragments from the five strains with a mean maximum OD of 1.3 ± 0.2 for 10 μg per well spore equivalents *A. versicolor* (data not shown). *A. versicolor* DAOM 235361 had the highest response to that antibody, with an OD of 2.0 ± 0.2. Using an indirect assay, the detection limit for the pure protein was ~500 ng/well (Fig. 7). Contents of both the 43 and 41 kDa proteins (w/w) in spores were 1 ng per $10^3$ spores, approximately $10^5$ times more when compared to the mycelium.

The results of cross reactivity tests with Mab 6E2 are shown in Fig. 8. This antibody was also very specific to the target protein.

## 4. Discussion

*A. versicolor* is one of the most common fungi degrading materials in the built environment. It is able to degrade various components in paper and wood, plastic polymers and animal proteins as well as starch and proteins in grains. As with the case of other fungi common on gypsum board, manufactured wood and related products (Miller et al., 2008), it is common in the concentrated gypsum saline environments such as the Dead Sea (Kis-Papo et al., 2001) and very tolerant to those conditions (Kis-Papo et al., 2003). It is also common in high salt soils and salt marshes (Chen, 1964; Abdel-Hafez et al., 1977).

Using *Penicillium*- and *Aspergillus*-reactive human sera, it was possible to identify two antigenic, excreted proteins, 43 kDa and 41 kDa, from mycelium and culture. Since these sera were not from occupational exposures, these were produced during degradation of substrates in the built environment (Xu et al., 2007; Wilson et al., 2009) in sufficient amounts to generate exposures necessary to raise antibodies in humans. The use of multiple human sera to

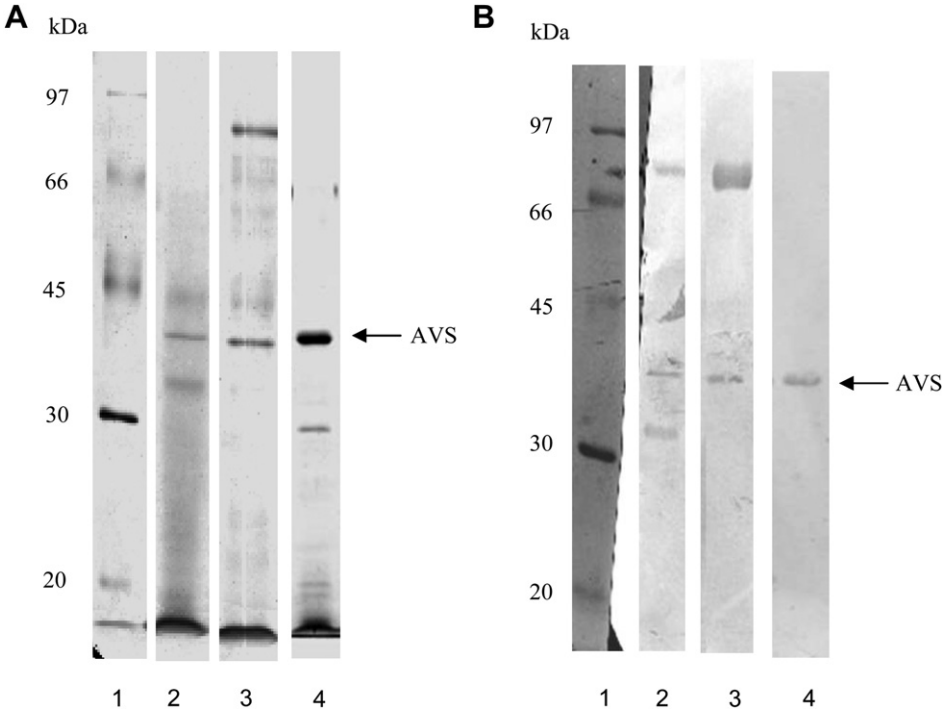


**Fig. 4.** Silver stain (A) and Western blot (B) comparing the target protein after each purification step for Avs 41; pAb Molecular weight marker; 2, 55%–65% ammonium sulphate precipitation; 3, ion exchange fraction; 4, gel filtration fraction.

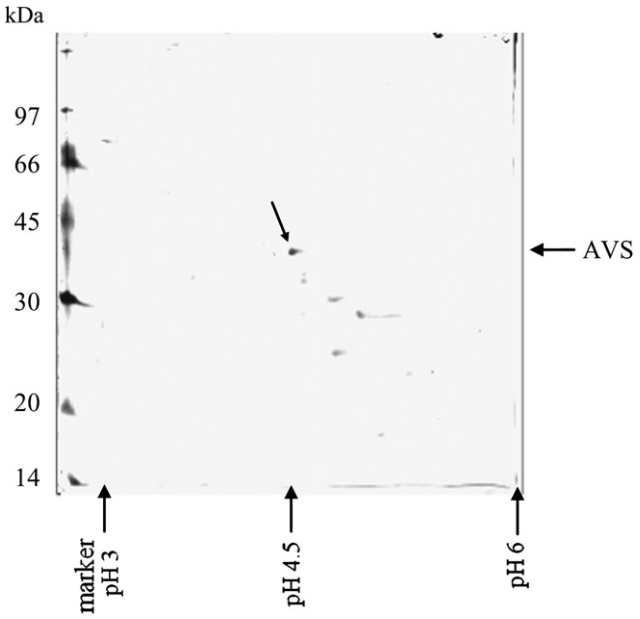


**Fig. 5.** 2-D gel electrophoresis analysis of purified Avs 41 protein (7.5 μg). IPG strip pH 3–6 was used and the gel silver stained marker: LMW-SDS marker from GE healthcare; AVS: the target protein from *A. versicolor* culture supernatant.

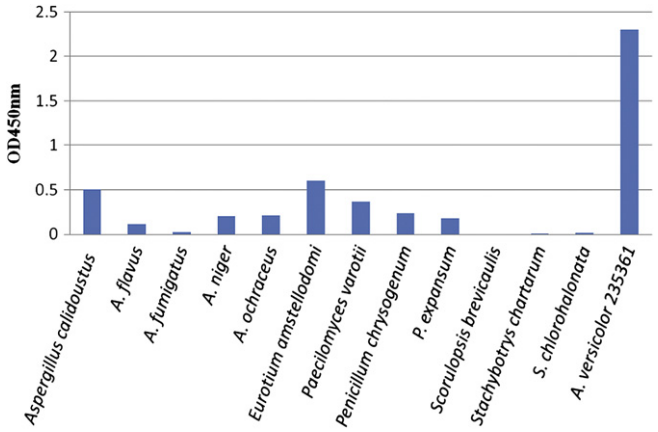


**Fig. 6.** ELISA response of the enhanced pAb to various fungal spore extracts $10^5$ spore equivalents, except *S. brevicaulis*, *S. chartarum* & *S. chlorohalonata* ,$10^4$ spore equivalents.

identify antigens produced consistently by different strains of the fungus narrowed the choice to proteins of greatest potential interest for environmental measurements. One of the proteins (43 kDa) was isolated from electrophoretic gels and used to demonstrate that antibodies had been produced that recognized the protein in a goat previously immunized with spores of *A. versicolor*. Boosting the previously immunized goat with 1 mg of this protein was found to expand one or more of the most specific antibody lines induced by the spores. In turn, this allowed demonstration that Avs 43 and Avs 41 are closely related proteins, of similar chromatographic properties and which are immunogenic in humans, goats and mice. This approach shows that the proteins involved were present in "natural" fungal cells and in strains collected across USA and Canada. This reflects the previous experience with the deteriogens *S. chartarum* (Xu et al., 2007, 2008) and *P. chrysogenum* (Wilson et al., 2009; Luo et al., 2010).

The N-terminal sequence of the 41 kDa protein had a 69% similarity with a 34 kDa protein from *A. fumigatus*, a 62% similarity with 34 kDa proteins from *A. flavus* and *A. oryzae*. The three 34 kDa proteins are alkaline serine protease (Ramesh et al., 1994; Yu et al., 1999; http://www.allergen.org/Allergen.aspx). The protease from *A. flavus* is 83% similar to the protein from *A. fumigatus* (Jaton-Ogay et al., 1992) and 82% to the *A. oryzae* protein (Tatsumi et al., 1989). The cDNA has been reported for all three proteases. The proteins contain a signal sequence (amino acid 1–21), a prosequence (amino acid 22–121) and a mature protein (amino acid 122–403). All have an open-reading frame that contains 1212 nucleotides. The *A. versicolor* sequence encodes a total of 403 amino acids which has a predicted molecular mass of 42 kDa. The proteins from the other species are processed only at the N-terminus (Tatsumi et al., 1989). The first 21 amino acids have the properties of signal sequence of secreted fungal proteins (Periman and Halvorson, 1983). The proteases contain three introns, and the N-terminal sequence for the mature protein was determined to be very similar to the protein sequence deduced from the DNA sequence. The N-terminus of the mature protein started at amino acid 122 and the first 121 amino acids possess the properties of the pre-pro sequence of the protease.

The mature proteins of these proteases from *A. fumigatus*, *A. flavus* and *A. oryzae* comprise 282 amino acids with a predicted molecular weight of 34 kDa, consistent with their molecular weight by SDS-PAGE. The alkaline serine protease are synthesized as a pre-proprotein with a signal sequence and processed when the enzyme is active (Ramesh et al., 1994).

A majority of serine proteases have a long propeptide that functions as a precursor structure. This structure can keep the protease from autolysis until it is processed when the protein is active (Yu et al., 1999). The propeptide from *A. fumigatus* has an identical 100 amino acid segment with propeptide of the *A. oryzae* serine protease, suggesting that these propeptides are strongly conserved within the same species. Since the N-terminal sequence for the *A. versicolor* protein is similar to these three species mentioned, it is possible that the 41 kDa protein is an alkaline protease that has a signal sequence, a prosequence and a mature protein. Based on the calculated molecular weight difference based on this hypothesis between Avs 41 and Avs 43, the latter is the unprocessed protein with a signal sequence of approximately 21 amino acids. Each of the homologous proteins discussed has a secreted peptide sequence in the N-terminus, which will be cut by

**Table 4**
N-terminal amino acid sequences of Avs 41 and proteins from other *Aspergillus* spp.

| Protein | N-terminal sequence | Identity with Avs 41 | Positives with Avs 41 |
|---|---|---|---|
| ADE74975.1 (AVS41) | ALTTQSDAPPGLGAISHQGDAS(/A)SSYI | 100% | 100% |
| XP_663162 (*A. nidulans*) | ALTSQSGAPWGLGAISHKGEAS TTYV | 69% | 92% |
| ACX47962 (*A. clavatus*) | ALTTQSGAPWGLGSISHKGQAS TNYV | 69% | 88% |
| XP_001213078 (*A. terreus*) | DLTTQSDAPWGLGSISHKGQPS TDYI | 72% | 84% |
| CAA75804 (*A. fumigatus*) | ALTTQKGAPWGLGSISHKGQAS TDYI | 69% | 80% |
| P35211 (*A. flavus*) | DLTTQSDAPWGLGSISHKGQPS TDYI | 72% | 84% |
| CAA38527 (*A. oryzae*) | GLTTQKSAPWGLGSISHKGQQS TDYI | 64% | 76% |

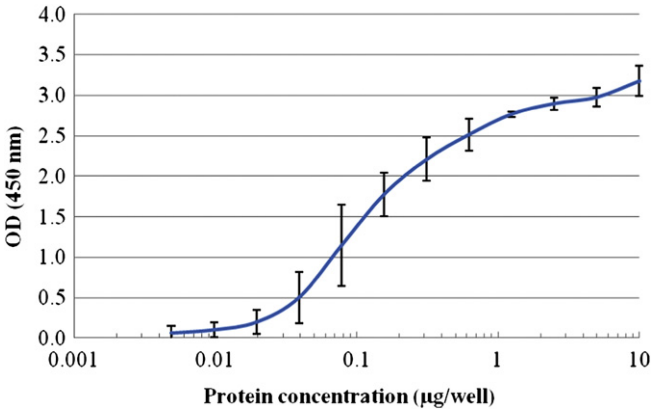


**Fig. 7.** ELISA response for mAb 6E2 against Avs 41.

protease after secretion. The signal peptide is around 20 amino acids (1.67 kDa), which explains the 2 kDa molecular weight difference between the 41 and 43 kDa proteins, given the chromatographic similarity of the two. It is interesting to note that there was another 33 kDa protein from *A. versicolor* that was recognized by two Mabs on immunoblot (data not shown). This may have resulted from autolysis (Liang, 2008). The 41 kDa protein is not glycosylated and the three 34 kDa *Aspergillus* proteins are also not glycosylated.

Aside from molecular weight and that apparent nature of the enzyme, pI values of the four proteins are quite different. The pI of the serine protease was 7.9 for the *A. fumigatus* (Reichard et al., 1990), 7.0 for *A. oryzae* (Shen et al., 1998) and 6.8 or 6.3 for *A. flavus* (Chou et al., 1999; Yu et al., 1999). All these alkaline serine proteases are either slightly acidic or slightly basic, whereas in this study, the protein identified as a serine protease by sequence from *A. versicolor* has a pI of 4.5. Based on partial sequence, the overlapping protease from *Aspergillus nidulans* is a hypothetical protein and from *Aspergillus terreus* is similar to that from *A. oryzae* and has a molecular weight of 32.5 kDa. The entire sequence has recently been completed using the methods described by Shi et al. (2011) which confirms that the protein is new (Shi, Smith, Miller, unpublished data).

A number of allergens from *Penicillium* species are alkaline serine protease allergens in the range of 34 kDa. However, the N-terminal amino acid sequence only shared approximately 40% identity with *A. versicolor*. The total number of amino acid and their cleavage sites are quite different. For example, the pre-proprotein for *P. chrysogenum* was predicted as 397 amino acids with a mature protein starting from amino acid 115. Shen et al. (1999) reported the pI for the *P. chrysogenum* alkaline serine protease allergens was ca. 6.7–7.2, similar to those of the other 34 kDa allergens but with a different pI from that of *A. versicolor*.

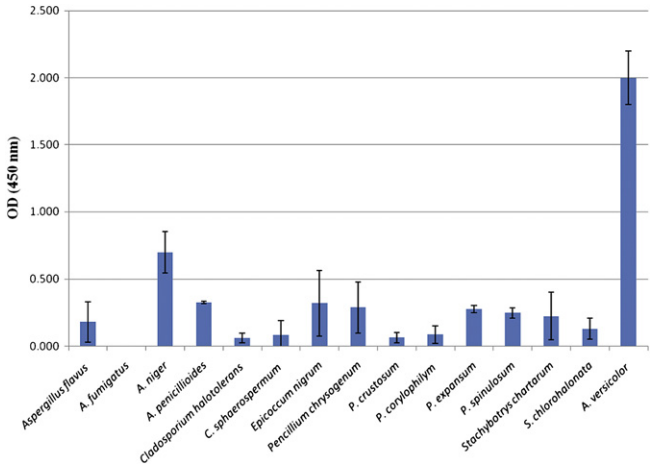


**Fig. 8.** Indirect ELISA response of mAb 6E2 to various spore extracts $10^6$ spore equivalents, except *E. nigrum. S. chartarum* & *S. chlorohalonata*, $10^5$ spore equivalents.

The isolated 43 kDa protein was used to produce Mabs in four mice. Two of them produced antibodies to the 43 kDa and 41 kDa proteins. As noted, both were present in very high relative concentrations in or on the spores similar to the situation for *S. chartarum* (Rand and Miller, 2008). When this process was completed, ca. 1000 clones were screened on ELISA and using a dot matrix protocol, on immunoblot. In contrast to similar studies with *S. chartarum* (Xu et al., 2008) and *P. chysogenum* (Luo et al., 2010), the *A. versicolor* mAbs were almost exclusively the IgM isotype. Using washed, unpurified spore fragments as immunigens, Schmechel et al. (2005) found that those murine antibodies to *A. versicolor* were also dominated as the IgM isotype.

It is unknown whether these two related proteins are proteases as is predicted by some domains in the sequence. However, the responses of both the enhanced polyclonal antibody and monoclonal antibody indicate that the protein is different from the spore–borne proteins which are reported to be mostly 34 kDa alkaline serine proteases (e.g. *A. flavus*, *A. niger*, *P. chrysogenum*). In our hands, these species have little material cross reactivity to the Avs – reactive antibodies.

Benndorf et al. (2008) studied allergens found in *A. versicolor* spores which were all common fungal proteins. In contrast to our approach which focused on excreted proteins present in high concentrations on spore surfaces (Rand and Miller, 2008; Xu et al., 2008; Luo et al., 2010), these authors extracted proteins after the spores were lysed. By mass spectroscopy, they identified proteins that are common in many related species (glyceraldehyde-3-phosphate dehydrogenase, a putative sorbitol/xylose reductase, catalase A, enolase and malate dehydrogenase). None of these were similar to Avs 41. Using washed spore fragments, Schmechel et al. (2005) produced antibodies that did not show species specificity, possibly because, as with Benndorf et al. (2008), the extracts contained mainly common fungal proteins.

In summary, we have isolated two related proteins with molecular weights of 41 and 43 kDa, both are human antigens and are immunogenic in goats and mice. The smaller protein is unglycosylated and has an acidic pI and based on the partial sequence obtained, a protease. By virtue of antibodies found in exposed people, it is apparently accumulated as a relatively high proportion of total protein on natural substrates of this biodeteriogenic fungus. The proteins are found in high concentrations on spores. Based on use of a pAb and a number of mAbs that have been developed to screen for the protein in a taxonomically diverse group of fungi, it appears to be useful as a biomarker for the fungus in nature and the built environment.

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