

Characterization of Human Antigenic Proteins SchS21 and SchS34 from *Stachybotrys chartarum*

Chunhua Shi^a Myron L. Smith^b J. David Miller^a

Departments of ^aChemistry and ^bBiology, Carleton University, Ottawa, Ont., Canada

Key Words

SchS21 • SchS34 • DNase • *Stachybotrys chartarum* • Antigen • Allergen • cDNA • Recombinant protein

Abstract

Background: SchS21 and SchS34 are proteins from *Stachybotrys chartarum sensu lato* that are antigenic in goats, mice and humans. Monoclonal antibodies to these proteins react with spores of *S. chartarum* and *S. chlorohalonata* but do not cross-react with a diverse taxonomic and ecological array of other fungi. **Methods:** Based on partial sequences of the 21- and 34-kDa proteins, obtained from tandem mass spectra and Edman degradation, degenerate primers were designed for touchdown PCR and the resulting amplicons were sequenced. Subsequently, inverse-PCR was used to obtain genomic DNA sequences encoding SchS21 and SchS34. RT-PCR products were sequenced to predict the mature protein sequences of SchS21 and SchS34. Based on the speculation that SchS21 protein was a DNase, the enzymatic properties were investigated. **Results:** Sequences of 435 and 666 bp in length were obtained from SchS21 and SchS34 cDNAs. The SchS21 open reading frame encodes a mature protein of 144 amino acids, while that of SchS34 is 221 amino acids in length. SchS21 is a secretory, alkaline, Mg-dependent exodeoxyribonuclease, while SchS34 is a secretory protein of un-

known function. His-tagged forms of the mature SchS21 and SchS34 proteins were separately overexpressed in *Escherichia coli* and purified using Ni-NTA columns (0.5 mg/l yield). **Conclusions:** Based on Western blots, the expressed proteins were similar in molecular weight and bound to the respective monoclonal antibodies to SchS21 and SchS34 proteins from *S. chartarum*. Interactions with human sera IgE confirmed the expressed forms of SchS21 and SchS34 as naturally occurring allergens.

Copyright © 2010 S. Karger AG, Basel

Introduction

There is an association between respiratory illness and indoor fungal contamination that is based on occupant reports for both symptoms and, with few exceptions, exposure assessments [1]. Expert panels from the United States National Academy of Science in 2000 and 2004 and Health Canada in 2004 found that mold and dampness were associated with increased upper respiratory disease and with increased asthma in mold-sensitive asthmatics. This prompted our interest in using sera from atopic individuals to screen fungal proteins. This process has involved a survey of many sera and fungal strains from across North America to ensure that the proteins detect-

ed were common antigens for multiple atopic individuals and unique to the fungi concerned [2–6]. Such proteins produced by indoor molds that are antigenic in humans may be relevant to human respiratory illness.

Spores as well as spore and hyphal fragments [7, 8] contain many proteins that are common to many fungi [9]. Atopic individuals typically react to more than one fungus and thus produce antibodies to many of these common proteins [2, 4, 10]. In this context, there have been a number of reports of proteins from *Stachybotrys chartarum sensu lato* to which antibodies in sera from atopic patients bind. The list includes 65-, 52-, 50- and 48-kDa proteins; assays with the latter 2 proteins revealed antibodies that were present in >80% of sera screened [11–13]. Subsequent analyses demonstrated that the 48-kDa protein is homologous to many fungal cellulases. Proteins of 34 and 21 kDa were also discovered using the screening process described above. Among the multiple fungi examined, these two proteins were present only in *S. chartarum sensu lato* [5, 6], both occur in cell filtrates and are glycosylated at an approximately 15% weight ratio [5]. SchS34 was shown by immunogold staining to be mainly present on the surface of spores and spore/hyphal fragments [14]. A partial sequence of the 21-kDa protein based on Edman degradation and tandem mass spectra indicated that the protein is related to an extracellular DNase from *Nectria haemolytica*. Partial sequences of the 34-kDa protein were similar to a hypothetical protein of unknown function from *Gibberella zeae* [5]. Antibodies to these proteins have been successfully used for the development of immunological methods for detecting *S. chartarum* in environmental samples [6].

In this paper, we report the use of inverse PCR and RT-PCR to obtain the cDNA sequences encoding SchS21 and SchS34. Both proteins were expressed in *Escherichia coli* and were indistinguishable from SchS21 and SchS34 proteins isolated from *S. chartarum* based on Western blot analyses. Additionally, information is reported on the enzymatic activity of SchS21.

Materials and Methods

Sequence Determination and Protein Expression

The strategy for DNA sequence determinations of SchS21 and SchS34 involved three PCR steps. The first step used degenerate primers, designed from partial protein sequences, to amplify and obtain DNA sequences of internal portions of each gene. In the second step, inverse PCR was done with self-ligated genomic DNA fragments as the template and using primers designed from

the internal DNA sequences obtained in step 1. From step 2, genomic DNA sequences were obtained for both genes. In the last step, reverse transcription of mRNA was followed by PCR (RT-PCR) to obtain cDNAs of both genes.

Degenerate Primer Design and PCR

Partial sequences of SchS21 and SchS34 were obtained by tandem mass spectrometry with an in-gel digestion using a Z-spray Q-TOF Mass (Micromass) in positive ion mode (BMSL, University of Western Ontario) and by Edman degradation (Sick Kids Hospital, University of Toronto) [5]. Degenerate primers were designed from these peptide sequences and using conserved cDNA sequences of proteins that aligned to the mass-based sequences (tables 1, 2). The details of the primers used are given in table 3. Touch-down PCR was used [15] to reduce non-specific reactions by decreasing annealing temperatures from 63 to 58°C in the first 10 PCR cycles, followed by 30 cycles with an annealing temperature of 58°C. Functional degenerate primer combinations used in these touch-down PCRs were S21-N-F/-M-R and S21-N-F/-C-R for amplifications within the SchS21 gene and SchS34-a-F/-e-R for amplification within the SchS34 gene. The PCR template used was 100 ng of genomic DNA extracted using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.) from *S. chartarum* DAOM 235557 mycelium stored at –80°C. The translated partial protein sequences from PCR fragments were then compared with the available partial peptide sequences from tandem mass spectra, Edman degradation and aligned proteins for confirmation.

Inverse PCR

S. chartarum genomic DNA was digested separately with the endonucleases *Pst*I, *Bam*HI or *Eco*RI (Invitrogen) for 1 h at 37°C. The digested samples were purified with a Qiaprep spin miniprep kit (Qiagen) to eliminate the restriction enzyme, and DNA concentration was determined using a Nanodrop instrument (Thermo Scientific). The digested genomic DNA was diluted to 2 ng/μl and ligated with T4 DNA ligase at room temperature overnight for self-ligation. After heating at 65°C for 10 min to denature the ligase, the self-ligated DNA sample was used as the template for inverse PCR. Touchdown PCR was again applied as described above for inverse PCR. The primers used for inverse PCR amplifications were SchS21-In-F/-In-R and SchS34-In-nR1/-In-nF1 for SchS21 and SchS34 genes, respectively (table 3).

RT-PCR

Total RNA was purified from –80°C frozen *S. chartarum* mycelium using the TRI® reagent (Sigma). Purified RNA (200 ng) was used for first-strand cDNA synthesis using 2.5 μM poly-T primer (table 1), 2 mM DTT (Sigma), 2.5 mM dNTP mix and 1 μl MMLV reverse transcriptase (New England Biolabs) at 42°C for 90 min. The primer pairs SchS21-N-F/-C-R and SchS34-N-F/-C-R (table 3) were used to amplify the cDNA regions that corresponded to the entire, mature SchS21 and SchS34 proteins, respectively, with the above first-strand cDNA synthetic product used as template. The RT-PCR conditions incorporated an annealing temperature of 58°C and 45 cycles.

DNA Cloning

Taq polymerase (Bioshop) was used in all PCR and amplicons were purified after electrophoresis in 1.5% agarose gels using a

Table 1. SchS21 peptide fragment sequences from MS data and Edman degradation with BLAST results

Peptide fragment			BLAST analyses		
number	sequence	mass, Da	primary hit	bit score	E value
1	TYFDVSA(L/I)VN PTDHDNVK	678.30 (3+)	DNase1 CAB63906 (<i>M. anisopliae</i>)	49	5×10^{-5}
2	VTFWT(L/I)DNVDR	762.38 (2+)	DNase1 CAB63906 (<i>M. anisopliae</i>)	28.6	73
3	ASVTFWTLDNVDRTL	N-terminal	DNase1 CAB63906 (<i>M. anisopliae</i>)	35.8	0.49

Table 2. SchS34 peptide fragment sequences from MS data and Edman degradation

Peptide fragment			BLAST analyses		
number	sequence	mass, Da	primary hit	bit score	E value
1	(L/I)DAGPGTTGVR	522.28 (2+)	hypothetical protein CBF82099 (<i>Aspergillus nidulans</i> FGSC A4)	24.0	138
*2	YPASSTSK	687.86 (2+)	hypothetical protein ABY87952.1 (<i>S. elegans</i>)	21.0	1,147
3	SDVVSTAR	586.96 (2+)	hypothetical protein ABY87952.1 (<i>S. elegans</i>)	24.0	147
4	(L/I)FYQDGTAAE(L/I)R	721.3 (2+)	hypothetical protein ABY87952.1 (<i>S. elegans</i>)	31.6	0.92
5	GFHLVVDVTDPSAD	Int N-term	hypothetical protein ABY87952.1 (<i>S. elegans</i>)	32.5	0.51

Table 3. Primer names, sequences and purpose in this study

Primer name	Sequences	Purposes
S21-N-F	GTCACCTTCTGGACCCTCGATAAYGTNGAYCGCACC	fragment sequence
S21-M-R	GTCNGTGGGRTTGACGATGGCNGAGACRTCGAAGTANG	fragment sequence
S21-C-R	CGTCRGSAGSYAGTAGSMGTTGTGTCARGGG	fragment sequence
SchS21-In-F	GTCCAATTCGGCGGCTGGAACG	inverse PCR
SchS21-In-R	GGGATAGGCATACCAGTTGCCTACC	inverse PCR
SchS34-a-F	CAACGSATCTTCTAYCAGAACGGMACMGCNGARGAG	fragment sequence
SchS34-e-R	CKCSGAGACATCCTYRTAGCACTKRGAGTYGGCRGCAAACYGTG	fragment sequence
SchS34-In-nR1	TGGACTGGGCGTAGCGGATC	inverse PCR
SchS34-In-nF1	CCCGAGGATGCTACGCC	inverse PCR
PolyT	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTV	RT-PCR
SchS21-N-F	GCCACCATGGCGTCCGTGACCTTCTGGACTCTTG	whole cDNA
SchS21-C-R	GAAGGTCAAGCCGGTGGATCCGG	whole cDNA
SchS34-N-F	ACCATGGGCCCATCGCCTCTCGCCAG	whole cDNA
SchS34-C-R	AGGGCCGTACTCGCTCCAGTTGAGG	whole cDNA

QIA quick Gel Extraction Kit (Qiagen). PCR fragments were ligated to the PCR2.1 TOPO TA cloning vector using the manufacturer's instructions (Invitrogen) and sequenced with M13F and M13R universal primers by a CEQ™ 8000 Genetic Analysis System in CAREG, University of Ottawa. RT-PCR products were cloned into the pET101 vector (Invitrogen) for further protein overexpression in *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene).

Homology Analysis

Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>) searches were carried out for the mass-based sequences of SchS21 and SchS34 trypsin-digested fragments and from Edman degradation sequences. Protein sequences were acquired from the non-redundant reference database at SwissProt (Swiss Institute of Bioinformatics). The alignments of SchS21 and SchS34 with their respective homologous proteins were done by T-COFFEE (<http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi>) [16, 17] with mature protein sequences (without signal peptide in the N terminus). Signal peptide analyses were performed using the SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>) with proteins homologous to SchS21 and SchS34.

SchS21 and SchS34 Overexpression, Purification and Characterization

Expression of SchS21 and SchS34 proteins from the pET101 vector (Invitrogen) in host strain BL21-CodonPlus(DE3)-RIL was induced using 0.2 mM IPTG and allowed to continue overnight at 23°C. The preparation of inclusion body and the purification of expressed proteins were as previously described [18]. The purified expressed SchS21 and SchS34 proteins were then characterized by 12% SDS-PAGE with SeeBlue® Plus2 Pre-Stained Standard (Invitrogen). Protein bands were visualized with GelCode Blue Stain Reagent (Pierce) according to the manufacturer's instructions. The identity of expressed SchS21 and SchS34 proteins was confirmed with immunoblots using the previously reported monoclonal antibodies 4G10 (specific for SchS21), 12D3 (for SchS34), anti-His antibody (Invitrogen) or representative human sera from USA patients shown to be IgE/IgG positive by Immunocap [5, 19]. Nonatopic human serum from Sigma (S2145) was used as a negative control. The corresponding secondary antibodies used were anti-mouse IgG (for 4G10, 12D3 and anti-His) and anti-human IgE, conjugated with alkaline phosphatase. BSA was loaded beside lanes with SchS21 or SchS34 proteins in all immunoblot experiments as the negative control. The secondary antibodies were detected by incubating membranes with BCIP/NBT (Sigma) for 5–10 min as above.

Enzyme Activity

DNase Activity Assays

Purification of native SchS21 and SchS34 from *S. chartarum sensu stricto* DAOM 235557 was done after Xu et al. [5]. DNase activity of SchS21 was assayed for exodeoxyribonuclease activity using the linear λ -DNA and for endodeoxyribonuclease activity using circular PCR2.1 plasmid DNA as the substrates [20]. Both λ -DNA and circular plasmid DNA were dissolved in TE buffer (50 mM Tris-HCl buffer at pH 7.5 with 1 mM EDTA). The assays were performed separately in 50- μ l reaction volumes with 10 μ g/ml DNA, 0.5 μ g/ml protein, 5 mM calcium chloride and 5 mM mag-

nesium chloride in 100 mM glycine-NaOH pH 9 at 37°C. After 0, 1, 2, 4, 6 and 8 h, the samples were electrophoresed in a 1% agarose gel and stained using ethidium bromide to qualitatively examine for DNA digestion.

DNase activity was further monitored using the method of Choi et al. [21]. Salmon testes dsDNA (Sigma) was dissolved in TE buffer (50 mM Tris-HCl buffer at pH 7.5 with 1 mM EDTA) and diluted to a 100 μ g/ml working solution with Milli-Q water. SchS21 enzyme was diluted to 10 μ g/ml with Milli-Q water. The DNase reaction was performed in 200 μ l containing 10 μ g/ml DNA, 0.5 μ g/ml protein, 5 mM calcium chloride and 5 mM magnesium chloride in 100 mM glycine-NaOH pH 9 at 37°C. At specified times, each reaction was brought to room temperature by addition of 1.8 ml of 200 $\times$ diluted PicoGreen reagent and fluorescence intensity was measured with a Cary Eclipse Fluorescence Spectrophotometer (Varian) in a 3.5-ml quartz cuvette (1 cm path length) 5–10 min after the addition of the PicoGreen reagent. The excitation and emission wavelengths used were 480 and 520 nm, respectively. Control reactions containing no enzyme were used as negative controls in all experiments.

SchS21 DNase activity in relation to 9 different cation solutions was determined after Fraser [22] as follows. Separate ion solutions (5 mM) of aluminum chloride (Al^{3+}), cobalt chloride (Co^{2+}), ferric chloride (Fe^{3+}), manganous chloride (Mn^{2+}), zinc sulphate (Zn^{2+}), cupric sulfate (Cu^{2+}), strontium chloride (Sr^{2+}), calcium chloride (Ca^{2+}) and magnesium chloride (Mg^{2+}) were each prepared in Milli-Q water. Reaction mixtures contained 10 μ l salmon testes dsDNA (100 μ g/ml), 10 μ l of SchS21 enzyme (10 μ g/ml), 20 μ l of a 50 mM cation solution, 20 μ l 100 mM pH 9 buffer (glycine-NaOH), and the final volume was brought to 200 μ l using ultrapure water. The reaction time was 4 h at 40°C. Optimal magnesium ion concentration for SchS21 activity was determined using 0.5, 2.5, 5.0, 7.5 and 10 mM magnesium chloride in addition to 10 mM calcium chloride. Divalent ion effects on SchS21 activity were determined using different calcium and magnesium chloride solutions. Reactions containing either 5 mM calcium chloride, 5 mM magnesium chloride, both 5 mM calcium chloride and magnesium chloride (+ control) or no ion supplemented (– control) were incubated for 5 h at 40°C.

Optimal pH was determined across a pH 3–10 range (glycine-HCl pH 3, K_2HPO_4 -citric acid pH 4–5, Tris-HCl pH 6–8 and glycine-NaOH pH 9–10), with incubation for 4 h in a shaking water bath (Fisher Scientific) at 37°C. Optimal temperature was determined following 6 h incubation at 5°C increments between 25–55°C. The SchS21 reaction kinetics were determined by varying substrate DNA concentrations from 2, 3, 4, 5 and 6 μ g/ml using an incubation time of 6.5 h at 37°C. All the tests were at least repeated in triplicate.

Results

Sequence Determination and Protein Expression Partial Amino Acid Sequences

The N-terminal amino acid sequences and internal fragment sequences of SchS21 and SchS34 proteins purified from SDS-PAGE were determined by Edman degradation and by in-gel digestion followed by tandem mass

spectra (tables 1, 2). SchS21 has the N-terminal sequence of ASVTFWTLDNVDRTL (table 1; peptide 3) from Edman sequencing. Two additional fragment sequences of TYFDVSA(L/I)VNPTDHDNVK (table 1; peptide 1) and VTFWT(L/I)DNVDR (table 1; peptide 2) were determined by tandem mass spectra with in-gel digestion; the latter peptide 2 fragment corresponds to the Edman degradation sequence. BLAST analysis [23] of all three SchS21 peptide sequences obtained showed highest scores with a fungal DNase1 protein from *Metarhizium anisopliae* var. *anisopliae* (CAB63906.1), and peptide 1 showed similarities with other fungal extracellular proteins such as hypothetical protein FG06779.1 from *G. zeae* (XP_386955.1). These *M. anisopliae* and *G. zeae* amino acid sequences were 88% similar to each other, suggesting a shared homology to SchS21 (data not shown).

Four internal peptide sequences (peptides 1–4; table 2) and an internal N-terminal sequence (peptide 5) were obtained from purified SchS34 protein. Of significance, peptides 3, 4 and 5 obtained for SchS34 had highest scores with ABY87952, a hypothetical protein from *Stachybotrys elegans*. Peptide 2 is relatively short and its sequence showed homology to ABY87952 with a relatively high E value. However, taken together, the multiple fragment matches between SchS34 and ABY87952 suggested that these proteins were homologous.

The SchS21 and SchS34 peptide sequences as well as the conserved regions of the putative SchS21 and SchS34 homologs identified by sequence alignment were used in the design of the respective degenerate primers for SchS21 and SchS34 (table 3). After sequencing of the touchdown PCR products, approximately 50% of SchS34 and 70% of SchS21 continuous sequences were revealed (data not shown).

cDNA Sequence of SchS21 and SchS3

Using the above PCR fragment sequences, inverse PCR primers were designed (table 3). Inverse PCR was done to obtain genomic DNA sequences of the genes encoding SchS21 and SchS34. For this, genomic DNA was separately digested with the restriction enzymes *Pst*I or *Bam*HI or *Eco*RI and the restriction fragments were self-ligated for use as inverse PCR templates. The largest inverse PCR amplicons were then ligated to PCR2.1 and sequenced. Based on the sequences from the inverse PCR amplicons, the genomic sequences encoding SchS21 and SchS34 from N-terminal first amino acids to the stop codon were inferred. RT-PCR was subsequently done with N-terminal forward primers and C-terminal reverse primers based on these inverse PCR sequences (table 3;

SchS21-N-F/-C-R and SchS34-N-F/-C-R). RT-PCR products were sequenced to reveal the cDNA sequences of the mature SchS21 and SchS34 proteins (without the extracellular protein signal peptide). Comparisons of the genomic and cDNA sequences indicate that neither SchS21 nor SchS34 contain introns (data not shown). Mature SchS21 and SchS34 proteins are predicted to comprise 144 and 221 amino acid residues and to have molecular weights of approximately 16 and 24 kDa, respectively. These sizes are in accordance with SchS21 and SchS34 isolated from *S. chartarum* that, with posttranslational glycosylation (15% by weight), were determined to be 21 and 34 kDa by SDS-PAGE, respectively [5]. The predicted amino acid sequences of SchS21 and SchS34 contained all fragments characterized from tandem mass spectra and Edman degradation (tables 1, 2; boxes in fig. 1, 2), which confirm the correct identities of both cDNAs. Figures 1 and 2 show the nucleotide and predicted amino acid sequences of SchS21 and SchS34 cDNAs that have been deposited in GenBank as GQ258854 and GQ258855.

The inverse PCR products obtained did not extend into the 5' untranslated region of either gene. However, signal peptide analyses of the homologous proteins to SchS21 (DNase1 from *M. anisopliae*, CAB63906) and SchS34 (hypothetical proteins FG06779.1 from *G. zeae* and ABY87952 from *S. elegans*) suggested the unprocessed forms of these proteins contain an N-terminal signal sequence. By inference, both SchS21 and SchS34 may contain signal sequences which would be consistent with the observations that both SchS21 and SchS34 are localized to the extracellular fraction. In addition, the amino acid sequences of SchS21 and SchS34 contained 1 and 4 putative N-glycosylation sites, respectively (dashed boxes in fig. 1, 2). Glycosylation is a co- or posttranslational modification that is common to secreted and membrane proteins.

Similar to BLAST analysis of its peptide fragments, BLAST analysis of the full-length SchS21 sequence had significant similarity values (E values $< 1 \times 10^{-27}$) with a DNase from *M. anisopliae* and several hypothetical proteins from other filamentous fungi. These included the hypothetical protein FG06779.1 from *G. zeae* PH-1 (XP_386955.1, E value 3×10^{-48}), DNase1 protein from *M. anisopliae* var. *anisopliae* (CAB63906.1, E value 4×10^{-47}), extracellular putative DNase from *Nectria haematococca* (AAD53090.1, E value 1×10^{-39}), hypothetical protein BC1G_02922 from *Botryotinia fuckeliana* (XP_001558258.1, E value 2×10^{-32}), hypothetical protein from *Sclerotium sclerotiorum* 1980 (XP_001591044.1, E value 5×10^{-31}) and unnamed protein product from

```

gcgtccgtgaccttctggactcttgataaacgttgaccgtaccctggcttctcactggcaac
A S V T F W T L D N V D R T L V F T G N
cccggtcccgccgcatcgaaacatcactgtggcccgccgaaacacacacgttgag
P G S A A I E T I T V G P A E N T I V E
ttccctgggtcttggtaggcaactggtatgcctatcccacgacgcccaggagcctct
F P G S W V G N W Y A Y P T D A E D V P
ggcatgctcgccgaagtccaattcgccggtggaacggcctcacctactttgacgtctct
G M L G E V Q F G G W N G L T Y F D V S
gccattgtcaacccaccgaccacgacaacgtcaagcagatgtggcccgccgaatccgc
A I V N P T D H D N V K Q M W P A E S R
aaacccatgtccggtcgaggtcttccctgacgacaacgctactggctccctgatgat
K P M S G C E V F P C D N A Y W L P D D
atccagaccaaggtcaccacgaggtcgacctctggaccacctggcgccgagatccacc
I Q T K V T H E V D L W T T L G A G S T
ggcttgaccttctaa
G L T F -

```

1

```

gcgggccccatcgctctcgccagatcggtcccaactaccccgctcgtccacctccaagggcttccacctcgtggtcaacgtgacggac
A G P I A S R Q I V P N Y P A S S T S K G F H L V V N V T I D
ccctcgccgacttcacgctccatcaac aacttctacgtcaacagcatccacgtcgccgcccctcaactacgtcgccggtgactgcc
P S A D F T P S I N N F Y V N S I H V G A A L N Y V G V T A
gtgcccggccgcatcttctaccagaacggcagccgaagatccgctacgccagtcacacgtcatcagcagcggcgccactcctccc
V P G R I F Y Q N G T I A E E I R Y A Q S T V I S D G A T P P
gtgcccttggctctgctgcgcccgatgagggctccgacgtcgtctccaccgcccgcctcgacgcgggtcccggaaccacgggtgtg
V P F G L S L R P D E G S D V V S T A R L D A G P G T T G V
ccggtgtcccgcttccccgagccgtaccgcttctccagcccgagacgttctcgctgcaacgagagcctgacctactaccagggcgac
R V S R F P E P Y R F L Q P E T F L A C N E S L A Y Y Q G D
tacttcaccgtcatcaagcagccgagctgacggtcgccgacgacggtccatcgactacaacgtgcccgcacactgcactctctgcgc
Y F T V I K Q A D V T V G D D G S I D Y N V P D N C I S V R
ctgatccccgagtgaccgagctcaacgagcttccgaggtgctacgcccagccaggtttgcccgcgacacccagtgctacgacgat
L I P E C T E L N E L P E D A Y A S H E F A A D T Q C Y D D
gtttcgccctcaactggagcgagtagcccttaa
V S A L N W S I E Y G P -

```

2

Fig. 1. SchS21 cDNA and deduced amino acid sequences. The peptide sequences from tandem mass spectra and Edman degradation characterization are boxed. The putative site(s) of glycolysis is identified with a dashed box. Translation termination codon is indicated with a dash.

Fig. 2. SchS34 cDNA and deduced amino acid sequences (labeling as for fig. 1).

Podospira anserina (E value 6×10^{-27}). T-COFFEE was used to compare the SchS21 sequence with these protein homologs (fig. 3). All of these proteins are secretory proteins based on sequence analysis by SignalP. Strong identity is evident over 136 residues between SchS21 and each of these sequences, ranging from 46 (*P. anserina*) to 66% (*M. anisopliae*; data not shown).

Similarly, BLAST analysis (fig. 4) showed that the full-length SchS34 sequence has significant matches with hypothetical proteins from *S. elegans* (ABY87952.1, E value 5×10^{-96}) and *G. zeae* (XP_386306, E value 9×10^{-60}). As with SchS34, these homologs are predicted to be secretory proteins based on SignalP analyses (data not shown). The predicted amino acid sequence of SchS34 shares 74% identity over 221 residues with the homolog from *S. elegans* and 49% identity over 211 residues with the *G. zeae* homolog.

Expression of SchS21 and SchS34

The overexpressed SchS21 and SchS34 proteins were localized in inclusion bodies (data not shown) and solubilized with 6 M guanidine hydrochloride. His-tagged SchS21 and SchS34 were purified from *E. coli* by Ni-NTA column affinity chromatography and subsequently determined to be 34 and 30 kDa by SDS-PAGE, with yields of 0.5 mg/l for both. Western blots verified that these expressed proteins were detected by the respective SchS34- and SchS21-specific antibodies (fig. 5, 6); monoclonal antibody 12D3 and anti-His both bound to the expressed form of SchS34 (fig. 5b), and monoclonal antibody 4G10 and anti-His both bound to the expressed form of SchS21 (fig. 6b). Also using Western blots, the expressed forms of SchS34 and SchS21 protein reacted when representative IgE/IgG positive human sera [5, 19] were used as the primary antibody and anti-human IgE was used as the sec-

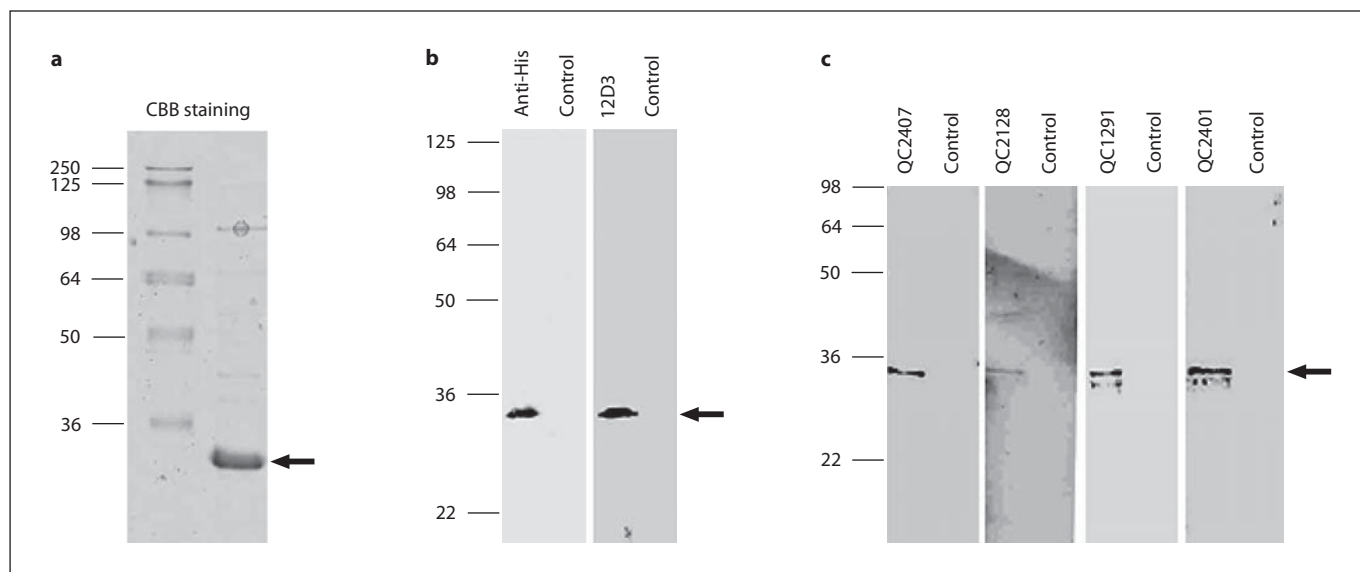


Fig. 5. Western blot analyses of SchS34 protein over-expressed in *E. coli* cells. **a** Coomassie Brilliant Blue (CBB)-stained gel of SchS34 overexpressed in DE3-RIL host cells and purified by Ni-NTA. **b** Western blot of purified expressed SchS34 with anti-His and 12D3 as the primary antibody and anti-mouse IgG as the secondary antibody. **c** Binding to SchS34 protein by IgE from each of 4 atopic individuals human sera labeled across top. Lanes labeled 'Control' in **b** and **c** were loaded with BSA as a negative control protein. The arrows show the position of the expressed SchS34 protein. Values at left in each panel indicate molecular weights in kDa.

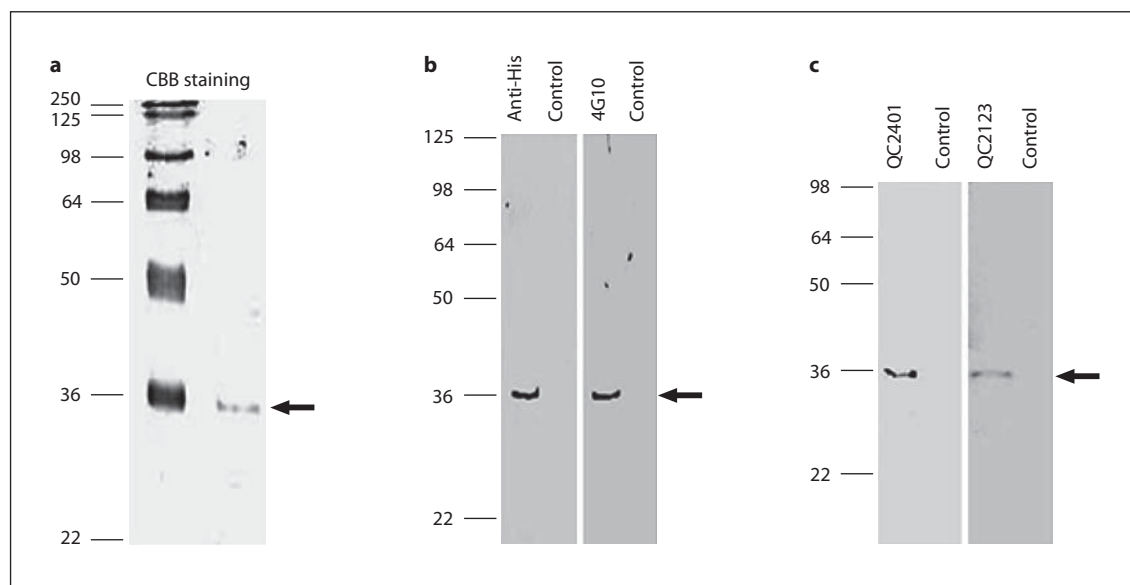
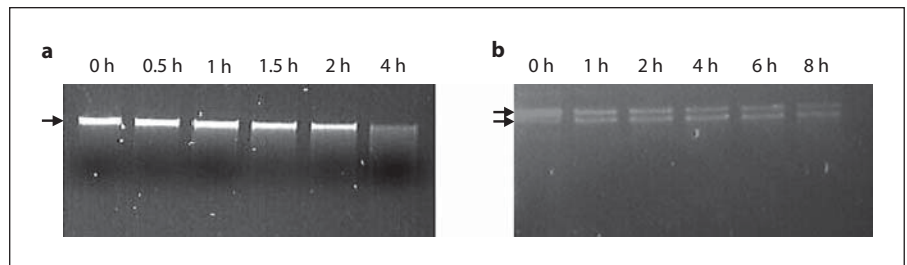


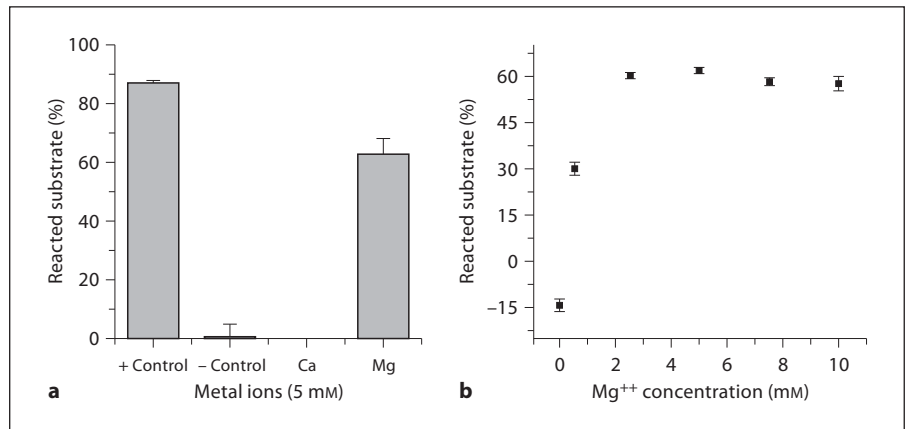
Fig. 6. Western blot analyses of SchS21 overexpressed protein in *E. coli* cells. **a** Coomassie Brilliant Blue (CBB)-stained gel of SchS21 protein overexpressed in DE3-RIL host cells and purified by Ni-NTA. **b** Western blot of purified expressed SchS21 with anti-His and 4G10 as the primary antibody and anti-mouse IgG as the secondary antibody. **c** Binding to SchS21 by IgE in sera from each of 2 atopic individuals labeled across top. The control lanes in **b** and **c** are loaded with BSA as a negative control protein. The arrows show the position of expressed SchS21 protein which is inferred to migrate as a dimer based on size. Labeling is as in figure 5.

Fig. 7. Effects of native SchS21 protein on linear λ -DNA and circular plasmid DNA. **a** Time course of digestion of λ -DNA by SchS21 protein. Lane 1, λ -DNA alone; lanes 2 to 6, digestion with SchS21 protein for 30 min, 1, 1.5, 2 and 4 h, respectively. **b** Time course of digestion of circular plasmid DNA by SchS21 protein. Lane 1, circular plasmid DNA alone; lanes 2 to 6, digestion of circular plasmid DNA with SchS21 protein for 1, 2, 4, 6 and 8 h, respectively (see Materials and Methods). The arrows indicate open circle (top) and supercoiled (bottom) plasmid DNA.



7

Fig. 8. SchS21 DNase activity dependence on divalent ions. **a** Positive control contained 5 mM Mg^{2+} and 5 mM Ca^{2+} , negative control solution lacked divalent ions, Ca assays contained 5 mM Ca and Mg assay contained 5 mM Mg^{2+} . **b** Optimal Mg^{2+} concentration for DNase activity by native SchS21 protein.



8

ondary antibody (fig. 5c, 6c). Indicative of their specificity, none of these above-listed antibodies bound to BSA, which was used in all Western blot experiments as a negative control. Likewise, binding artifacts from human serum can be ruled out since primary antibodies from non-atopic human serum did not bind to either of the expressed SchS34 or SchS21 proteins based on Western blots (data not shown). Overall, the Western blot data with monoclonal, anti-His and human sera indicates that both SchS34 and SchS21 proteins represent common human allergens.

Enzyme Activity

SchS21 DNase Characterization

After 4 h incubation, λ -DNA largely disappeared when incubated with SchS21 protein isolated from *S. chartarum* (fig. 7a). No low molecular weight fragments were observed in the agarose gels, suggesting that SchS21 is an exodeoxyribonuclease. This was further confirmed when it was observed that little or no degradation of circular plasmid DNA occurred after 8 h incubation with SchS21 (fig. 7b).

The greatest DNase activity by SchS21 was obtained when both Mg^{2+} and Ca^{2+} were included in the solution. Most of the activity (72%) was retained using only Mg^{2+} ,

with an optimal value of 2 mM. DNase activity was not observed when SchS21 was assayed in the presence of Ca^{2+} only (fig. 8a). This suggests that SchS21 is a Mg^{2+} -dependent exodeoxyribonuclease. Assays with any one of the other metal ions tested, including 5 mM Cu^{2+} , Zn^{2+} , Sr^{2+} , Mn^{2+} , Fe^{3+} , Co^{2+} and Al^{3+} , did not result in SchS21 DNase activity (data not shown).

Optimal DNase activity for SchS21 was observed at circa pH 9 (ANOVA, Tukey Test, $p < 0.001$; fig. 9a); pH 3 to 6 resulted in no detectable activity (data not shown). This suggests that SchS21 is an alkaline DNase. Optimal DNase activity was at 45°C (ANOVA, Tukey Test, $p < 0.001$; fig. 9b). However, temperature effects were limited in the range of 35–50°C; at 35 and 50°C activity was circa 80% of the optimum at 45°C. The K_M of SchS21 was 3.29 $\mu g/ml$, with a V_{MAX} of 0.17 $\mu g/ml/min$ at pH 9 and 37°C (fig. 9c).

Discussion

S. chartarum sensu lato readily grows on water-absorbent building materials such as paper faced-gypsum board and has been associated with various respiratory and non-respiratory problems [24]. There is currently ad-

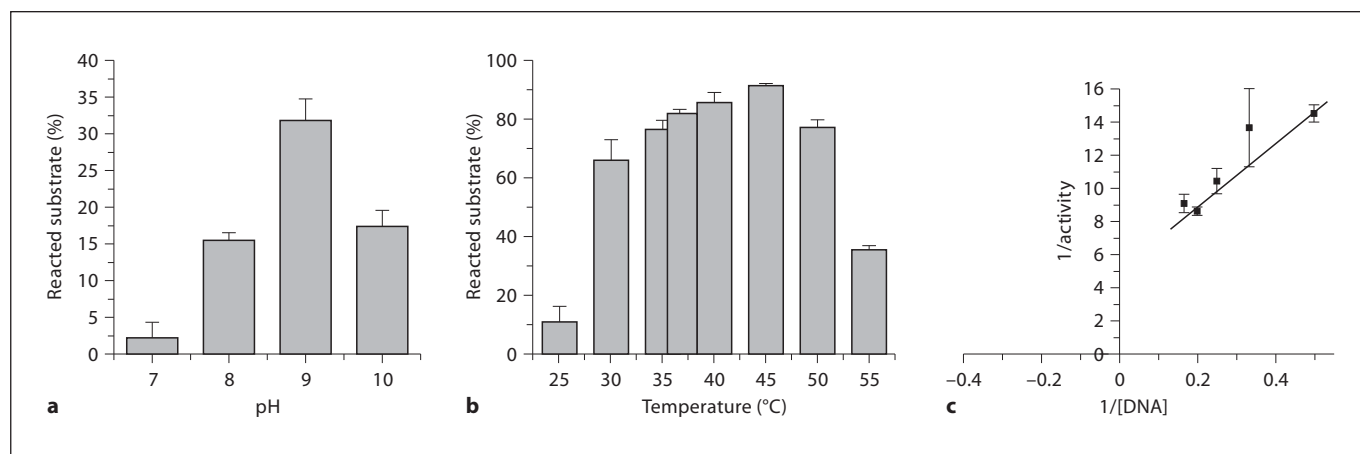


Fig. 9. Assay results demonstrating optimal pH (a) and temperature (b) conditions for enzymatic activity for native SchS21 protein. c Lineweaver-Burk plot showing SchS21 enzyme kinetics.

equate evidence to demonstrate that removing mold-damaged building materials from buildings improves occupant health [25].

Two proteins, SchS21 and SchS34, both human IgG/IgE-binding antigens from *S. chartarum sensu lato*, were fully characterized in this study. Both proteins are encoded by genes that lack introns and are predicted to encode peptides of 144 (SchS21) and 221 (SchS34) amino acids. The 21-kDa SchS21 has a pI of 3.9, and SchS34 has a pI of 4.2. Both proteins are secreted from the fungus and inferred to be glycosylated. There was no appreciable cross-reactivity of monoclonal antibodies raised against SchS21 and SchS34, and these antigens were not detected in the spores or mycelium from diverse fungi aside from *S. chartarum* [5]. These monoclonal antibodies have been used to measure the presence of these proteins in house dust and have a detection limit comparable to those for house dust mite allergens and the *Aspergillus fumigatus* allergen, Asp f1 [6]. SchS21 and SchS34 both occur primarily in the culture filtrate and are therefore likely secretory proteins, which is consistent with the SignalP characteristics of their respective homologs. The amino acid sequences of SchS21 and SchS34 contained 1 and 4 putative N-glycosylation sites, respectively. This is consistent with a previous report that both SchS21 and SchS34 comprised circa 15% carbohydrate [5]. As suggested from the more limited sequences reported by Xu et al. [5], homology with CAB63906 from *M. anisopliae* suggested that SchS21 was a possible DNase. Further enzymological characterization from the present study showed that SchS21 is an extracellular Mg-dependent alkaline exode-

oxyribonuclease with an optimal temperature of 45°C, optimal pH 9.0 and K_M value of 3.29 $\mu\text{g/ml}$.

DNase is an enzyme that catalyzes the hydrolytic cleavage of phosphodiester linkages in the DNA backbone. These enzymes can be divided into 2 classes: endonucleases that hydrolyse the deoxyribose phosphodiester backbone within the DNA strand and exonucleases that hydrolyze the phosphodiester bonds at the DNA ends [26, 27]. Differing in their substrate specificities, chemical mechanisms and biological functions, a wide variety of DNases are known [26, 28, 29]. An extracellular DNase (AAD53090.1) from *N. haematococca* was homologous and has similar properties to SchS21, such as a molecular weight of 20 kDa, isoelectric point of 4.2 and optimal temperature of 45°C [5, 20]. However, maximal activity for SchS21 was pH 9.0 and SchS21 did not show appreciable DNase activity in the acidic pH range, while the DNase from *N. haematococca* has a pH optimum of around 5.0–6.0. DNase activity from *N. haematococca* in solutions containing various divalent cations was highest in Mn^{2+} followed by $\text{Ca}^{2+} > \text{Co}^{2+} > \text{Mg}^{2+}$ with a maximal concentration of around 10 mM [20]. In contrast, SchS21 DNase showed a specific Mg^{2+} dependency with maximum activity at a concentration of 2 mM. Other metal ions, including Ca^{2+} , Cu^{2+} , Zn^{2+} , Sr^{2+} , Mn^{2+} , Fe^{3+} , Co^{2+} and Al^{3+} resulted in limited or no DNase activity. Interestingly, the combination of Ca^{2+} and Mg^{2+} in the reaction mix resulted in a higher SchS21 DNase activity than when only Mg^{2+} was present. This suggests Ca^{2+} may stabilize a favorable active conformation of SchS21.

DNases comprise an essential family of proteins that maintain genomic stability and are involved in processes that include DNA repair, replication and recombination [26, 27]. Some acidic DNases are also involved in other important cell cycle functions such as apoptosis, during which genomic DNA is degraded to oligonucleosomes [30]. DNases from the extracellular fraction of filamentous fungi also function in autolysis when fungal cells reach maximal dry weight [31]. Other functions of DNases of filamentous fungi, including that of *N. haematococca*, may also result in the stimulation of pathogenesis-related genes and phytoalexin accumulation in plants [20, 29, 32]. The DNase from *N. haematococca* shares 58% identity with SchS21. Given that *S. chartarum* is a saprophyte, it is more likely that the physiological function of SchS21 in *S. chartarum* is to degrade environmental DNA, and thus mobilize nitrogen for uptake.

At present, we cannot suggest a function for SchS34 despite having a complete mature protein sequence. In addition to being readily isolated from cell filtrates, this protein is localized on the surface of conidia and hyphae of *S. chartarum* based on immunogold staining [14], so its function is evidentially extracellular in nature. SchS34 shares 74% identity over 221 amino acids with the hypothetical protein ABY87952 from *S. elegans*, not including the putative signal peptide regions. ABY87952 was identified as an upregulated gene of unknown function from a screen for mycoparasite-related transcripts that are induced when *S. elegans* interacts with *Rhizoctonia solani* [33].

The prevalence of human response to SchS21 and SchS34 proteins is not known. Most work on fungal al-

lergens has been conducted on fungi for which there is near universal exposure, such as to *Alternaria alternata*, *A. fumigatus*, and *Cladosporium herbarum*. Among other implications, study of these common allergenic fungi is associated with inherently large numbers of patients with reactive sera. For example, just over 50% of the prescreened patients were shown to react to a 54-kDa antigen from the indoor clade of *Penicillium chrysogenum* [4], 10% of sera originally tested were positive to the relevant 34-kDa protein from *S. chartarum* [5], and for *A. versicolor*, the comparable value was 20% of all prescreened sera tested positive [2].

The expressed forms of proteins SchS21 and SchS34 both reacted with representative human sera from patients who had a IgE/IgG positive response for *S. chartarum*, *Aspergillus* spp. or *Penicillium* spp. (fig. 5c, 6c). These particular sera were also demonstrated to react with native SchS21 and SchS34 protein from culture filtrate, indicating that a common epitope(s) occurs in expressed and natural SchS21 and SchS34, despite the absence of posttranslational modifications in the expressed proteins. Therefore, these expressed proteins can be used in future research for T cell/B cell epitope mapping, and have potential applications in the development of new diagnostics.

Acknowledgements

This work was funded by an NSERC IRC to J.D.M. We are grateful for the contributions of Don Belisle and Javid Khanand as well as the advice of Robert Beecroft (ImmunoPrecise Antibodies) who made the antibodies.

References

- 1 Fisk WJ, Lei-Gomez Q, Mendell MJ: Meta-analyses of the associations of respiratory health effects with dampness and mold in homes. *Indoor Air* 2007;17:284–296.
- 2 Liang Y, Zhao W, Xu J, Miller JD: Characterization and production of antibodies against two related exoantigens from *Aspergillus versicolor* isolated using sera from atopic patients. *Int Biodeterior Biodegrad* 2011, in press.
- 3 Luo W, Wilson AW, Miller JD: Characterization of a 52 kDa exoantigen of *Penicillium chrysogenum* and monoclonal antibodies suitable for its detection. *Mycopathologia* 2010;169:15–26.
- 4 Wilson AW, Luo W, Miller JD: Using human sera to identify a 52 kDa exoantigen of *Penicillium chrysogenum* and implications of polyphasic taxonomy of anamorphic ascomycetes in the study of antigenic proteins. *Mycopathologia* 2009;168:213–226.
- 5 Xu J, Jensen JT, Liang Y, Belisle D, Miller JD: The biology and immunogenicity of a 34 kDa antigen of *Stachybotrys chartarum sensu lato*. *Int Biodeterior Biodegrad* 2007;60:308–318.
- 6 Xu J, Liang Y, Belisle D, Miller JD: Characterization of monoclonal antibodies to an antigenic protein from *Stachybotrys chartarum* and its measurement in house dust. *J Immunol Methods* 2008;332:121–128.
- 7 Green BJ, Sercombe JK, Tovey ER: Fungal fragments and undocumented conidia function as new aeroallergen sources. *J Allergy Clin Immunol* 2005;115:1043–1048.
- 8 Green BJ, Tovey ER, Sercombe JK, Blachere FM, Beezhold DH, Schmechel D: Airborne fungal fragments and allergenicity. *Med Mycol* 2006;(suppl 1):S245–S255.
- 9 Horner WE, Helbling A, Salvaggio JE, Lehrer SB: Fungal allergens. *Clin Microbiol Rev* 1995;8:161–179.
- 10 Benndorf D, Müller A, Bock K, Manuwald O, Herbarth O, von Bergen M: Identification of spore allergens from the indoor mould *Aspergillus versicolor*. *Allergy* 2008;63:454–460.

- 11 Barnes C, Buckley S, Pacheco F, Portnoy J: IgE-reactive proteins from *Stachybotrys chartarum*. *Ann Allergy Asthma Immunol* 2002;89:29–33.
- 12 Kärkkäinen M, Raunio P, Rautiainen J, Auriola S, Hinke K, Pasanen AL: Partial amino acid sequence of a cellulase-like component with IgE-binding properties from *Stachybotrys chartarum*. *Int Arch Allergy Immunol* 2004;133:136–144.
- 13 Raunio P, Kärkkäinen M, Virtanen T, Rautiainen J, Pasanen AL: Preliminary description of antigenic components characteristic of *Stachybotrys chartarum*. *Environ Res* 2002;85:246–255.
- 14 Rand TG, Miller JD: Immunohistochemical and immunocytochemical detection of SchS34 antigen in *Stachybotrys chartarum* spores and spore impacted mouse lungs. *Mycopathologia* 2008;165:73–80.
- 15 Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS: 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res* 1991;19:4008.
- 16 Poirot O, O'Toole E, Notredame C: Tcofee@igs: a web server for computing, evaluating and combining multiple sequence alignments. *Nucleic Acids Res* 2003;31:3503–3506.
- 17 Notredame C, Higgins D, Heringa J: T-Coffee: A novel method for multiple sequence alignments. *J Mol Biol* 2000;302:205–217.
- 18 Eriksson TLJ, Whitley P, Johansson E, Hage-Hamsten MV: Guro gafvelin identification and characterisation of two allergens from the dust mite *Acarus siro*, homologous with fatty acid-binding proteins. *Int Arch Allergy Immunol* 1999;119:275–281.
- 19 Halsey JF, Jensen JT, Miller JD: Immunological responses to *Stachybotrys chartarum*. *Allergy Clin Immunol* 2000;107:s317.
- 20 Gerhold DL, Pettinger AJ, Hadwiger LA: Characterization of a plant-stimulated nuclease from *Fusarium solani*. *Physiol Mol Plant Pathol* 1993;43:33–46.
- 21 Choi SJ, Szoka FC: Fluorometric determination of deoxyribonuclease I activity with PicoGreen. *Anal Biochem* 2000;281:95–97.
- 22 Fraser MJ: Alkaline deoxyribonucleases released from *Neurospora crassa* mycelia: two activities not released by mutants with multiple sensitivities to mutagens. *Nucleic Acids Res* 1979;6:231–246.
- 23 Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: 'Gapped BLAST and PSI-BLAST: a new generation of protein database search programs', *Nucleic Acids Res* 1997;25:3389–3402.
- 24 Miller JD, Rand TG, Jarvis BB: *Stachybotrys chartarum*: cause of human disease or media darling. *Medical Mycology* 2003;41:271–291.
- 25 Jacobs DE, Brown MJ, Baeder A, Sucusky MS, Margolis S, Hershovitz J, Kolb L, Morley R: A systematic review of housing interventions and health: introduction and methods. *J Public Health Manag Pract*, in press.
- 26 Kavanagh D, Spitzer D, Kothari PH, Shaikh A, Liszewski MK, Richards A, Atkinson JP: New roles for the major human 3'-5' exonuclease TREX1 in human disease. *Cell Cycle* 2008;7:1718–1725.
- 27 Hubscher U, Maga G, Spadari S: Eukaryotic DNA polymerases. *Annu Rev Biochem* 2002;71:133–163.
- 28 Martínez Valle F, Balada E, Ordi-Ros J: Vilardell-Tarres M, DNase I and systemic lupus erythematosus. *Autoimmun Rev* 2008;7:359–363.
- 29 Klosterman SJ, Chen J, Choi JJ, Chinn EE, Hadwiger LA: Characterization of a 20 kDa DNase elicitor from *Fusarium solani* f. sp. *phaseoli* and its expression at the onset of induced resistance in *Pisum sativum*. *Mol Plant Pathol* 2001;2:147–158.
- 30 Counis MF, Torriglia A: Acid DNases and their interest among apoptotic endonucleases. *Biochimie* 2006;88:1851–1858.
- 31 Reyes F, Villanueva P, Alfonso C: Nucleases in the autolysis of filamentous fungi. *FEMS Microbiol Lett* 1990;69:67–72.
- 32 Hadwiger LA, Chang MM, Parsons MA: *Fusarium solani* DNase is a signal for increasing expression of nonhost disease resistance response genes, hypersensitivity, and pisatin production. *Mol Plant Microbe Interact* 1995;8:871–879.
- 33 Morissette DC, Dauch A, Beech R, Masson L, Brousseau R, Jabaji-Hare S: Isolation of mycoparasitic-related transcripts by SSH during interaction of the mycoparasite *Stachybotrys elegans* with its host *Rhizoctonia solani*. *Curr Genet* 2008;53:67–80.