



Research paper

Characterization of monoclonal antibodies to an antigenic protein from *Stachybotrys chartarum* and its measurement in house dust

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Abstract

Using sera from atopic patients we have isolated an extracellular protein, which is antigenic in humans, from *Stachybotrys chartarum* *sesu lato*. Here we report the production of monoclonal antibodies to the protein and the development of a sensitive and specific assay to the target protein as well as analyses in house dust samples spiked with spores. The detection limit for the target antigen in house dust was ~0.2 ng/g dry weight house dust. This detection limit is comparable to those for house dust mite allergen and the allergen of the fungus *Aspergillus fumigatus* but lower than that for the fungus *Alternaria alternata*.

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1. Introduction

Health Canada among other cognizant authorities has considered the relationship of exposure to fungi growing in damp buildings to respiratory disease. Mold growth indoors is associated with exacerbation of asthma in mold-sensitized individuals and increased upper respiratory disease. Reliable tools for exposure assessment methods are critical to resolving causality and attributable risk of dampness contaminants (Health Canada, 2004; NAS, 2000, 2004). The NAS panel that wrote ‘Clearing the air’ indicated that the best way to assess the causality of allergic disease associated with fungi in

the environment would be to develop “standardized methods for assessing exposure to fungal allergens... preferably based on measurements of allergens rather than culturable or countable fungi”. Acquisition of these data is a necessary step before the role of fungal allergen in asthma can be documented” (NAS, 2000).

We have responded to this agenda by working with a large random collection of human sera from atopic patients previously screened for fungal allergy by a clinical laboratory. The human sera were used to perform Western blotting on extracts of cultures of building-associated fungi collected from across North America with a view to isolating proteins that are (1) antigenic in humans and (2) diagnostic for the species. This has permitted the identification of fungal proteins antigenic in humans including one from *Stachybotrys chartarum* *sesu lato*. This fungus is characteristic of wet lignocellulosic

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materials in nature and in the modern built environment and represents a community of ecologically-similar fungi on moldy materials (Miller et al., 2003).

The target is a 34 kDa protein which is antigenic in humans, mice and goats. This protein is present on the surface of spores from *Stachybotrys chartarum* grown on rice (Rand and Miller, in press). It is excreted from mycelium when grown in culture, on paper-faced gypsum board, as well as the preferred natural substrate of the fungus, straw (Xu et al., 2007). The protein is characteristic of *Stachybotrys chlorohalonata* and *S. chartarum* (both formerly *S. chartarum*; Andersen et al., 2003). The objective of this work was to produce and characterize monoclonal antibodies to this protein.

2. Materials and methods

2.1. Production of monoclonal antibodies

The SchS protein, which is comprised of SchS34 (34 kDa) was purified as described by Xu et al. (2007). Monoclonal antibodies (mAb) were prepared as follows. Four female BALB/c mice were immunized by intraperitoneal injections with 25 µg of SchS protein per mouse, in Complete Freund's Adjuvant. Four subsequent boosts were administered as above, spaced at 3 week intervals, with Incomplete Freund's Adjuvant. When the serum titer had risen more than 10-fold from a pre-immune serum sample, as determined by ELISA, the sera was tested for response to the pure SchS protein on Western blotting (Xu et al., 2007). The two highest responders with a clear response on Western blotting were each boosted intravenously with 10 µg of the SchS protein 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.

Fusion of the splenocytes with SP2/0 BALB/c parental myeloma cells was performed after Kohler and Milstein (1975), except that one-step selection and cloning of the hybridomas was performed in Clonacell™-HY medium (Stemcell Technologies Inc., Vancouver, BC; Davis et al., 1982). The resulting clones were screened by ELISA for antibody activity on plates coated with 1 µg/well of the target antigen using both anti-mouse IgG and anti-mouse IgM conjugates as secondary antibodies, respectively. Putative positive hybridomas were further characterized on Western blotting, and ascites were obtained from selected hybridoma cell lines. Ascites were prepared in BALB/C mice after IP, with 0.50 ml of Pristane (Sigma, P9622), 7 days prior to inoculating them with the hybridoma cell line (Harlow and Lane, 1988).

Murine monoclonal IgG (mAb) and goat polyclonal IgG (pAb) antibodies were purified using affinity

chromatography. The production and characteristics of the goat polyclonal antibody are described in Xu et al. (2007). Briefly, sera or tissue culture supernatant was made to 50% saturation ammonium sulfate by adding equal volume of 100% saturation ammonium sulfate solution and kept at 4 °C overnight. The pellet was separated by centrifugation and dissolved in PBS buffer and subjected to ultrafiltration with several buffer changes to remove extra ammonium sulfate. Antibodies were then purified on Hitrap Protein G affinity columns (Amersham Biosciences) according to the manufacturer's instructions.

The affinity of the selected mAbs to the target protein was determined by a non-competitive enzyme immunoassay (Briguet et al., 1985). Calibration curves were established for determination of the concentrations of mAbs 12D3 and 4G10 in microwell plates coated with SchS (10 ng/well) by indirect ELISA. In separate tubes, a range of concentrations of the antigen solution (SchS, 10 µg/mL to 10 ng/mL) were mixed with each antibody at a constant concentration (0.25 µg/mL). The concentration of free antibody was determined by indirect ELISA. The K_d values were calculated using the Scatchard equation.

2.2. Affinity protein purification

Affinity chromatography was used for the further purification of SchS protein. An affinity column was made by coupling mAb 12D3 or mAb 4G10 to CNBr-activated Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's instruction. The SchS protein purified by ion exchange, hydrophobic and then gel filtration columns was dissolved in PBS buffer and applied to a 12D3-Sepharose column. The column was washed with PBS to remove unbound proteins and the SchS protein was eluted with 0.1 M glycine, pH 2.7. Fractions (1 mL) were collected in tubes containing 50 µL of 0.5 M Tris buffer, pH 8.8, as neutralization buffer. The SchS protein fractions were pooled, and subjected to ultrafiltration against PBS buffer and stored at −20 °C.

2.3. SDS-PAGE and Western blotting

Proteins were separated by SDS-PAGE as described by Laemmli (1970) using a low molecular weight marker (Amersham Biosciences, 14.4–97 kDa). Protein bands were visualized with the PlusOne silver staining kit (Amersham BioSciences) or GelCode Blue Stain Reagent (Pierce) according to the product procedures.

The proteins separated on SDS-PAGE gel were transferred to a Hybond-PVDF membrane (Amersham Biosciences) with an electrotransfer unit (Hoefel

miniVE, Amersham Biosciences). The electrotransfer was carried out at a constant current of 40 mA. Then the membrane was incubated with a primary antibody solution (pAb or mAb) followed by corresponding secondary antibody (anti-mouse IgG or anti-goat IgG conjugated with alkaline phosphatase). The proteins with immunological activities were stained by incubating with 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) for 5–10 min.

2.4. Capture ELISA

For the capture ELISA assay, purified monoclonal antibody 4G10 was labeled with biotin. Biotinylation was carried out using (EZ-Link NHS-LC-LC-Biotin, Pierce) with 80 molar excess according to manufacturer instructions. The activities of Biotin-4G10 were confirmed by both indirect ELISA (for antibody activity) and direct ELISA (for biotin activity).

Microplates were coated with 100 μ L/well of mAb 12D3 (1 μ g/mL) in 50 mM carbonate–bicarbonate buffer, pH 9.6 and incubated at 4 °C overnight. The plate was washed three times with 150 μ L of PBST between incubations and blocked with 150 μ L of 2% BSA/PBS solution for 1 h at room temperature. A volume of 100 μ L *Stachybotrys* allergen solution or test samples diluted in 1% BSA/PBST was added to each well and the plate was incubated for 1 h. After washing, 100 μ L/well of biotinylated anti-SchS monoclonal antibody 4G10 (1 μ g/mL) in 1% BSA/PBST was added and the plate was incubated at room temperature for 1 h. After washing with PBST buffer, the plate was incubated with 100 μ L of streptavidin-peroxidase (Sigma) in PBST at a dilution of 1/1000 for 1 h. The colour was developed by adding 100 μ L of substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) and incubated for 10 min. The absorbance was read in a microplate reader (Molecular Devices) at 450 nm. A checkerboard analysis was performed to optimize the antigen and antibody concentrations.

2.5. Culture of fungal spores and mycelium

Liquid cultures and spores that were not wetted prior to testing were produced for specificity tests. The fungi tested included *Alternaria alternata* 27-1*, *Aspergillus ochraceus* 12-7-3, *A. flavus* 12-7-4, *A. fumigatus* 31, *A. niger* 5869-6, *A. penicillioides* 5053-15, *Eurotium amstelodami* 9507-5, *E. rubrum* 21-4, *A. ustus* 12-7-3, *A. versicolor* DAOM** 235361, *A. versicolor* 43-2, *Cladosporium cladosporioides* 48-1, *Epicoccum nigrum* 51-2, *Chaetomium globosum* 317-1, *Memnoniella echinata* DAOM 235365, *Paecilomyces variotii*

DAOM 235357, *Penicillium commune* 21-1, *P. expansum* DAOM 235358, *P. corylophilum* 12-7-3, *P. chrysogenum* DAOM 234056, *P. spinulosum* 12-7-1, *Scopulariopsis brevicaulis* 5869-3, *S. chartarum* (DAOM 235364), *S. chlorohalonata* (***ATCC 201863), *Wallemia sebi* 12-7-2. *Paracel Laboratory number; **DAOM; National Mycological Herbarium, Ottawa; ***American Type Culture Collection, Manassas, VA.

Liquid cultures were produced on a medium designed to favour protein/peptide production (Xu et al., 2007). The mycelia were filtered and freeze dried. The production of spores was based on the method of Murad et al. (1993). Rice (50 g Uncle Bens™) 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 then air dried. Spores were harvested by physical removal of spores from the rice grains and stored in vials at –20 °C. Some species did not produce spores in sufficient quantities for this assay.

One milligram of spores or mycelium of each available species was carefully weighed into two 2-mL vials, respectively. In the case of the spores, one vial of dry spores was milled with a polystyrene bead for 15 min using a Spex-Certiprep mixer mill (model 5100, Metuchen, NJ). Then, 0.1 mL of PBS buffer was added and allowed to stand for 10 min. Spore concentrations were determined in the vial not treated with the mixer mill with a hemacytometer. The remaining vial was frozen until analysis. The resulting protein contents were then measured with the Micro BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. A defined number of spore equivalents and 20 μ g of total protein mycelial extracts were analyzed with the capture ELISA.

2.6. Analysis of *S. chartarum* spores in house dust

Fine dust was used from samples collected as part of a study where all floors in six houses were repeatedly vacuumed with a HEPA filter equipped vacuum (Miele model S558, Vaughan, ON). These houses did not have moisture problems and the cleaning was done by a professional cleaner following a strict protocol (Salares et al., 2006). Samples were transported and stored under air-dry conditions, and the dust was sieved to <300 μ m (stainless steel test sieve model 50, Fisher Scientific) and weighed using a Sartorius balance (A120-S 4; accurate to 0.1 mg).

S. chartarum (DAOM 235364) spores were collected from the heavily colonized straw by gently scraping the surface using a microspatula (straw is a natural substrate of

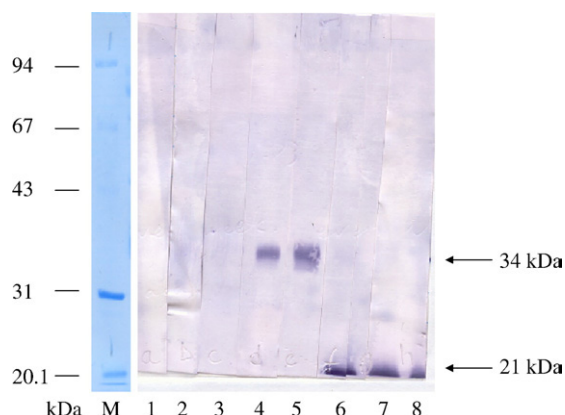


Fig. 1. Western blotting of the active hybridomas. 50 μ g SchS was applied in each lane. After electrophoresis, the membrane was cut into strips and hybridoma tissue culture supernatant (1/200 dilution) was applied; anti-mouse IgG-AP (1/2500 dilution) was used as secondary antibody. Lanes 1–8 are hybridoma cell lines 2B11, 5A3, 5D1, 8B12, 12D3, 4G10, 5B1 and 5B2, respectively.

S. chartarum; Xu et al., 2007). Approximately 6 mg were suspended in 15 mL 1% BSA/PBST. A 5 mL aliquot of the suspension was reserved for further analysis, while the remaining 10 mL was doubly diluted 7 times which resulted in 8 spore suspensions with a range of 2 ~ 0.0156 mg spores/5 mL. Each spore suspension was then added into 0.5 g of <300 μ m fraction of settled house dust with a ninth dust sample containing only 5 mL 1% BSA/PBST to act as a negative control. The dust/spore suspensions were allowed to shake at room temperature for 1 h followed by centrifugation at 10,000 $\times$ g for 10 min at 4 $^{\circ}$ C to remove debris. The supernatants from both the initial 5 mL aliquot (no dust) and the dust/spore suspensions were tested in triplicate on three separate occasions using the SchS capture ELISA. Pellets were resuspended in 5 mL 1% BSA/PBST and spores enumerated.

To determine the presence of culturable fungal propagules in the dust, an aliquot (1 g) was serially diluted to extinction in sterile 0.5% Tween over four log dilutions using the spread plate method on 2% malt extract agar plates and DG 18 (both Difco) for more xerophilic species. Plates were incubated at 25 $^{\circ}$ C for 7 days, counted and representative colonies were transferred to 2% malt extract agar and Czapek–Dox agar plates for identification according to the procedures described (Hung et al., 2005).

3. Results

3.1. Characterization of monoclonals

A total of 34 hybridoma lines were obtained from the fusions with a very high reactivity in the ELISA assay, all

of which were identified as IgG. However, on Western blotting, only 13 monoclonal antibodies strongly recognized the target protein, SchS34. Unexpectedly, 6 monoclonal antibodies recognized SchS21 (a putative subunit) which was uncommonly recognized by human sera and goat sera (Xu et al., 2007). The remaining clones did not show bands on Western blotting (Fig. 1). Thus, two different classes of mAbs, anti-SchS34 mAb and anti-SchS21 mAb, were identified from thirty four active clones. No cross reactivity was observed between these two classes (Fig. 1), indicating that the monoclonal antibodies bound exactly with its corresponding antigenic protein. The dissociation constants of mAb 12D3 and mAb 4G10 were 0.0222 nM and 0.0427 nM, respectively (Fig. 2).

To separate the two proteins, mAb 12D3 and mAb 4G10 immunoaffinity chromatography was attempted. However, the protein recovered from the mAb 12D3-immunoaffinity column (i.e. that targeted to SchS34) contained the intact SchS protein, SchS34 and SchS21 subunit (Fig. 3A). Both subunits retained their expected response to the two mAbs (Fig. 3B). When mAb 4G10 was used in the immunoaffinity chromatography, comparable results were obtained (data not shown).

3.2. Capture ELISA methods

The combination of mAb 12D3 as the capture antibody (coated on the microplate), a biotinylated mAb 4G10 as the primary antibody and the streptavidin-horseradish peroxidase conjugate as the detection reagent were used in a SchS capture ELISA. A typical titration curve was

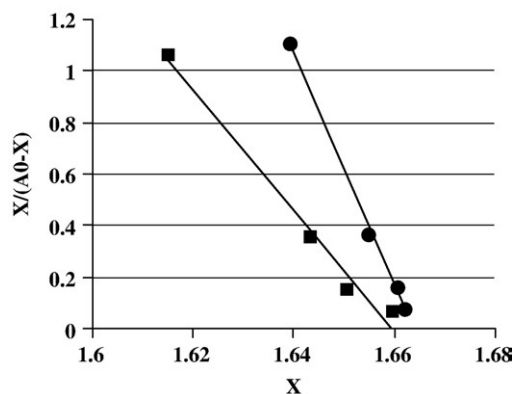


Fig. 2. Scatchard plots* of the binding of SchS4S protein to mAb 12D3 (●) and mAb 4G10 (■). X (nM): the concentration of bound antigen (Ag)/antibody (Ab) complex A0 (nM): the initial antibody concentration. Scatchard equation: $X/(A0-X) = (-1/Kd)X + A0/Kd$; Linear equations: $y = -45.074x + 74.99$, $R^2 = 0.9973$ (●); $y = -23.411x + 38.851$, $R^2 = 0.9839$ (■). * Results of triplicate experiments.

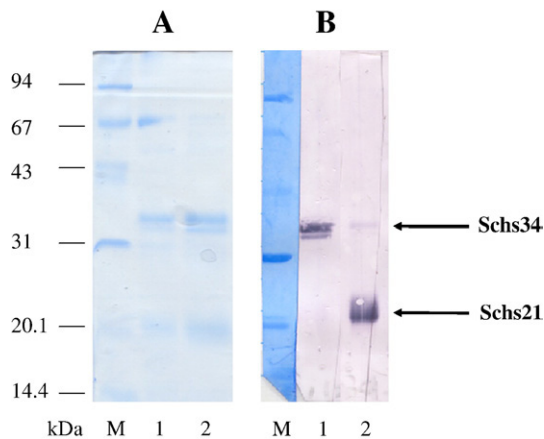


Fig. 3. SDS-PAGE analysis of SchS preparations. A: Preparation from: gel filtration column (lane 1), further purified by affinity chromatography with mAb 12D3 (lane 2). The proteins were stained with Coomassie brilliant blue. B, SchS protein recovered from mAb 12D3-affinity column was visualized on Western blotting against mAb 12D3 (lane 1) and mAb 4G10 (lane 2).

established with serial dilutions of affinity purified SchS protein under optimized capture antibody and primary antibody concentrations (Fig. 4). The detection limit of this assay was 0.02 ng/mL, with a linear range of 0.04–0.6 ng/mL.

The capture ELISA assay was used to compare the response of SchS protein purified by affinity chromatography (present experiments), ion exchange chromatography and culture extract (Xu et al., 2007). All three preparations showed similar shaped curves, in the correct order of sensitivity considering their relative purity, but with different linear ranges and detection limits (Fig. 4). These data also indicate that the antibodies selectively captured the target protein and that the protein was not

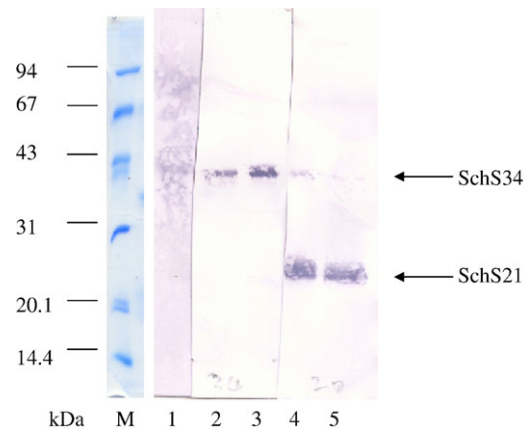


Fig. 5. Responses of the polyclonal and monoclonal antibodies to *S. chartarum* antigen preparations. Both partially purified extract of *S. chartarum* (10% SchS; lanes 1, 2 and 4) and pure SchS protein (lanes 3 and 5) strips were used in the Western blotting against polyclonal antibodies (lane 1), mAb12D3 (lanes 2 and 3) mAb4G10 (lanes 4 and 5).

altered during the purification steps, as also shown on the Western blotting in Fig. 5.

Using the capture ELISA, the response to spore (Fig. 6) and mycelial extracts (data not shown) of some fungi were tested. The culture extracts of *S. chartarum*, and the closely related, *S. chlorohalonata* gave high responses. Western blotting of the mycelial extracts against mAbs 12D3 and 4G10 showed that both SchS34 and SchS21 proteins were present in these taxa (data not shown). However, spores or mycelium of the remaining species showed limited reactivity in the capture ELISA tests (Fig. 6) indicating that there are no proteins with the same (or similar) antigenic properties as that of SchS protein in those fungi.

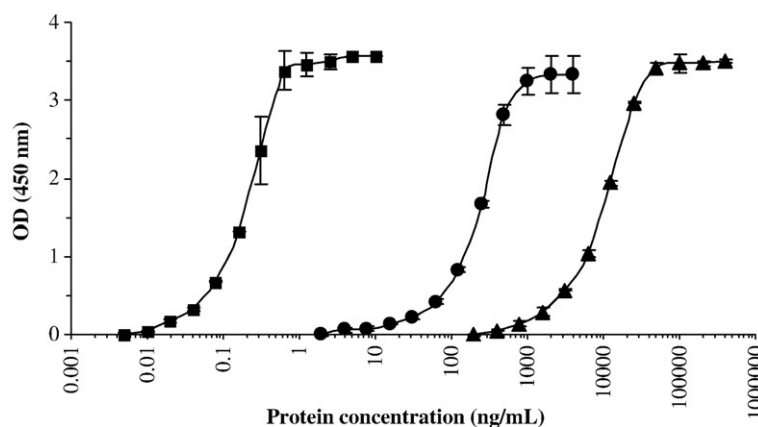


Fig. 4. Capture ELISA response for different preparations of SchS*. *affinity purified (■), ion exchange (●) *S. chartarum* culture extract (▲).

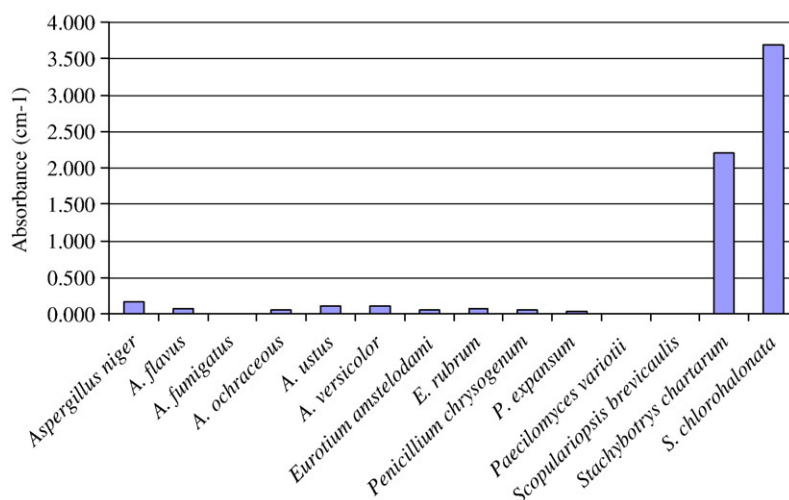


Fig. 6. Response of capture ELISA to spores of selected fungi*. * Relative spore numbers: *S. chartarum* and *S. chlorohalonata*, 1 (10^5 spore equivalents); others: $10\times$ to $200\times$ more; values of triplicate experiments.

3.3. Analysis of house dust samples spiked with *S. chartarum*

The recovery of the antigen was $93.6\% \pm 7.5$ while the quantification limit of SchS34 was 0.2 ng/g determined by LSD after ANOVA of the data (Fig. 7). No response was observed for the control, i.e. un-spiked dust sample. The results of the culturable plating analysis indicated the dust sample contained $>10^6$ CFU/g based on the results from the two culture media of differing water activities (data not shown). The species present mainly comprised moderate xerophiles such as *P. variotii*, *P.*

chrysogenum, *A. versicolor* and the teleomorphic *Aspergillus* species.

4. Discussion

Our previous studies had shown that the SchS protein is closely related to an extracellular DNase from *Nectria haematococca* (Xu et al., 2007) which is comprised of a 20 kDa associated with a larger protein (Klosterman et al., 2001). Using mAbs that recognize SchS34 protein, we found that response and sensitivity were similar regardless of which epitope pattern was being determined. This indicates that there are very strong interactions between two subunits of SchS, although they are not covalently bound through disulfide bonds (Xu et al., 2007).

Andersen et al. (2003) found that *S. chartarum sensu lato* could be circumscribed as a species with two chemotypes (*S. chartarum*) and one new species with different morphological and cultural characteristics, *S. chlorohalonata*. The metabolite profile of the latter species was the same as *S. chartarum* chemotype A. The two had different *tri5*, *chs1* and *tub1* gene fragment sequences. We report that these two species also share the SchS protein.

Based on the tests with human sera thus far (Xu et al., 2007; Jensen, unpublished data) we found only one clinical sample that responded to the 21 kDa protein as well as the 34 kDa protein. The response to the 34 kDa protein was characteristic of patients displaying IgG or IgE antibodies for *S. chartarum* as determined using the Immunocap™ system (see Halsey et al., 2000). However, during the production of mAbs in mice, antibodies were

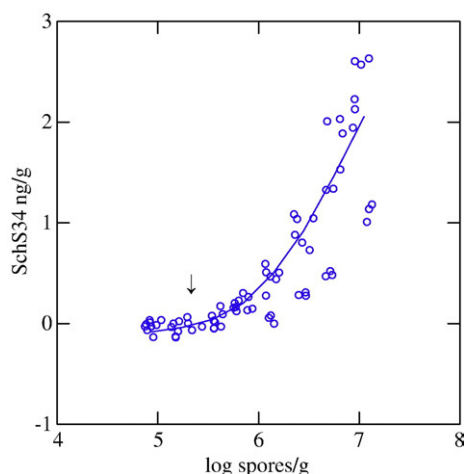


Fig. 7. Analysis of *S. chartarum* spore spiked dust samples using the SchS capture ELISA, arrow indicates limit of quantification.

produced in a ratio of 2:1. This demonstrates that the protein and the putative subunit are potentially immunogenic and the relative response in both goats (the pAb) and humans suggests that the smaller subunit is less so.

There are few studies of the production of monoclonal antibodies to isolated individual proteins of filamentous fungi from environmental fungi using human sera (e.g. Karkkainen et al., 2004). In the case of the phylloplane species and *A. fumigatus*, workers have taken the same basic approach as presented here, i.e. isolate a fungal protein for which there is a human distinct and consistent immunological response and create antibodies from the isolated or cloned materials (see Horner et al., 1995). Other approaches used have depended on *in vitro* techniques to find characteristic protein(s) for the purpose of environmental measurements. Since fungi make numerous, common antigenic proteins including in humans (Horner et al., 1995; see Rydjord et al., 2005, their Fig. 1), this approach might reasonably be expected to produce antibodies that widely cross react. The cross-reactivity information from the present study involves a variety of genetically diverse fungi. Essentially no material cross reactivity was observed, despite the use of an exquisitely sensitive assay for the target protein. As noted, since environmental fungi that occur in the built environment are known to produce many proteins in common, it is therefore unlikely that the SchS protein is found in other taxa. Additionally, this species is not closely related to most of the fungi common on damp building materials or the fungi dominant in outdoor air (Andersen et al., 2003; Koster et al., 2004; Miller et al., 2003).

We spiked a house dust sample with unwashed spores of *S. chartarum* grown on a natural substrate and were able to detect ~0.2 ng SchS34 antigen/g sieved dust. This is comparable to values found in settled dust for dust mite allergens (Earle et al., 2007) and the *Aspergillus fumigatus* allergen Aspfl (Dillon et al., 2007) but much lower than reported values for the *A. alternata* allergen Alt a1 (0.14 µg/g sieved dust; Salo et al., 2005). The detection of ~0.2 ng SchS34 represented ca. 10⁵ intact spores/g house dust (Fig. 7). These experiments do not give a reliable impression of the sensitivity in terms of spore equivalents because the majority of fungal allergen in house dust (>70%) is contained in particles that are smaller than intact spores (e.g. Foto et al., 2005; Gorny et al., 2002; Green et al., 2005), many of which are not viable nor contain intact DNA. Although SchS34 is mainly on the surface of the spore cell wall, there is additional protein embedded (Rand and Miller, *in press*) that becomes more accessible as the spores fragment. By way of comparison, an analysis of Asp fl in *A. fumigatus* spiked in the same

dust sample using the same methods was performed. This indicated that that the detection limit of 1 ng/g dust associated with 10⁷ intact spores/g (Belisle, 2007; Indoor Biotechnology #EL-AF1).

In summary, we report the production of a number of monoclonal antibodies that have provided additional information on the nature of an extracellular protein from *S. chartarum sensu lato*. The antibodies offer the basis for a sensitive assay for the environmental occurrence of *S. chartarum* based on the measurement of a protein that elicits a human response.

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