A partial sequence of the 34 kDa antigen from *Stachybotrys chartarum* and studies of the recovery of fungal antigens from particulate samples

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1. Abstract

Detection and characterization of fungal antigens is an important step towards developing an understanding of fungi related disease. Using mass spectrometry and ELISA, a fungal antigen from *Stachybotrys chartarum sensu lato* and an allergen from *Aspergillus fumigatus* has been characterized. The *S. chartarum* protein SchS21 has been identified as a DNase while the SchS34 is of unknown function. Mass spectrum analysis of the SchS21 and SchS34 proteins has identified that these proteins are glycosylated, possibly with high mannose glycans. The SchS34 ELISA was optimized to provide the lowest achievable detection limit. ELISA analysis in dust has demonstrated that 0.2 ng SchS /g sieved house dust (10^5 spores) is the limit of detection for the SchS34 assay while 1 ng Asp f1/g sieved house dust (10^7 spores) for the commercially available Asp f1 assay. Detection of SchS on *S. chartarum* contaminated drywall and swab was accomplished for both experimental and field samples. Lastly, it was shown that EDTA, THP and conidial disruption have a positive effect on Asp f1 recovery from *A. fumigatus* spores.

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6. List of Abbreviations

3Q- triple quadrapole mass spectrometer
μg - microgram
μL - microlitre
ABPA - allergenic bronchopulmonary aspergillosis
a_w – water activity
BSA – bovine serum albumin
CF – cystic fibrosis
CID – collision induced disassociation
Da - Dalton
DNase – deoxyribonuclease
DTT – 1,4-dithiothreitol
EDTA - ethylenediaminetetraacetic acid
ELISA - enzyme linked immunosorbant assay
ESI – electrospray ionization
ESI-3Q – electrospray ionization triple quadrapole mass spectrometer
ESI-IT – electrospray ionization ion trap mass spectrometer
ESI-QTOF – electrospray ionization quadrapole time of flight mass spectrometer
FTICR - Fourier transform ion cyclotron resonance mass spectrometer
g - gram
HIV – human immunodeficiency virus
HP- hypersensitivity pneumonitis
HPLC – high performance liquid chromatography
IA – invasive aspergillosis
IAQ – indoor air quality
IgE – immunoglobulin E
IgG – immunoglobulin G
HRP – horse radish peroxidase
hrs – hours
kDa - kiloDalton
eV –electron volts
LSD ANOVA – least significant difference analysis of variance
M – Molarity
mbar - millibar
mM - milliMolarity
MALDI - matrix assisted laser desorption/ionization mass spectrometer
MALDI-TOF – matrix assisted laser desorption/ionization time of flight mass spectrometer
mg – milligram
min - minutes
mL – millilitre
mm - millimeter

ng - nanogram
MRM – multiple reaction monitoring
MS/MS – tandem mass spectrometry
OD – optical density
ODTS-organic dust toxic syndrome
PBS - phosphate buffered saline, pH 7.4
PBST – phosphate buffered saline, pH 7.4, 0.05% Tween 20
PBST1%B - phosphate buffered saline, pH 7.4, 0.05% Tween 20 1% bovine serum albumin
PBST2%B - phosphate buffered saline, pH 7.4, 0.05% Tween 20 2% bovine serum albumin
PCR – polymerase chain reaction
QPCR – quantitative polymerase chain reaction
U – enzyme units
RAPD - random amplified polymorphic DNA
RIP – ribosome inactivating protein
RNA – ribonucleic acid
RT-PCR – real time polymerase chain reaction
S/N – signal to noise ratio
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
Strep-HRP - streptavidin-HRP
TFA - trifluoroacetic acid
TH - helper T cells
TH1 – helper T1 cells
TH2 – helper T2 cells
THP - tris(hydroxypropyl)phosphine
TLR – toll like receptor
TMB - 3, 3', 5, 5'-tetramethylbenzidine
UWO-BMSL - University of Western Ontario Biological Mass Spectrometry Laboratory

7. Introduction

7.1. The fungi

Fungi are common eukaryotic saprobes found in a wide variety of ecological niches. Fungi were originally taxonomically categorized as plants due to their cell wall and non-motile nature. The number of fungal species has been estimated to be between 500,000 to 9 million with 74,000 of these being described (Hawksworth 2001). Fungi are characterized by their semi-rigid cell wall which is organized to form hyphae which, in turn, is organized into the mycelium. Fungi excrete enzymes into the substrate and reabsorb the nutrients for metabolism. Fungi are capable of reproducing sexually and/or asexually. Traditionally fungi have been divided into 3 major phyla: Ascomycetes, Basidiomycetes and Zygomycetes, with each being capable of sexual reproduction. Fungal classification schemes are under constantly restructured, with another classification system being recently published (Hibbett et al. 2007).

For many fungi, the most important propagule is a spore. Spores can be passively or actively disseminated, which allow the fungi to "move". Asexual reproduction produces mitospores while sexual reproduction produces, meiospores such as basidiospores, ascospores or zygospores.

Throughout history features of fungi have been exploited by man for food and medicine. The antibiotic penicillin from the fungus *Penicillium chrysogenum* was famously and purportedly accidentally discovered by Flemming (Fleming 1929). As history would dictate this discovery revolutionized modern medicine and played an integral part in the outcome of World War II. Fungi are also used in the fermentation of foods such as mizo, soy sauce and alcoholic beverages.

7.2. Fungi and the built environment

Growth of fungi in the indoor environment is often preceded by high water intrusion of some form. For fungi to proliferate, a minimum water requirement must be met. Different fungi have different water requirements, therefore one can expect different arrays of fungi depending on the amount of water present. The water content in building materials is known as water activity or a_w. Water activity is a measure of free water in the building material. This number is a ratio of the vapour pressure of the water in the building material compared to the vapour pressure of pure water (Flannigan and Miller 2001). This water to support fungal growth can come from a variety of sources including condensation, standing water or flooding.

Fungi that grow on building materials can be grouped into categories based on the water activity that is required to support their growth. These groups are the: extremely xerophilic, moderately xerophilic, slightly xerophilic and hydrophilic species. Hydrophilic fungi require an a_w of greater than 0.9, slightly xerophilic 0.8-0.9, moderately xerophilic 0.75-0.79 and extreme xerophiles less than 0.75 (Flannigan and Miller 2001). Extreme xerophiles include the *Eurotium* species and *Wallemia sebi*, the moderately xerophilic include *Penicillium brevicompactum* and *Aspergillus versicolor*, the slightly xerophilic include the *Cladosporium* species, *Penicillium crustosum* and *Aspergillus fumigatus*, while the hydrophilic fungi include *Stachybotrys chartarum* and *Memnoniella echinata* (Flannigan and Miller 2001).

Fungi can proliferate on a wide array of substrates, ranging from paper side of gypsum board to paint, and especially drywall. These building materials allow for selection of certain fungi, which result in unnatural concentrations of uncommon fungi in the indoor air. This unnatural concentration and species distribution facilitates use of outdoor/indoor comparisons for determination of the extent of fungal contamination. Fungi that are commonly found as contaminants in an indoor environment include: *Chaetomium globosum*, *S. chartarum*, *Aspergillus versicolor*, *Penicillium chrysogenum*, *Ulocladium chartarum* and *Cladosporium sphaerospermum* (Flannigan and Miller 2001; Miller et al. 2008).

Remediation of fungal contaminated building relies on the expertise of a qualified building engineer. An inspection is performed and the level of contamination determined. If the mold is hidden then air sampling maybe required to locate the fungal source. Once the contamination is found, the size of the mold contamination determines the remediation approach. Small areas may require a cleaning with water and detergent while large areas of mold contamination requires considerable effort to clean, including removal of porous and cleaning of non-porous material (Shaughnessy and Morey 1999). Remediation of these situations are often costly and commonly result in insurance and/or legal claims.

7.3. Health effects of fungal growth indoors

Growth of fungi in indoor environments has been attributed to modern building design while it is commonly noted in the literature that reports of fungal exposure go as far back historically as biblical times (Leviticus 14:33-45). Fortunately modern science has allowed us to evaluate the reasons for and causes of fungal exposure, and will continue to reduce the health effects attributed to exposure.

Exposure to types of fungi that grow indoors has a wide variety of health effects. This is due to the variety of biological material present in inhalable fungal materials. This includes species-specific low molecular weight compounds (such as mycotoxins), antigens or allergens, ß 1-3 D glucan and possibly volatile organic compounds (VOCS; Health Canada 2004). Mycotoxins are secondary metabolites produced by the fungi usually produced as a defence mechanism. Routes of contact with mycotoxins maybe through inhalation, ingestion or skin contact while their health effects include skin rashes, dizziness, nausea and immunosuppression (Health Canada 2004). Glucan is the main structural component found in fungal cell walls. These are known to produce health effects similar to endotoxin exposure, such as fever like symptoms (Health Canada 2004). VOCS are released from actively growing fungi, but no specific symptoms have been associated with exposure (Burge and Otten 1999). Each of these toxins likely have a synergistic health effect in promoting disease which in turn cause difficulties when attempting to set exposure limits (Miller et al. 2003).

The principle diseases caused by high fungal exposure include allergenic disease, organic dust toxic syndrome (ODTS) and hypersensitivity pneumonitis (HP). The allergenic diseases comprise: rhinitis, sinusitis as well as exacerbation of asthma (NAS

2000). Allergic symptoms include: wheezing, sneezing, itchy eyes and coughing. HP is characterized by immune mediated inflammation of the lungs due to large exposures of chemical or biological products (Hodgson and Flannigan 2001). Symptoms of HP include chest tightness, coughing and wheezing. ODTS is thought to be a non-specific immune response that does not require sensitization, with the symptoms including fever and malaise (Sorenson 2001). While HP and ODTS both involve inflammation of the lung, HP differs from ODTS in that ODTS is not a specific immune response (Hodgson and Flannigan 2001). Allergic disease is described in greater detail below.

Exposure to fungi indoors is associated with a number of other conditions including increased upper respiratory disease (NAS 2004). The mechanism for these outcomes are not clear, however may relate to effects of glucan and toxins on lymphocytes (Rand et al. 2005; Rand et al. 2006).

7.4. Allergy

A variety of allergic diseases exist, including asthma, eczema, and hay fever. The dollar cost associated with asthma care in Canada in 1990 was estimated to be between $3-4 billion, (4.1-5.6 billion in 2007 dollars) this cost appears to continue to rise based increasing allergy incidence (Krahn et al. 1996; Statistics Canada 2007). In 1998/99 approximately 8% of Canadians and 12% of children suffered from asthma (Statistics Canada 2001). Asthma is considered one of the top 5 reasons for hospital visits with 20% of absenteeism from work and school being associated with this disease (Statistics Canada 2001).

The term allergy was first coined by the Austrian pediatrician, Clemens von Pirquet in 1910 (Silverstein 2000). He observed that second injections of horse serum in patients with small pox resulted in more severe reactions than the primary injection. In von Pirquet's famous drawing, he showed that at the first injection the circulating antigen concentration slowly decreased until antibody production began. Then after a second injection of antigen, the antigen concentration decreased rapidly. This experiment elegantly demonstrated the sensitization and hypersensitive adaptation due to allergen exposure. von Pirquet suggested that this hypersensitive exposure was due to antibody-antigen reaction, this was later demonstrated to be the case due to the involvement of IgE in allergic response (Gould et al. 2003).

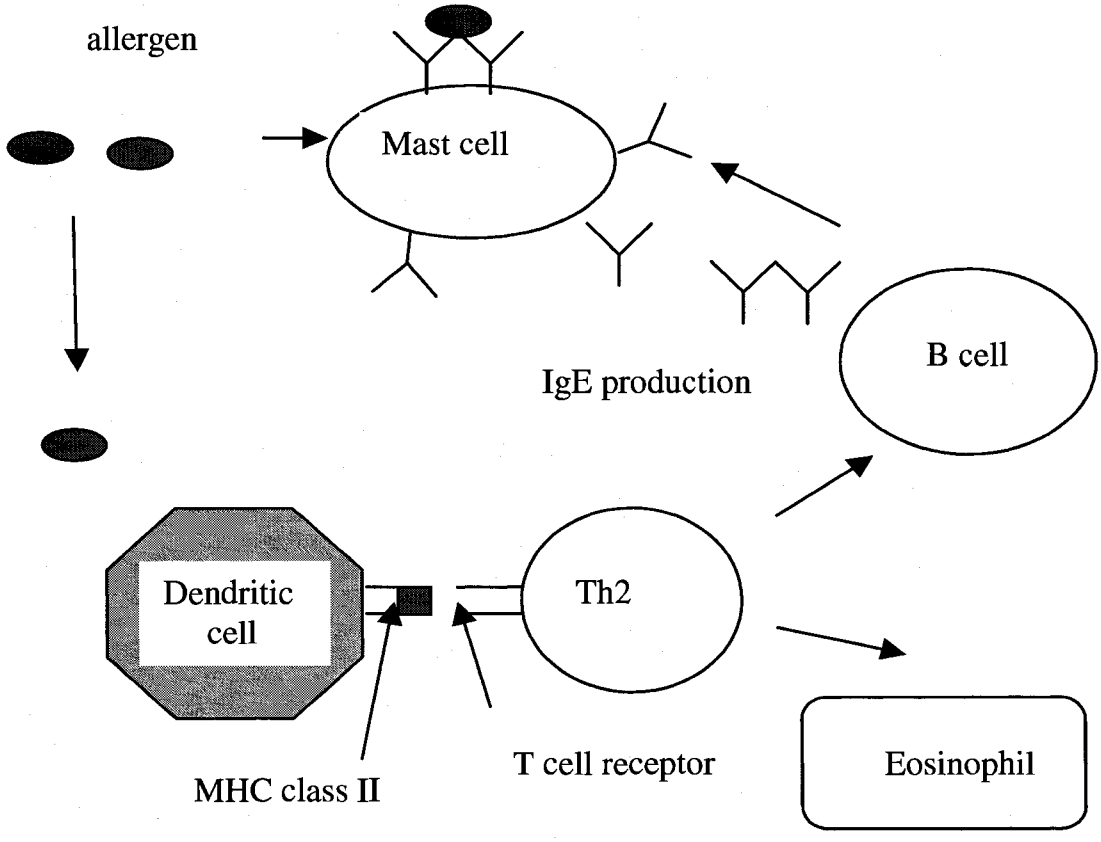


Figure 1. Mechanism of allergic response, adapted from Kay (2001)

The mechanism of allergic response revolves around this IgE response to foreign antigens (Figure 1). IgE concentrations in allergenic individuals have been shown to be approximately 1000 times higher than in healthy patients (Gould et al. 2003).

IgE has high affinity for the FcεRI receptor found in mast cell membranes. When IgE is bound to this receptor, binding of the allergen to the IgE antibody facilitates degranulation of the mast cell. Degranulation results in release of a complex number of mediators such as histamine, prostaglandins, leukotrienes, proteases, chemokines and cytokines. This, in turn, results in accumulation of eosinophils, antigen presentation to helper T-cells (Th) and increased production of Th1 class of helper T-cells directed by dendritic cells. This biochemical cascade is responsible for the variety of symptoms associated with allergy.

The development of allergic response involves both environmental and genetic factors (von Mutius 2002). Much attention has been focused on the "hygiene hypothesis". This hypothesis states that allergy is developed due to lack of exposure to infectious disease at a young age, and the immune system does not develop properly. This concept was first introduced by Strachan (1989), where he noticed family size and age had an effect on hay fever incidence. He then postulated "allergenic diseases" were prevented by infection in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally from a mother infected by contact with her older children" (Strachan 1989). A recent Canadian study comparing children raised on farms, children living in rural communities but not living on farms and urban children demonstrated that the farming environment might be protective against allergenic disease (Midodzi et al. 2007). It has also been shown that different environmental factors on the farm have been

associated with different effects on children in relation to development of asthma. The same study found that endotoxin was associated with reductions in the development of asthma (Eder et al. 2004).

One of the prevailing mechanisms for the hygiene hypothesis is: living in a hygienic environment places the TH1 and TH2 helper T-cell distribution out of balance, predisposing an individual to develop TH2 cells (Schaub et al. 2006). Also thought to be involved in this process is the innate immune system, particularly the Toll-like receptors (TLR; Schaub et al. 2006). TLRs are thought to influence the immune system to engage the adaptive immune system or a TH1 response, therefore a lack of early exposure or genetic factors may predispose an individual to a TH2 response (Schaub et al. 2006).

Another piece of the allergy puzzle is the genetic portion. Individuals who are genetically predisposed to develop allergies are known as atopic. TLRs and CD14 are thought to be involved in the genetic component of allergenic disease. Another study of farmers' children looked at genetic polymorphism in TLR genes and found that genetic variances could explain susceptibility to allergenic disease (Eder et al. 2004). Aside from TLR gene polymorphisms, it has also been shown that CD14 levels were higher in the farmers' children (Lauener et al. 2002). The significance of this finding is still being discussed in the literature. One study has found no link between CD14 promoter polymorphism and IgE levels while another study has found a correlation between serum CD14 levels and IgE levels (Jackola et al. 2006; Kabesch et al. 2004).

One of the most interesting questions in allergy/immunology is "what makes an allergen an allergen?". Protein allergens must be capable of causing an IgE response and must have 2 IgE binding sites to cross-link 2 IgE antibodies bound to mast cell to

facilitate mast cell degranulation. For IgE antibodies to develop, the allergen must first be processed by antigen presenting cells and the epitope presented to a T cell. Therefore allergens must also contain epitopes that favour the production of Th2-type T cell, implicated in allergic disease. Experiments where these T-cell epitopes were altered changed the T-cell response. Other allergen characteristics thought to be involved in allergenicity include: resistance to proteolysis, disulphide bonds presence, glycosylation and enzymatic activity (Huby et al. 2000). With regards to enzymatic activity, Der p1 has been thoroughly studied. The proteolytic activity of this allergen is thought to facilitate increased uptake of the allergen in the bronchial epithelium (Huby et al. 2000). This allergen is thought to directly cleave a low affinity IgE receptor (CD23; Hewitt et al. 1995). This CD23 receptor is thought to be involved in a feedback loop, which reduces IgE levels when the levels are too high (Hewitt et al. 1995).

Allergenicity may be due to certain structural similarities found between allergens (Fedorov et al. 1997). Cross-reactive allergens have been shown to have similar structural motifs as well as epitope amino acid sequences (Fedorov et al. 1997). These similarities may also explain why an individual is allergic to many allergens as they maybe cross-reactive. An individual is not allergic to many different allergens but in reality allergic to a group of allergens which share a common epitope or motif. Some researchers are now utilizing this new found knowledge to develop *in silco* techniques to predict allergenicity (Schein et al. 2007).

Many sources of allergens exist in the environment; these include biological as well as chemical. The most common allergens sources are fungi, pollen, and insect with other sources including food and latex. A nomenclature system is applied to named

allergens, the first 3 letters of the genus name, followed by the first letter of the species then a number in the chronological order of discovery (Chapman et al. 2007). A list of accepted allergens is maintained International Union of Immunological Societies (IUIS) and the World Health Organization (WHO) at www.allergen.org.

Many fungal allergens exist in nature, but fungi tend to be the least understood. Fungal spores can be found in large concentrations in the outdoor environment, even more so than the much better studied pollens. The most thoroughly studied fungi with regard to allergenicity include *Cladosporium herbarum*, *Alternaria alternata* and *Aspergillus fumigatus* with many allergens from Basidiomycetes and Ascomycetes having been discovered as well (Horner et al. 1995). All three of these fungi are commonly found in outdoor air.

Exposure to *Alternaria alternata* has been implicated to cause asthma attacks. O'Hollaren et al. (1991) found that 10-11 patients with asthma were positive for skin prick test against *A. alternata* extracts and also showed elevated levels of IgE to this fungus. There are ten different recognized allergens from this fungus. These extracts have been difficult to characterize due to their large variability. Alt a1 is the major allergen from *A. alternata* and has 5 different variants, complicating characterization of this allergen (Horner et al. 1995). The concentration of this allergen varies in extracts and it has been suggested to be involved in plant pathogenicity (Cramer and Lawrence 2003; Horner et al. 1995).

C. herbarum is the best characterized species of the Cladosporia. *Cladosporium* spores are the most abundant fungal spore type found in the outdoor environment (Baxter et al. 2005). Two major allergens have been purified and characterized from *C.*

herbarum, Cla h1 and Cla h2. A study where a deglycosylated form of Cla h2 was studied for IgE binding ability determined that the deglycosylated protein had larger IgE binding ability than the native form (Horner et al. 1995).

The best-studied indoor allergens include the group 1 dust mite allergens, Der p1 and Der f1, the cockroach allergen, Bla g1 and cat allergen Fel d1. A study of over 1000 children in the US and Canada demonstrated that exposure levels to the group 1 dust mite and cockroach allergens in house dust correlated with sensitization while fungi, cat and dog did not correlate (Huss et al. 2001).

While little is known about fungal allergens even less is known about fungi that grow on building materials. The database at www.allergen.org indicates that there are about 100 fungal allergens in their database with only 7 allergens being derived from fungi that commonly grow on building material. The database includes 2 allergens from *P. brevicompactum* and 5 from *P. chrysogenum* (Sevinc et al. 2005; Shen et al. 1999). A few studies on the exposure to the *P. chrysogenum* allergens have been completed. Shen (1999) showed that 24% of asthmatics in their study (n=70) had IgE activity towards *P. chrysogenum* protein extracts. Another study by Shen showed that IgE activity to the *P. chrysogenum* allergen, Pen ch13 increased with age (Chou et al. 2003). Both the P ch13 and Pen ch18 were shown to be cross reactive with *P. citrinum* and *P. brevicompactum* (Shen et al. 1996). These allergens were determined to be serine proteases that were also found in *P. citrinum*, *P. brevicompactum* and *P. oxalicum* (Shen et al. 1999). While the *A. alternata* allergen, Alt a1, is sometimes measured in homes, this fungus is not commonly found growing on building material (Miller et al. 2008).

While intact spores are usually considered the main source of fungal allergens, fungal fragments are known to contain allergens (Green et al. 2005). These fragments can penetrate deeper into the lung than intact spores. A study of over 100 homes in PEI showed that ~ 30 % of all fungal biomass is made up of fine spore and mycelial fragments (Foto et al. 2005). A study which analyzed different size fractions from a multistage sampler, found no viable conidia in the small size fractions but did find a large portion of human allergens to *C. herbarum* and *A. alternata* (Fluckiger et al. 2000). They found that, in the indoor environment, the amount of viable fungi and allergens did not correlate, which they attributed to fragmentation. The authors concluded that sampling for viable fungi underestimates the allergen load.

7.5. Detection of fungi in the environment

To estimate exposure levels, accurate detection of fungi is necessary. Various approaches are available to investigators today but a few are routinely used. The traditional approach was by the use of viable sampling whereby collection of either air or dust is cultured on agar media, the representative viable fungi are counted and identified (Hung et al. 2005). The inherent problems encountered with this approach include; not all fungi present grow on the agar media chosen and considerable experience is required to identify the fungi correctly (Hung et al. 2005). In addition the analysis takes approximately a month to complete. It has been estimated that less than 1% of all propagules sampled are cultured on viable fungal media (Hung et al. 2005). This low number is due to a decrease in fungal viability as they age, the media type used as well as damage to the fungal propagules due to the sampling itself (Hung et al. 2005). Viable air

samples, have been called "grab" samples due to the small volume of air taken, in a short time frame. This small sample size has been found to play a part in the temporal variability that exists between samples. The three main factors in this variability are: the amount of activity in room prior to sampling, pressure changes and amount of settled dust present (Hung et al. 2005). The advantage of this method is that species information is obtained which allows an understanding of the biological and ecological properties of each fungus detected.

Another common approach is the use of sticky surface samples in air sampling. The most commonly used commercial product is the Air-O-Cell cassette, which contains a slide with an adhesive to capture the fungal propagules. In this case a known volume of air is pumped on to the slide containing an adhesive, the spores are then counted and identified directly (Hung et al. 2005). This method has been found to capture 10-75% of fungal propagules found in the air, depending on the spore size. A relative high percentage of spores are captured but provides little taxonomic information due to the lack of identifiable features on the collected spores. It does however allow a limited estimate diversity of unculturable fungi present in the air.

PCR, a molecular approach is gaining popularity as a detection method for fungi. PCR involves using small segments of DNA known as primers, which anneal to melted DNA (Saiki et al. 1985). A DNA polymerase then binds to the primer/DNA complex and makes a copy of the DNA. This process is repeated many times, which results in an amplified part of DNA. The primers can be made species specific so when an amplified gene product is found, this indicates the species is present. To make this methodology quantitative, a fluorescent dye is incorporated in the amplified products, therefore as

more copies are made, the larger the fluorescent signal (Hung et al. 2005). This variety of PCR is known as QPCR. This procedure is rapid and sensitive but the quantitative number provided is difficult to interpret and primers are not always species specific (Dillon et al. 2007; Keswani et al. 2005).

Vesper et al. (2005) have developed a large array of primers that are deemed capable of detecting a wide variety of fungi. Studies using a large panel of PCR primers designed against many fungi have demonstrated that they are capable of discriminating fungal contaminated from clean buildings (Meklin et al. 2004). A statistical method, known as the Relative Mold Index (RMI) was developed which allows one to differentiate between clean and moldy homes. The use of RMI was suggested to be useful in predicting respiratory illness (Vesper et al. 2007). PCR is known to be adversely affected by certain matrices, incapable of quantifying fungal propagules without DNA and DNA extraction efficiencies are known to vary between various fungi, all of these problem likely reduce the accuracy of PCR quantification which in turn minimizes the usefulness of the RMI in predicting disease. Lastly, QPCR maybe cost prohibitive for routine investigations.

Biochemical methods used to quantify total fungal biomass include the cell wall component β-glucan and the membrane sterol, ergosterol (Hung et al. 2005). Some studies have found an association between respiratory health and glucan levels (Hung et al. 2005). Ergosterol levels in homes have been shown to correlated with viable data, it has also been shown to not correlate with respiratory illness in children (Hung et al. 2005). These methods are non-specific but provide information on the total fungal biomass present in the environment.

The enzyme linked immunosorbant assay (ELISA) methods of quantify antigens are starting to be employed in IAQ investigations. ELISA has commonly been used to quantify dust mite, cat and cockroach allergens, but due to the unavailability of antibodies, fungi are rarely detected using this approach. ELISA uses antibodies raised to specific antigens to act as biomarkers. If the antigen being quantified is an allergen then this methodology is quantifying material that causes a known health effect or biological response in humans (NAS 2000).

ELISA methods have been used in large studies to quantify allergens to gain a sense of exposure, which hopefully will predict allergic outcomes. With allergen avoidance the best method to alleviate allergen symptoms, accurate environmental analysis is required. House dust is generally used in allergen sampling because it acts as a reservoir for allergens but it also provides a long-term look at exposure levels. Dust samples taken from mattresses and floors are routinely collected then analyzed for allergen content. Dust samples may contain biological and inorganic components. This includes such components as: skin cell, fungal materials, proteins, soil, traffic pollution and fibres of many kinds. Allergens find there way into dust by either being produced in the dust, such as dust mite allergen or by circulation (O'Meara and Tovey 2000). Air sampling of allergens has also been performed. This method is attractive as it can demonstrate breathable exposure. A study of cat, dog and dust mite allergens found that airborne quantification of cat and dog allergens maybe useful for assessing exposure while dust analysis would still be better for dust mite allergens exposure (Custovic et al. 1999).

Allergen concentration has been found to vary within rooms as well as between rooms (O'Meara and Tovey 2000). Two studies have shown that large variations (>50%) of dust mite concentrations exist between dust samples taken in the same location as well as over time (Hirsch et al. 1998; Loan et al. 2003). Storage of dust samples has also been studied, it was found that Der p1 and Der f1 were stable when stored at –20°C while Fel d1 concentrations decreased by approximately 40% over a 90 day period ($p < 0.05$) (Fahlbusch et al. 2003).

A study investigating childrens' hospital visits related to asthma found that 75% of these children had greater than 10 μg of Der p1 per gram of house dust (Sporik et al. 1993a). A similar study in Melbourne found comparable results, where high levels of dust Der p1 levels correlated to asthma incidence they also found that carpeting was a risk factor for developing asthma (Hill et al. 1997). A study on the concentration of the cockroach allergen, Bla g1, in dust found that concentrations great than 1U/g were strongly correlated with IgE presence (Chew et al. 2007). A large study in the USA of almost 1000 homes with asthmatic children analysed dust concentrations of group 1 dust mite, cockroach cat and dog allergens (Leaderer et al. 2002). For each allergen, they set a lower cut off point and an upper cut off point. The cut off points were as follows: >2.0 μ/g and > 10 μ /g of group 1 dust mite, > 1.0 U/g and > 4.0 U/g Bla g1, > 1.0 μg /g and > 8.0 μg/g of Fel d1, and > 2.0 μ g/g and > 10.0 μg/g for Can f1. They found that 86% of homes in the study had detectable amounts of the lower cut off point for at least one allergen and at least 58% had at least one allergen in the upper cut off point.

While dust sampling has been shown to be an effective measure of estimating exposure, it can be a very involved process. Home test kits have been developed,

whereby the homeowner can test allergen levels themselves which facilitates improved allergen avoidance. The dust is collected by a vacuum, some of the dust added to a buffer, then the buffer is added to the home test kit. The home test kits tend to be lateral flow tests, where the dust sample is mixed with a solution containing a specific detection antibody bound to gold, as the added sample moves across the membrane by diffusion, it collects on another specific antibody bound to the membrane generating a positive line (Chapman et al. 2001; Sercombe et al. 2005). A study by Sercombe et al. (2005) found that these home test kits correlated with the laboratory ELISA results.

While the usefulness of using ELISA for detection of specific fungi in the environment has been debated, a variety of ELISAs have been proposed for a variety of fungi (Schmechel et al. 2003; Schmechel et al. 2005). These include *P. brevicompactum*, *P. chrysogenum*, *A. versicolor* and *S. chartarum*, none of these have demonstrated to be useful for practical applications and the protein detected not known or clear (Portnoy et al. 1998; Schmechel et al. 2003; Schmechel et al. 2005; Shen et al. 1990; Xu et al. 2007). In the case of *A. fumigatus* and *A. alternata*, the allergens are known and these tests are useful in environmental analysis (Abebe et al. 2006; Asturias et al. 2003; Dillon et al. 2007; Sporik et al. 1993b).

7.6. Aspergillus fumigatus

7.6.1. *A. fumigatus* biology

A. fumigatus is a common fungus associated with composting organic matter . It is a thermotolerant mold that can grow at temperatures between 20 and 50°C (Figure 2). It is frequently isolated in outdoor air samples with concentrations tending to peak in late

fall (Mullins et al. 1984). When found in hospitals, it can cause fatal infection of susceptible patients (Chazalet et al. 1998; Goodley et al. 1994; Hospenthal et al. 1998). *A. fumigatus* is also found in occupational environments such as forestry and composting facilities wherein exposure can constitutes a workplace hazard. The disease allergic bronchial pulmonary aspergillosis (ABPA) can result in chronically exposed people (see following) (Beffa et al. 1998; Dillon et al. 2007; Sanchez-Monedero et al. 2005).

Given the importance of *A. fumigatus*, considerable attention has been given to its taxonomy. A variety of taxonomic methods have been applied to delineate the species, these include morphological, phyllogenic as well as chemotaxonomy. *A. fumigatus* formally belongs to the fumigati section of *Aspergillus*. It is morphologically characterized by its green conidial colour, its uniseriate clavate vesicle and its relative fast growth (Klitch 1992). There are a few closely related species in the Ascomycete phylum, including *Neosartorya* (Hong et al. 2005). Analysis of clinical isolates isolated from patients suffering from *Aspergillus fumigatus*-related-diseases showed that each isolate was indeed *A. fumigatus sensu stricto* (Schmidt and Wolff 1997). Some evidence suggests that certain *A. fumigatus* related diseases maybe caused by a specific strain while the other *A. fumigatus* diseases are caused by a variety of strains (Bertout et al. 2001; Mondon et al. 1997).

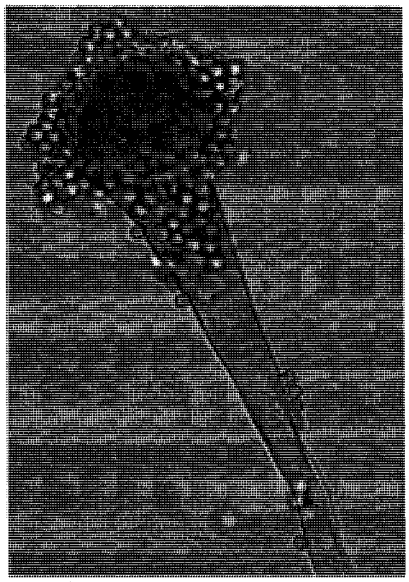


Figure 2. Photograph of an *A. fumigatus* conidiophore, taken by author

The entire genome of *A. fumigatus*, (29.4 mega base), has been sequenced (Nierman et al. 2005). The genome is broken into 8 separate chromosomes. It has generally been considered an asexual species, yet recent evidence from the analysis of the genome indicates that it contains genes thought to be involved in sexual reproduction, indicating it may reproduce sexually in nature (Paoletti et al. 2005).

7.6.2. Diseases associated with *A. fumigatus*

A. fumigatus is also known to produce a wide array of potent toxins. These mycotoxins include gliotoxin, fumagillin, fumigatin, fumigaclavine, trypoquivaline, verruculogen and helvoic acid (Fischer et al. 1999). Gliotoxin is the most important mycotoxin because it is believed to be involved in immune suppression while trypoquivaline and verruculogen are tremorigens (Amitani et al. 1995). A study of clinical isolates and outdoor isolates showed both produced verruculogen (Kosalec et al. 2005). Two separate studies have shown that verruculogen and gliotoxin were present in the conidia of *A. fumigatus* (Khoufache et al. 2007).

Research into *A. fumigatus* has grown due to its pathogenic and allergenic nature. It is responsible for causing ABPA, invasive aspergillosis (IA) and aspergilloma. ABPA is characterized by colonization of the mucus in the lung cavity by *A. fumigatus* whereby allergy develops. ABPA can occur in individuals who have had prolonged and high exposure to *A. fumigatus* (Dillon et al. 2007). ABPA is most commonly found in asthmatics and patients suffering from cystic fibrosis. Approximately 2% of patients with cystic fibrosis and 5-7% of asthmatics are known to have this condition (Basich et al. 1981; Geller et al. 1999; Grammer et al. 1986).

Invasive aspergillosis occurs when an *A. fumigatus* infection spreads from the point of infection into surrounding tissues within the body. Generally, there are four varieties: pulmonary, brachial, nasal and disseminating which involves the eye, brain etc. (Latge 1999). This disease usually occurs in severely immuno-compromised individuals such as AIDS, transplant or leukemia patients. Invasive aspergillosis occurs in about 5-6% of patients with acute leukemia with a mortality rate of approximately 40-50% (Pagano et al. 2001). Invasive aspergillosis is a major concern for AIDS patients with less than 50 CD4 cells/mm^3 and has a very high mortality rate of 90% (Mylonakis et al. 1998).

Diagnosis of invasive aspergillosis is difficult and many approaches have been investigated. These include detection of circulating antigens, detection of circulating antibodies to *A. fumigatus* antigens, PCR and β 1-3 glucan with each have positives and negatives and none being definitive in diagnosis (Johnson et al. 1989; Latge 1999; Obayashi et al. 1992).

Another form of disease that occurs with *A. fumigatus* is a fungus ball or aspergilloma, which forms at the site of infection, usually in the lung. An aspergilloma is characterized by the formation of a proteinacous hyphal ball with conidiophores on its surface (Latge 1999). Aspergillomas tend to occur in patients with pre-existing lung conditions such as sarcoidosis and tuberculosis as well as those which are HIV infected (Addrizzo-Harris et al. 1997; Tomlinson and Sahn 1987).

7.6.3. *A. fumigatus* allergenicity

A. fumigatus produces a wide array of antigens. Antigenicity in this fungus was originally studied with fungal extracts but these extracts varied widely in protein and carbohydrate content, thus the need for standardized purified allergens was realized (Turner et al. 1980).

More than 20 allergens from *A. fumigatus* have been purified and expressed (Kurup 2005). The strongest IgE responses have been shown from the Asp f1, f2, f3, f4 and f6 allergens (Kurup 2005).

Asp f1 or mitigillon is an 18 kDa allergen from *A. fumigatus*. Asp f1 was originally detected in the urine of patients diagnosed with invasive aspergillosis where it was demonstrated to be excreted into neighbouring tissue (Haynes et al. 1990; Latge et al. 1991). Asp f1 functions as a ribotoxin, and is included as a member of the ribosome inactivating protein (RIP) group. Initially, ribotoxins were studied for their anti-cancer properties until it was demonstrated that the major allergen of *A. fumigatus*, Asp f1, was also a ribotoxin (Arruda et al. 1990; Olson and Goerner 1965). Other members of this group include restrictocin and *a*-sarcin. Mitogillin differs from restrictocin by a single methionine residue while *a*-sarcin shows 86% homology (Fernandez-Luna et al. 1985; Rodriguez et al. 1982). The 3 dimensional structure of Asp f1 is not known but the structures for restrictocin and *a*-sarcin have been elucidated (Perez-Canadillas et al. 2000; Yang and Moffat 1996).

Functionally, ribotoxins specifically cleave a single phosphodiester bond of the 28S ribosomal RNA of the large ribosome subunit (Lamy et al. 1991; Schindler and Davies 1977). Cleavage by these enzymes results in the complete cessation of protein

synthesis and has been shown to reduce total protein synthesis by 90% *in vitro* (Arruda et al. 1990; Fando et al. 1985; Madan et al. 1997).

The Asp f1 gene is 686 base pairs, has been cloned and subsequently expressed in *E. coli* (Moser et al. 1992). The gene encodes for a 149 amino acid protein which consists of 2 disulfide bridges (Fernandez-Luna et al. 1985). The allergen has also been cloned into an insect cell line where it did demonstrate post-translational glycosylation (Priyadarsiny et al. 2003) The recombinant protein has been shown to have IgE activity as well as produce positive results from the skin prick test (Moser et al. 1992).

Asp f1 has been found in high concentration in *A. fumigatus* liquid culture filtrate with lower concentrations found the in hyphae and spores (Arruda et al. 1992a). This is an indication that the allergen is excreted by the fungus. A recent study of conidial cell wall surface proteins of *A. fumigatus*, did not detect any Asp f1 (Asif et al. 2006). This further supports the hypothesis that Asp f1 transiently passes through the cell wall where it is excreted.

Asp f1 is known to produce elevated levels of both IgG and IgE antibodies and interacts with these antibodies in patients with ABPA (Arruda et al. 1990; Arruda et al. 1992b; Knutsen et al. 2004; Kurup et al. 1994). Approximately 85% of patients with ABPA had IgG antibodies to Asp f1 (Arruda et al. 1992b). Forty one percent of patients with CF have been found to have anti-Asp f1 IgG antibodies by 5 years of age while 98% have these antibodies by the time they reach 10 years old (el-Dahr et al. 1994). While ABPA patients tend to show high titres of IgE and IgG to Asp f1, asthmatics tend to show much lower levels (el-Dahr et al. 1994). Deletion of a hair-pin loop from the Asp f1 protein has been shown to dramatically reduce IgE activity (Garcia-Ortega et al. 2005).

Clinical diagnosis of *A. fumigatus* infection can be performed by screening for circulating antibodies raised against Asp f1 via a recombinant form of Asp f1 (Almeida et al. 2006; Knutsen et al. 2004; Weig et al. 2001). Aside from detecting circulating antibodies, PCR has been investigated as a method of detection. PCR primers developed against the Asp f1 gene was been shown to detect *A. fumigatus* in some patients with IPA but did not detect it in all cases (Urata et al. 1997).

Asp f1 has been shown to not be necessary for infection in invasive pulmonary aspergillosis (Paris et al. 1993; Smith et al. 1993; Smith et al. 1994). Smith et al. (1994) deleted the Asp f1 gene and found no difference in number and size of *A. fumigatus* colonies in mouse models. However it has been shown that Asp f1 may play a role in suppression human polymorphonuclear leukocytes where this cell type is involved in preventing mycelial growth (Ikegami et al. 1998).

7.6.4. Cell wall of *A. fumigatus*

The cell wall of fungi provide a structural barrier and protection from the outside world. At the same time, the cell wall allows materials to pass back and forth, whether for excretion of digestion enzymes or uptake of nutrients. With the cell wall biodiversity being unique to fungi, the biosynthesis of cell wall components has been a target for drug development against invasive aspergillosis (Beauvais and Latge 2001).

The cell wall of *A. fumigatus* mycelium is predominately composed of linked polysaccharide chains comprising: β-1,3-glucan, β-1,4-glucan, galactomannan and chitin (Fontaine et al. 2000). The galactomannan component in the cell wall is composed of

mannose sugar residues linked in the following pattern: 6manα1-2manα1-2manα1-2manα1 (Bernard and Latge 2001). The β-glucan portion is linked as follows: 3Glcβ1-4Glcβ1 with the 3Glcβ1 being further branched with 6Glcβ1 (Bernard and Latge 2001). Chitin, a β-1,4-n-acetylglucosamine polymer, is linked to the β -1,3 -glucan via a β 1-4 bond while galactomannan is linked to β-1,3 -glucan by an α-1-6 bond (Bernard and Latge 2001).

Aside from the polysaccharide matrix, the mycelial cell wall also contains approximately 3.5% protein (Bernard and Latge 2001). The majority of cell wall proteins, are proteins on transit to be excreted but some are cell wall associated. These proteins in transit tend to be highly glycosylated enzymes while the cell wall associated proteins are largely involved in cell wall synthesis (Bowman and Free 2006). Bernard et al. (2001) also found that enzymatic treatment with the cell wall with β -1,3- glucanase, α-1,3-glucanase and a chitinase did not release anymore protein than buffer extraction.

Little is known about the conidial cell wall of *A. fumigatus* in contrast to the hyphal cell wall. On the surface of the thick cell wall is a rodlet layer while within the cell wall are proteins as well as melanin (Bernard and Latge 2001; Dague et al. 2007). Some of the cell wall proteins and the melanin are thought to function together to protect the spore from host defences in humans. The melanin protects the spore from reactive oxygen species produced from human monocytes and polymorphonucleur leukocytes (Jahn et al. 2000; Pastor et al. 2006). While the proteins include receptors which bind various human proteins, such as fibrinogen, laminin, complement, fibronectin, albumin, immunoglobulins, surfactant proteins and collagen (Bernard and Latge 2001; Gil et al. 1996).

A variety of methods have been employed to detect *A. fumigatus* in the environment. These include PCR, ELISA and viable culturable methods (Dillon et al. 2007; McDevitt et al. 2004; Ryan et al. 2001; Sporik et al. 1993b). PCR generally involves random amplified polymorphic DNA (RAPD) or Q PCR while ELISA quantifies the Asp f1 allergen. A study of 369 homes in Wallaceburg, ON by Scott (2001) using culturable dust methods found approximately 10% of the homes had detectable levels of *A. fumigatus*. A comparison of culturable spores, total spore counts, Asp f1, ergosterol and β 1,3 D glucan by Dillon (2007) revealed that several of the methods correlated well with Asp f1 quantification, PCR did not correlate with any of the methods studied.

Only a few studies have been performed where environmental Asp f1 levels have been quantified in both dust and air samples using the same methods. Detectable Asp f1 was seldom found in air samples and dust samples in office buildings and homes studied (Ryan et al. 2001; Sporik et al. 1993b). *A. fumigatus* was found in approximately 65% of house dust samples cultured (Sporik et al. 1993b). A study at a wood chipping facility found 0.9 ng/m^3 in the outdoor air (Dillon et al. 2007).

7.7. *Stachybotrys chartarum* and *Stachybotrys chlorohalonata*

The natural habitat of *Stachybotrys chartarum* is the root system or woody stems of plants (Li et al. 2002; Miller et al. 2003). It is routinely found as a contaminant in indoor environments where considerable water intrusion has occurred and has been isolated from wet straw (Miller 2008; Miller et al. 2003; Murtoniemi et al. 2003). *S.*

chartarum was detected in ca. 5% of homes studied in Wallaceburg (Scott 2001). It has gained much attention due to being a primary suspect in a pulmonary hemodsiderosis outbreak in the USA (Etzel et al. 1998; Miller et al. 2003).

Morphologically *S. chartarum sensu lato* is characterized by its black to blackish green colony colour (Figure 3; Samson et al. 2000). The hyphae and conidia are black with the conidia produced on slimy heads of phalids. Its conidia are ellipsoidal and coarsely roughened. On malt extract agar at 25°C, it reaches a diameter of 2.5-3 cm. The optimal temperature for growth is 23°C (Samson et al. 2000).

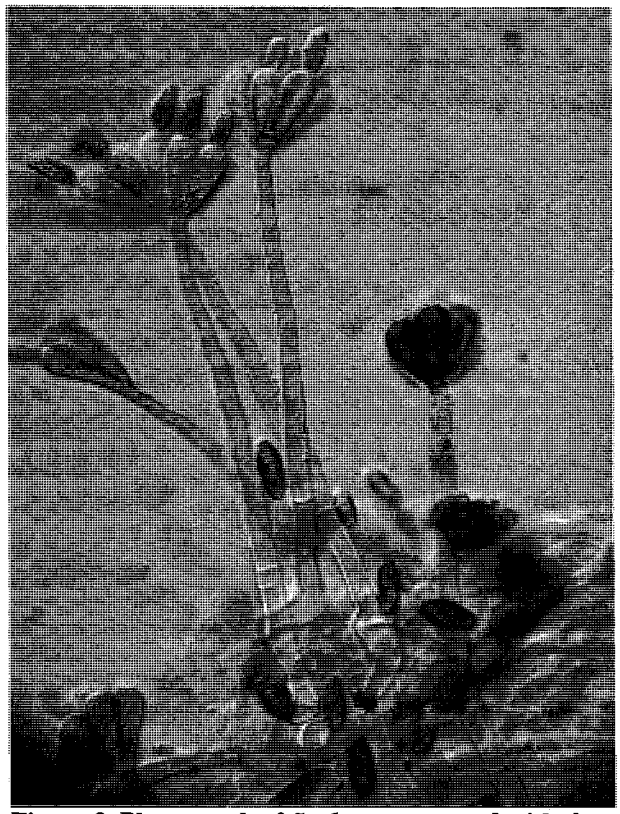


Figure 3. Photograph of *S. chartarum*, used with the permission of Dr. R. Samson, CBS

Genetic analysis of *S. chartarum* demonstrated that it is in the Ascomycete order, the Hypocreales (Castlebury et al. 2004). This order includes genera such as Hypomyces, *Nectria*, *Hypocrea* and *Stachybotrys* species. *S. chartarum* has been separated into 2 species, *S. chlorohalonata* and *S. chartarum* based on mycotoxin production, phyllogenetic and morphological differences (Andersen et al. 2003). *S. chlorohalonata* is morphologically differentiated from S. *chartarum* by its ovoid, smooth conidia, smaller colony diameter and the presence of a green soluble pigment on potato dextrose agar (Andersen et al. 2003). These fungi are closely related to

Memnoniella echinata which occupies a similar econiche except in sub-tropical environments (Flannigan and Miller 2001).

In indoor environments, *S. chartarum* can be found growing in buildings, which have had significant amounts of water damage. Due to its cellulytic nature it is often isolated from the paper side of wallboard. It requires a water activity or a_w above 0.97 to out compete other fungi (Flannigan and Miller 2001).

Environmental exposure to *S. chartarum* has been attributed to a wide variety of symptoms. These symptoms include headache, sore throat, general malaise, diarrhoea, muscle aches, rashes, sinusitis, bronchitis, allergy and asthma. More extreme symptoms such as neurotoxicity, vomiting, skin lesions and infant pulmonary hemosiderosis have been reported (Miller et al. 2003; NAS 2004). Recently, satratoxin-protein adducts have been identified in infants with infant pulmonary hemosiderosis (Yike et al. 2006).

The pathophysiology of exposure to *S. chartarum* has been studied. *S. chartarum* conidia have been shown to cause lung damage in mice. The effects include changes in composition of lung surfactant, inflammation, changes in lung cell organelle structure, granuloma formation and haemorrhage (Mason et al. 2001; McCrae et al. 2007; Rand et al. 2002; Rand et al. 2003; Rand et al. 2006; Vesper and Vesper 2002).

S. chartarum sensu lato is known to produce an array of mycotoxins. These include satratoxin G, atranones A-G and phenylspirodirmanes (Hinkley et al. 2000; Jarvis et al. 1995). Satratoxin G is cytotoxic and a potent inhibitor of protein synthesis, atranones are proinflammitory while the phenylspirodrimanes are known to be immunosuppressive (Hinkley et al. 2000; Jarvis et al. 1995; Rand et al. 2006). Satratoxin G, a macrocylic trichothecene, has been isolated from the conidia of *S. chartarum*

(Gregory et al. 2004). Different strains of *S. chartarum* and *S. chlorohalonata* have been shown to produce different mycotoxins. Both are considered chemotypes, one produces trichothecenes and the other produces atranones respectively (Andersen et al. 2003).

The antigenicity of *S. chartarum* has been poorly studied. Extracts of *S. chartarum* have shown IgG and IgE activity from individuals exposed to the fungus (Barnes et al. 2002; Raunio et al. 2001). Barnes (2002) demonstrated that almost 50% of the individuals in their study had IgG activity while almost 10% showed IgE activity.

A few reports describe some specific antigens as well as a few allergens from this species. The antigens include an uncharacterized hemolysin (stachylysin), protease (stachyrase-A), a cellulase and a DNase (Karkkainen et al. 2004; Xu et al. 2007). The claim that stachylysin and stachyrase-A are true allergens has been contested in the literature, due to improper methodology (Page et al. 2005).

The *S. chartarum* cellulase isolated by Karkkainen et al (2004) is a 48 kDa glycoprotein, demonstrated to have IgE activity. IgE activity was determined by immunoblotting using sera obtained from teachers who were not working in a moldy school. The *S. chartarum* DNase is a heterodimer, named SchS34, which is composed of 2 puntative subunits, 34 and 21 kDa, and is also glycosylated (~15%;Xu et al. 2007). SchS34 was discovered by screening *S. chartarum* conidia grown on rice cultures using sera from patients shown to be IgE/IgG positive by immunocap (Xu et al. 2007). The DNase has also been shown by immunogold staining to be present on the surface of spores and excreted into the surroundings (Rand and Miller 2008).

The *S. chartarum* antigens noted above have been used for the development of immunological methods for detecting *S. chartarum* (Schmechel et al. 2006; Xu et al.

2007). However, the ELISA to the DNase (SchS) is the only one to date, which meets the criteria proposed by the National Academy of Sciences Institute of Medicine whereby it quantifies a human antigen (NAS 2000). The ELISA developed by Schmechel et al (2006) is based on surface antigens. The published data suggests that this methodology can be species-specific, but the protein is not described and therefore not known to be a human antigen. Cross-reactivity studies were performed in this study but they failed to test *S. chlorohalonata* as well as *Memnoniella echinata*, also spore preparations were not normalized based on protein content or spore number (Schmechel et al. 2006). Without normalization it is difficult to compare responses, for example tested species may give a low response, but may have a relatively low spore number or protein concentration. Therefore the lack of cross-reactivity maybe just due to a relatively lower spore number tested, so it is difficult to evaluate the level of cross-reactivity in this assay. Xu (2007) showed cross reactivity to the closely related *S. chartarum*; *S. chlorohalonata* and *Memnoniella echinata.*

7.8. Protein preparation in fungi

Due to the rigidity cell wall of fungi, extraction of protein in a qualitative manner is difficult while a quantitative manner even more cumbersome. The most commonly employed approaches include: chemical, physical or enzymatic treatment (Nandakumar and Marten 2002). Many of these methods are derived from fungal genomics, particularly yeast (Fredricks et al. 2005; Haugland et al. 1999; van Burik et al. 1998).

Chemical treatment usually involves a detergent or a mild acid or base proceeded by boiling. Detergents often used include Triton X, SDS and Tween 20. The detergents increase the solubility of proteins, hence allowing them to enter solution and allowing the antigens to be available for the ELISA.

Common physical approaches are bead milling, French press or mortar and pestle. Bead milling uses either a single large bead or many smaller beads, these methods are commonly used for DNA extraction from fungi. Bead mill extraction of *A. versicolor* was shown to increase AVS antigen content (Zhao 2006). The French press is a large machine which forces the spore through a very small opening, which in turn disrupts the spores. A mortar and pestle is used to grind fungal material, often in liquid nitrogen, this has the advantage in that it keeps samples cool, reducing protein degradation. These physical methods would be difficult to employ when used to analyse field or small samples.

Lastly, a variety of enzymatic methods are used, most commonly β-glucanases and/or chitinases, which usually involves sphaeroplasting followed by subsequent osmotic lysis (Shimizu and Wariishi 2005). These enzymes digest the cell wall, thereby releasing any proteins found therein. This would allow the intercellular proteins to be available for the ELISA This method is difficult to perform and usually requires specific growth conditions; also the enzymes used tend to contain protease impurities.

A few of these approaches are useful in quantitative proteomics for filamentous fungi particularly where ELISA is used. ELISA requires that the antigen of interest to be quantified while its 3D structure is maintained for the antibodies to recognize it, therefore non-denaturing extractive techniques are required. Most proteomic studies have focused

on protein extraction from the mycelium with very few reports exist from fungal spores (Asif et al. 2006).

7.9. Mass spectrometry and proteomics

Mass spectrometry is becoming the tool of choice with regard to protein characterization (Aebersold and Mann 2003; Domon and Aebersold 2006; Nesvizhskii 2007). This popularity can be attributed to technological advances in mass spectrometers as well as the short period of time required for identification. One of these technological advances includes soft ionization techniques such as matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI; Fenn et al. 1989; Karas and Hillenkamp 1988). The applications of mass spectrometry in proteomics are many, these include *de novo* sequencing of peptides, post-translational modifications, protein interactions and protein quantitation (Kinter and Sherman 2005; Morelle et al. 2006; Yan and Chen 2005).

Various mass spectrometers are available, each having its own strengths and weaknesses. The most commonly used machines in proteomics are the quadrapole time of flight (ESI-QTOF), ion trap (ESI-IT) and matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) instruments with the Fourier transform ion cyclotron resonance mass spectrometer (FTICR) gaining much popularity. The triple quadrapole mass spectrometer (ESI-3Q) is seldom used in proteomics due to its lack of mass accuracy and resolving power but due to its multiple reaction monitoring (MRM) capabilities it is sometimes used for protein quantification (Domon and Aebersold 2006).

The ion trap mass spectrometers tend to have a short duty cycle which makes them attractive to researchers doing whole proteome analysis. It also is very sensitive, but the mass accuracy and resolution is not as good as a QTOF instrument (Domon and Aebersold 2006). MALDI-TOF is commonly used to analyze entire proteins for molecular weight determination. These machines tend to have good resolution and mass accuracy (Domon and Aebersold 2006). The QTOF instrument is often used for *de novo* sequencing and protein identification due to its high mass accuracy and resolution (Domon and Aebersold 2006). The FTICR is under study intensely because it has the capability of top-down characterization of proteins (McLafferty et al. 2007). The FTICR also has very high mass accuracy and resolution but the machines are difficult to use and tend to produce few fragment ions (Aebersold and Mann 2003).

7.9.1. *De novo* sequencing of peptides

Before mass spectrometry methods for sequencing proteins were available, protein were partially sequenced using Edman degradation or N-terminal sequencing. This method traditionally involves cleavage of the N-terminal amino acids and each analysed one by one using UV spectroscopy (Edman and Begg 1967). N-terminal sequencing is deemed time consuming and would only allow the analysis of a few amino acids due to decreased efficiency after each analyzed amino acid. In many cases this method cannot be applied because the N-terminal is blocked (Eng et al. 1994).

Initially proteins were identified using mass spectrometry based on their "mass finger print" (James et al. 1993). This method involved digesting proteins, then

measuring the mass of the peptides, then searching a data base for proteins which had similar peptide mass profile. Mass finger printing is highly data base dependant, if the target protein was not in the database it cannot be identified. In addition protein must be very pure to avoid erroneous peaks in the mass spectrum (Eng et al. 1994).

To develop more accurate identification of proteins, pieces of the protein of interest were sequenced using tandem mass spectrometry, to generate sequence tags. Specifically, this method uses very short sequences, 2 or 3 amino acids, and their location with in the peptide, to search a database (Mann and Wilm 1994). Longer sequences can also be obtained and these search against the NCBI database to find homologous proteins. PCR primers can then be designed to find the cDNA clone to obtain the entire protein sequence and clone the gene (Shevchenko et al. 2002).

To further increase accuracy of sequencing information, programs were developed which would convert known proteins into peptides, these peptides would then be converted into expected mass spectra. This approach allowed conformation of sequences obtained by mass spectrometry but it also allowed searches of databases using mass spectral data (Eng et al. 1994).

De novo sequencing of proteins and peptides via mass spectrometry relies on the ability of a mass spectrometer to select an ion, then introduce that ion into a collision cell whereby the peptide is fragmented, followed by measurement of the daughter fragment masses. To make the peptides small enough to fall within the mass spectrometers mass window, a protein is usually digested with a protease, commonly trypsin. These tryptic peptides can be separated using an HPLC coupled to the mass spectrometer. For increased sensitivity a nano-HPLC is generally used which uses flow rates in the

nanolitre/min range which facilitates use of small diameter HPLC columns (Mitulovic and Mechtler 2006). A reverse phase column is often used in proteomics, whereby proteins are eluted based on increasing hydrophobicity (Mitulovic and Mechtler 2006).

Once fragment spectra are acquired, interpretation is required. Many fragment series of the peptide are generated by collision-induced disassociation but 2 series are the

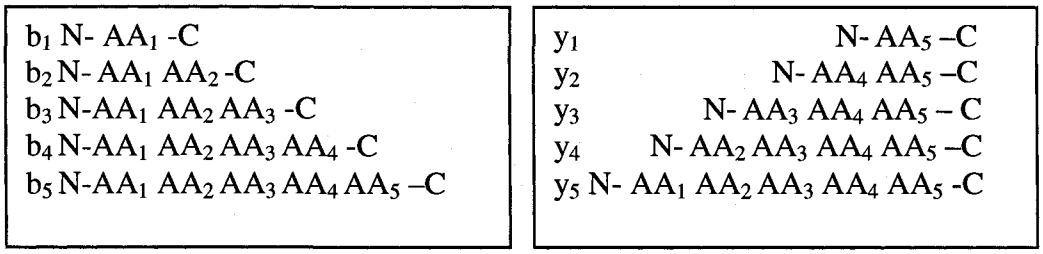


Figure 4. Diagrammatic representation of y and b-ion series generated from mass spectrum analysis of peptides. AA = amino acid, C = C- terminus, N = N-terminus

most commonly used for interpretation. These series are named the y and b-ions, with the y-ion series generated from N-terminal fragmentation and the b-series generated from C terminal fragmentation (Figure 4). Fragments from each series are labelled numerically from either the N or C terminus depending on the respective ion series. Therefore the b-ion series would expand at the C-terminus while the y-ion series would expand from the N-terminus. For example the y_2 ion would contain the 2 C-terminal amino acids of the peptide being studied. While the peptide breaks into 2 pieces only one is seen by the mass spectrometer as only one ion is charged with the H^+.

With there being 20 amino acids and their masses known, the amino acid sequence can be deduced by comparing mass differences of fragments within an ion series. For example if the y-2 ion had a mass of 211 Da and the y1 ion had a mass of 97 Da, then the difference (211-97 Da) would be 114 Da which is the mass of asparagine.

Also, since it is known that proline has a mass of 97 Da, we now know the first 2 C-terminal amino acids; ProAsn.

To further aid in interpretation, the enzyme trypsin is regularly used for digestion of proteins. Trypsin specifically cleaves C-terminal to arginine and lysine in the protein which results in digested peptides having either of these amino acids at the C-terminus (Olsen et al. 2004). Therefore the last amino acid in the b-ion series or the first ion in the y-ion series would be either an arginine or lysine.

To further facilitate in mass spectra interpretation, low molecular weight ions are generated from the collision-induced dissociation of peptides. The most useful of these ions are known as immonium ions. These ions are single amino acids from the peptide being investigated. These ions provide compositional information but do not provide information with regard to amino acid orientation.

In an ideal situation, both of the y-ion and b-ion series will be present, therefore each can reinforce the puntative sequence generated. Unfortunately this is rarely the case, but using the available data can often produce sequences with great confidence.

Lastly, other tools commonly used for *de novo* sequencing are computer software programs and databases. With mass spectra interpretation often being the bottleneck in the *de novo* sequencing procedure, computer software such as PEAKS will automatically generate sequences from generated mass spectra (Domon and Aebersold 2006). The advantage of this is that it allows many peptides to be sequenced in a given time followed by parent protein identification. In some cases whole proteomes can be analyzed by high-through put mass spectrometry (Washburn et al. 2001). Commonly used software

programs such as SEQUEST and Mascot are online databases that can be searched using mass spectrum data.

8. Objectives

The objectives of these studies reported in this thesis were: (1) to optimize the SchS34 ELISA, (2) to test the recovery of SchS34 from settled house dust, (3) to test methods for improving Asp f1 recovery and (4) apply mass spectrometry to obtain partial sequence information of the SchS proteins.

9. Materials and Methods

9.1. Reagents

Product	Supplier	Catalogue #	Lot #
Acetic Acid	Anachemia	00598-468	503345
Acetonitrile	Fisher	A21-4	000964
Acrylamide/Bis solution	Biorad	161-0156	L16100156
Asp f1 ELISA Kit	Indoor Biotechnologies	EL- AF1	30045/2766
Beads	Spex Certiprep	3112	NA
Benzonase (25U/μL)	Novagen	70746	N61197-2
Bovine serum albumin	Sigma	A7030	086K1230
Bradford reagent	Biorad	500-0205	4307755
Bromophenol Blue	USB	12370	111590
C-18 macrospin column	Nestgroup	SS04 –SS18V	NA
Carbonate-Bicarbonate buffer	Sigma	C3041-100CAP	076K8206
Chitinase C (250 U/g)	Interspex	0440-2	3601
DTT	Sigma	D-9163	15H0728
EDTA	Fisher	S-311	731136
Formic Acid	Fisher	A119-4	990767
Galactomannase	Interspex	0435-1	14-933221-01
GelCode Blue Stain Reagent	Pierce	24592	1F113673
Glycine	USB	US16407	112491
GTTP membrane filters, 0.2 μm 26mm	Millipore	GTTP04700	NA

Horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG	Jackson Laboratories	111-036-046	55036
Iodoacetamide	Sigma	A3221	NA
Low Molecular weight marker	GE Healthcare	17-0446-01	337610
Methanol	Fisher	A421-1	D0371
Microbeads 212-300 μm acid washed	Sigma	G9143-250G	115K0017
Microtitre plate	Nunc	439454	630139
Potassium chloride	NA	NA	NA
Potassium phosphate (monobasic)	Sigma	P-5379	89H0030
Quantazyme, 10,000 U	Q Biogene	QUA0501	404Q3792
Protease inhibitor cocktail (complete)	Roche	1697498	2890601
Protein G sepharose fast flow	GE Healthcare	17-0618-01	10009446
Rice, converted	Uncle Ben's	NA	NA
Skim Milk Fat Free	Nestle Carnation	NA	NA
Streptavidine-Horse Radish Peroxidase	Sigma	S5512	NA
Sodium chloride	Sigma	S988-10KG	064K0021
Sodium dodecyl sulphate	Biorad	161-0300	L1610300
Sodium phosphate	Fisher	S-374	720607
Sodium sulphate	Caledon	8220-1	56631
Swabs (BBL Culture Swab Plus, Ames media, without charcoal)	Fisher		B4320093

THP solution	Novagen	71190	N61150
Tris Base	Sigma	T1503-1KG	116K5449
Tris(hydroxypropyl)phosphine (TMB)	Sigma	T0440-1L	047K0713
Trifloroacetic acid (TFA)	Fisher	04901-500	992162
Trypsin	Sigma	T-6567	044K6003
Tween 20	Sigma	P7949-500mL	9005-64-5
Urea	Mallinckrodt	8648	8648 KDSH
YeastBuster Protein Extraction Reagent	Novagen	71186-3REF	N63059

9.2. Fungal strain information

The *Aspergillus fumigatus* strain B2 used for all the below experiments was obtained from a liquid impinger sample taken at a wood chip processing plant in Alabama. This isolate came from a sample with high Asp f1 concentrations (Dillon et al. 2007). *S. chartarum* DAOM 235364 used for the following studies was taken from an indoor sample.

9.3. Optimization SchS34 capture ELISA

9.3.1. SchS34 ELISA

A schematic representation of the SchS34 ELISA is found in Figure 5 The mouse anti-SchS IgG 12D3 monoclonal antibody (0.25 mg/mL) was diluted 1:1000 (1 µL/mL) in 50mM carbonate-bicarbonate, pH 9.6. An aliquot (100 µL) was added to each well of microtire plate and incubated overnight at 4°C. The plate was then washed 3 times with 200 µL phosphate buffered saline 0.05% Tween 20, pH 7.4 (PBST) per well. Blocking was performed using 100 µL PBS- 2%BSA (PBS2%B) per well for 1 hr at room temperature, followed by 3 times washing with 200 µL PBST per well. The SchS standard curve was prepared by adding 190 µL PBST- 1%BSA (PBST1%B) to the first well followed by 100 µL to nine other wells in the row. An aliquot (10 µL) of the SchS standard (100 ng/mL) was added to the 190 µL of PBST1%B, this was then diluted using doubling dilutions across the row, to give a standard curve with a range of 5-0.01 ng/mL. This was then repeated to produce a duplicate curve. An aliquot (100 µL) of each sample to be tested were added to each well, usually in triplicate, also 4 wells with

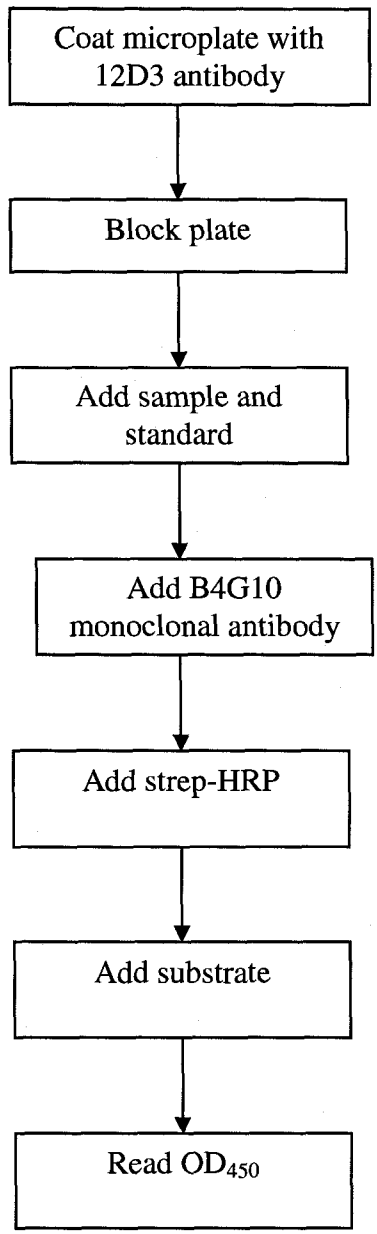


Figure 5. Schematic representation of SchS34 ELISA

PBST1%BSA were used for controls. The microplate was then incubated at room temperature for one hour on a plate shaker. Again the plate was washed 3 times using 200 µL PBST per well. To each well a 100 µL aliquot of 1:1000 diluted biotin conjugated mouse IgG monoclonal antibody 4G10 (0.5 mg/mL) was added and was then incubated for 1 hr at room temperature on plate shaker. The plate was washed 3 times using 200 µL of PBST per well. Horse radish peroxidase (HRP) conjugated streptavidin (0.250 µg/mL, 0.5 mL ddH_2O:0.5 mL glycerol) was diluted 1:1000 and 100µL added to each well. After 30 min incubation at room temperature on plate shaker the plate was washed 3 times with PBST for the final time. One hundred microlitres of the HRP substrate, Tris(hydroxypropyl)phosphine (TMB), was added to each well. The plate was left to develop at RT for 10 minutes and the enzymatic reaction was subsequently stopped using 50 µL 0.5M sulphuric acid. Optical densities were read at 450 nm using a Molecular Device Spectramax 340PC plate reader (Sunnyvale, California) and data analyzed using the Softmax pro software 4.8. SchS34 concentrations were determined from linear range of standard curve with regression values above 0.9 (Xu et al. 2008).

9.3.2. Purification of 12D3 monoclonal antibody

The 12D3 antibody was precipitated by slowly adding 1 mL of saturated ammonia sulphate to 1mL 12D3 mouse ascites. The mixture was left shaking over night at 4°C. The 2 mL solution was centrifuged 10,000 x *g* for 10 min at 4°C, the supernatant was discarded and the pellet resuspended in 1 mL PBS. Particulate matter was removed from the resuspended pellet by centrifugation at 10,000 x *g* for 10 min at 4°C, in this case the

pellet was discarded. The buffer was changed to PBS by washing sample 3 times with PBS using a 40 kDa molecular weight cut-off centrifuge tube. The washed sample was brought up to 1 mL and applied to protein G column (Protein G Sepharose 4 fast flow, GE Healthcare). The column was stopped and shaken for 2 hr at room temperature. The column was opened and the sample allowed to flow out. Flow through was collected and passed through again to ensure all antibody had chance to bind to protein G. The column was then washed at least 10 times bed volume with PBS and OD_{280} checked to ensure unbound proteins were washed off. Antibody was eluted using 0.1 M glycine buffer, pH2.7. Aliquots of 1 mL were eluted into 1.5 mL Eppendorf tubes containing 50 μL Tris-HCL pH 8.8. The OD_{280} of each aliquot was determined, aliquots with positive optical densities being pooled and concentrated to 1 mL using 40 kDa molecular weight cut-off centrifuge tubes. Antibody concentrations were estimated by OD_{280}/1.4 and by Bradford microplate method. Antibody purity was determined by Coomassie blue stained SDS-PAGE (Figure 8).

9.3.3. Micro bradford assay for protein quantification

An aliquot of the Bradford reagent was allowed to warm to room temperature. Once warmed, 150 μL of reagent was added to each well in a microplate. Using the appropriate sample dilution or dilution series, 150 μL of sample was applied to the reagent in the well. Also a blank composed of 150 μL ddH_2O and reagent was used, this blank was subtracted from sample data. The mixture was shaken at room temperature for 10 min. Using the plate reader the OD was read at 595 nm and compared to a standard curve (0.25 - 500 ug/mL).

9.3.4. Optimization of reagents for SchS34 ELISA.

Various concentrations of the 12D3 capture antibody were explored in preliminary experiments. The SchS34 ELISA was preformed as above with the exception of the concentrations of the 12D3 antibody. The newly purified 12D3 antibody was coated in the following concentrations 500, 250, 125 and 61.25 ng/mL. Each was tested in duplicate against the standard curve (5-0.01 ng/mL). Signal to noise ratios were calculated and evaluated.

To reduce background noise, the effectiveness of the following blocking agents were tested: 2%BSA, 5% Skim Milk powder, 2%BSA new batch, Blotto and no blocking solution. BSA and skim milk powder blocking was evaluated in both PBS and PBST buffers. SchS34 ELISA was performed as above with the exception of the blocking solutions. Blocking was performed for 1 hr at room temperature for all blocking solutions. Comparison of standard curve signal to noise values were performed.

With high background being a problem, streptavidin-HRP (strep-HRP) concentration and incubation times and were analyzed. For strep-HRP concentration optimization, concentrations tested included 250, 125 and 62.5 ng/mL. With regard to incubation time, both a 30 and 60 min strep-HRP (250 ng/mL) incubation times were explored. In all cases SchS34 ELISA was carried out as above with the exception strep-HRP treatment. Signal to noise comparisons were carried out with respect to the standard curve.

9.3.5. Preparation of *A. fumigatus* conidia

A. fumigatus conidia were obtained by inoculation of rice cultures as described by Murad et al. (1993). Rice cultures were prepared as follow: 50 g of Uncle Ben's converted rice was mixed with 30 mL deionized water and autoclaved for 30 min at 121°C (Xu et al. 2007). Rice cultures were allowed to cool overnight. A 2% malt extract culture plate of *A. fumigatus* was flooded with 10 mL sterile ultra pure water. The flooded plate was gently rocked then 5 mL added to 2 separate rice cultures. Inoculated rice cultures were allowed to grow for 30 days at 24°C. The resulting culture was cut in a half and place in disposable plastic petri dishes. Conidia were gently scraped into as pre-weighed glass vial. The remaining unused material was autoclaved.

9.4. Analysis of settled dust samples using SchS34 capture ELISA

9.4.1. Culturable analysis of settled house dust

One gram of the <300 μm fraction of the settled house dust sample was serially diluted to extinction in sterile 0.5% Tween 20 over four log dilutions using the spread plate method on 2% malt extract agar plates. Plates were incubated at 25° 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 in the AIHA methods manual (Hung et al. 2005).

9.4.2. Asp f1 ELISA

The Asp f1 ELISA is a two site capture ELISA that uses a monoclonal antibody as the capture and a polyclonal as the secondary antibody (Figure 6). This method is used to quantify the Asp f1 allergen produced by *A. fumigatus*. Asp f1 analysis was carried out using the EL-AF1 kit purchased from Indoor Biotechnologies. The mouse anti-Asp f1 IgG mAB 4A6 was diluted 1:1000 (10 ug/mL) in 50mM carbonate-bicarbonate, pH 9.6. A 100 µL aliquot was added to each well of microtitre plate and incubated over night at 4°C. The plate was then washed 3 times with 200 µL PBST per well. Blocking was performed using 100 µL PBST1%B per well for 1 hr at room temperature, followed by 3 times washing with 200 µL PBST per well. The Asp f1 standard curve was prepared by adding 180 µL PBST1%B to the first well followed by 100 µL PBST1%B to nine other wells in the row. An aliquot (20 µL) of the Asp f1 standard (400 ng/mL) was added to the 180 µL of PBST1%B, this was then diluted using doubling dilutions across the row, to give a standard curve with a range of 40-0.4 ng/mL. This was then repeated to produce a duplicate curve. An aliquot (100 µL) of each sample to be tested were added to each well, at least in triplicate; 4 wells with PBST1%BSA were used for blanks. The microplate was then incubated at room temperature for one hour on a plate shaker. Again the plate was washed 3 times using 200 µL PBST per well. To each well, a 100 µL aliquot of 1:1000 diluted rabbit anti-Asp f1 polyclonal antibody were added and the plate incubated for 1hr at room temperature on plate shaker. The plate was washed 3 times using 200 µL of

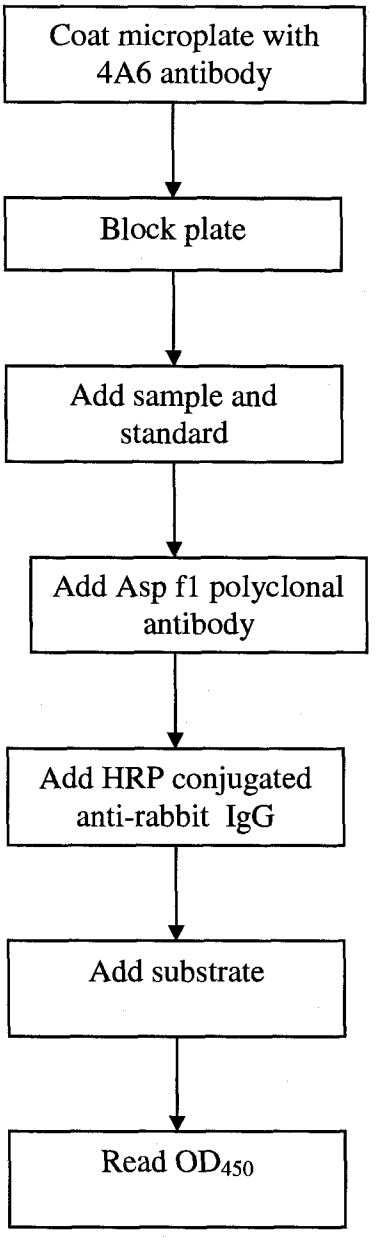


Figure 6. Schematic representation of the Asp f1 ELISA

PBST per well. HRP conjugated goat anti-rabbit IgG was diluted 1:1000 and 100 µL added to each well. After 1hr incubation at room temperature on plate shaker the plate was washed 3 times with PBST for the final time. An aliquot (100 µL) of the HRP substrate, TMB, was added to each well. The plate was allowed to develop at room temperature for 10 minutes and in some instances the enzymatic reaction was subsequently stopped using 50 µL 0.5 M sulphuric acid. Optical densities were read at 680 nm for unstopped and 450 nm for stopped reactions using the plate reader (Sunnyvale, California). Data were analyzed using the Softmax pro software 4.8. Asp f1 concentrations were determined from linear range of standard curve with regression values above 0.9. Values were compared by ANOVA with Fishers least significant difference test using Systat 8.0 software (San Jose, California).

9.4.3. SchS34 ELISA analysis of *S. chartarum* contaminated samples

Sieved house dust was initially tested to determine the limit of detection for *S. chartarum* conidia. *S. chartarum* conidia (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 conidia/5 mL. Each conidial suspension was then added to 0.5 g of <300 µm 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 1hr followed by centrifugation at 10,000 x *g* for 15 min at 4°C using the microcentrifuge (Micro 18 Centrifuge, Beckman Coulter, Mississauga,

Ontario). The supernatants from both the initial 5 mL aliquot (no dust) and the dust/spore supernatant were tested in triplicate on three separate occasions using the SchS34 capture ELISA.

Pellets were resuspended in 5 mL PBST1%B and spores enumerated. The number of conidia in the suspensions was counted using a Neubaur ruled haemocytometer. Sixteen squares were counted which covered a volume of 1mm x 1mm x 0.1mm. This was then multiplied by 10,000 to give spore number per millilitre, according to manufacturer's instructions.

A. fumigatus spiked dust samples were also analysed for comparison. Analysis was carried out the same as the *S. chartarum* samples, except the *A. fumigatus* conidia were initially used at a 15 mg/15 mL and the Asp f1 ELISA kit was used for the ELISA analysis. Conidia were enumerated using a haemocytometer and limit of detection determined by ANOVA analysis.

The SchS34 ELISA response to intact *S. chartarum* and *S. chlorohalonata* spores was explored. An aliquot (1 mg) of *S. chartarum* and *S. chlorohalonata* conidia, respectively were weighed into two separate 2 mL Eppendorf centrifuge tubes and suspended in 1 mL PBST1%B and vortexed for 1 min. These were doubly diluted greater than 10 fold range (1-0.078 mg/mL) and analyzed in triplicate with the SchS34 ELISA

The response of the SchS34 ELISA to drywall contaminated with *S. chartarum* spores and fragments was tested. Experimentally inoculated drywall was prepared using

20 x 20 cm pieces of drywall wetted with sterile ultra pure water. A fraction of a *S. chartarum* DAOM 235364 culture plate was macerated in sterile ultra pure water and 1 mL of the mixture added to each piece of drywall. The pieces were then placed inside a large Tupperware container, allowed to grow for 1 month at 24 °C and were then freeze dried. Aside from experimentally inoculated drywall samples, two samples taken from the field provided by Paracel Laboratories Ltd., were analyzed. A portion (0.5 g) of drywall paper was weighed and added to a 20 mL centrifuge tube. An aliquot (5 mL) of PBST1%B was added to each tube and vortexed for 1 min. Each sample (100 uL) was then analyzed by the SchS34 ELISA.

In an attempt to removed extraneous debris (e.g. drywall dust, paint, paper fibres) from the samples, experimentally-inoculated and field drywall samples were wiped with wet swabs. Once the cotton tip of the swab was black (with spores), the cotton end was broken and added to 5 mL of PBST1%B in a 20 mL centrifuge tube. The sample was vortexed for 1 min and a 100 uL aliquot was analyzed by the SchS34 ELISA.

9.5. Asp f1 recovery

9.5.1. Recovery of *A. fumigatus* spores from membrane filter

A spore suspension of *A. fumigatus* conidia was prepared in 0.1% Tween 20. A 26 mm, 0.2 μm membrane filter was placed onto the bottom portion of a 3 piece air cassette. The membrane was added to a 20 mL centrifuge tube and 1 mL 0.1% Tween 20 was used

to rinse filter cassette. The filter was then vortexed for 5 min, the conidia counted and percent recovery calculated.

To calculate the number of conidia per milligram, 2 mg of conidia were suspended in 2 mL phosphate buffered saline 0.05% Tween 20, pH 7.4 (PBST). Conidia were counted with the haemocytometer and conidia/mL were determined.

9.5.2. Enzymatic treatment of *A. fumigatus* conidia

The following treatments were tested for their effect on Asp f1 recovery: ß 1-4 glucanase, chitinase C, galactomannase, large bead disruption, microbead disruption, Tween 20, protease inhibitor cocktail, EDTA, "Yeast Buster" protein extraction kit, THP, temperature and time

Initially, the effect of incubation temperature and duration was tested for their effect on Asp f1 recovery. Nine aliquots of 15 mg of *A. fumigatus* spores were weighed into 2 mL Eppendorf tubes (7.5 mg/mL). To each tube 2 mL of PBST was added. Three tubes were incubated at room temperature, 5°C and 36°C. A 200 µL sample was taken from each sample at 0, 1, 2, 3, 4, 5 and 24 hr and frozen at -20°C. All samples were analyzed by the Asp f1 ELISA

Quantazyme is a protease free ß 1-3 glucanase. An aliquot (10,000 units) of this enzyme were resuspended in 100 µL PBST giving a solution of 100 U/µL. *A. fumigatus* conidia was tested with 0.1 U, 1 U and 10 U/mL of Quantazyme. Solutions of the enzymes were prepared in PBST at the above activities and added to conidia at a

concentration of 7.5 mg/mL. A PBST only preparation was also made to act as a control solution. All experiments were performed in triplicate with aliquots taken at 0, 1, 2, 3 and 4 hr at room temperature. These samples were then analyzed using the Asp f1 ELISA.

Chitinase C, an enzyme which degrades the cell wall polysaccharide, chitin was similarly tested as Quantazyme. Chitinase C concentrations used were 0.01 and 0.1 mg/mL with sampling times of 0, 2.5, 5 and 24 hr at room temperature.

Galactomannase was also tested the same as Quantazyme, except with different enzyme concentrations, in this case the sample times were the same. Galactomannase concentrations tested were 0.01, 0.1 and 1 mg/mL.

9.5.3. Effect of bead disruption and Tween 20 on Asp f1 recovery

Initially a single large bead was tested. Four samples of 7.5 mg *A. fumigatus* conidia were accurately weighed, with two being weighed into 2 mL Eppendorf tubes and the other two in small plastic containers. Conidia to be disrupted were smashed for 10 min in a Spex-Certiprep mixer mill (model 5100, Metuchen, NJ) with one glass bead per plastic container. One disrupted sample and one intact spore sample were resuspended into 1 mL PBS, while the other samples were resuspended in 1 mL PBST. Each were vigorously vortexed and doubling dilutions were analyzed by the Asp f1 ELISA.

Microbeads are commonly used for the extraction of DNA from fungi and plants. In this case they were used to determine their effectiveness on Asp f1 recovery from spores. Three amounts of beads were tested: 1, 5 and 10 g with a fourth sample having no

beads. Four 5 mL solutions of *A. fumigatus* conidia (7.5 mg/mL in PBST) were prepared in 20 mL centrifuge tubes. To one tube, 1 g of microbeads (212-300 μm acid washed), to another 5 g, to the third 10 g and no beads in the last tube. Only a 1 mL aliquot was taken from the no bead sample at 0 min as it was difficult to pipette with the small beads. All four samples (no bead, 1 g, 5 g and 10 g) were vortexed for 2 min, then centrifuged using the microcentrifuge at 10,000 x *g* for 10 min at 4°C. For each sample the supernatants were collected and the beads discarded. The supernatants were analyzed by the Asp f1 ELISA in triplicate using the microplate reader.

9.5.4. Effect of Yeast Buster Protein Extraction Kit.

The effectiveness of the "Yeast Buster" protein extraction kit in Asp f1 recovery was determined for intact and disrupted *A. fumigatus* conidia. This kit was originally designed for high yield extraction of protein from yeast. It uses the combination of a surfactant (extraction reagent), reductant (THP), protease inhibitor (PMFS) and a DNase (benzonase) to extract yeast proteins. A variant of this method was published by the manufacturer and used below (Okpuzer et al. 2007). Intact and disrupted samples were prepared at conidial concentrations of 7.5 mg/mL as above. Control samples contained PBST while Yeast Buster samples contained 978 μL of Yeast Buster Protein extraction reagent, 10 μL 0.5M THP, 1μL benzonase (25 U) and 11 μL PMSF per mL. Each sample was prepared in triplicate with 200 μL aliquots taken at 0, 2.5, 5 and 24 hr and stored at -20 °C. All samples were analyzed by Asp f1 ELISA.

9.5.5. Effect of protective methods on Asp f1 recovery

Fungi excrete large amounts of proteases which can have a negative effect on protein isolated from fungal materials. In protein purification these cocktails are added to reduce protease digestion of target proteins and maximize protein yields (de Vries et al. 2004). Two protease inhibitor cocktail tablets (Roche) were diluted in 10 mL PBST, this solution was then diluted 1:10 with PBST, to give undiluted and 10 fold dilution. *A. fumigatus* conidia (7.5 mg/mL) was then suspended with each respective inhibitor cocktail dilution with the third sample being suspended with PBST only to act as a control. Each sample was prepared in triplicate with 200 µL aliquots taken at 0, 2.5, 5 and 24 hr at room temperature and stored at -20 °C. All samples were analyzed by Asp f1 ELISA.

Ethylenediaminetetraacetic acid (EDTA) is typically used in biochemical preparations due to its metal chelating capabilities. Free metals may oxidize proteins, changing their conformation; also EDTA is known to inactivate metallic proteases (de Vries et al. 2004). Three concentration of EDTA (0.1, 0.01, 0.001 M) were tested to determine their effectiveness on Asp f1 recovery from intact *A. fumigatus* spores. In this case, 7.5 mg of intact spores were suspended with 1 mL of 0.1, 0.01, 0.001 M EDTA and PBST. Aliquots at 0, 1, 2 and 3 hr were tested in triplicate by Asp f1 ELISA.

The Tris(hydroxypropyl)phosphine (THP) supplied with the "Yeast Buster" kit was tested independently on intact spores and disrupted spores (7.5 mg/mL). THP concentration tested included 5 mM, 0.5 mM and 0.05 mM with an untreated sample

containing PBST. A suspension (7.5 mg/mL) of *A. fumigatus* conidia was prepared from 1 mL of each of the THP solutions (5 mM, 0.5 mM and 0.05 mM) and PBST. At 0, 1, 2, 3 and 4 hr 200μL aliquots were taken. All aliquots were then tested in triplicate using Asp f1 ELISA.

9.6. Mass spectrum analysis of proteins

9.6.1. Mass spectrum analysis of BSA peptides

Mass spectrum analysis was carried out with a Z-spray 3Q Micromass machine in positive ion mode found at the Univesirty of Ottawa mass spectrometry centre. Due to its inert nature helium was used as the collision gas for all experiments. Initial experiments were carried out with the readily available and well-studied protein, bovine serum albumin (BSA) before analyses were attempted with the SchS protein. Mass spectral data were analyzed using Masslynx 5.0.

Preliminary experiments involved a high performance liquid chromatography (HPLC) system coupled to the mass spectrometer. HPLC was initially carried out using a Phenomenex Jupiter Proteo Column (C12, 90 Å, 250 x 4.6mm) (Torrance, California) with a splitter valve directing the peptides to the mass spectrometer and the UV detector. UV detection was performed at 220nm. The HPLC conditions were as follows: solvent A = ddH_2O 0.1%TFA, solvent B = acetonitrile 0.1%TFA, start 95% A, 5 min 95% A, 35 min 5% A, 40 min 5% A. A flow rate of 1 mL/min was used and injections consisted of 20 μL from a 1 mg/mL BSA digest. This approach was abandoned because the splitter valve resulted in not sending enough protein to the mass spectrometer. The HPLC was

then directly attached to the mass spectrometer with no splitting of the flow, therefore no UV trace was acquired. HPLC conditions were the same as above. This method did not work due to the high flow rate. The majority of the solvent did not evaporate and collected just prior to entering the mass spectrometer.

It was then decided to perform direct injections. Peptide samples were injected directly into the mass spectrometer and the first quadrapole used to select which ion would be subjected to collision induced dissociation (CID). Tubing was connected directly from the injector of the HPLC to the mass spectrometer, essentially using the HPLC as pump. The solvent used was 50% ddH_2O:50% acetonitrile 0.1% formic acid. The apparatus was thoroughly cleaned with ddH_2O 0.1% formic acid after each injection. Three principle parameters were manipulated in an attempt to produce the best MS/MS spectra. These included: solvent flow rate, collision gas pressure and collision energy. Flow rate was varied from 1 mL-50 μL, collision gas pressure tested between 1.0×10^{-4} - 8.0×10^{-4} mbar and collision energy from 30-55 eV.

9.6.2. In-solution tryptic digestion of BSA

A mass of 10 mg of bovine serum albumin (BSA) was resuspended in 1 mL 6 M Urea, 100 mM Tris buffer. A 100μL aliquot of the protein suspension was mixed with 5 μL reducing agent (200 mM DTT in 100 mM Tris) in 1.5 mL centrifuge tube and vortexed. This mixture was then incubated at room temperature for 1hr. After the reduction was completed, 20 μL of alkylating reagent (200 mM Iodoacetamide in 100 mM Tris) was added to the mixture, this was also vortex and incubated at room temperature for 1hr. An aliquot (10 μL) of reducing agent was then added to the mixture

and incubated at room temperature for 1hr. Urea concentration was reduced to 0.6 M by adding 775 μL to the reaction mixture. A 100 μL of trypsin solution (25 μL 0.4 M Tris and 20 μg sequencing grade trypsin) was added to the mixture and incubated at 37°C over night. The following morning the tryptic reaction was stopped by reducing the pH to less than 6 by adding 6 drops of concentrated acetic acid.

After trypsin digestion, the protein sample was cleaned-up using a C-18 macrospin column. The column was washed with 500 μL of 100% acetonitrile and centrifuged for 1 min. at 110 x *g* using the microcentrifuge. The flow-through was discarded, then 500 μL of ddH_2O was added to the column and centrifuged at 110 x *g* for 1 min using the microcentrifuge. This was then repeated and the flow-through was discarded. An aliquot (100 μL) of digested sample was applied to column and centrifuged at 110 x *g* for 1 min using the microcentrifuge. The column was then washed 3 times with 500 μL of ddH2O and the flow through discarded. The peptides were then eluted into a new 1.5 mL Eppendorf centrifuge tube with 100 μL of 80% methanol.

9.6.3. Mass spectrometry analysis of intact SchS proteins

A schematic representation of mass spectrometry analysis of SchS protein is shown in Figure 7These mass spectrometry experiments were done at UWO-BMSL using a Z-spray Q-TOF mass spectrometer (Micromass) in positive ion mode. Liquid chromatography was completed using a C4 PepMap300 5μm, 300Å, 1 mm x 15 cm (LC Packings) on a

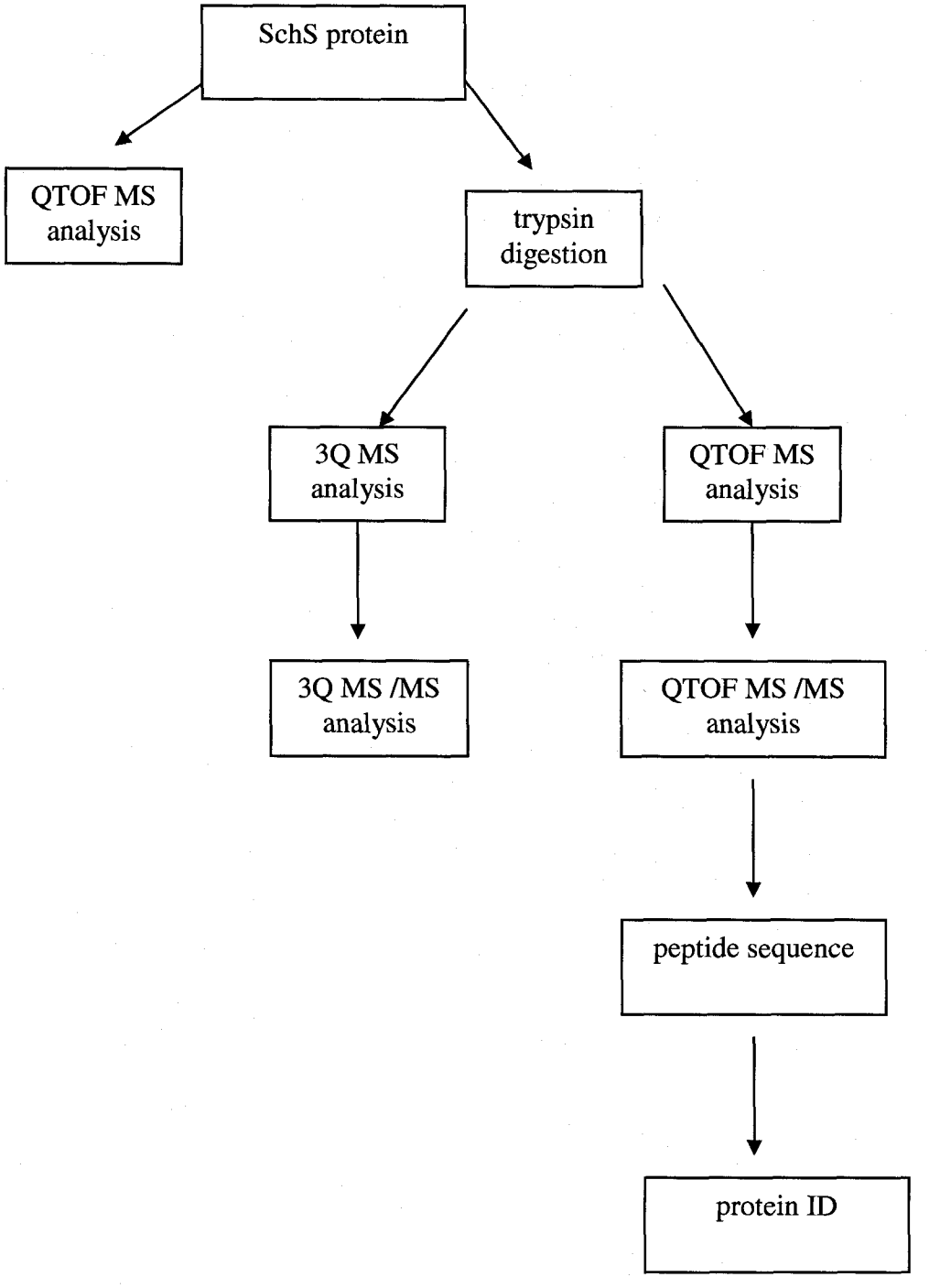


Figure 7. Schematic representation of analysis of SchS proteins using mass spectrometry

Waters CapLC system. Chromatography was carried out as follows: Solvent A= 0.1 % formic acid in HPLC grade water and solvent B=0.1 % formic acid in HPLC grade acetonitrile; flow rate 40 nL/min; 0 min 95% A; 5 min 95% A; 50 min 5% A; 55 min 5% A and 60 min 95% A. Data was processed and deconvoluted using the maximum entropy algorithm (MaxEnt1) in Masslynx 4.0.

9.6.4. In-solution digestion of Schs34 and SchS21 proteins

The SchS protein sample was adjusted to 100 μL in 50mM NH_4HCO_3. The solution was then reduced by the addition of 5 μL 200 mM DTT and boiled for 10 min. Alkylation of cysteine residues was accomplish by the addition of 4 μL 1 M iodoacetamide in 100 mM NH_4HCO_3. This reaction was then stopped by the addition of 20 μL 200mM DTT. The digestion solution (1 mg sequencing grade trypsin/ 20 mg protein in 25 mM NH_4HCO_3 and 2.5mM $CaCl_2$) was then incubated overnight at 37°C.

In an attempt to separate the SchS 21 and 34 proteins, the proteins were treated with urea. A 20 μL of in-solution sample was adjusted to an 8 M urea concentration then incubated on ice for 1hr. The DTT concentration was adjusted to 5 mM and incubated for 1 hr at 37°C. Mass spectrum analysis and liquid chromatography was performed as above.

An in-solution digestion was then performed as above on selected fractions from intact protein liquid chromatography. Peptides were then analyzed by HPLC-QTOF as indicated above.

9.6.5. SDS- PAGE

Proteins to be analyzed were mixed with loading buffer and boiled for 5 min. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 8.5 x 8 cm vertical slab gels) in a 10% acrylamide gel using Laemmli buffer system. Electrophoresis was carried out at 160 V for 20 minutes followed by 200 V for 1 hr. The protein bands in the gel were visualized with the PlusOne silver staining kit or GelCode Blue Stain Reagent according to the manufacturer's protocol. A low molecular weight calibration kit for SDS electrophoresis was used as molecular weight marker.

9.6.6. In-gel analysis of SchS34 and SchS21

Digestions were performed as follows: Coomasie gel was washed twice with water, then the gel band was cut using clean scalpel. The gel band was then further cut into 1 mm x 1 mm cubes and placed into a 1.5 mL Eppendorf tube. An aliquot (100 μL) of water was added and incubated for 15min, the water was then discarded. The gel pieces were washed 3 times with 40 μL aliquots of a 50 water: 50 acetonitrile solution, incubated for 15 min and the solution discarded. Then, 40 μL of acetonitrile was added and incubated for 5 min before solution was discarded. Following this a 40 μL aliquot of 100 mM NH_4HCO_3 was added, this was incubated for 5 min before 40 μL of acteonitrile was added. The solution was discarded and the gel pieces allowed to dry.

After gel pieces were dry, 40 μL of 10 mM DTT, 100 mM NH_4HCO_3 was added and incubated at 56°C for 45 min. The solution was discarded and the gel pieces allowed to cool. A 40 μL sample of 55 mM iodoacetamide, 100 mM NH_4HCO_3 was added and incubated for 30 min at room temperature in dark. The gel pieces were then washed with 100 μL of 100 mM NH_4HCO_3 and incubated for 5 min. The solution was discard and a 1000 μL aliquot of 50% acetonitrile added, this was incubated for 15 min before the solution discarded, then 40 μL of acetonitrile was added. This was incubated for 3 min and discarded. A 40 μL aliquot of 100 mM NH_4HCO_3 was added to the gel pieces and incubated for 5 min before 40 μL of acetonitrile was added. The solution was incubated for 15 min then discarded. The gel pieces were allowed to dry before digestion proceeded.

For digestion, approximated 20 μL of the digestion solution (25 ng sequencing grade trypsin/ μL in 25 mM NH_4HCO_3 and 2.5mM $CaCl_2$, pH 7.8) was added and incubated for 45 min at 4°C. The excess solution was removed and approximately 20 μL of digestion solution without enzyme added. This was then incubated overnight at 37°C. The next day the digestion was sonicated in cool water, then the supernatant was removed and kept. Following this, 20 μL of 25 mM NH_4HCO_3 was added and incubated for 15 min. To this solution 20 μL acetonitrile was added and incubate for 15 min. The supernatant was collected and kept. Then 20 μL of a 5% formic acid solution was added and incubated for 15 min. An equal aliquot of acetonitrile was added before another incubation of 15 min and the supernatant was kept. All supernatants were pooled and the solution was adjusted to 1 mM DTT. The solution was then dried in a speed vac for

approximately 5 hr. Lastly, 5 mL of 0.1% trifluoroacetic acid (TFA) solution was added to the dried material.

9.6.7. Mass spectrometry of peptides

Mass spectrometry of peptides was carried out at UWO-BMSL using a Q-TOF Global Ultima mass spectrometer (Micromass) equipped with a Z-spray source and run in positive ion nanospray mode. Peptides were separated using a C18 75 μm x 150 mm column from LC packings using a Waters CapLC system. Liquid chromatography was carried out as follows: Solvent A: 0.1 % formic acid in HPLC grade water; B: 0.1 % formic acid in HPLC grade acetonitrile; flow rate was 2.4 nL/min; 0 min 95% A; 3.10 min 95% A, 40 min 50% A, 45 min 5% A; 53 min 95%A; 60 min 95% A. All data was processed using the peptide auto function of MassLynx 4.0 (Waters, Mississauga, Ontario.) and PEAKS online (Bioinformatic Solutions Inc, Waterloo, Ontario). Peptide sequences with good b or y ion series coverage and signal strength were further verified by hand.

De novo sequencing was carried out using the PEAKS software and verified by hand. All peptide sequences were subjected to BLAST search analysis. The Blastp algorithm was used to search the NCBI non-redundant database against other fungi. Significant hits were aligned using the ClustalW program (EMBL-EBI, Cambridge, UK).

10. Results

10.1. Optimization of the SchS34 capture ELISA

10.1.1. Purification and characterization of 12D3 monoclonal antibody

The SchS34 ELISA was optimized to ensure the greatest amount of sensitivity was achieved. The parameters optimized included: 12D3 purity, 12D3 concentration, strep-HRP concentration, strep-HRP incubation time and block reagent.

Initially, it was found through SDS-PAGE that the coating antibody (12D3) was not very pure, and likely to have an adverse effect on the background signal (Figure 8). This antibody was re-purified, tested by performing the SchS34 ELISA and subsequently found to produce a lower background. After 12D3 purification, IgG light chain (25 kDa) and IgG heavy chain (50 kDa) bands were prominent and few contaminant bands were present. Also, various concentrations of 12D3 were tested, and 0.25 μg/ mL was shown to produce the best signal to noise ratio; followed by 0.06125, 0.125 then 0.5 mg/mL (Figure 9 & Table 1).

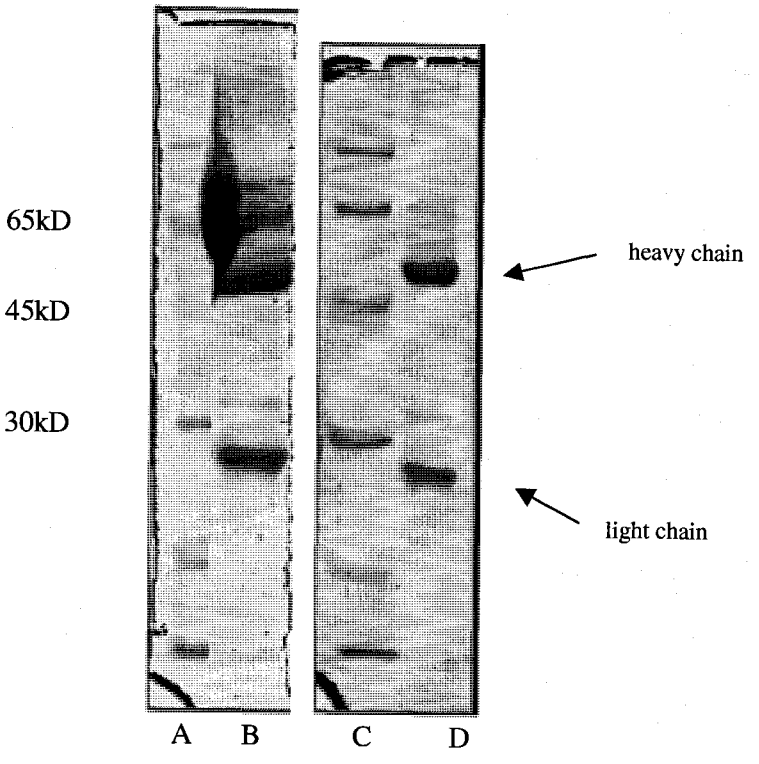


Figure 8. SDS-PAGE of 12D3 antibody. A & C molecular marker, B partially purified 12D3, D purified 12D3

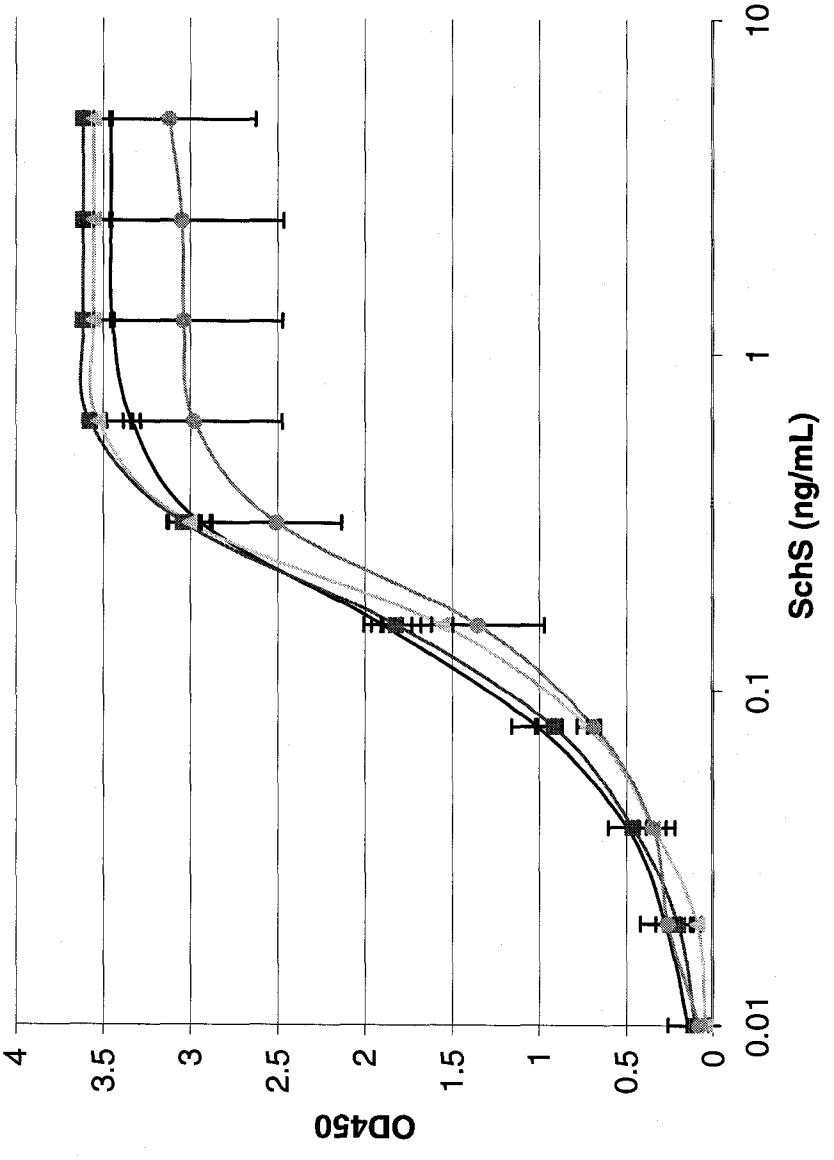


Figure 9. Optimization of 12D3 antibody concentration for SchS34 ELISA — 0.5 µg/mL ■ 0.25 µg/mL▲ 0.125 µg/mL ● 0.6125 µg/mL

Table 1. Optimization of 12D3 antibody concentration

12D3 (mg/mL)	signal	noise	s/n
0.5	3.45	0.54	6.33
0.25	3.61	0.38	9.37
0.125	3.55	0.44	7.98
0.06125	3.46	0.37	9.23

10.1.2. Comparison of blocking agents for SchS34 ELISA

The blocking material and Tween 20 were optimized for the SchS34 ELISA. (Figure 10& Table 2). Two different batches of BSA were tested to ensure, that the high background was not caused by a bad batch of BSA. The best performing blocking agent was 2%BSA in PBS (from both batches of BSA), while as expected the worst performer was no blocking. This data also showed that Tween 20 had a negative effect on background, in each instance it was used the signal-noise ratio was lower.

Table 2 Optimization of various blocking agents used in SchS34 capture ELISA

blocking agent	signal	noise	s/n
no block	3.61	0.38	9.47
2% BSA PBS	3.73	0.26	14.12
2% BSA PBST	3.67	0.321	11.43
5% Skim Milk PBS	3.72	0.28	13.28
5% Skim Milk PBST	3.78	0.33	11.26
2% Paracel BSA PBS	3.46	0.25	13.67
2% Paracel BSA PBST	3.66	0.33	10.90
Blotto	2.92	0.26	10.86

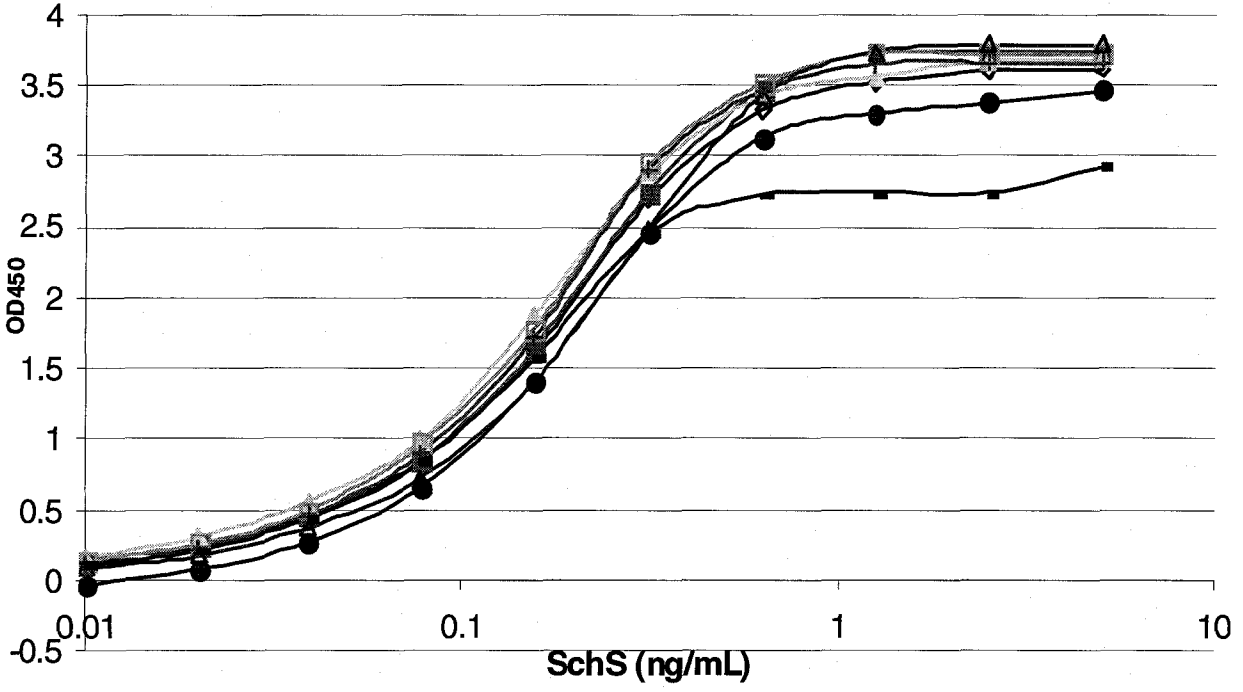


Figure 10. Comparison of blocking agents for SchS capture ELISA ◊ no block ■ 2% BSA PBS▲ 2% BSA PBST□ 5% Skim Milk PBS △ 5% Skim Milk PBST ● 2% Paracel BSA PBS + 2% Paracel BSA PBST — Blotto

10.1.3. Optimization of streptavidin-horse radish peroxidase conjugate

The concentration of strep-HRP must also be optimized ensure the best signal noise ratio. Three concentrations were tested and it was found that 62.5 ng/mL had the best signal to noise (Figure 11 & Table 3). Even though 62.5 ng/mL appeared the best, the 250 ng/mL concentration was chosen as it appeared to have the best shape curve, as well as the highest response for many concentrations of SchS.

Table 3. Optimization of streptavidin-HRP concentrations used in SchS34 capture ELISA

Strep-HRP (ng/mL)	signal	noise	s/n
250	3.38	0.49	6.88
125	3.56	0.41	8.77
62.5	3.53	0.32	10.88

Aside from concentration, incubation time of the Strep-HRP must also be considered. It is known that the longer the incubation time of this conjugate the higher the background. It was found that a 30 min incubation time produced a better signal-noise ratio when compared to a 60 min period ratio (Figure 12 & Table 4).

Table 4.Optimization of incubation periods for streptavidin-HRP used in SchS34 capture ELISA

Time (min)	signal	noise	s/n
strep-HRP 250 ng/mL	3.86	0.26	14.87
strep-HRP 250 ng/mL	3.33	0.52	6.35

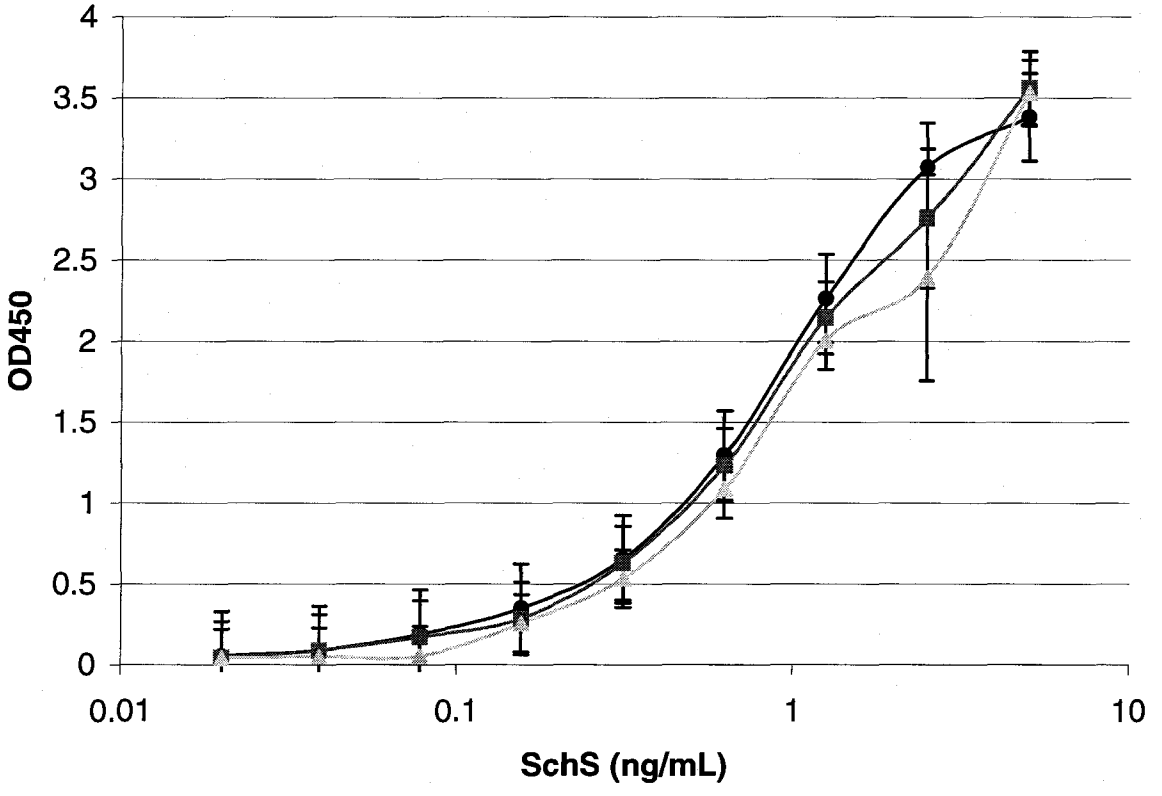


Figure 11. Optimization of streptavidin-HRP concentration for SchS34 capture ELISA ● 250 ng/mL ■ 12.5 µg/mL▲ 62.5 µg/mL

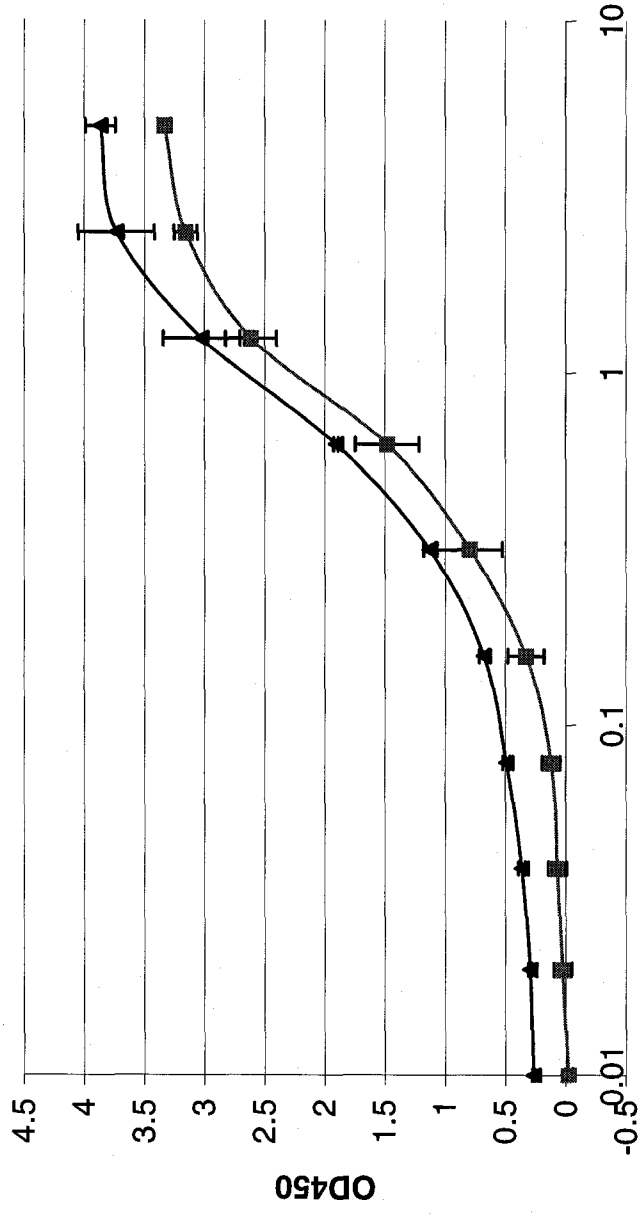


Figure 12 Optimization of streptavidin-HRP incubation time▲ 30 min ■ 60 min

10.2. Analysis of field samples using the SchS ELISA

10.2.1. Culturable analysis of house dust

The dust samples used in the spiking samples were dilution plated in series to determine the fungi present. The predominant species found were *Paecilomyces variotii*, various *Penicillium* species as well as some *Aspergillus* species and its teleomorphs (Table 5) Greater than 10^5 CFU/g were found while this methodology was capable of detecting 10^2 CFU/g for some isolates.

10.2.2. Analysis of house dust samples using SchS34 capture ELISA

A house dust sample spiked with various amounts of *S. chartarum* conidia was analyzed using the SchS34 capture ELISA (Figure 13). House dust is generally analyzed as it provides a historical perspective of the fungi circulating through the home. This experiment was done to determine the limit of detection as well as determine if the dust has an effect on recovery. The limit of quantification was found to be 0.2 ng/g of dust while the limit of detection for spore number was approximately 10^5 /g of sieved dust. The SchS34 recovery was found to be 93.6± 7.5% and the control dust sample with no *S. chartarum* conidia had no measurable response.

Table 5. Culturable analysis of sieved house dust sample

Malt Extract Agar

species	CFU x 10^3/g
Paecilomyces variotii Bainier	420
Penicillium citrinum Thom	333
P. crustosum Thom	85.1
non sporulating isolates	45.8
white yeasts	21.2
Pithomyces chartarum M.B. Ellis	1.7
Cladosporium sphaerospermum Penz.	0.7
Aspergillus sydowii Thom & Church	0.3
Mucor species	0.3
P. chrysogenum Thom	0.3
Phoma species	0.3

Total 9.1 x 10^5 CFU/g

DG18 Agar

species	CFU x 10^3/g
P. citrinum	350
white yeasts	117
P. variotii	88.3
P. crustosum	34.6
non sporulating isolates	5.7
C. sphaerospermum	2.0
pink yeasts	2.0
Phoma species	1.0
A. versicolor (Vuill.) Tirab.	0.7
E. nidulans (Eidam) Vuill.	0.3
E. herbariorum Link	0.3
P. chrysogenum	0.3
P. chartarum	0.3

Total 6.0 x 10^5 CFU/g

A comparable analysis of Asp f1 in *A. fumigatus* spiked dust samples was also performed (Figure 13). The limit of detection was found to be approximately 10^7 spores/g which equated to approximately 1 ng/g dust. The recovery of Asp f1 from dust was found to be 26.6±7.5% when dust samples were compared to in-solution samples

10.2.3. Detection of *S. chartarum* and *S. chlorohalonata* intact spores using the SchS34 ELISA

Before field samples were tested, intact spores from *S. chartarum* and its close relative *S. chlorohalonata* were analyzed using the SchS34 capture ELISA. The detection limit for *S. chartarum* was approximately 5000 spores/mL while for *S. chlorohalonata* it was 7500 intact spores/mL. *S. chlorohalonata* produced a higher response for comparative number of spores (Figure 14). This indicates that the ELISA reliably detects SchS from intact spores and has potential to detect spores from environmental samples.

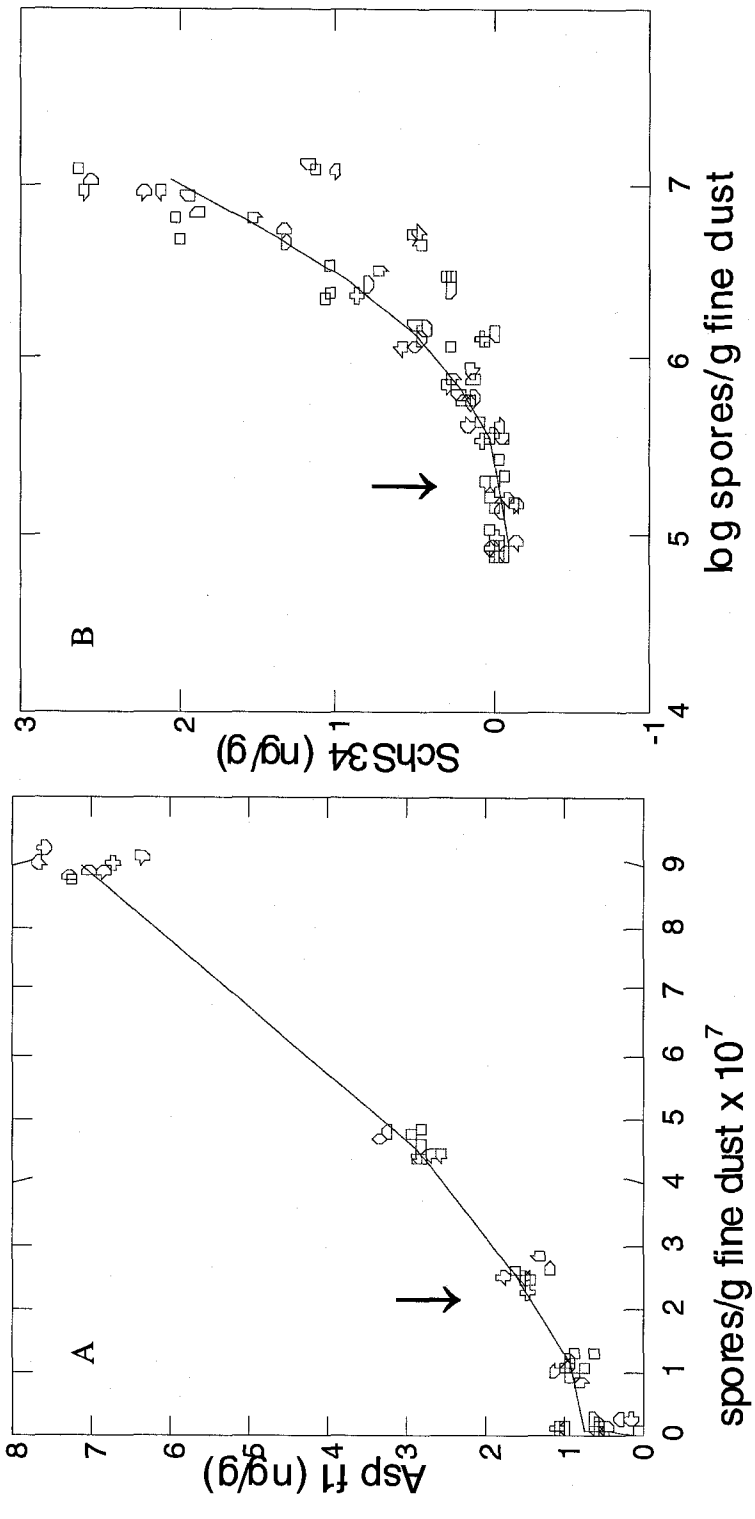


Figure 13. Analysis of *A. fumigatus* (A) and *S. chartarum* (B spores in sieved house dust samples. *A. fumigatus* conidia measured in x10^7 spores while *S. chartarum* using log scale

10.2.4. Analysis of field samples

The first set of IAQ samples analyzed were pieces of *S. chartarum* contaminated drywall. Field samples as well as a sample that was experimentally inoculated with *S. chartarum* were analyzed. Both samples were visibly moldy while the field samples appeared darker black, the experimental sample appeared grey in colour. A direct microscopic examination was performed on the field drywall samples, and only *S. chartarum* spores were visible. The field samples out performed the experimental sample using the SchS34 ELISA. Field sample 1 had a SchS concentration of 130 ng/g, field sample 2, 69 ng/g and the experimental sample was less than 10 ng/g. This analysis demonstrated that field samples can be examined by the SchS34 ELISA

Swab samples from the drywall materials were also prepared. It was thought that the swabs would concentrate the spores as well as reduce drywall debris mixing into the suspension. Again in this case the field drywall samples showed a much higher response than the experimental sample, the experimental sample was non-detectable. The swab of the second swab produced the highest response (0.95 ng/swab), which is similar to the high response it produced when the comparative drywall was tested. While the swabs gave good responses in these preliminary studies, a direct analysis of drywall paper give a much higher responses.

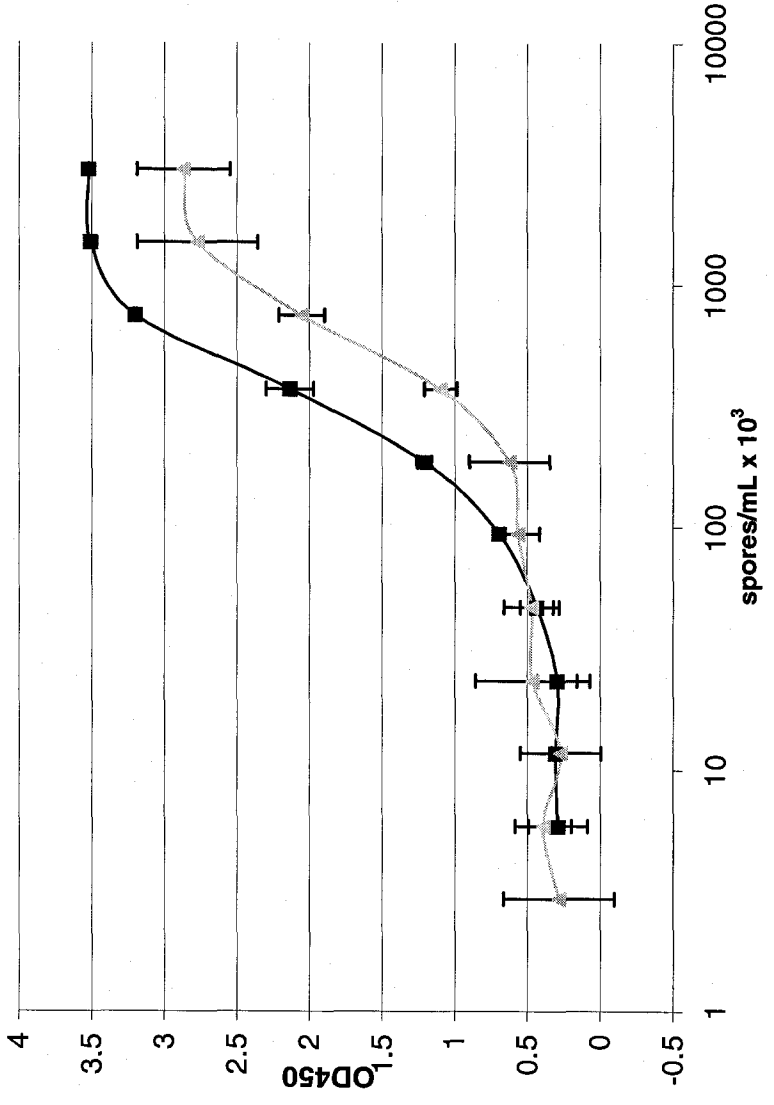


Figure 14. Analysis of ▲ *S. chartarum* and ■ S. *chlorohalonata* conidia using SchS34 capture ELISA

10.3. Methods to increase Asp f1 recovery

10.3.1. Recovery of *A. fumigatus* spores from membrane filter

A known quantity of spores were applied to a 0.2 μm membrane filter it was found that conidial recovery from the filter encased in a 3-piece cassette was 86%.

10.3.2. Effect of enzymes on Asp f1 recovery

The effect of Qanatazyme on Asp f1 recovery is shown in Table 6. There was no statistical difference when untreated conidia were compared to Quantazyme treated conidia. As the Quantazyme concentration changed there was also no change in the recovery of Asp f1. Longer incubations with this enzyme did not show an increase of Asp f1.

The effect of chitinase C on Asp f1 recovery is shown in Table 7. Chitinase was found not to increase the recovery of Asp f1. As the concentration increased, the amount of Asp f1 recovery decreased. Increase incubations times with this enzyme had a negative effect on Asp f1 recovery

The effect of galactomannase is shown in Table 8. One sample showed a statistical significant ($p < 0.05$) difference from the comparative untreated sample, 0.1 mg/mL at 0 hr, but the numerical difference was small.

Table 6. Effect of Quantazyme on recovery of Asp f1 from intact *A. fumigatus* conidia

Quantazyme (U/mL)	time (hr)	optical density	standard deviation
untreated	0	1.42	0.24
	1	2.41	0.15
	2	2.70	0.08
	3	2.16	0.08
	4	1.98	0.11
0.1	0	1.14	0.25
	1	2.35	0.12
	2	2.42	0.09
	3	2.02	0.34
	4	2.08	0.31
1	0	0.86	0.06
	1	2.04	0.27
	2	2.22	0.25
	3	2.23	0.14
	4	2.01	0.06
10	0	1.61	0.14
	1	2.29	0.11
	2	1.95	0.19
	3	2.02	0.20
	4	1.98	0.09

values followed by a different letter are significant ($p < 0.05$), after ANOVA, LSD, comparisons between treated and untreated sample at the same incubation time

Table 7 Effect of chitinase C on Asp f1 recovery from *A. fumigatus* spores

chitinase C g/mL	time (hr)	optical density	standard deviation
untreated	0	2.02	0.077
	2.5	2.34	0.181
	5	2.12	0.127
	24	2.10	0.135
0.01	0	1.34	0.127
	2.5	1.59	0.137
	5	2.13	0.142
	24	1.95	0.130
0.1	0	0.80	0.038
	2.5	1.05	0.073
	5	1.49	0.024
	24	0.79	0.207

values followed by a different letter are significant ($p < 0.05$), after ANOVA, LSD, comparisons between treated and untreated sample at the same incubation time

Table 8 Effectiveness of galactomannase on Asp f1 recovery from *A. fumigatus* conidia

galactomannase (mg/mL)	time (hr)	optical density	standard deviation
untreated	0	0.97	0.057
	1	1.02	0.020
	2	1.09	0.106
	3	0.95	0.039
	4	0.50	0.019
0.01	0	0.91	0.165
	1	0.93	0.038
	2	0.96	0.050
	3	0.50	0.047
	4	0.40	0.061
0.1	0	1.14	0.109[a]
	1	0.80	0.026
	2	0.90	0.054
	3	0.99	0.041
	4	0.49	0.006
1	0	1.02	0.068
	1	1.04	0.122
	2	0.83	0.057
	3	0.88	0.054
	4	0.52	0.014

values followed by a different letter are significant ($p < 0.05$), after ANOVA, LSD, comparisons between treated and untreated sample at the same incubation time

10.3.3. Effect of bead extraction on Asp f1 recovery

Extraction of Asp f1 using the large bead is shown in Table 9. Use of the larger bead had a statistical significant ($p < 0.05$) increase of Asp f1 recovery. The use of Tween 20 did not have an effect for either the intact or disrupted conidia.

The effect of microbeads on Asp f1 recovery is shown. Microbeads did not have a positive effect on Asp f1 recovery (Table 10). The small beads showed a higher recovery when compared to the untreated sample at 0 hr, but when compared to the untreated sample at 2 hr, the recovery was lower. Also as the amount of small beads added increased, the recovery decreased.

10.3.4. Effect of protective methods on Asp f1 recovery

The first protective method tested was a protease inhibitor cocktail, the data for this analysis is shown in (Table 11). The protease cocktail showed some effect on Asp f1 recovery. A statistically significant difference was seen for the undiluted cocktail at times 0 and 24 hr but this difference is smaller than the largest value for the untreated spores.

The protective effect of EDTA was shown to have a moderate effect on the recovery of Asp f1 with the lower concentrations of EDTA producing greatest effect (Table 12). The 0.001, 0.01 and 0.1 M EDTA samples did show a statistical difference when compared to the untreated samples for all incubation times tested.

Table 9. Effect of large bead disruption of *A. fumigatus* conidia on Asp f1 recovery

buffer	treatment	mean (ng Asp f1/mg conidia)	standard deviation
PBST	intact	1.095	0.149[b]
	disrupted	1.897	0.309[ac]
PBS	intact	0.815	0.140[bd]
	disrupted	1.612	0.012[c]

Table 10. Effect of microbeads on recovery on Asp f1 from A. *fumigatus* conidia

treatment	time (min)	optical density	standard deviation
untreated	0	1.21	0.062[e]
	2	2.29	0.171[ade]
1g beads	2	2.07	0.013[ae]
5g beads	2	1.86	0.067[ae]
10g beads	2	0.069	0.009

values followed by a different letter are significant ($p < 0.05$), after ANOVA, LSD

Table 11. Effect of protease inhibitor cocktail on Asp f1 recovery from *A. fumigatus* conidia

treatment	time (hr)	optical density	standard deviation
untreated	0	1.47	0.11
	2.5	2.05	0.17
	5	1.75	0.06
	24	1.08	0.04
PIC	0	1.82	0.13[a]
	2.5	1.74	0.10
	5	1.30	0.22
	24	1.46	0.15[d]
1/10 PIC	0	1.74	0.22
	2.5	1.36	0.31
	5	1.92	0.18
	24	0.89	0.13

values followed by a different letter are significant ($p < 0.05$), after ANOVA, LSD, comparisons between treated and untreated sample at the same incubation time

The effectiveness of THP, as a protectant against oxidation was tested. This allows Asp f1 to be extracted in a reducing extraction environment. THP displayed a positive effect on the recovery of Asp f1, the lower the THP concentration, the higher the recovery (Table 13). The incubation times of 1, 2 and 3 hr for all THP samples were higher ($p < 0.05$), while 0.5 and 5 mM also showed a difference at 4 hr.

10.3.5. Effect of temperature and time on Asp f1 recovery

The effect temperature and extraction duration on Asp f1 recovery were tested (Table 14). Cooler temperatures were hypothesized to reduce degradation while higher temperatures may increase the extraction rate. No statistical difference was observed when recoveries at each time point for each temperature were compared to each other.

10.3.6. Effect of the Yeast Buster extraction kit on Asp f1 recovery

The Yeast Buster kit is designed to extract protein from yeasts. This kit contained a reductant (THP), nuclease (bezonase) as well as a proprietary extractant, presumably a buffer. In this case a modified version, designed for filamentous fungi was used. The use of this methodology resulted in no increase in the recovery of Asp f1. For both the disrupted spores and undisrupted spores, the untreated spores displayed a higher recovery of Asp f1 than the treated spores ($p < 0.05$;Table 15).

Table 12. Effect of EDTA on the recovery of Asp f1 from *A. fumigatus* conidia

EDTA (M)	time (hr)	optical density	standard deviation
untreated	0	1.09	0.07
	1	1.30	0.10
	2	1.35	0.07
	3	1.29	0.09
0.001	0	1.64	0.06[a]
	1	1.87	0.03[b]
	2	1.93	0.01[c]
	3	1.83	0.01[d]
0.01	0	1.72	0.04[a]
	1	1.78	0.14[b]
	2	1.84	0.05[c]
	3	1.72	0.13[d]
0.1	0	1.45	0.10[a]
	1	1.56	0.10[b]
	2	1.60	0.08[c]
	3	1.49	0.08[d]

values followed by a different letter are significant ($p < 0.05$), after ANOVA, LSD, comparisons between treated and untreated sample at the same incubation time

Table 13. Effect of THP on the recovery of Asp f1 from *A. fumigatus* conidia

THP (mM)	time (hr)	optical density	standard deviation
no treatment	0	1.54	0.31
	1	1.54	0.51
	2	1.72	0.47
	3	1.80	0.30
	4	1.98	0.15
0.05	0	2.16	0.11[a]
	1	2.17	0.21[b]
	2	2.38	0.08[c]
	3	2.21	0.12[d]
	4	2.09	0.10
0.5	0	1.75	0.10
	1	2.18	0.16[b]
	2	2.40	0.10[c]
	3	2.42	0.10[d]
	4	2.45	0.12[e]
5	0	0.37	0.03
	1	1.99	0.14[b]
	2	2.30	0.02[c]
	3	2.16	0.09[d]
	4	2.36	0.15[e]

values followed by a different letter are significant ($p < 0.05$), after ANOVA, LSD, comparisons between treated and untreated sample at the same incubation time

Table 14 Effect of temperature on recovery of Asp f1 from *A. fumigatus* conidia

temperature	time (hr)	optical density	standard deviation
RT	0	0.84	0.16
	1	1.84	0.25
	2	2.32	0.12
	3	2.23	0.18
	4	2.10	0.14
	5	1.79	0.22
	24	1.89	0.08
5 °C	0	0.90	0.24
	1	1.94	0.23
	2	2.28	0.15
	3	2.07	0.12
	4	1.93	0.07
	5	2.13	0.08
	24	2.16	0.13
36 °C	0	0.74	0.54
	1	1.76	0.13
	2	1.88	0.13
	3	1.98	0.13
	4	2.08	0.13
	5	1.96	0.13
	24	1.21	0.29

values followed by a different letter are significant ($p < 0.05$), after ANOVA, LSD, comparisons between treated and untreated sample at the same incubation time

Table 15 Effect of Yeast Buster on extraction of Asp f1 from *A. fumigatus* conidia, intact and disrupted

treatment	time (hr)	optical density	standard deviation
PBST intact	0	0.79	0.31
	2.5	1.18	0.28
	5	0.71	0.23
	24	0.55	0.29
Yeast Buster intact	0	0.50	0.12
	2.5	0.96	0.23
	5	0.65	0.19
	24	0.50	0.05
PBST disrupted	0	1.19	0.30
	2.5	0.94	0.21
	5	0.47	0.12
	24	0.39	0.13
Yeast Buster disrupted	0	0.67	0.14
	2.5	0.41	0.24
	5	0.15	0.06
	24	0.07	0.06

values followed by a different letter are significant ($p < 0.05$), after ANOVA, LSD, comparisons between treated and untreated sample at the same incubation time

10.4. Mass spectrum analysis of proteins

10.4.1. Mass spectral analysis of BSA

Mass spectrum analysis was initially carried out using BSA (Figure 15). This protein was tryptic digested and analysed using HPLC -3Q mass spectrometry. Initially an HPLC was used for peptide separation, but shortly it was found that little of the sample was reaching the mass detector. The first quadrapole was used as a mass selector while the third was used as the detector of its fragments. This also dramatically reduced the analysis time from about 1 hr to about 5 min which allowed more analyses to be completed in a given period of time. The 653 (2+) peptide was the most abundant peptide in this analysis, but many other peptides were detected, these include the 582, 740 and 820 Da peptides which are all doubly charged.

The lowest flow rate (200 μL/min) was chosen to optimize the concentration of the protein in the solvent, while at the same time ensure a consistent flow of solvent. The lower the flow rate, the better the signal to noise tended to be. The energy and gas pressure needed to be optimized so that only peptide bonds were broken not other bonds. However, gas pressure and energy needed to be high enough to ensure most of the parent ion was fragmented to produce informative peaks.

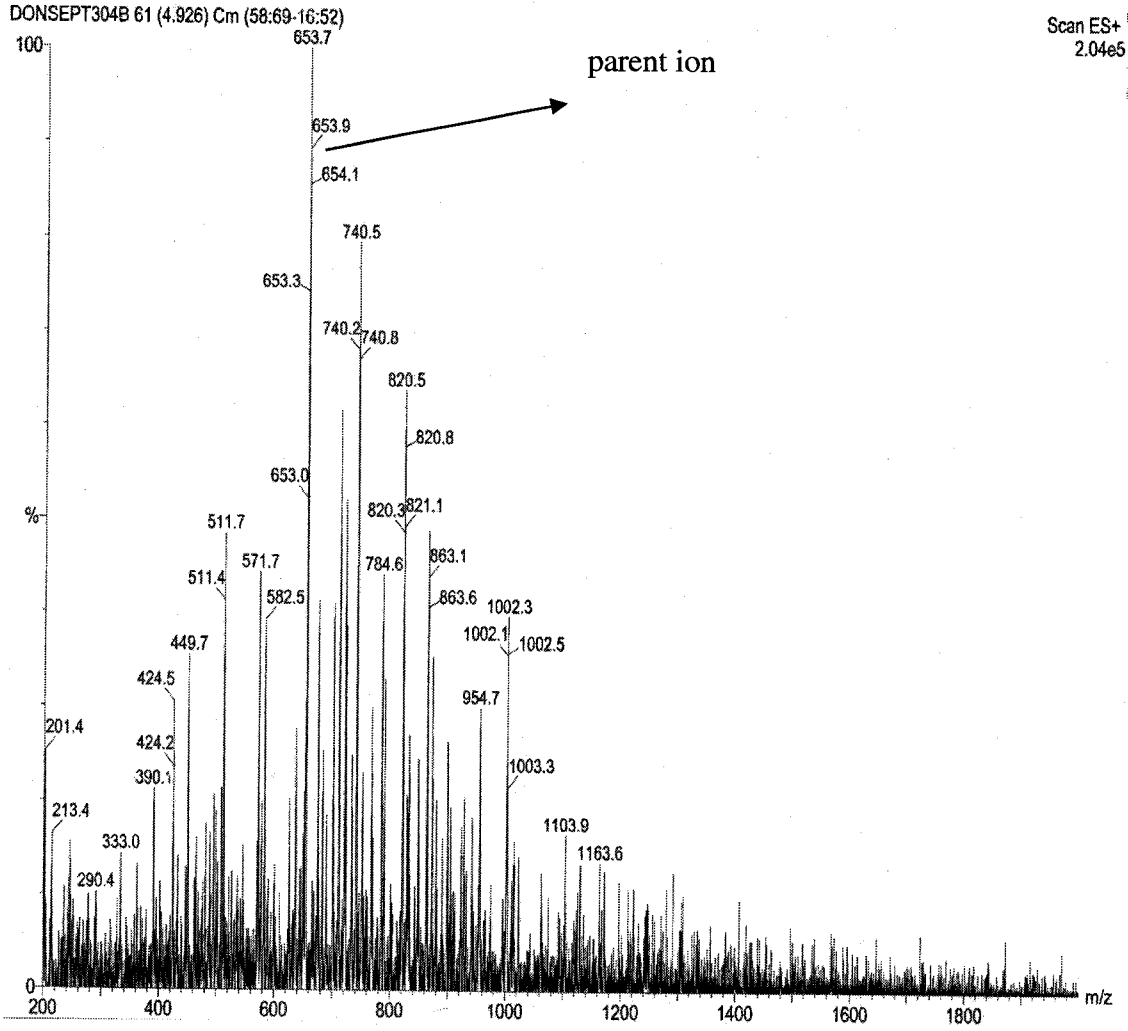


Figure 15. Mass spectrum of bovine serum albumin tryptic digest using 3Q mass spectrometer

In the mass spectrum of BSA it can be seen the double charged ion, 653 Da, had the most abundant ion from the BSA digest (Figure 15). This ion was studied extensively as it was thought higher abundance would translate into more fragment ions. If this was successful lower abundance ions could be studied.

The expected amino acid sequence of the 653 Da peptide was HLVDEPQNLIK (Table 16). In mass spectrum analysis of peptide, the b-ion series and the y-ions series are the most sequence informative, the predicted immonium ions are displayed (Table 17). These ion are the individual amino acids and their fragments, while they do not provide sequence orientation they provide sequence compositional information.

The first condition tested was a 35 eV collision energy and a 5.5 x 10-4 mbar gas pressure (Figure 16). Using these conditions the parent ion (653 Da) was still apparent. Three b-ions; b2, b5 and b6 and three y-ions; y6, y8 and y9 were generated. Also present were the histidine and isoleucine/leucine immonium ions. Given that 2 adjacent y-ions (y8 & y9) were present one amino acid can be elucidated from this spectrum. The y8 ion has a mass of 957.1 Da and the y9 ion has a mass of 1055.7, if these are subtracted a mass of 98.6 Da is obtained. With the sequence of this peptide known, the answer is valine which has a mass of (99.13) but proline has a mass of 97.11 Da. Given that valine has the closer mass of the two it would be selected but this demonstrates the requirement for high mass accuracy.

Table 16. The b and y-ion series for the 653 Da (2+) peptide from tryptic digest of BSA

b ions	---	251.15	350.22	465.25	594.29	691.34	819.4	933.44	1046.5	1159.61		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	
N -	**H**	**L**	**V**	**D**	**E**	**P**	**Q**	**N**	**L**	**I**	**K**	**- C**
	11	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**	
y ions	---	1168.66	1055.57	956.5	841.48	712.44	615.38	487.32	373.28	260.2	147.11	

Table 17. Potential immonium ions generated from MS/MS analysis of 653 Da (2+) peptide from tryptic digest of BSA

H	L	V	D	E	P	Q	N	L	I	K
110.07, 138.07	86.1	72.08	88.04	102.05	70.07, 126.05	84.08, 101.07, 129.07	87.06	86.1	86.1	84.08, 101.07, 129.07

Next, the collision energy was raised (40 eV) while the gas pressure was maintained (5.9 x 10-4 mbar; Figure 17). The parent ion under these conditions is still dominant but when compared to the previous conditions, it has reduced in relative abundance. Four y ions were produced (y2 y3, y6, y8) and 4 b-ions (b2, b3, b5, b8). The histidine, leucine/isoleucine immonium ions were present. In this case, 2 amino acids can be deduced, from the 2nd and 3rd ion from each series. Subtraction of the b-ions gives 99.0 Da which corresponds to valine, the same was found above except deduced from the other end of the peptide. Subtraction of the 2 and 3rd y-ions gives 113 Da which corresponds to leucine/isoleucine. The mass of these two amino acids is undistinguishable using mass spectrometry. The immonium ion for leucine/isoleucine was present in this spectra so this y-ion series data corresponds with the immonium ion data. Also present in this spectra is the parent ion subtracted from the mass of a terminal lysine, (653.8 *2) - 1H+ - 147 = 1159.6 Da. Trypsin cleaves C-terminal to either arginine or lysine, in this case we now know we have lysine at the C-terminal. This ion also equates to the b10 ion.

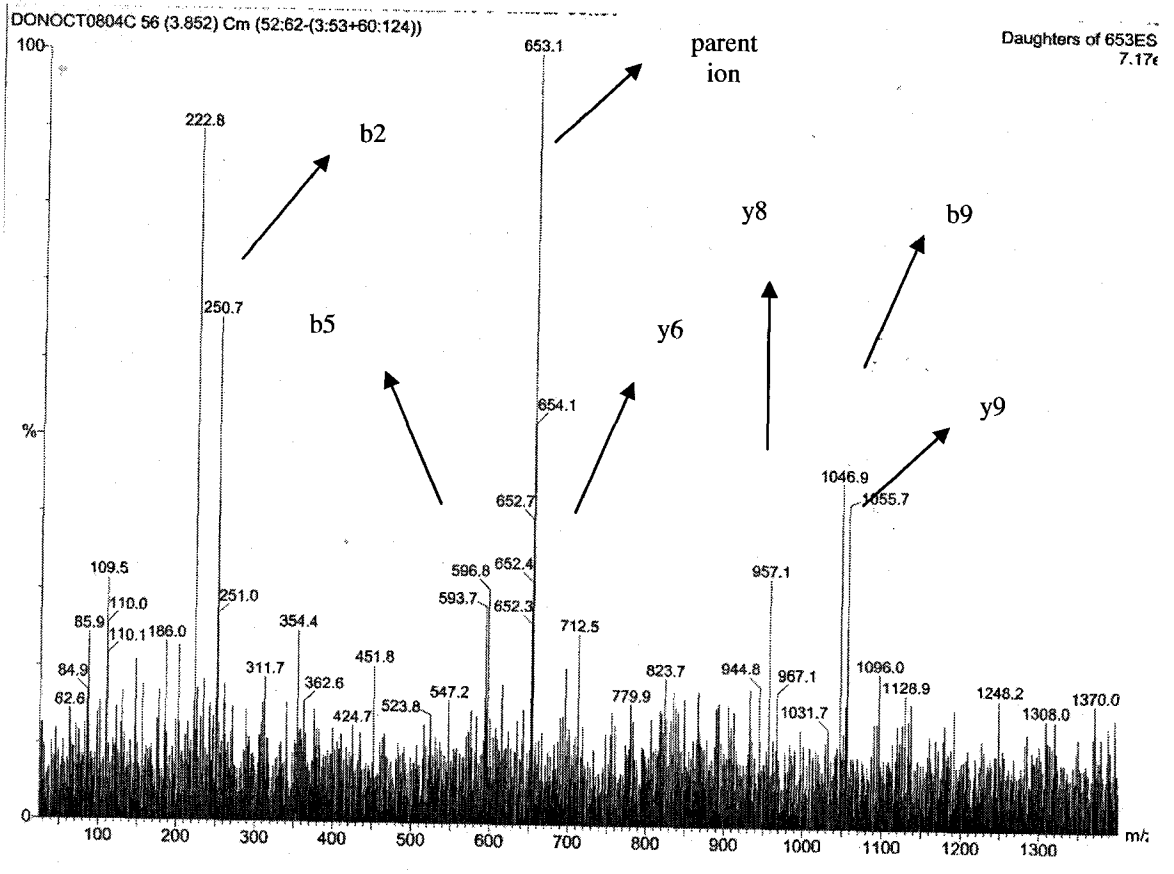
The mass spectrum generated using a collision gas energy of 35 eV and a pressure of 7.2 x 10-4 mbar is found in Figure 18. Again the parent ion has been reduced, even more so than the above conditions. Under these conditions the b2 and b8 ion and y5, y6 and y7 were generated, also the immonium ions for histidine and glutamine. Each of these peaks are very weak and would be very difficult to assesses as peaks if this was an unknown peptide. The y7 ion, in this instance is doubly charged. Two amino acids can be determined, by the three y ions present. First the difference of the y5 and y6 is 97, which

is the mass of proline. The next amino acid in the series is aspartic acid which can be determined by subtraction of y6 from y7, which gives 129 Da.

The final collision conditions tested, had the highest collision conditions (7.2 x 10-4 mbar and 40 eV (Figure 19). The 653 Da ion has a low relative abundance, as expected. No y-ions were generated, 4 b ions (b2, b6, b7, b8) and 3 immonium ions (histidine, leucine/isoleucine, lysine). Using the b6, b7 and b8 ions an amino acid pair of glutamine and asparagine can be deduced.

To summarize, the immonium ions have indicated there are leucine/isoleucine, histidine, lysine and glutamine in this peptide. Also known is that the C-terminal amino acid is lysine, as well pairs of peaks indicate the following amino acids; a valine and isoleucine/leucine are present. Lastly, there is a proline, aspartic acid and glutamine asparagine amino acid pairs in this peptide.

Through the above experimentation it was found that, using a gas energy of 40 eV and gas pressure at 5 x 10-4 mbar provided the most amount of sequence information (Table 18). In this case 12 of the possible 26 ions were present while the other three conditions displayed fewer ions with one missing an entire ion series. While this appeared promising, insufficient information was generated to obtain a complete sequence. Even though many ions are present, some of the ions are in low concentrations making it difficult to discern from the background. In addition there was incomplete ion-series information to perform a confident *de novo* sequencing of an unknown peptide


DONOCT0804C 56 (3.852) Cm (52:62-(3:53+60:124))
Daughters of 653ES
7.17e
100
%
0
653.1
parent
ion
222.8
b2
y8
b9
250.7
b5
y6
654.1
y9
1046.9
1055.7
652.7
596.8
652.4
957.1
109.5
110.0
593.7
652.3
251.0
354.4
85.9
110.1
186.0
712.5
451.8
84.9
311.7
362.6
823.7
944.8
967.1
1096.0
62.6
547.2
779.9
1128.9
1248.2
1370.0
424.7
523.8
1031.7
1308.0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
m/z

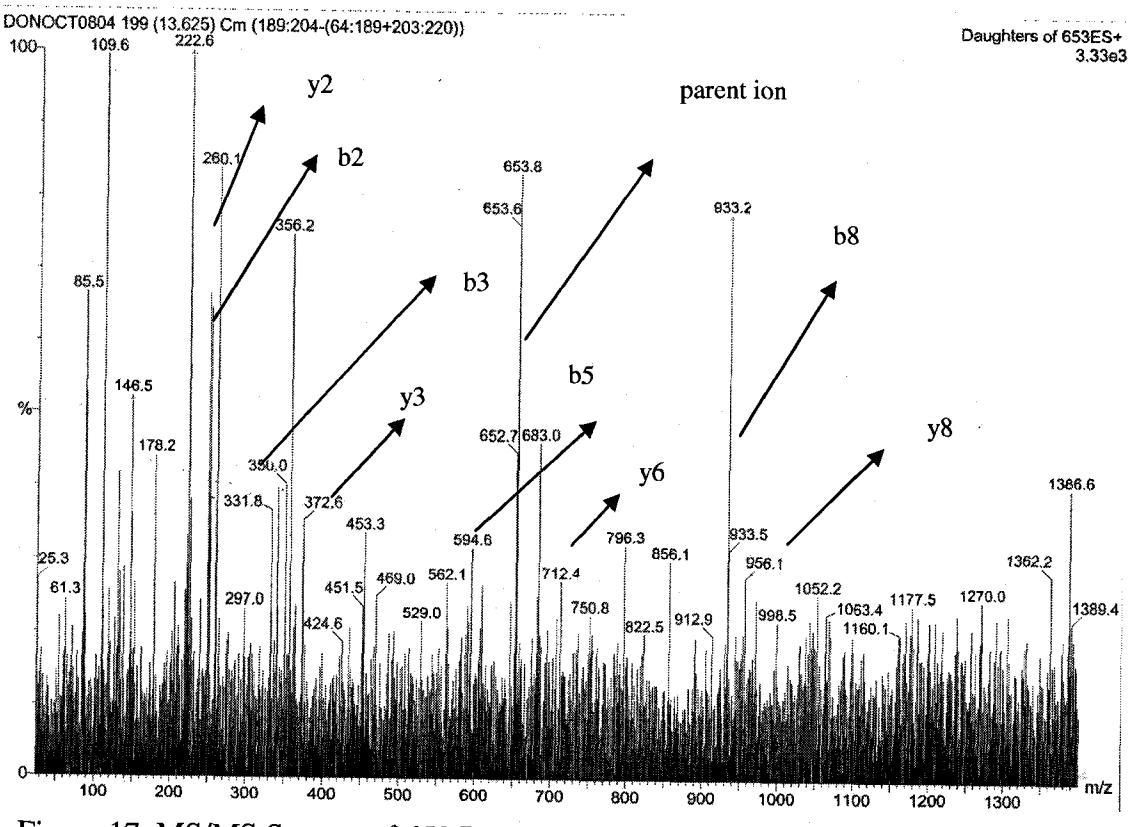


Figure 17. MS/MS Spectra of 653 Da peptide from tryptic digest of BSA using a collision energy of 40 eV and gas pressure of 5.9 x 10^{-4} mbar

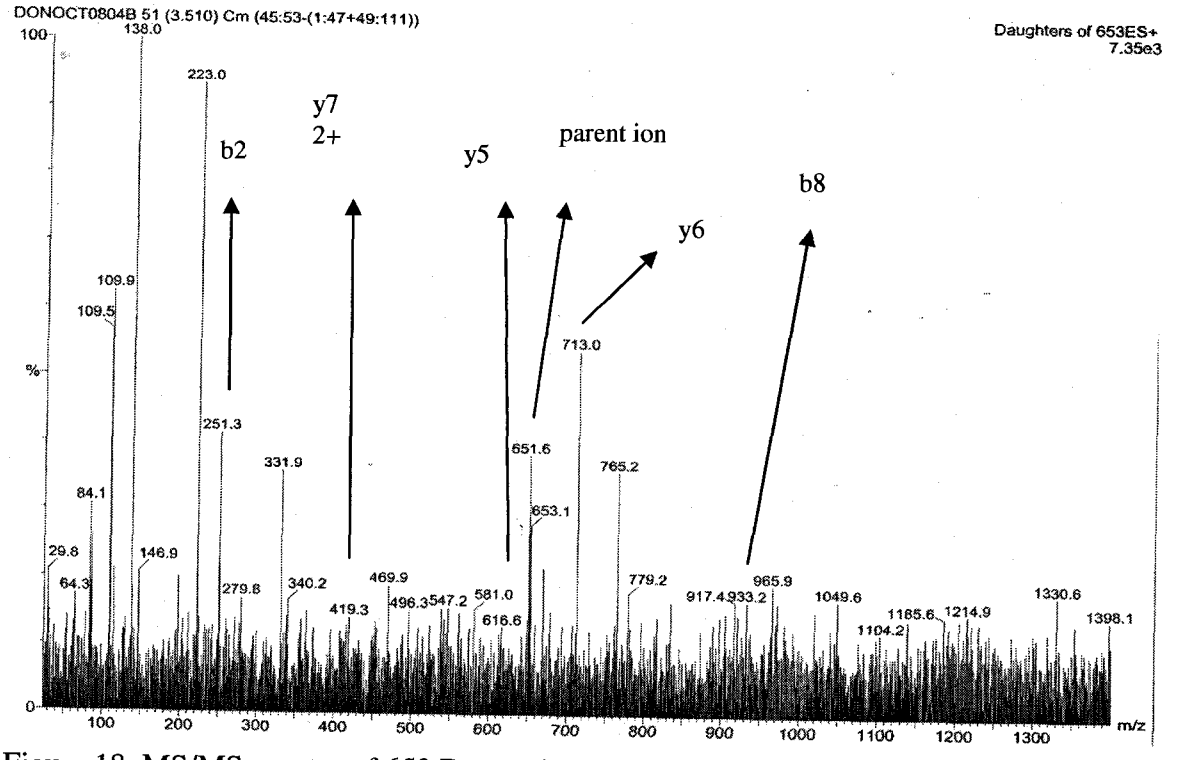


Figure 18. MS/MS spectra of 653 Da peptide from BSA using a collision gas energy of 35 eV and gas pressure of 7.2 x 10^{-4} mbar

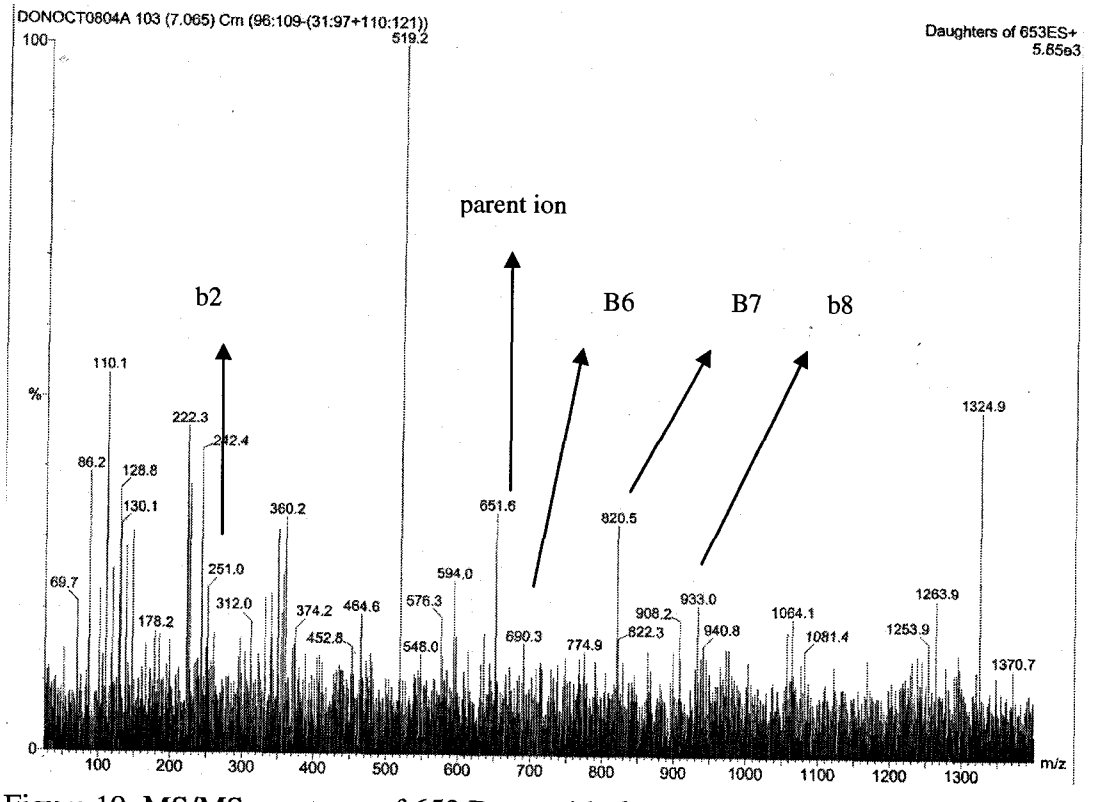


Figure 19. MS/MS spectrum of 653 Da peptide from BSA using a collision gas energy of 40 eV and gas pressure of 7.2 x 10^{-4} mbar

Table 18. Ions generated from MS/MS analysis of 653 Da (2+) peptide while varying collision energy and gas pressure

35 eV 5 x 10-4 mbar			35 eV 7.2 x 10-4 mbar						40 eV 5 x 10-4 mbar			40 eV 7 x 10-4 mbar		
immonium Ion	y-ion	b-ion	immonium Ion	y-ion	b-ion	y-ion		b-ion	immonium Ion	y-ion	b-ion	immonium Ion	y-ion	b-ion
√	-	-	√	-	-	11	H	1	√	-	-	√	-	-
√		√			√	10	L	2	√		√	√		√
	√					9	V	3			√			
	√					8	D	4		√				
		√		√		7	E	5			√			
	√			√		6	P	6		√				√
			√	√		5	Q	7						√
					√	4	N	8			√			√
√		√				3	L	9	√	√		√		
√						2	I	10	√	√		√		
	-	-		-	-	1	K	11		-	-	√	-	-
4/9	3/9	3/9	2/9	3/9	2/9		total		4/9	4/9	4/9	5/9	0/9	4/9

10.4.2. Mass spectrum analysis of in-gel tryptic digests of SchS using triple quadrapole mass spectrometer

Even though no partial *de novo* sequencing information was obtained using the 3Q machine, the mass spectra between the 2 proteins were compared for similarities. An in-gel digestion was performed on both SchS21 and SchS34, and both were analyzed by ESI-3Q mass spectrometry. Three peaks were identifiable from the SchS21 mass spectrum, 2 trypsin auto digestion peaks (522 & 421 Da) and 1 keratin (1164 Da) peak (Figure 20). Due to the high noise, it was difficult to discern noise from signal. With the sensitivity of the 3Q machine being limited, the protein concentration was inadequate for MS/MS analysis.

The in-gel digestion of the SchS34 protein produced five identifiable peaks (Figure 21). A trypsin peak at 842 Da and a keratin peak at 1164 Da were identified. A ion at 522 Da possibly either SchS34 or trypsin as both have tryptic peptides very close to that mass. Lastly, the peak at 687 Da was later identified by Q-TOF experiments to be a SchS34 peptide. No useful MS/MS spectra were obtained due a combination of low protein content and inadequate sensitivity of the 3Q machine.

Many peaks were found to be similar between the 2 proteins, some were trypsin auto digestion peaks while many were unidentifiable peaks (Table 19). Common identified peaks include the trypsin auto digestions peaks of 421 Da and 522 Da as well as the keratin peak (1164 Da). One common unidentifiable peak was found at 927 Da. No good MS/MS spectra could be produced from the SchS21 or SchS34 in-gel digestions using the Micromass 3Q mass spectrometer, which prevent identification of unknown ions.

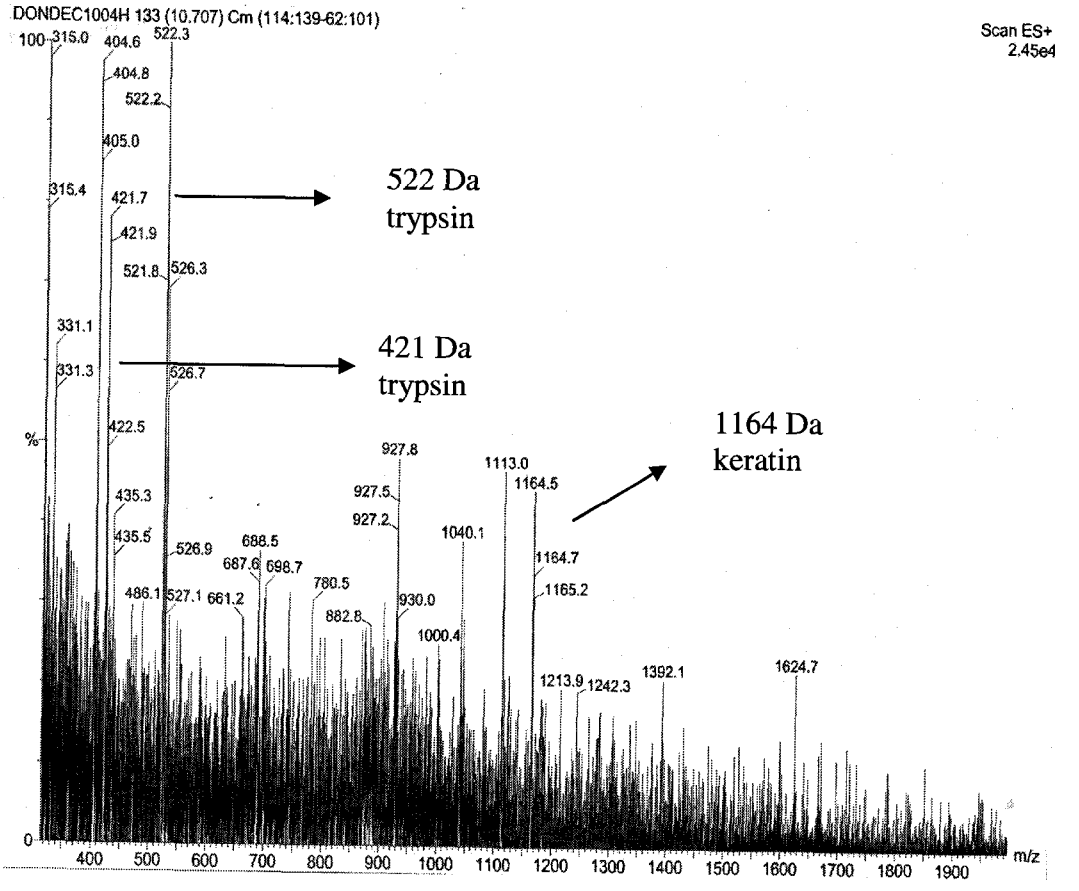


Figure 20. MS spectrum of SchS21 in-gel tryptic digest using 3Q mass spectrometer

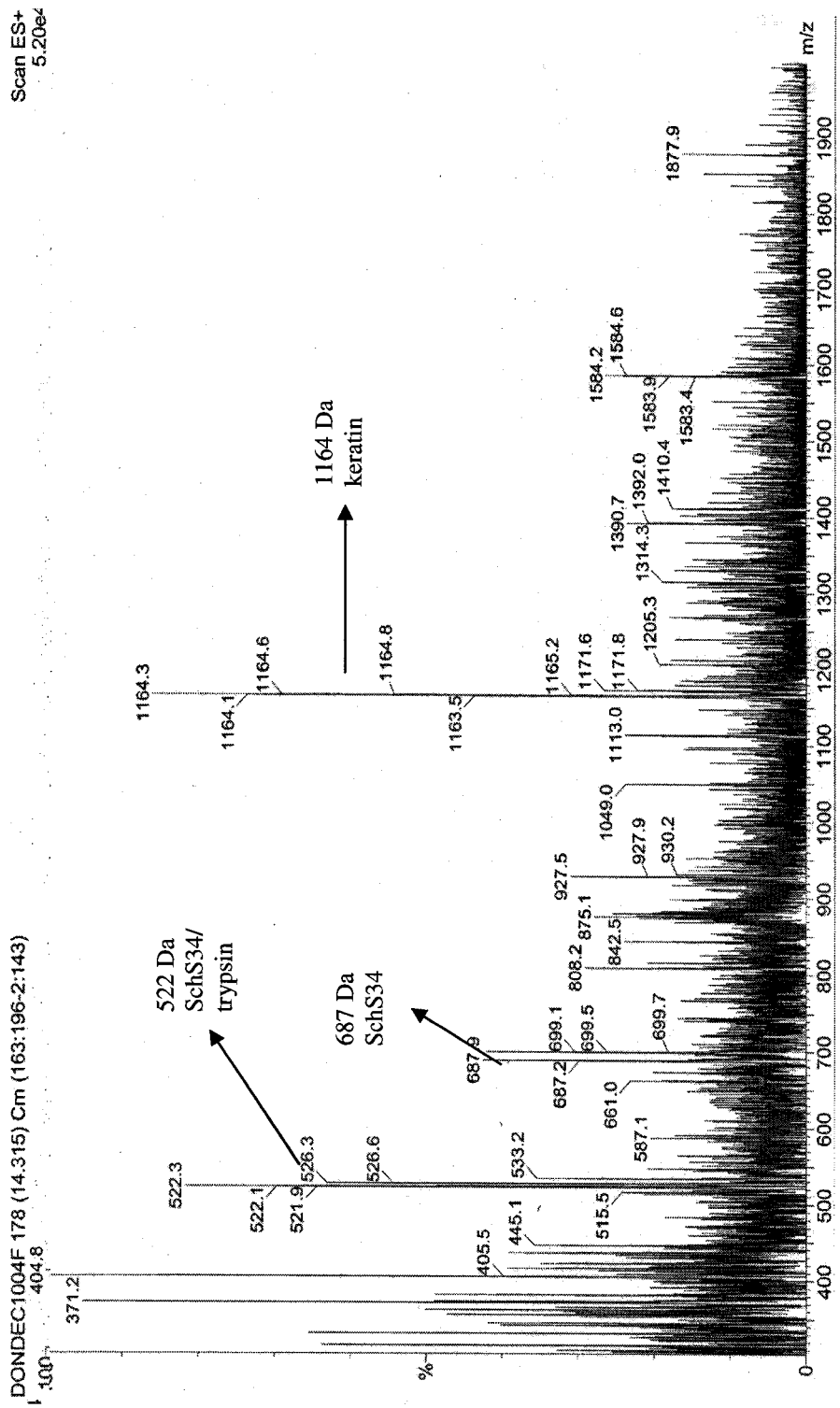


Figure 21. Mass spectrum of SchS34 in-gel tryptic digest using 3Q mass spectrometer

Table 19. Comparison of peptides from in-gel digestions of SchS21 and SchS34 using 3Q mass spectrometer

peak	SchS21	SchS34	identity
1	315	NA	unknown
2	404	NA	unknown
3	421 (2+)	842 (1+)	trypsin
4	522 (2+)	522 (2+)	SchS34/ trypsin
5	NA	687 (2+)	SchS34
6	688	NA	unknown
7	NA	699	unknown
8	NA	875	unknown
9	927	927	unknown
10	1164(1+)	1164(1+)	keratin
11	NA	1390	unknown
12	NA	1584	unknown

10.5. Mass spectrum analysis of in-solution SchS

Initially intact SchS21 and SchS34 in-solution were analysed before trypsin digestions were preformed. It was found that both the 21 kDa protein and the 34 kDa proteins co-eluted during HPLC analysis (Figure 22). Each protein showed a shift in molecular mass, the 21 kDa to 16 kDa and the 34 kDa to 27 kDa, when compared to electrophoretic data. This mass spectrum indicated that each of the proteins exist in a variety of glycoforms. The 34 kDa protein showed two major glycoforms at 26287.46 and 27342.53 Da. These two glycoforms may represent the difference of an entire glycan side chain. The other minor glycoforms of the 27 kDa protein were 27180.71, 27504.76, 27667.02, 27829.13, 27991.87, 28153.81 Da and for the 26 kDa 26126.06 and 26450.57 Da. For the 21 kDa protein the detected glycoforms include 16648.09,16810.62 and 16973.21 Da. For both of the SchS proteins, the minor glycoforms differ each other by 162 Da, this mass represents the difference of a hexose sugar (180 Da – Water). The large number of the 162 Da differences is indicative of high mannose glycans. This data corroborates with previous deglycosylation experiments which also indicated both proteins were glycosylated.

From the in-solution tryptic digestion a useful peptide sequence was obtained. The peptide had a mass of 678 kDa (3+) with the following sequence, TYFDVSA(L/I)VNPTDHDNVK. The mass spectrum of this peptide contained almost the entire y-ion series but only one ion from the b-ion series was present (Figure 23).

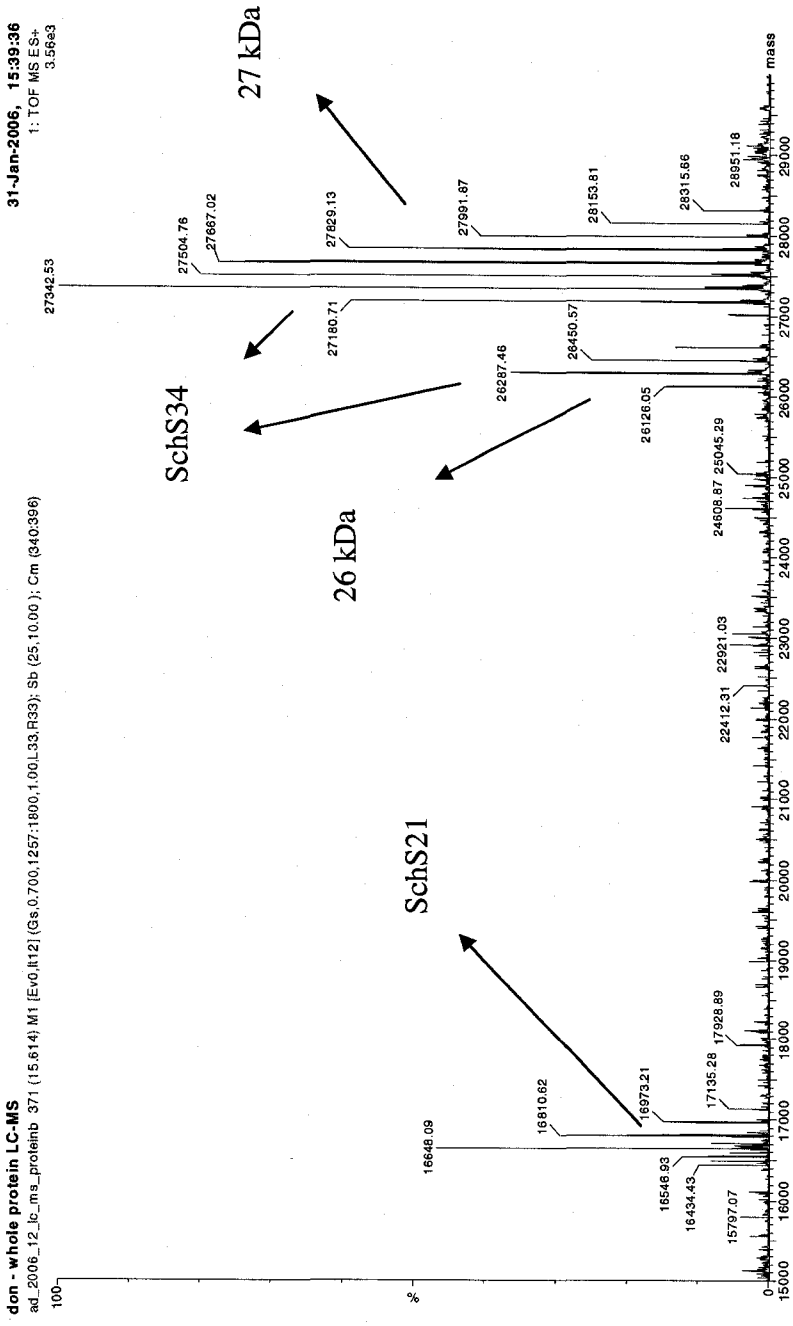


Figure 22. MaxEnt deconvoluted mass spectrum of in-solution SchS protein at 15.5 min

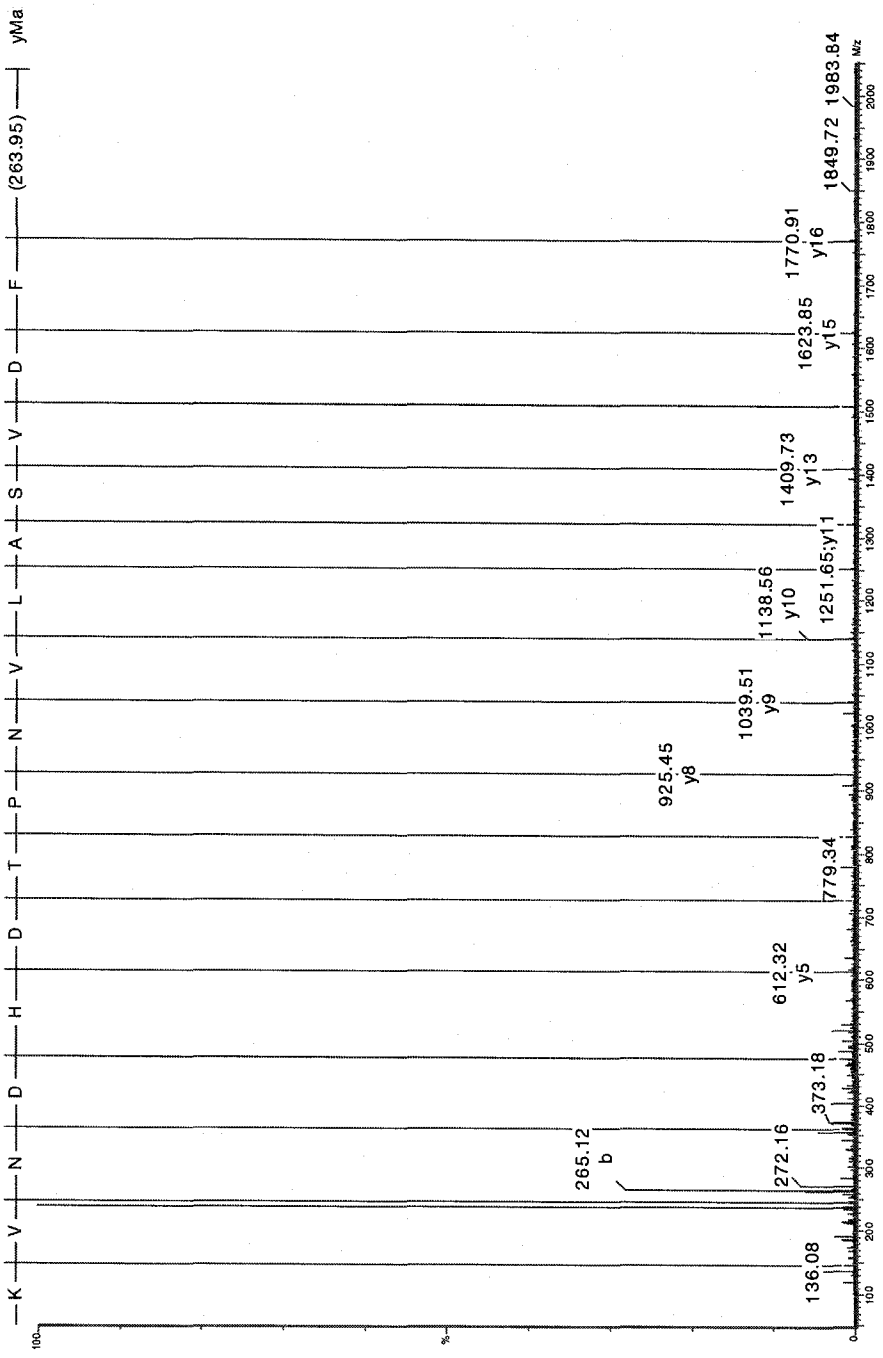


Figure 23. MS/MS spectrum of 678 Da (3+) peptide from SchS21

Peptide	Sequence	Position in Protein
SchS21	TYFDVSAXVNPTDHDNVK	1-18
1	**TYFDVSAIV**D**P**K**D**Q**N**N**VK**	94-111
2	**TYFDVSAIV**D**P**N**D**K**DNVK**	94-111
3	**TYFDVSAIV**V**P**G**D**I**N**N**VK**	193-210
4	**TYFDVSAI**D**D**P**G**D	106-118
5	**Y**Y**DVSA**L**ENP**--N**DNV**	107-120

Figure 24. Alignment of 678 (3+)Da peptide sequence from SchS21 with homologous proteins. 1. DNase1 protein *Metarhizium anisopliae*, 2. hypothetical protein FG06779.1 *Gibberella zeae*, 3. extracellular putative DNase *Nectria haematococca* 4. hypothetical protein MG08644.4 *Magnaporthe grisea* 5. related to DNase I protein *Neurospora crassa*

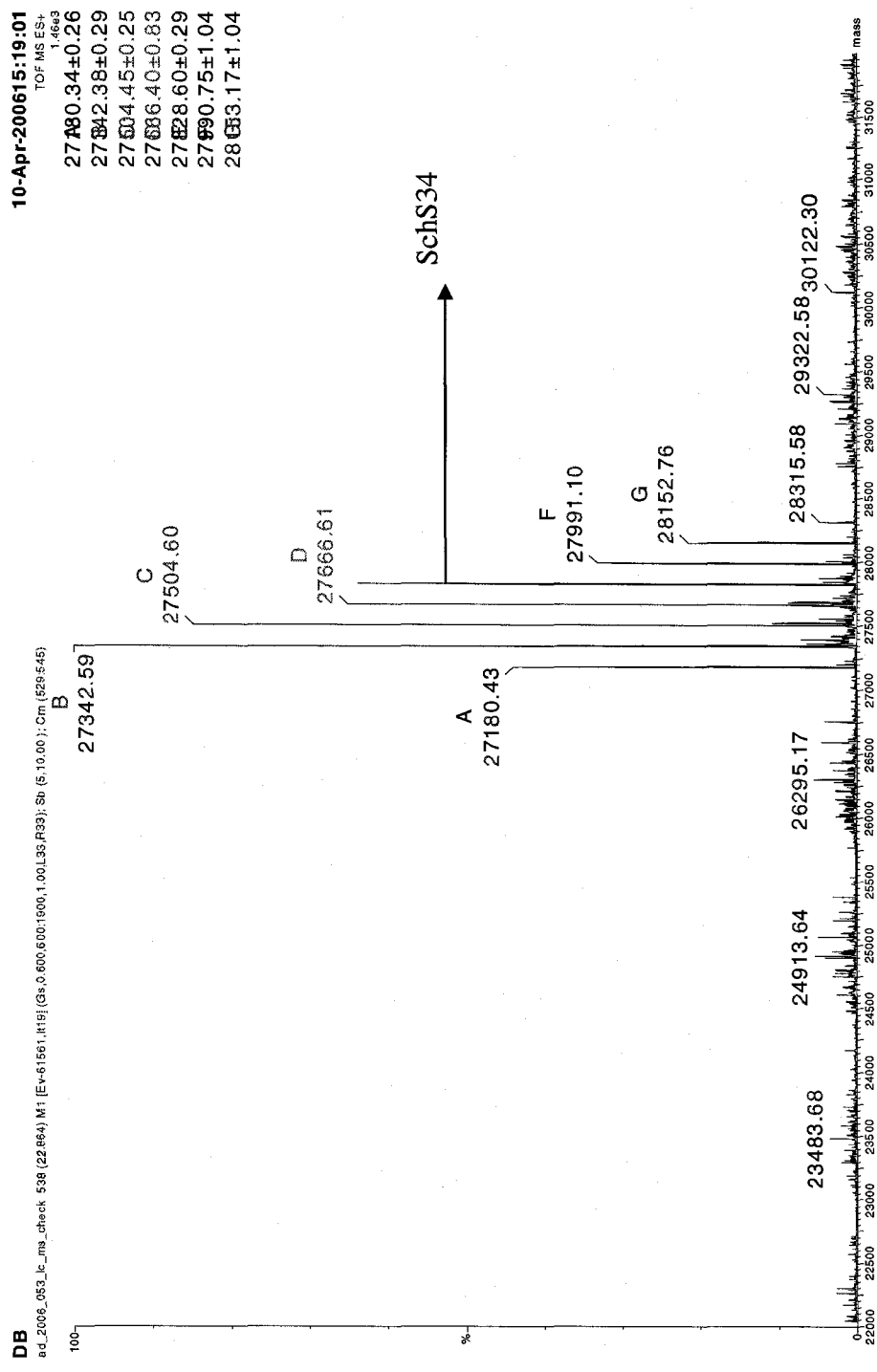


Figure 25. MaxEnt deconvoluted mass spectrum of urea treated SchS protein at time 22.86 min

Upon BLAST searching this peptide against the NCBI non-redundant database, it was found that homologous protein hits matched hits from a previous search using a N-terminal sequence. This peptide was subsequently attributed to the 21 kDa protein and upon alignment with homologous proteins the function thought to be a DNase. These homologous proteins included other ascomycetous fungi, hypothetical proteins from *Gibberella zeae* and *Magnathorpe grisea* as well as DNases from *Nectria haematococca, Neurospora crassa* and *Metarhizium anisopliae* (Figure 24).

When analysing BLAST search hits, 2 parameters are used to determine significance, the BLAST score and the expect value. The BLAST score is used to quantify how close the compared sequences match, where sequence gaps and substitutions contribute to the score, the higher the score the better. The expect value is used to determine the possibility the sequence match occurring by chance, the lower the score the better. The BLAST score was relatively high and expected values were relatively low for the above sequence when compared to the *Metarhizium anisopliae* DNAase (Table 20).

In an attempt to separate the 2 putative subunits, urea was added to the protein solution. The urea was successful in isolating the 34 kDa protein but was unsuccessful in isolating the 21 kDa protein (Figure 22). This method did not lead to acquiring any useful sequence information. This mass spectrum also displayed all the same 27 kDa glycoforms found in the previous intact protein mass spectrum of SchS34 (Figure 25).

10.5.1. Mass spectrum analysis of in-gel tryptic digests SchS proteins by nano-HPLC-QTOF

After some success at separation by urea, separation via polyacrylamide electrophoresis was attempted, followed by an in-gel digestion (Figure 26). The SDS-PAGE gel used for the analysis showed two clear SchS21 and SchS34 bands.

The in-gel digestion provided no new information for the SchS21 protein (Figure 27), with known peptide sequences summarized in Table 20. Two peaks were identified to be trypsin auto digestion peaks (1045 and 2211 Da).

On the other hand, many good sequences were obtained from the in-gel digestion of SchS34 (Figure 28). Three SchS34 peptides were identified as 1043, 1374 and 1758 Da, also three trypsin peaks were identified. Consistent homologous proteins could not be found by BLAST searching the NCBI database. Consistent sequences obtained by more that one mass spectra analysis of SchS34 are summarized in Table 21. All primary BLAST search hits resulted in hypothetical proteins. None of the primary hits were considered to be significant based on BLAST score.

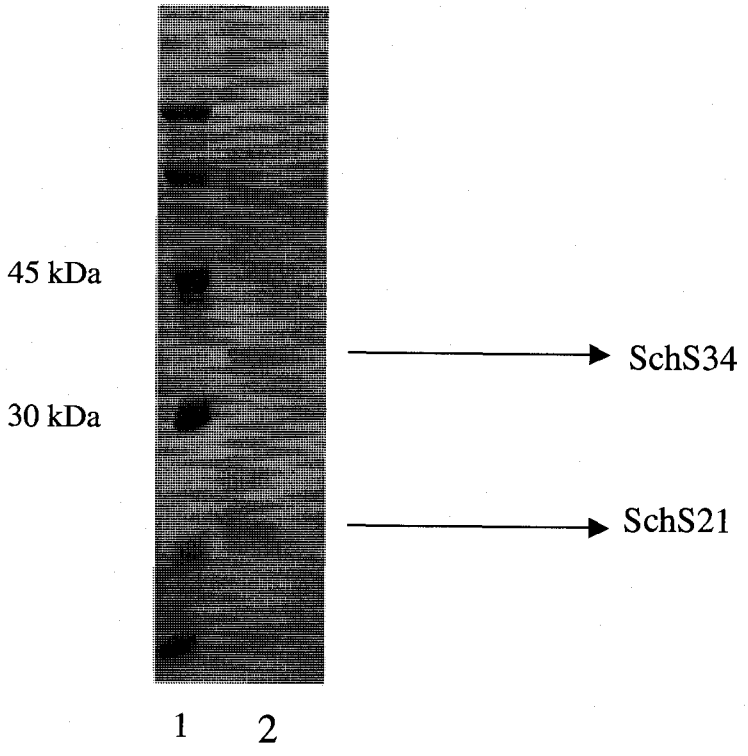


Figure 26. SDS-PAGE used for tryptic digestion of SchS21 and SchS34, lane 1 molecular marker, lane 2 SchS protein

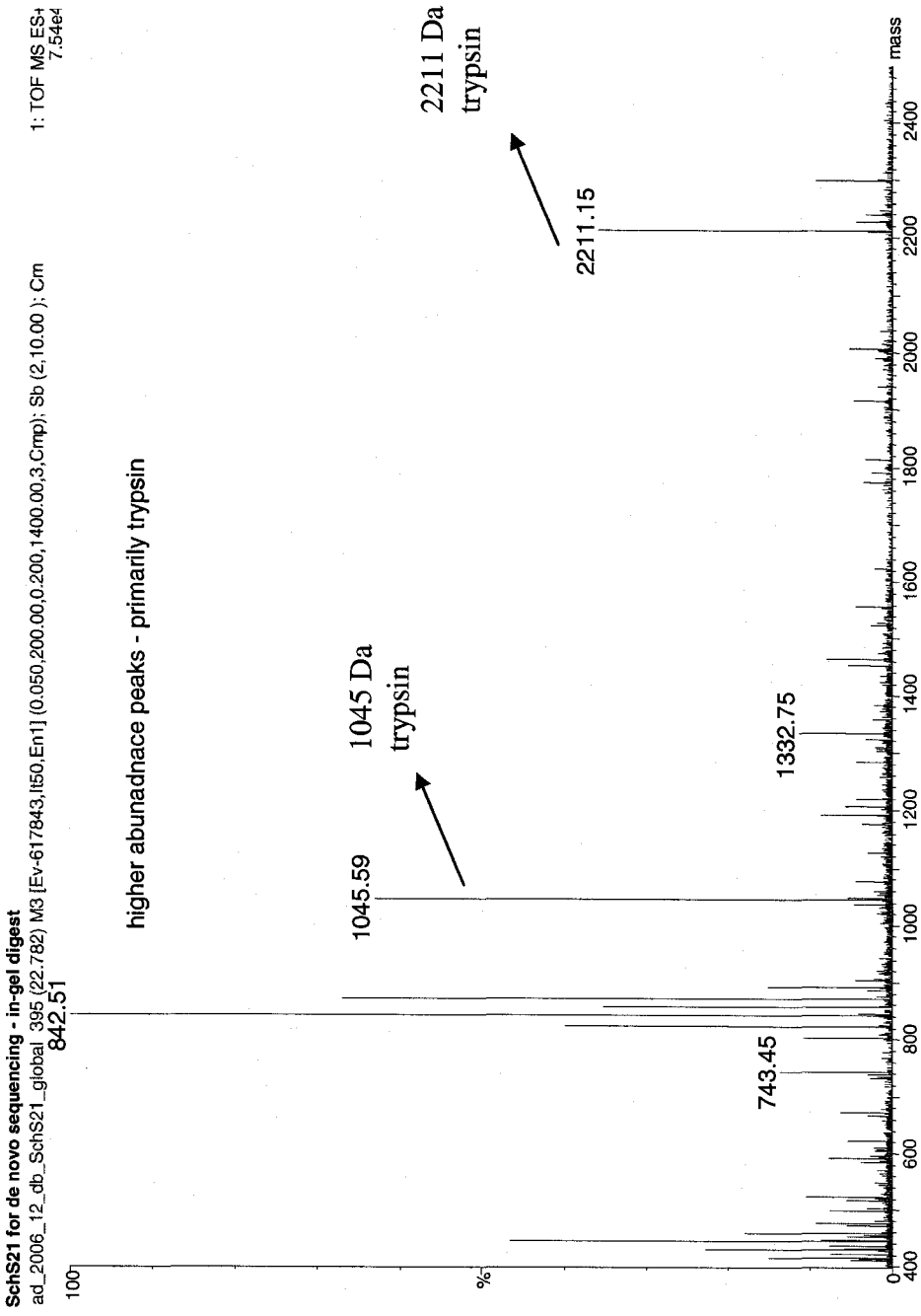


Figure 27. MS spectrum of SchS21 in-gel tryptic digest using QTOF mass spectrometer

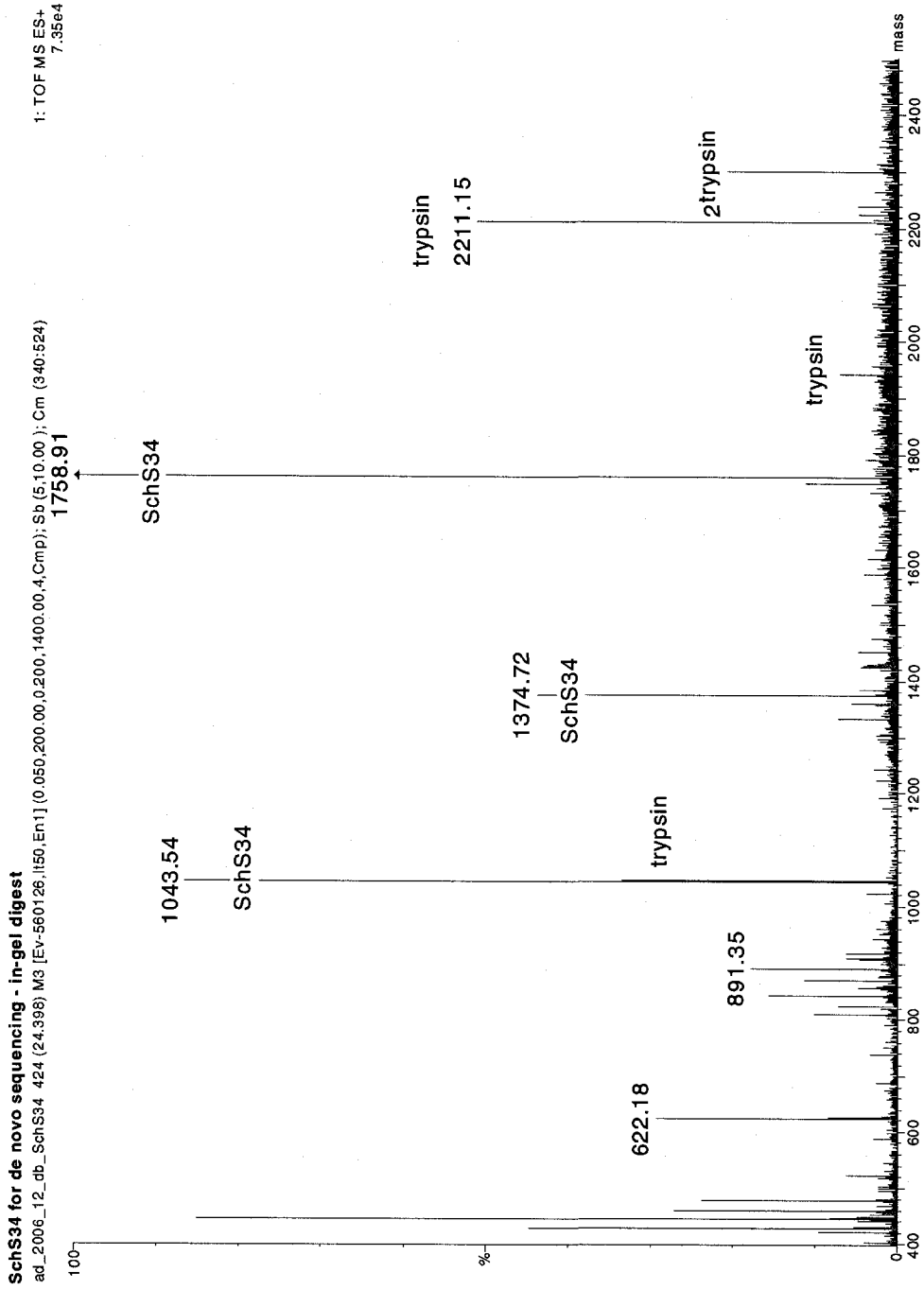


Figure 28. Mass spectrum of SchS34 in-gel tryptic digest using Q-TOF mass spectrometer

When multiple SchS34 peptides were BLAST searched simultaneously, it was found that peptides 4 & 5 showed homology to a hypothetical protein from *Gibberella zeae* (hypothetical protein FG06130.1; Table 21). Although this protein has no known function/identity it did have a molecular mass of 26 kDa which is similar to the mass of deglycosylated SchS34.

The comparison of generated spectra between the 3Q and QTOF instruments for SchS21 and SchS34 showed a dramatic difference in signal to noise (Figure 20, Figure 27, Figure 21 & Figure 28). This difference is facilitated by the much longer averaged runs on a nanospray machine as well as the fact the protein solution is suspended in much less solvent. The reduced noise in the QTOF instrument also provides a clearer signal which facilitated *de novo* sequencing.

When the peptide profiles for each protein are compared between the 2 machines (Table 22 & Table 23), some ion similarities are apparent. For SchS21 both the 3Q and QTOF instruments had a single trypsin peak in common (1045 Da). For SchS34, 2 SchS34 ions were in common, 1043 and 1374 Da. Many unknown peaks were present, these peptides did not provide enough information to be sequenced and identified.

Table 20. Primary BLAST hits for SchS21

peptide number	peptide sequence	peptide mass (Da)	primary BLAST hit	Blast Score	Expected Value
1	TYFDVSA(L/I)V NPTDHDNVK	678 (3+)	emb|CAB63906.1| DNase1 protein *Metarhizium anisopliae*	49.0	2×10^{-6}
2	VTFWT(L/I)DNV DR	762 (2+)	emb|CAB63906.1| DNase 1 protein *Metarhizium anisopliae*	28.6	3.6
3	ASVTFXTLDNV DRTLV	int N-term	emb|CAB63906.1| DNase1 protein *Metarhizium anisopliae*	29.9	1.5

Table 21. Primary BLAST hits for SchS34 peptide sequences

peptide number	peptide sequence	peptide mass (Da)	primary BLAST search hit	Blast Score	Expected Value
1	(L/I)DAGPGTTGVR	522 (2+)	hypothetical protein AN5324.2 [*Aspergillus nidulans* FGSC A4]	24.0	91
2	YPASSTSK	687 (2+)	hypothetical protein SS1G_09313 [*Sclerotinia sclerotiorum* 1980]	24.0	73
3	SDVVSTAR	587 (2+)	hypothetical protein UM05045.1 [*Ustilago maydis* 521]	24.0	73
4	(L/I)FYQDGTAEE(L/I)R	722 (2+)	hypothetical protein CHGG_06011 [*Chaetomium globosum* CBS 148.51]	29.9	1.5
*5	GFHLVVDVTDPSAL	int N-ter	hypothetical protein FG06130.1 [*Gibberella zeae* PH-1]	32.9	0.19

Table 22. Comparison of mass spectra data of in-gel digests of SchS21 from 3Q and QTOF instruments

peak	ESI-3Q (Da)	nESI-QTOF (Da)	identity
1	315	NA	unknown
2	404	NA	unknown
3	NA	678 (3+)	SchS21
4	688	NA	unknown
5	NA	762 (2+)	SchS21
6	NA	743	unknown
7	NA	842	unknown
8	927	NA	unknown
9	522 (2+)	1045	trypsin
10	1164(1+)	NA	keratin
11	NA	1332	unknown
12	NA	2211	trypsin

Table 23. Caparison of mass spectra from in-gel tryptic digestion of SchS34 from 3Q and QTOF instruments

peak	ESI-3Q	nESI-Q-TOF	identity
1	522.3 (2+)	1043 (1+)	SchS34/ trypsin
2	NA	587 (3+)	SchS34
3	687 (2+)	1374 (1+)	SchS34
4	NA	622	unknown
5	699	NA	unknown
6	842 (1+)	NA	trypsin
7	875	NA	unknown
8	NA	879 (2+)	SchS34
9	927	NA	unknown
10	1164(1+)	NA	keratin
11	1390	NA	unknown
12	1584	NA	unknown
13	NA	2211 (1+)	trypsin
14	NA	2299 (1+)	trypsin

11. Discussion

Detection of indoor fungi using ELISA and characterization of their human antigens is rare to non-existent in the scientific literature. The majority of fungi studied have been of the outdoor variety. With large fungal exposures occurring from fungal contaminated indoor environments, a greater understanding of their allergenicity as well as more accurate measures are needed. Accurate detection of fungal allergens would facilitate development of causality for building related illnesses.

Detection of antigens with ELISA requires an extraction to be performed, whether from an air sample or a dust sample. Characterization of antigens can be accomplished by identification of the antigen type, using previous studies of similar proteins to gain an understanding of the antigen. This study explored methods for extraction of allergens from spores, detection of the SchS34 antigen in a dust sample and optimized the SchS34 ELISA to increase the sensitivity.

Detection of fungi using ELISA has many advantages over methods commonly used today. These include fast turn around, capability of analyzing dust samples, and potentially analyzing long duration air samples. The Air-O-Cell, for example, allows quick turn around but little taxonomic value, additionally the sampling time must be limited or the slide becomes too crowded to view under the microscope. The culturable methods provide species identification but have very short sampling times, long turn around times and require very specialized knowledge to perform analysis. Lastly PCR, is very sensitive and has short turn around time but produces a quantitative number with little taxonomic meaning, and it may not detect spore and hyphal fragments (Dillon et al. 2007).

11.1. Optimization of SchS34 ELISA

Optimization of reagents for the SchS34 ELISA is required to obtain the best possible detection limit. Proper quantity of the enzyme conjugate and antibody is necessary to ensure a low background while maximizing signal. Too much antibody may lead to non-specific binding, thereby increase background noise, while too little will leave some epitopes undetected decreasing signal. Both the 12D3 antibody (0.25 μg/mL) and HRP-conjugate (250 ng/mL) concentrations were maximized through dilution and analyzing the response to the standard curve. The criteria used for determination of optimum concentration were the signal to noise ratio, curve shape and standard deviation.

Purity of the antibody can also have a large effect on background signal. If contaminant proteins are coated onto the well of a plate, non-specific binding occurs, this could increase background signal. Some of the contaminant proteins in the antibody preparation may be antibody binding proteins, if these are coated onto the well of a microplate, they will bind the secondary antibody and produce a false positive signal and, in turn, increase background signal.

The incubation time of the HRP-conjugate is also important to optimize an ELISA assay. Longer incubations times can result in increased background noise while too short of incubation times while not allow for complete binding of the conjugate to the biotin. Two commonly used incubation times are 0.5 and 1 hr, it was found in this study that 0.5 hr produced the optimal signal. This short incubation time also has the advantage as it shortens the amount of time required to perform the assay.

Lastly, the blocking material can also affect the performance of an immunoassay. In some cases, it is not optimal to use serum proteins for blocking because they may

contain antibody binding proteins or contaminant antibodies. Common blocking reagents include; calf serum, skim milk power, BSA, gelatine or casein. BSA and skim milk powder were tested in this study. BSA was found to produce the lowest background even though it is a serum protein. Tween 20 was demonstrated to have a negative effect in the blocking solution. Tween 20 may prevent the blocking protein from binding to the non-specific binding sites which may increase the background signal.

11.2. Analysis of field samples using SchS34 ELISA

After the SchS34 ELISA was optimized, environmental samples were analysed. A serial dilution of house dust was first to be analyzed using the culturable plate method. This approach allowed us to gain an understanding of the ecology of the fungi found in the dust. No *Stachybotrys chartarum* was found in the culturable analysis while the predominant species included various Penicillia species, *Paecilomyces variotti* and the Aspergillus telomorphs. *S. chartarum* is rarely found in culturable dust samples due to its slow growth and short half-life (Hung et al. 2005). A study by Scott (2001) found that only 5% of homes had detectable levels of culturable *S. chartarum* in dust samples.

Very few studies have been performed where ELISA has been used to quantify fungi in an indoor environment. The two fungi commonly studied are *A. fumigatus* and *A. alternata*, due to the availability of commercial ELISA kits. Both of these fungi are not commonly found growing on building materials (Miller et al. 2008). In two *A. fumigatus* studies, virtually no Asp f1 was detected in the environment using an Asp f1 ELISA. While *A. fumigatus* is commonly found in the environment, it is generally found at low levels. In a study by Mullins, they found on average 13.5 cfu/m^3 in St. Louis and 11.5 cfu/m^3 in Cardiff while Scott found 10% of homes in Wallaceberg had detectable levels

of *A. fumigatus*. A study by Dillon et al. (2007), where they looked at compost facilities demonstrated that Asp f1 could be detected in the air clearly demonstrating the usefulness of ELISA when high concentrations of spores are found. In our study, we found that the detection limit of the ELISA is 10^7 spores/g for *A. fumigatus* or 1 ng Asp f1/g of sieved dust. With regards to *A. alternata*, a study by Salo et al. (2005) found that the detection limit of their *Alternaria* antigen assay was 140 ng/g of sieved dust. The authors were capable of detecting *Alternaria* antigens in 95% of homes with the geometric mean of 4.5 μg *Alternaria* antigens/g sieved house dust.

The analysis of *S. chartarum* spores in dust demonstrated that it had a lower detection limit when compared to the *A. fumigatus* and *A. alternata* ELISAs. The *S. chartarum* detection limit was 10^5 spores or 0.2 ng SchS34/g of sieved dust. This represents a 100-fold increase in sensitivity when compared to the *A. fumigatus* ELISA. Even though *S. chartarum* spores are larger than *A. fumigatus* spores, this large difference cannot be explained by this alone. This increase in sensitivity maybe due to a larger amount of SchS protein is present per weight of spores or the antibodies used may have a higher affinity. It should also be noted that the negative control produced no signal. This exemplifies the specificity of the SchS34 ELISA, given the culturable analysis demonstrated a wide variety of fungi were found in the dust sample.

S. chartarum contaminated swab and drywall were also tested using the SchS34 ELISA. It was found that the field samples produced a higher response than the experimental samples. The experimental samples were freeze-dried to stop further fungal growth, it is possible that a large portion of the spores were removed by the vacuum

suction. The field samples were darker black in colour which is an indication of a greater density of fungal growth.

For the swab samples analysed, again the experimental samples displayed the higher response. By using a swab sample, contamination from drywall material could be avoided; also the spores could be concentrated on the tip of the swab. When the swab analysis is compared to the drywall sample, the drywall sample generated a higher response. This greater response is due to the larger size of the sample when compared to a swab, therefore more *S. chatarum* material is available for detection by ELISA. The results from these analyses indicate when field sampling it would be better to take a piece of the drywall if possible.

Whether or not the ELISA has a sufficient detection limit to detect *S. chartarum* contamination is a valid question. A study of non-viable fungal spores, in 625 buildings in southern California found that a clean building typically contained greater than 4000 counts/m^3 of air while a mouldy building could contain 200 to greater than 2 million spores (Baxter et al. 2005). They also noted that they rarely found greater than 1000 counts/m^3 of *Stachybotrys* spores. It is difficult to ascertain the quantity of *S. chartarum* conidia in dust samples by the culturable method, due to its slow growth rate and short half-life. It is likely often over looked by these kinds of analysis. In this study, 10^5 CFU/g house dust were found for both *P. variotii* and *P. citrinum*, therefore fungi are found in the range in which the SchS34 ELISA can detect fungi. The culturable method only detects a fraction of the total fungal material, as it only detects viable propagules. One must remember that the above analysis are detecting intact spores, where these only represent 1/3 of the fungal biomass found indoors. ELISA is capable of detecting these

fragments which provides it a distinct advantage over PCR. A study by Fluckiner (2000), where they analyzed air samples for *C. herbarum* and *A. alternata* allergens, they easily detected the fungi at concentrations of 100-1000's of CFU/m^3. Interestingly in this study, they also used a multistage liquid impinger sampler which demonstrated that the ELISA could detect allergen in the much smaller pore sizes when compared to the viable. This finer fungal material is not being measured by culturable analysis but is available to the ELISA. This study did however demonstrate that the allergen load is different when one compares the outside air to the inside air, where the inside environment is higher than the outside.

11.3. Methods to increase Asp f1 recovery

In an attempt to decrease the detection limit, methods of extracting the antigen/allergen from spores were explored. The hypothesis was if more protein per spore weight was released, the ELISA response per weight would increase which would then decrease the detection limit. The limitation was bypassing the rigid cell wall of fungi, which turned out to make extraction of intercellular protein difficult. Through millions of years of evolution, spores have developed resistance to the environment as well as other organisms which regard them as a food source. Spores with short life span in the environment results in no propagation of that fungi which ends in extinction. Only spores which can propagate and grow can continue to thrive.

Many methods have been applied but few of these have been shown to be useful for quantitative proteomics. Approaches developed for genomics do not necessarily apply to quantitative proteomics because they may use conditions not favourable for ELISA

detection of delicate proteins. The most commonly employed methods in genomics/proteomics are disruption by beads, grinding by mortar and pestle and enzymatic digestion.

A. fumigatus and its allergen Asp f1 were used as a model fungus due to the commercially available ELISA kit. The cell wall of *A. fumigatus* is known to contain polysaccharide chains composed of β –1,3 glucan, galactomannan and chitin, thereby making these plausible enzymatic targets (Bernard and Latge 2001). Many commercial enzyme preparations commonly provided are impure, and have protease activity. This may not be a concern for genomics researchers, but for proteomics it is a major problem.

With β-1,3 glucan being the major component of fungal cell walls a protease free β-1,3 glucanase, Quantazyme, was first used. This enzyme did not show an increase in Asp f1 recovery. This may indicate that the enzyme was not functioning optimally under the given conditions suitable for ELISA or only digested part way through the cell wall. Partial digestion would probably release some protein held in transit. After 1 hr, the optical density values did not change or vary significantly between enzyme concentrations or time periods, these maybe indicative of the protease free preparation. Bernard et al (2001), also found that treatment of *A. fumigatus* mycelium with a β-1,3 glucanase did not result in an increase in extracted protein. They suggested, that unlike yeast, most of the protein in the cell wall is either passing through or non-covalently bound.

With both chitin and galactomannan also occurring in *A. fumigatus* cell walls further enzymatic experiments were completed in this study using chitinase and galactomannase, which were not protease-free preparations. A single sample showed a

statistical difference from the untreated control, that sample was 0.1 g galactomannase/mL at time zero. This difference may have occurred due to poor solubility of *A. fumigatus* when initially mixed in buffer. Poor mixing may have resulted in clumps of *A. fumigatus* conidia being sampled at time zero.

In most instances where one is attempting to digest the cell wall of fungi, the fungus must be grown using specific media for it to be susceptible to enzymatic digestion, particularly for spheroplast production (Vicentefranqueira et al. 2005). To obtain enzymes capable of digesting the appropriate cell wall, fungi are often grown on cell wall material from the fungus to be digested. For example, *Trichoderma harzianum* is commonly used, it would be grown on media with *A. fumigatus* cell wall material as the only carbohydrate source and the excreted enzymes extracted. In other cases a panel of enzymes are used, but for each unpure enzyme preparation used, the proteolytic activity increases.

Physical methods for Asp f1 extraction were also tested. While these approaches are not ideal for use in field samples, they may provide insight into whether more Asp f1 is available for extraction. Using large beads were shown to increase AVS1 protein recovery from *A. versicolor* and large beads were shown to increase Asp f1 extraction in this study (Zhao 2006). Small beads however negatively effected Asp f1 recovery, possibly by non-specific binding of the protein to the beads. The discrepancy between large bead and small beads maybe due to the smaller beads having higher surface area to volume ratio, allowing more protein binding per unit of volume. Some data from a commercial bead retailer (BT&C/OPS Diagnostics) has indicated that low non-specific binding beads produce higher recoveries than commonly used acid washed beads. This

maybe worth exploring but it will be difficult to apply bead disruption to environmental samples such as a dust sample or bulk sample. Each of these sample types would greatly reduce the efficiency of bead method. Therefore chemical or enzymatic methods would be more conducive to obtain increased recovery as they can be applied to these sample types.

The disruption of spores with beads did demonstrate that there is some Asp f1 that is not being extracted by buffer alone. If enzymes or chemicals could be used to extract the allergen from the inside of the spore, this would increase the relative signal for a given amount of spores and hence increase the sensitivity of the method. For this to work the chemical/enzyme must digest the cell wall and free any allergen trapped inside the wall or cell while at the same time not be too harsh to effect allergen structural conformation. This is where the difficulty lies. Most enzymes require specific conditions such as reductants, pH and buffers but these may not work with ELISA and were shown to not be successful at extracting Asp f1. So it is a delicate balancing act whereby the extractant is strong enough to get the protein out but not too strong to denature the protein or negatively affect the ELISA.

This led to the idea of using protective methods, to increase Asp f1 recovery. The initial approach involved using a protease inhibitor cocktail to over come proteases. Again these cocktails may work to protect the protein but may in turn negatively affect the ELISA. In this case the higher concentration of protease inhibitor cocktail appear to have a positive effect on Asp f1 recovery but it did not display the overall highest optical density value, which was shown by the untreated sample at 2.5 hr. This result can be interpreted in 2 ways, either there is little protease activity and the PIC has little effect on

the ELISA or there is significant protease protection but the PIC negatively affects the ELISA.

Propriety protein extraction kits are available which are touted to be designed to increase recovery from a variety of tissues; the Yeast buster kit is such a product. This kit contains an extraction solution, a reductant (THP) and benzonase (DNase). The THP is purportedly used to protect the proteins from oxidation while the benzonase is used to digest DNA, thereby reducing the viscosity of the mixture and increase mixing. In both cases, disrupted and undisrupted spores treated with the Yeast buster showed a decrease in recoveries. The lack of a positive effect from this kit maybe due to it was developed specifically for yeast. Yeast have different cell wall make up than filamentous fungi. For example, *Saccharomyces cerevisiae* cell wall is composed of mostly β1-4 glucan, chitin and β1-6 glucan while *A. fumigatus* cell wall is composed of chitin, galactomannan, β1-3 glucan, α 1-6 glucan, β1-4 glucan (Lipke and Ovalle 1998; Bernard and Latge 2001).

The THP effectiveness was examined by itself to determine if it could protect the allergen, while at the same time not effect the ELISA. In many cases, the THP did have a positive effect on Asp f1 recovery, this may indicate that the protein is susceptible to oxidation during buffer extraction and this oxidation affects the ability for the ELISA to bind it. This, in turn, led to the investigation of EDTA which acts as a metal chelator to protect proteins from oxidative damage. EDTA is also known to inhibit metalloprotease activity. EDTA is commonly used in extraction buffers for a wide variety applications, including fungal allergen extraction (Simon-Nobbe et al. 2006). EDTA was found to have a positive effect on Asp f1 recovery, which indicates that Asp f1 maybe subject to protease activity or oxidative damage.

Detergents are also commonly used to extract proteins from tissues. The most commonly employed detergent is SDS. SDS was used by Bernard et al. (2001) to extract protein from the mycelium of *A. fumigatus*. They found it removed the bulk of the protein but these authors did not use ELISA downstream. SDS is an ionic detergent which tends to disrupt the 3D structure of proteins, therefore the non-ionic detergent Tween 20 was used. Tween 20 did not display a positive net effect on extraction of Asp f1 from both disrupted and undisrupted spores. While Tween 20 likely acts as a surfactant facilitating dissolution of the allergen into solution, it is not necessary for extraction of Asp f1 from spores.

Length of antigen extraction should also be considered when optimizing recoveries. A too short extraction time can result in incomplete extraction of the target protein while too long of an extraction period can result in antigen degradation. It required at least 1 hr to see an increase in protein extraction, with 2 hr being optimal. This was consistently seen with all untreated samples for each analysis. The delay has been suggested in the literature as an indication of germination. The more likely answer would be that it takes some time for the very hydrophobic spores to be wetted and enter solution. When first mixed the spores and buffer tend to form 2 phases. After about an hour of mixing the spores are in solution. Once in solution, this facilitates washing the spores and extraction of the Asp f1. This change in hydrophobicity was also demonstrated by Dague (2007). They also showed that cell wall was still intact after 2 hr, therefore the increase in Asp f1 cannot be explained due to germination.

Aruda et al (1992) found 1000 times more Asp f1 in culture filtrate than in disrupted spores and mycelium, where they reasoned that growth was required for Asp f1

production. The data in this paper indicates that the allergen is indeed excreted which was also demonstrated by Latage et al. in 1991 using immunolabelling experiments. The data produced herein indicates that Asp f1 is detectable at time 0 where it maximizes at approximately 2 hrs while spore ultra structural data shows that the spores have not opened after 2hrs (Dague et al. 2007). This indicates that some Asp f1 must be contained within the cell wall/intercellular space or synthesis and excretion from intact conidial walls is rapid after suspension in aqueous solution. Lastly, a proteomic study of conidial surface proteins from *A. fumigatus* did not detect any Asp f1, which demonstrates that this allergen is not surface-associated (Asif et al. 2006).

The temperature can also have a large effect on extraction efficiency. Temperature has an effect on Brownian motion of the water, which in turn facilitates diffusion. As temperature increases, Brownian motion increases, which increases diffusion of the allergen out of the spore. Temperature also has an effect on protease activity and microbial degradation. The higher temperature may also increase the chances that a molecule of Asp f1 bumps into a protease or microbe, which would negatively affect recovery. A cooler temperature would have the opposite result, increasing viscosity of the fluid thereby decreasing diffusion at the same time suppressing microbial and protease activity. Room temperature would likely represent a middle ground between the two extremes. The results of this studying indicated that there was no statistical difference between 4 °C, 37 °C and room temperature, but the highest optical density value was obtained for room temperature. This data indicates that room temperature is a viable option when performing the ELISA, the inconvenience of refrigeration or incubation in an incubator is not necessary.

11.4. Mass spectrum analysis of proteins

Mass spectrum analysis of proteins is a convenient method for identifying proteins. This method is become more commonplace as the price per analysis decreases as well as the data generated becomes more accurate. Mass spectrum analysis also allows for *de novo* sequencing of peptides where the N-terminal has been blocked.

Mass spectrum analysis of intact SchS protein, resulted in 2 major peaks that co-eluted. Both of these peaks displayed a shift in mass, when compared to the electrophoretic masses (21 & 34 vs 16 & 26 kDa). Also both the SchS proteins exhibited a variety of ions indicating both proteins exist in an assortment of glycoforms. This result was expected as both proteins had previously been shown to be glycosylated (Xu et al. 2007). Xu et al (2007) demonstrated that both the SchS21 and SchS34 were approximately 15% glycosylated, by comparing masses of glycosylated and deglycosylated forms by electrophoresis. This represents a shift in mass to 17 kDa and 29 kDa respectively. Mass spectral analysis in this paper demonstrated that the mass of the glycosylated forms of each protein was approximately 16 kDa and 26 kDa respectively. The unglycosylated masses of these proteins were not determined by mass spectrum analysis so no comparison can be made with the apparent electrophoretic mass. It is known that glycosylation can have an effect on electrophoretic mobility, when change in mass has been taken into account, it can change mobility by as much as 20-100% (Segrest et al. 1971). This discrepancy is hypothesized due to charge distribution and hydrodynamic shape (Segrest et al. 1971). In this case, it is likely incorrect to say that the protein is 15% glycosylated based on electrophoretic mobility alone. A more accurate

method would be to examine a deglycosylated version of the protein and compare it to a glycosylated form by mass spectrometry (Kleinert et al. 2007).

Glycosylation of proteins come in 2 varieties, O- linked where the carbohydrate is linked to a serine or threonine amino acid residue or N-linked where the carbohydrate is linked to an asparagines residue. Glycosylation of extracellular protein, particularly O-linked, is deemed necessary for secretion (Kubicek et al. 1987). Glycosylation has also thought to provide extracellular proteins more stability, increase solubility and decreased susceptibility to proteolytic attack (Peberdy 1994). The glycosylation pattern found in this report is indicative of high mannose glycans. High mannose glycans are commonly found in fungi, in either the N-linked or O-linked forms (Maras et al. 1990). The structure of these glycans in fungi generally consists of 5-10 mannose residues bound to a N-acetylglucosamine residue (Maras et al. 1990). The heterogeneity of the glycans found in fungi has been theorized due to the excretion of glycolytic enzymes (Maras et al. 1990). For both the SchS proteins a variety of glycoforms were observed. It has also been shown that growth conditions can affect glycosylation patterns (Maras et al. 1990). Elucidation of exact glycan structure usually requires careful NMR or mass spectroscopy analysis.

With the co-elution of both SchS21 and Sch34 by HPLC this further suggested that the proteins interact, possibly as subunits. A similar phenomenon was demonstrated by Louie et al.(1996), where they also observed 2 proteins co-elute when reversed phase HPLC was used as the separation method. The possibility of both SchS proteins interacting was previously demonstrated by gel filtration experiments as well as affinity chromatography (Xu et al. 2007, Xu et al. 2008). In each instance both SchS21 and SchS34 co-eluted. The HPLC evidence alone may indicate the SchS proteins maybe of

similar hydrophobic character or isoform, hence the co-elution. Given the evidence from Xu el al. (2007 & 2008) where it was demonstrated that both proteins were found to elute from a size exclusion column together at approximately the 65 kDa mark as well they both eluted from a monoclonal antibody affinity purification column where the individual monoclonal was shown to be specific for SchS34, supports the hypothesis that they are subunits. Also, d*e novo* sequence data indicated that both proteins are subunits, rather than fragments of an intact protein, due to peptides found in one are not found in the other. The treatment with urea, which denatures proteins, had some success in separating these proteins while Xu et al. (2007) showed that these proteins were not covalently bound.

De novo sequencing of peptides by mass spectrometry from digested proteins has almost reached "routine" status. While reports of using this method to sequence entire proteins exist in the literature, this has not become routine. The reason for lack of coverage could be due to lack of ionization as well as generation of poor spectra.

The data generated from the 3Q machine and BSA indicated that peptides could undergo CID and produced fragment spectra. The difficulty lies in the interpretation of these spectra. In each case incomplete ion series made interpretation very difficult, but partial sequencing information was obtained. Also a poor signal to noise ratio made it difficult to discern true signals from background noise. Lastly, the mass accuracy on a 3Q is considered poor. High mass accuracy is necessary to make accurate interpretations of spectra. Plus or minus a Dalton can have dramatic effect on when deciding which amino acid to choose.

The 3Q machine analysis of the tryptic peptides for SchS peptides demonstrated that the protein concentration was too low for the sensitivity of the machine. While many peaks were visible, there was insufficient protein to produce good MS/MS spectra. The ions which were identified were either done so using the QTOF instrument or they were well known contaminant peaks. A few contaminant peaks were identified, primarily trypsin with one keratin. Trypsin is used for the digestion of intact proteins to produce peptides of suitable size to be analyzed by mass spectrometry. Trypsin is capable of digesting itself, therefore producing known as tryptic auto digestion contaminant peaks. Also keratin is a very common contaminant. Keratin is found in hair and skin, has a long half-life and is ubiquitous in the environment.

When the 3Q was compared to the QTOF, a significant increase in signal to noise was observed. Also, this machine was coupled to a nano-spray HPLC which allowed the use of small sample sizes such a gel bands, but at the same time, long acquisition of peaks. If an HPLC peak is large, this allows long acquisition times of fragmented peptide ions. When these are averaged, the background tends to be "averaged" out.

Peptide sequences from SchS21 generated from the QTOF instrument matched fungal DNases upon a BLAST search. The top 5 hits were all Ascomycetes which is consistent with the taxonomy of *S. chartarum*. Interestingly research previously done on the *Nectria haematococca* DNase also indicated it had a subunit (Klosterman et al. 2001). *N. haematococca* is also a member of the Hypocreales order, indicating it is closely related to *S. chartarum*. Klosterman et al (2001) also found that this DNase was involved in the plant pathogenicity of the fungus. This also appears to be the case for the Alt a1 allergen. It has long been a mystery as to the function of Alt a; recent evidence suggests

that it is involved in the plant pathogenicity (Cramer and Lawrence 2003). Alt a1 is also known to be a heterodimer.

DNase I is a group of enzymes which cleave double and single stranded DNA resulting in 3'-OH and 5' phospho tri and/or tetranucleotides (Laskowski 1971). This group of DNase is known to be excreted and is thought to be involved in digestion of extracellular DNA for nutrition. It is known to require Ca^{2+} and Mg^{2+} or Mn^{2+} and to be inhibited by Zn^{2+}. DNase I is known to have an optimal pH of 7.0 and have a pI of approximately 4. Most of the work on DNases has been carried out on mammals which include human, rat, pig and cow.

Extracellular DNases have been identified from a variety of fungi, including *Nectria haematococca*, *Neurospora crassa* and *Colletotrichum coccodes* (Gerhold et al. 1993; Watkins and Hadwiger 1998). The *Nectria haematococca* DNase was found in high concentrations in conidia which is the same for SchS in *S. chartarum*. Also the DNase from *Nectria haematococca* was found to be excreted which is also the same for SchS34 (Gerhold et al. 1993; Klosterman et al. 2001). SchS is known to be excreted when *S. chartarum* is cultured in liquid medium (Xu et al. 2007). With fungi digesting its nutrition in the environment, rather than internally, it is no surprise to find an extracellular DNase. Digestion of DNA from plant material or other sources could be used as a nutrition source or could play a part in pathogenesis.

No consistent match was obtained for BLAST searching of SchS34 peptides sequenced using the QTOF instrument. All of the primary BLAST hits were of unknown function, which exemplifies that genes sequences are of little value if the function of the transcript is unknown. The majority of the BLAST hits were obtained from fungi that

have had their entire genome sequenced, such as *Neurospora crassa* and *Gibberella zeae*. This indicates that the smaller protein maybe found among a number of Ascomycetes, particularly the Hypocreales order, yet its function has still to be determined.

When the peptides from SchS34 were blast searched together, two of the peptides produced a similar hit, hypothetical protein FG06130.1 from *Gibberella zeae* PH-1. *Gibberella zeae* is also member of the Hypocreales order, in which *S. chartarum* resides. This protein has a mass of 26 kDa, which is similar to the mass found by mass spectrometry of SchS34. While mass spectrometry is particularly good at identifying known proteins, it still relies heavily on databases.

Peptide sequencing by mass spectrometry can generate inaccurate sequences, when these inaccurate sequences are BLAST searched, they may generate a false identity for the protein. For example, the sequences shown in this report include only the consistent portion of the sequence, different runs of the QTOF instrument produced varying sequence results. These inconsistence sequence results maybe due to weak signal from some fragment ions, thereby making interpretation ambiguous.

11.5. Future prospects

With regard to sequencing the SchS proteins, the known peptide sequences can be used to produce PCR fragments which in turn can be used to screen a cDNA library. The appropriate cDNA can be sequenced and expressed. Also digestion with an alternative enzyme such a chymotrypsin could be used.

An increase in sensitivity of the SchS34 ELISA could be further increased by using different substrates, using Xmap technology or even immunoPCR. Sigma-Aldrich sells a supersensitive version of TMB which is advertised as having 40% higher kinetics than standard TMB. Xmap technology has demonstrated to increase sensitivity 10 fold when compared to traditional ELISA (Earle et al. 2007). ImmunoPCR has shown incredible sensitivity. This method has been known to quantify antigens expressed as molecule number/reaction while increasing sensitivity by 10^5 fold (Sano et al. 1992). The authors of this study go on to suggest that this method can detect a single antigen per reaction.

To summarize, this study has demonstrated that an increase in recovery of Asp f1 can be accomplished by use of THP, EDTA or THP. It has also shown that SchS21 functions as a DNase while SchS34 is of unknown function. Lastly, theses antigens can be detected in *S. chartarum* contaminated dust as well as drywall and swab samples.

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