**Isolation and purification
of fungal proteins antigenic in humans from indoor
strains of *Penicillium chrysogenum***

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A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the
requirements for a degree of

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ABSTRACT

The goal of this research was to identify proteins antigenic to humans, produced by clade 4 strains of *Penicillium chrysogenum* from a wide geographic distribution. This fungus is common on damp materials in buildings and has been shown to cause symptoms of allergies and/or asthma. Proteins produced by *P. chrysogenum* were extracted from liquid media and screened with ELISA and immunoblotting against atopic human polyclonal sera. A 52 kDa protein, common to the clade 4 strains screened, was purified by protein precipitation, anion exchange, and gel filtration. Laboratory goats were immunized with *P. chrysogenum* spores and subsequently boosted with the target protein. This was confirmed that the target protein was present in spores and mycelium, and an antigen in goats as well as humans. Monoclonal antibodies were produced against the 52 kDa target protein. Test results indicated that the mAbs were sensitive and selective to the target antigen. The amino acid sequence of the target protein determined it to be a 52 kDa glucoamylase. Cross reactivity tests indicate other fungi to not produce a similar protein that reacts to the pAbs or the mAbs. This research could be incorporated into new tools for assessing exposure levels to *P. chrysogenum* in the indoor environment.

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ABBREVIATIONS

1° Ab - Primary antibody; the abbreviation used in both ELISA and immunoblotting protocol.

2° Ab - Secondary antibody; abbreviation used in both ELISA and immunoblotting protocols.

AP - alkaline phosphatase, enzyme which is conjugated to antibodies to amplify signal immunoblotting.

ATCC – American type Culture Collection, Manassas, VA

CBB - commassie brilliant blue stain. GelCode Blue Stain distributed by Pierce Biotechnology Inc, Rockford, IL, was used for all CBB staining.

CBS - The Centraalbureau voor Schimmelcultures (CBS), which is an institute of the Royal Netherlands Academy of Arts and Sciences, located in Utrecht.

DAOM - Department of Agriculture, Ottawa, Mycology, Ottawa ON

ELISA - Enzyme linked immuno sorbent assay.

GpAb – goat polyclonal antibody

HpAb – human polyclonal antibody; atopic patient serum

HRP - Horseradish peroxidase. In the presence of a developing agent (hydrogen donor), the molecule is converts the colourless solution to one with colour, detectable and quantifiable with OD spectroscopy.

kDa – kilodaltons (1000 Da), where 1 Da is equivalent to the mass of one hydrogen atom.

LMW – Low molecular weight marker, used for protein size determination in immunoblotting. Expressed in kilodaltons (kDa)

mAb – monoclonal antibody

MEA – Malt extract agar

MW – molecular weight, expressed in kDa

OD – optical density, measured at 280 nm when estimating protein concentration, 450nm for ELISA protocols.

pAb – polyclonal antibody

Pen ch – *Penicillium chrysogenum*

P. variotii – *Paecilomyces variotii*

INTRODUCTION

1.1 - Fungi

Fungi comprise a taxonomic kingdom of more than 150,000 described species from an estimated 1.5 million species yet to be described (Hawksworth and Rossman 1997). Fungi represent around one quarter of the total biomass on the planet (Miller 1992). They are ubiquitous in nature, generally have semi-rigid cell walls, lack chlorophyll and must obtain carbohydrates from their surrounding environment. Fungi have developed a role in nature as the Earth's "recyclers". This is performed in most cases by their aerobic decay of non-living organic matter with degradative enzymes, and up-taking the accessible carbohydrate and other nutrients.

The genus of fungi studied here, *Penicillium*, is part of the group Ascomycota, which have both anamorphic and teleomorphic stages, although both stages are generally not observed. The anamorphic stage utilizes mitotic reproduction through spores in reproductive structures called asci. Some of the genus may also reproduce through meiosis alone, known as the teleomorphic stage. For many genera of fungi, the connections between the anamorphic and teleomorphic stages have not been made (Kendrick 2001).

Penicillium species are called "filamentous" fungi, their cells being arranged in long filaments called hyphae (Bush and Portnoy 2001).

Humans and fungi have been sharing the same environments and interacting for as long as humans have been on earth. Many fungi are capable of colonizing the indoor environment, provided suitable environmental conditions exist. Being ubiquitous in the environment, fungal growth will generally occur provided there are sufficient levels of water and nutrients (CHMC 1993). Moisture problems in the indoor environment can be the result of several issues including water coming in from the outdoor environment through a poor or failing roof, floor or wall construction, plumbing leaks, moisture produced by the occupants, or daily activities like bathing, washing and cooking. Disregarding floods or chronic water leaks, the most severe fungal infestation problems often occur when moisture is allowed to accumulate in the home because of poor ventilation or insufficient air circulation. For many fungi, condensation within building materials is sufficient for colonization. Most fungal species that colonize the indoor environment belong to three groups of fungi: the Ascomycetes, the Basidiomycetes and to a small degree, the Zygomycetes. With more than 50 orders, Ascomycota is a large and diverse group of fungi. They are often the primary colonizers of sugar and starch materials that are generally abundant in the indoor environment. These rapid growing fungi include species associated with food spoilage and building material damage (Kendrick 1992; Burnett 2003). However, for the most part, the fungi that are most commonly associated with indoor air contamination are anamorphic Ascomycetes. This group includes fungi that are commonly thought of by the lay public as indoor “molds”, often growing on food or structural materials (Flannigan et al. 2001; Denizel 1974). Reproduction by the indoor fungi is generally asexual, in many cases allowing their

external spores to be dispersed through air currents, wind or agitation (Flannigan et al. 2001).

Fungi can cause health problems in many ways and often the effects are incompletely understood. Many mycotic diseases have been studied, these infections range from cutaneous, localized or of an invasive nature. Health problems from exposure to fungi are thought to be largely from exposure to their toxic metabolites. These metabolites and mycotoxins cause upper respiratory disease, and other building related illnesses (Crameri et al. 2006). The cell walls of fungi also contain (1→3)-β-D-glucan, which is a compound known to elicit inflammatory responses in humans. Mycelial fragments and spores expose people to known allergens, as well as cytotoxic compounds (Górny et al. 2002). The current knowledge of human health effects from exposure to fungi is extensive, however, most researchers believe that the full spectrum of illness is not fully understood.

1.2 - *Penicillium chrysogenum*

Penicillium chrysogenum is a cosmopolitan fungus with a panglobal distribution. Colonized substrates include: dried foods, cheese, fruit, bread and building materials. The distribution of *P. chrysogenum* spans almost all biologically accessible regions and climates, making it among the most common eukaryotic life-forms on the planet (Pitt 2000).

Figure 1 - *P. chrysogenum* colonies on MEA medium; *P. chrysogenum* conidiophores at 920 X magnification (Flannigan et al. 2001).

There are many industrial and biotechnological applications of *P. chrysogenum*. Some of these applications include: penicillin production, treatment of pulp mill waste, production of polyamine oxidase, polyamide oxidase phosphor-gluconate dehydrogenase, glucose oxidase and as well as the incorporation in several biotransformations (Samson and Frisvad 2004).

P. chrysogenum has a distinct morphology, typically developing colonies with white to yellowish, dull blue to near greyish turquoise to dull green tones on CYA medium (Pitt 2000, Scott et al. 2004). Growth on 2% malt extract agar (MEA) medium generally produces more granular colonies with a grayish turquoise to dull green mycelium. However, depending on the substrate and strain, the colonies can exhibit substantial variations in colour. This fungus grows quite quickly on standard media as well as on environmental substrates at 25°C (Pitt 2000). The conidia are smooth-walled, globose to subglobose to broadly ellipsoidal. The spores exhibit a large size range, which can be heavily influenced by moisture levels. Not unlike many other similar fungi, *P. chrysogenum* can distribute spores into the environment at an extraordinary rate. A single spore colony of *P. chrysogenum* is capable of producing 10^{12} spores in as short of period as a few days (Miller 1992). The aerodynamic diameter, or diameter of a spherical particle having a density of 1 g/m^3 , is an important characteristic to describe the ability of a particle to infiltrate lung tissue (US EPA, 2007). Approximately one third of *P. chrysogenum* spores have an aerodynamic diameter less than $10 \text{ }\mu\text{m}$, and thus are small enough to penetrate into the lungs (Sorenson et al. 1996), but spore particles are typically smaller (Green et al. 2006).

For many years, researchers have been debating the homogeneity of the species. Genetic analysis of many strains from culture collections around the world have allowed certain segments of the species to be removed and re-named, as well as allowed the creation of four haplotypes, or clades for *P. chrysogenum*. This haplotype (clade 4) represents a

large majority of the strains found in indoor environment, and has a large geographic distribution. *P. chrysogenum* strains from clade 4 were more or less exclusively found in the indoor environment in localities across North America and Western Europe (De La Campa et al. 2007). Some have hypothesized that clade 4 strains must have a competitive advantage over other lineages to exploit the indoor environment (De La Campa et al. 2007; Scott et al. 2004).

The genus *Penicillium* represents a small fraction of the fungi in the outdoor environment; however, they represent a large portion of the species in the indoor environment (Scott et al. 2004). *P. chrysogenum* is considered to be the most prolific species of this genus to inhabit buildings (Pitt 1980). Scott et al. (2004) found that of the 109 Wallaceburg (Ontario) homes examined, *P. chrysogenum* was detected in 52%. Indoor growth occurs readily on food and building materials such as gypsum board. *P. chrysogenum* is often present in household dust in North America and Western Europe (Chang et al. 1995). The introduction of *P. chrysogenum* into the indoor environment is generally facilitated from sources such as food and decaying vegetation (Frisvad and Samson 2004; Pitt 2000).

P. chrysogenum moderately is xerophilic, with a optimal water activity (a_w) between 0.75-0.85 at 25°C (Flannigan et al. 2001; Santour et al. 2001). The definition of a_w is a ration of the vapor pressure of the air in equilibrium with a substance to the vapor pressure of pure water. Water activity values range from 0.0 to 1.0 (Madigan et al. 2000). The a_w is also influenced by the temperature, as well as the nutrients available to

the fungus (Grant et al. 1989). *P. chrysogenum* is a mesophile, and thus has an optimum growth temperature range of 20-30°C, with minimal growth temperature of approximately 4°C, 38°C being the maximum, and 28°C the optimum (Flannigan et al. 2001).

Many metabolites from *P. chrysogenum* have been described; roquefortine C & D, chrysogine, penicillin F & G are among the most recognizable (Samson and Frisvad, 2004). *P. chrysogenum* has become synonymous with antibiotics since Sir Alexander Fleming's serendipitous discovery in 1928. Since this unprecedented discovery, penicillin has revolutionized medicine. These β -lactam antibiotics have now saved countless lives and initiated researchers to study other compounds to combat microbial infection. The three most prominent antibiotics produced by clade 4 *P. chrysogenum* are penicillin G, roquefortine and meleagrin. Accumulation of the antibiotic xanthocillin X appears to vary between strains of clade 4 (De La Campa et al. 2007; Samson and Frisvad 2004).

1.3 - Indoor air quality (IAQ)

In recent times indoor air contamination has provoked significant concern by both the scientific community and increasingly the public at large. Developed countries in the northern hemisphere such as Canada, have an increasing proportion of the citizens spending most of their time in the indoor environment. There are many factors that are known to cause deteriorating levels of air quality in the indoor environment. Moisture problems from poor structural components of buildings, condensation on cold surfaces,

fungal spores and mycelial fragments, levels of home cleaning, penetration of particulate matter from the outdoor environment and the creation of particulate matter in the indoor environment are some of the primary components responsible for poor indoor air quality (Health Canada 2004). Some of the common indoor air contaminants include volatile organic compounds, β -1,3-D-glucan endotoxins, dust mite allergens and fungal allergens (Miller et al. 2007). For many of these examples, the levels of contaminants are often at much higher levels in the indoor environment than in the outdoor environment. Particulate matter is one source of indoor air pollution that is almost always found to be considerably more concentrated in the indoor environment (Pellizzari et al. 2007)

Over the years, several definitions of indoor air quality (IAQ) have been proposed. One basic definition stated by Fanger in 2006 is “IAQ is the extent to which human requirements are met”. However, the author does go on to explain this further, in that IAQ can be defined in several other ways. “We all want to ensure that air in the indoor environment does not have a negative effect on our health, and that we aim to have an air quality level that can increase productivity in the workplace, or to increase performance of children in schools” (Fanger 2006).

Others have attempted to define the quality of the air in the indoor environment by the chemicals and compounds that are present. This objective has proven difficult to realize however, due to the fact that there are generally hundreds or thousands of chemicals as well as biological matter in the indoor environment. Also, to make the situation even more difficult, many of the chemical and biological components in the indoor air

environment have not been fully characterized for human health implications (Fanger 2006).

Illnesses that have been commonly linked recently with declining indoor air quality include immune and allergic conditions. These conditions cause significant increases in health care costs, and lost productivity due to absenteeism. In developed countries, the rates of allergic and asthmatic disease have increased two-fold over the past twenty years. Many explanations have been proposed for this startling increase, but one idea that is increasingly popular with the scientific community is that this situation is largely influenced by indoor air contaminants, including fungi (Bornehag et al. 2004; Dales et al. 2002). Scientists now believe that this increase in poor indoor air is connected to developed countries increasing concerns with high-energy costs, and in response, greatly reducing the rates of ventilation in the indoor environment. New building materials and the drastic increases in electronic devices and appliances have also been associated with poor indoor air quality to some extent (Health Canada 2004).

Bornehag et al. (2004) were able to link indoor dampness in relation to ventilation to cases of asthma and allergies in the study of 11,000 children. This review concluded that moisture problems in buildings were a risk factor for health effects among atopic and non-atopic people. The authors also went on to say that a multi-disipline assessment of the agents responsible for the observed health effects is needed. The 2004 assessment of fungi in the indoor environment by Health Canada also came to the same conclusions. When ventilation in the homes was improved there was a statistically significant decrease

in moisture, indoor fungi and children's symptoms of allergies and asthma (Bornehag et al. 2004; Health Canada 2004). These observations have been repeatedly made over the last two decades and are now regarded as the major conclusion by recognized authorities (IOM 2004; Health Canada 2004; WHO 2004).

1.4 - IAQ and fungi

Since the rise of concern regarding IAQ and building related illness in the early 1980s, more than 30 large studies have been conducted examining respiratory health and the links with dampness and fungi (Health Canada 2004).

Figure 2 – Fungal contamination in the indoor environment
(Photo: Architectural Diagnostics Ltd.)

Many of these studies have concluded that indoor moisture problems and the fungi that generally thrive in those environments are at least partially responsible for many of the symptoms hypothesized to be associated with building related illness (Miller and Dales 1999). The table below lists some of the most common fungi found on building materials.

Table 1 - Fungi commonly found in the indoor environment (Flannigan et al. 2001)

<i>Alternaria alternata</i>	<i>Memnoniella echinata</i>
<i>Aspergillus sydowii</i>	<i>Paecilomyces variotii</i>
<i>Aspergillus versicolor</i>	<i>Penicillium aurantiogriseum</i>
<i>Chaetomium globosum</i>	<i>Penicillium chrysogenum</i>
<i>Cladosporium cladosporioides</i>	<i>Penicillium crustosum</i>
<i>Cladosporium sphaerospermum</i>	<i>Penicillium citrinum</i>
<i>Eurotium herbariorum</i>	<i>Stachybotrys chartarum</i>
<i>Eurotium repens</i>	<i>Ulocladium chartarum</i>

Aside from pathogenic fungi, determining that building related illness is caused by fungi in the indoor environment has proven difficult. Often, exposure is assessed by the occupant response to questionnaires, or obvious fungal growth observed in the building. However, in many cases, fungal growth may not be visible since it is located inside walls, in the ceilings, under the carpet or in basements (Dales et al. 1997). As such, present methods of associating the symptoms of building related illness, asthma and allergy with indoor fungal growth has not been established in a quantitative or sensitive manner. Assessing the connection between indoor fungi presence and building related illness has

also been difficult for reasons such as reliance on short-term grab samples analyzed only by culture, and because of an incomplete understanding of fungal allergens and poor quality or unavailability of purified allergen standards. In addition, it is known that there is a large variation in human responses to fungi (Daisey et al. 2003; Health Canada 2004).

In the 2004 Health Canada report examining the health effects of fungi in the indoor environment, several principal findings were presented. Four of the eight cross-sectional studies reviewed found a significant link between exposure to fungi and either physician-diagnosed asthma or asthma related symptoms. These studies observed the outcomes of indoor exposure to fungi at one point of time. These studies varied, but generally included subject questionnaires, home investigation for dampness and visible fungi, and physician assessment of occupant health (Dales and Miller 1999; Cuijpers et al. 1995; Garrett et al. 1998; Koskinen et al. 1999; Kilpelainen et al. 2001; Pirhonen et al. 1996; Li et al. 1996; Hu et al. 1997). Seven case-controlled studies investigating the connection between fungi and asthma found associations between fungi/damp environment and asthma. The basis for this group of studies is to compare the living conditions of those with diagnosed asthma or similar health problems with those without the conditions of interest. The indoor fungi-asthma/allergies connection was confirmed in most of the studies; however there were some conflicting reports. This series of experiments does include several variables (residences characteristics, different questionnaires, different geography and climates), which can create some conflicting results (Verhoeff et al. 1995; Strachan et al. 1995; Williamson et al. 1997; Hessel et al. 2001; Zacharasiewicz et al.

1999). This report also found that many studies examining the exposure effects of animal models to fungi, antigens or constituents, displayed similar immune response as those suspected in humans (Health Canada 2004). A *P. chrysogenum* extract inhalation experiment with 24 albino guinea pigs showed that IgM and IgG antibodies were detectable after 7 weeks, indicating an antigenic response to the proteins inhaled (Alonso et al. 1998). Intravenous experiments with C57B1/6 mice and BAL cell lines had also displayed similar immune responses with *P. chrysogenum* exposure (Cooley et al. 2000). Several other experiments studying the antigenic effects of fungi that are reviewed in the Health Canada report came to similar conclusions (Wang et al. 1994; Fogelmark et al. 2001; Alonso et al. 1997).

Thus, the challenges that face researchers in this field are numerous. Developing fungal allergen standards and methods to test their concentrations in the indoor environment is a crucial first step in this process of establishing human exposure levels. In order to accomplish this, a greater understanding of the immunobiology of human exposure to indoor fungi is also a necessary.

1.5 – Indoor environment and illness

Illness related to fungi growing indoors is by no means a modern conclusion. In the Old Testament, Leviticus (14:33-45) described this problem in a concise fashion; *“If the mildew reappears in the house after the stones have been torn out and the house scraped and plastered, the priest is to go and examine it and if the mildew has spread in the house, it is destructive mildew; the house is unclean. It must be torn down - its stones*

timbers and all plasters – and taken out of the town to an unclean place.” What is quite remarkable about this statement is three millennia later, we are still combating indoor fungi problems in a similar fashion (Cooley et al. 2004).

Little more attention had been paid to this issue from the days of the Old Testament until perhaps as late as the 1970s. In the mid-1970s, reports began to surface regarding possible health implications that may be elicited by poor indoor air quality (IAQ). Several years later in 1982, Sick building syndrome (SBS) was first described in modern literature (Repace 1982). SBS was first described in that “*there is something inside said building that is actually making people sick*” (Straus 2001). In the most recent reports, the term *sick-building syndrome* has been replaced by the more accurate term, *building related illness*.

1.6 - *Penicillium chrysogenum* and human illness

In the 1998 study by Cooley et al. the association of certain fungi and human illnesses were observed. This study compared negative health problems in schools and the presence of fungi in those buildings. In the schools examined, individuals with most of the allergic symptoms were found to attend the institutions with the highest counts of *P. chrysogenum*. One should note, however, that other symptoms generally not associated with allergenic conditions were also documented, leading investigators to believe that factors other than *P. chrysogenum* may be responsible for some of the adverse affects (Cooley et al. 1998).

Recent studies have correlated the exposure to *P. chrysogenum* spores and mycelial fragments with symptoms commonly associated to building related illness. Laboratory animal models in this study provided evidence that exposure to high levels of *P. chrysogenum* conidia led to increased airway eosinophilia and neutrophilia, as well as increased levels of IgG antibodies (Schwab et al. 2004). The authors did note, however, that further studies should be performed to determine the exact mechanisms inducing these allergic effects. Garrett et al. (1998) found that asthma in children was significantly associated with exposure to *Penicillium* during the winter months. Several other researchers had also come to conclude that there is a significant correlation between asthma and *Penicillium* exposure (Licorish et al. 1985).

1.7 – Allergies and asthma

Modern, industrialized nations have up to 20% of their populations affected by atopic allergies, where close to 10% suffer significant or severe allergic diseases (Crameri et al. 2006; Health Canada 2004). Skin test surveys have concluded that 3-10% of the population on a worldwide basis is affected by fungal sensitization. Asthma affects 5% of the general population, and 7 to 10 % of children. The upper limit for the attributable risk of fungi-induced asthma in Canada was estimated to be 20% of cases. This value was derived after excluding other common indoor factors affecting respiratory health such as pets, household smoking, dust mites, endotoxins and socio-economic factors (Dekker et al. 1991). The rise of asthma in the last decades has now put the condition on

the top of the list of chronic illness in children (Carter *and* Platts-Mills 1998; Pate et al. 2005).

The WHO estimated that in 2005 there were more than 300 million people suffering from asthma, with more than 10% of those people living in Canada and the United States, having one of the highest rates in the world (WHO 2007; Masoli et al. 2004). Allergic rhinitis was also found to be a prevalent allergic disease, with somewhere between 8% and 16% of the US population being afflicted (Malone et al. 1997; NIH 1990). The direct and indirect costs associated with asthma in the US are also very large and increasing. In 1990 the cost of asthma in the US was estimated to be \$6.2 billion dollars, and the 1998 estimates had increased to more than \$12 billion dollars. Modern figures have the cost exceeding \$20 billion dollars for the US national cost attributed to asthma (Mudarri and Fisk 2007). While the hospitalization costs have decreased in this time period, the increase in patients and medication costs have risen sharply (Weiss and Sullivan 2007; Weiss et al. 1992).

Despite many years of intense research, the exact immunological pathways that lead to allergic hypersensitivity are not yet completely resolved (Cramer et al. 2006). Aside from the incomplete understanding of the exact nature of an individual's development of an allergenic condition, the symptoms associated with this hypersensitivity are well established and readily identifiable by the medical and scientific community.

The symptoms associated with allergies include but are not limited to allergic rhinitis, conjunctivitis, and bronchial asthma. There are many clinical symptoms that may be present when an individual is afflicted with an allergic condition. These symptoms include sneezing, nasal discharge, coughing, wheezing, shortness of breath, urticaria, angioedema and anaphylaxis. These symptoms are generally due to a hyper-response of IgE antibodies to environmental allergens, most notably pollen, food, dust mites, animal dander, insect venom and fungal spores (Kurup and Banerjee 2000). Aside from the typical IgE response, there are several other allergenic responses that may induce symptoms. A genetic predisposition, in combination with environmental exposures, are thought to be the prerequisites for developing an allergic condition.

Generally speaking, allergens can be classified into five main categories based upon their enzymatic responses. These proteins are hydrolytic enzymes such as proteases, non-hydrolytic enzymes such as enolases, inhibitors such as trypsin amylase inhibitors, transport proteins such as lipocalins, and regulatory proteins such as heat-shock proteins (Kurup et al. 2002; Aalberse 2000).

1.7.1 - Hypersensitivity

An immune hypersensitivity or allergic reaction, has been separated into four different types of immunological reactions. These categories are separated based on the clinical presentation of the symptoms and the underlying mechanisms involved in the response (Horner et al. 1995). The four main classifications of hypersensitivity are anaphylactic or allergic, cytotoxic, immune complex induced and cell mediated. Each one of these

immunological reactions is induced by a unique agent of the immune system. Anaphylactic or allergic reactions are induced by antigens to immunoglobulin-E (IgE) antibodies present on the surface of mast cells or basophils. One's genetic make-up determines the level of the IgE-mediated reaction to the exposure to an antigen. An atopic individual is one who has a sustained elevated IgE levels. The second category of hypersensitivity is referred to as a cytotoxic response. In this case, antigens that are bound to host cells are recognized by IgG or IgM antigen-specific antibodies, and subsequently cell lysis occurs. In the third reaction category circulating immune complexes attach to vessel walls and activate complement (C3a, 4a and 5a). Neutrophils then phagocytize the complexes and induce a diffuse inflammation called vasculitis. These immune reactions cause afflicted individuals to exhibit a wide variety of symptoms; however, there are some ailments that are common in allergenic disease. (Bush et al. 2006, 2006; Horner et al. 1995). Some of these conditions are outlined in the following sections.

1.7.2 - Hypersensitivity pneumonitis

Hypersensitivity pneumonitis is one kind of inflammation of the alveoli and the bronchioles of the lung. This affliction is caused by an allergic reaction to inhaled material. Many people are more familiar with the generic terms for the condition such as *farmer's lung*. Being an allergic reaction, most of the damage that occurs on the lung tissues is caused by lymphocytes. The damage is from T-lymphocyte-mediated inflammation, often causing permanent lung damage (Lacey and Cook 1988).

1.7.3 - Allergic rhinitis

Rhinitis is one of the most common and obvious symptoms exhibited in individuals with allergic sensitivity to fungi. This symptom occurs as an emergence of nasal mucous membrane inflammation leading to nasal discharge, sneezing, and congestion. Fungal spores are an important cause of nasal allergy because they are so widespread in the indoor and outdoor environment. Spore particles are considerably smaller than other allergens such as pollen, and thus are more readily inhaled and absorbed by the respiratory system. In a study of 3371 patients, 25.8 % of those with a diagnosis of rhinitis were sensitized to fungi. A combined diagnosis of asthma and allergic rhinitis was recorded (Flannigan et al. 2001).

1.7.4 – Conjunctivitis

The conjunctiva is a thin membrane covering the eye and the inner eye-lids. Allergic conjunctivitis is a clinical irritation of this membrane, often due to exposure to an allergen. This may lead to redness, irritation, and fluid discharge of the eyes as well swelling of the eye-lids (Simoni et al. 2007; Immonen et al. 2001).

1.7.5 - Bronchial asthma

This disorder is a chronic inflammatory condition in which the airway develops an increased responsiveness to external stimuli, often allergens. This condition is characterized by bronchial hyper-responsiveness, increased mucus production, inflammation and intermittent airway obstruction. These symptoms can have a large range of severity, from irritating to life-threatening (Flannigan et al. 2001). This chronic

inflammatory disease has increased substantially worldwide over the past three decades (CDC 2003).

1.8 - Allergic clinical syndromes and indoor fungi exposure

Human exposure to fungi, fungal spores and fungal metabolites and secondary compounds has been documented to induce a large array of hypersensitivity responses. Many of these symptoms and illnesses have been long associated with fungi, but only recently have they been established in more quantitative terms. Many of the conditions experienced by those allergic to fungi are similar to allergenic symptoms experienced by those with symptoms elicited from other well-documented sources. Fungal allergens appear to be important agents of respiratory allergies, most notably, asthma (Cramer et al. 2006). An American study of almost 5,000 children from northeastern cities concluded that there was a strong correlation between the growth of fungi, moisture problems and several observed respiratory symptoms (Brunekref et al. 1989). The people living in residences with fungal contamination have also shown a 50% relative increase in cases of asthma, and increases of 60% in upper respiratory disease (Dales et al. 1991a, 1991b). In the United States alone, 4.6 (2.7-6.3) million of the total 21.8 million people reported to have asthma are estimated to have symptoms attributable to exposure to damp housing and fungi (Mudarri and Fisk 2007). The estimated cost of these cases is \$3.5 (\$2.1 - 4.8) billion dollars.

1.9 - Fungal allergens

Fungi are among the earliest observed sources of allergy, first report by Blackley in 1873. These century-old observations concluded that *Penicillium* and *Chaetomium* spores were likely the causative agents of hay fever. Today it is now recognized that fungi play a role in inducing respiratory allergy and asthma. A significant proportion of the population exhibits allergy to fungi (6-15%), when tested with the skin prick allergy test (Miller 1992), however, as noted earlier, this is a underestimate because of poor testing methods (Miller and Day 1997).

The fungi causing these conditions are found in all major groups of the kingdom, however, the Ascomycetes, are primarily responsible for fungal allergies. This group represents a major proportion of the total airborne spore-load encountered in the indoor environment. More than 60 genera of fungi have now been associated with the symptoms of respiratory tract allergy (Shen et al. 1999; Crameri et al. 2006). Some studies of the aerobiology of allergenic fungi enumerate 10 to 20 of the more common genera, based on an individuals response to 3 or 4 available allergen extracts. This method of allergy identification has many severe problems for those assessing affected people and for the patients themselves (Green et al. 2005a). Spores have been associated with fungal allergy for decades; however, non-spore fungal material is now regarded to be more relevant. Spore and hyphal fragments and degraded substrate particles, which also contain allergens, outnumber intact spores by more than 300:1. These particles are often smaller than 1.5 μm , and thus fall into the respirable size range (Gorny et al. 2002).

One of the most valuable tools for researchers to measure the health outcomes of environmental agents is the use of biomarkers. Biomarkers should be selective, sensitive, biologically relevant, practical, and available (Metcalf and Orloff 2004). Many biomarkers have been examined for this purpose, including complete blood counts, surface receptors of immune cells, humoral mediators like complement, cytokines and antibodies (Rydjord et al. 2005). Immunoglobulin G (IgG) antibodies have been proven useful in determining the extent and pattern of fungal exposure in individuals. This is a sign of exposure though, not necessarily of disease, since IgG antibodies have a half-life of a few weeks, and are gradually eliminated from the body when people are no longer having exposure to the antigen. However, some studies have shown that IgGs to fungi remain in some individuals 5-10 years after the source was thought to be eliminated. Several studies have shown that healthy individuals may often have IgG antibodies to specific indoor fungi, well above the normal threshold, and do not display allergic symptoms (Taskinen et al. 2002; Korppi et al. 2003).

To date, major and minor fungal allergens have been purified from cultures or created with recombinant techniques for *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus oryzae*, *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium culmorum*, *Penicillium chrysogenum*, *Penicillium oxalicum* and *Penicillium brevicompactum* (Shen et al. 1999, Bisht et al. 2003). There are more than 70 allergens for which amino-acid sequences have been obtained (www.Allergome.org, 2007). In the US, seven allergen product companies now offer 236 fungal allergen extract products. These commercial allergens represent 45 genera and 75 different species of fungi (Esch

2004; Miller and Day 1998). Never the less, most researchers in the field will agree that the list of fungal allergens is far from complete.

In most cases, the companies producing the allergen extracts would be getting their source strains from culture collections such as CBS or ATCC, but many strains for each species exists. Over 100 strains of *A. alternata* and over 200 strains of *A. fumigatus* are available from just these two culture collections. Another problem is that fungi have a high rate of somatic mutation and may occur as of heterokaryons to produce new gene combinations. Manufacturers must be careful to ensure that cultures are produced from single spore isolates to ensure genetic purity. There also are differences in the methods of producing the fungal cultures and producing extracts between companies (Esch 2005). Some have chosen the route of beginning the process with liquid cultures on orbital shakers to maximize biomass, while others have used static cultures and special growth media to maximize sporulation. The nutrient composition of the media, light and dark cycles, temperature, pH, and growth period are all important to allergen production and sporulation of the cultures. Since none of the companies producing the fungal allergens use the same conditions for producing their cultures, there are modest to extreme differences between the same extracts produced by different companies, even when using the same fungal stock culture. Even greater diversity occurs in the purification processes of the allergen extracts. The final extracts are a complex mixture of proteins, glycoproteins, polysaccharides and other compounds. Protein content of these extracts may range by as much as 7-fold and most extracts have 5 to 25 times more carbohydrate material than protein. Overall, there is yet to be a universally accepted method by the

industry, and until that critical break-through is developed, it is doubtful that the current situation will improve (Esch 2005).

Many of the allergens listed by the International Union of Immunological Societies – Allergen Nomenclature Sub-Committee and sold by private companies have been criticized for several reasons. Many problems are encountered when determining which proteins antigenic to humans should be used in fungal standardization extracts. Over the years, many articles have discussed the extreme variation among commercially available fungal allergen extracts (Esche 2005; Aas et al. 1980). Many of the allergen extracts produced commercially are complex mixtures of proteins. This creates many problems when standardizing samples, often extreme differences occurring between samples from different sources, or even among samples from the same company (Bush and Yunginger, 1987). One of the most studied fungi, *Aspergillus fumigatus*, has been suggested to produce over 80 antigens, regardless of the known allergens. This large array of target proteins creates a situation where standards produced by commercial companies have an uneven array of allergens or allergen combinations. Not only do the *A. fumigatus* allergen standards vary immensely in protein content, even the concentrations were found to vary as much as two-orders of magnitude, as was the case with *A. alternata* extracts (figure 3, Kodzius et al. 2003, Esch 2005).

Figure 3 - SDS-PAGE of commercial *A. alternata* allergen extracts from 7 different US laboratories (Esch 2005).

A key element that these researchers appear to have disregarded is that the proteins they have isolated may not be present in all strains of the species. That is, if one were to examine the protein content of several strains of a species found over a large geographic area, there lies the possibility that only some of the strains produce particular allergens, while others do not (Shen et al. 1999). Therefore, until these problems are resolved, measuring the prevalence of fungal allergy across populations can only be determined in approximate terms (Cramer et al. 2006). Aside from the points above, if the original source of the fungal allergy in the building is not definitively identified, treatment or diagnosis will not be successful, without extensive luck (Tarlo et al. 1988).

Many of the fungal allergens observed and worked with thus far are degradative enzymes such as serine proteases. Fungi release many enzymes into their environment to degrade organic material to be absorbed as nutrients. These proteins usually are hydrolytic enzymes, non-hydrolytic enzymes, inhibitors, transport proteins or regulatory proteins. Exposure to these allergenic proteins may be via inhalation, skin contact, maternal or paternal introduction. These proteins are often present in aerosol matter in the indoor environment when there is the presence of fungi. These proteins can be found on both the spores and mycelial fragments (Kurup et al. 2002).

1.10 – *P. chrysogenum*, asthma and allergens

As noted, *P. chrysogenum* is one of the most prominent fungi in buildings, more precisely, clade 4 haplotype strains of the species (Scott et al. 2004; De La Campa et al. 2007). This fungus has been shown to induce allergic response in mouse models (Chung et al. 2005; Cooley et al. 2000). Many other research groups have found correlations between illness associated with building related illness and the presence of *P. chrysogenum* contamination (Cooley et al. 1998; Wilson and Straus 2002).

In the 1991 experiments conducted by Shen et al., eleven antigenic proteins were identified. The proteins had molecular weights ranging from 94,000 to 20,000 Daltons. The authors suggest that the major allergens were the 68 kDa protein, responding to 56% of the 39 sera analyzed and a 64 kDa protein, responding to 46% of the allergic patient sera. Considerable cross-reactivity between some *P. chrysogenum* allergens and other

fungi has been reported. For example, Pen ch 13 and 18 were shown to cross react with extracts of *P. citrinum* and *P. brevicompactum* (Shen et al. 1996). Currently, five different *P. chrysogenum* allergens have been accepted by the International Union of Immunological Societies – Allergen Nomenclature Sub-Committee (table 2).

Table 2 - List of accepted *P. chrysogenum* allergens

allergen	biochemical name	MW (kDa)
Pen ch 13	alkaline serine protease	34
Pen ch 18	vacuolar serine protease	32
Pen ch 20	N-acetyl-glucosaminidase	68
Pen ch 31	calreticulin	-
Pen ch 33	-	16

(adapted from <http://www.allergen.org/Allergen.aspx>, accessed July 2007)

The allergens presented in this table were isolated from various *P. chrysogenum* strains from mainly from Asia. The researchers involved have not verified that all indoor strains of *P. chrysogenum* produce these antigens. Pen ch 31 and 33 have only been identified recently and literature discussing the antigens has yet to be published.

1.11 - Pen ch 13 and Pen ch 18

The sequences of Pen ch 13 and Pen ch 18 indicate that these proteins are similar in function and size. These serine proteases are 34 and 32 kDa in size, respectively (Shen et al. 2003). Shen et al. (1997; 1999) stated that these two proteins are the major allergens

for *P. chrysogenum* as well as *P. citrinum*, *P. brevicompactum*, and *P. oxalicum*. The IUIS Allergen Nomenclature Sub-committee has designated the 34 kDa allergen to group 13 (alkaline serine proteases) and the 32 kDa allergen to group 18 (vacuolar serine proteases) for *Penicillium* species (Shen et al. 2003). These two allergens share more than 47% amino acid sequence similarity. IgE antibodies from asthmatic patient sera were used to identify these proteins as allergens. Asthmatic patients from Taipei, Taiwan were examined and they found that 15-30% of the patient's sera were positive for the presence of the IgE antibodies. Amino acid sequence alignments have shown these two proteins to share between 60-80% sequence identity with serine proteases from *P. oxalicum* (Pen o 18), *A. fumigatus* (Asp f 18), and *Saccharomyces cerevisiae* (PRB1; Shen et al. 1995).

To isolate Pen ch 18, *P. chrysogenum* CCRC 30568 was grown for 6 days in CzapekDox medium for 6 days then extracted with PBS buffer. Then, centrifugation and dialysis against 50 mM NH_4HCO_3 , pH 8.0 were used to make the allergen extracts. Mapping of this protein found that there were at least 9 IgE binding epitopes, however, one epitope near the N-terminal of the protein was recognized by 75% of the allergic patients tested (Yu et al. 2002). Pen ch 13 was grown in culture medium, and then the proteins precipitated with ammonium sulfate and separated by ion exchange columns (Chou et al. 2002).

Both of these allergens have been documented to be causative agents of disease in humans and animal models. Damage to human lung cells through induction of the

release of mediators that induce the immune and inflammatory process has been performed in vitro (Tai et al. 2005).

1.12 - Pen ch 20

Cloning and other immunology techniques have shown Pen ch 20 to be a 68 kDa beta-N-acetylglucosaminidase. Most of the work done identifying and characterizing this allergen was performed using information gained through immunological studies with IgE antibodies from asthmatic patients, and then later, with specifically produced mAbs. This allergen has several glycosylation sites. The molecular weight was reduced from 68 kDa to 62 kDa when treated with a deglycosylating agent, indicating a significant portion of the allergens molecular weight being represented by sugar residues. This allergen was sequenced with cDNA library methods and was found to cross-react with other *Penicillium* species as well as species of *Aspergillus* (Shen et al. 1995).

The allergenic extracts studied were freeze-dried fungal preparations obtained from the Swedish company Allergon AB. Preparation of the samples was done by extracting 5g of the extract with PBS solution, centrifugation, and then the supernatant dialyzed with phosphate buffer. IgE immunoassays were used to characterize the produced cDNA clones. Cross-reactivity to three of the four mAbs produced was found in nine different *Penicillium* and *Aspergillus* species. The remaining mAb had a different pattern of cross-reactivity, indicating that a minimum of two epitopes to Pen ch 20 exists (Shen et al. 1995).

1.13 - Fungal allergy diagnosis

The current methods for assessing fungal allergy are either made through direct or indirect detection of allergen-specific IgE or assessing the observable biological consequences. Routine assessment is generally accomplished with standard skin-prick tests (SPT) or intra-dermal skin tests. SPT is generally utilized more often, being a relatively safer and more rapid method of assessing allergic response. Aside from these *in vivo* methods, there are many other *in vitro* methods of assessing patient serum for allergen specific IgE antibodies. Several enzyme linked immunosorbent assays and Western blot analyses have been developed in specialized laboratories to assess patient serum. Pharmacia ImmunoCAP system is widely considered to be the *gold standard* for determining the presence of allergen specific IgE (Cramer et al. 2006). Established ELISA test kits for dust mite, cat, dog, cockroach, mouse and rat allergens have proven to be useful (Pate et al. 2005; NAS 2000). However, few fungi allergens have been purified at present, and thus, determining fungal contamination has proven difficult.

Since research on fungal allergens in the indoor environment began in the Miller laboratory several years ago, three species have been investigated. The fungi examined were selected as representatives of common indoor species based on their water activity (A_w) levels. The three species investigated were *Stachybotrys chartarum* (Xu et al. 2007), a hydrophilic species, *Aspergillus versicolor* (Zhao 2006) a xerophilic species and *P. chrysogenum*, a fungus with moderate A_w . These three species encompass the entire range of A_w for indoor fungi. Identifying the presence of one or more of these fungi allows researchers to understand the niche present in a particular building. With that

information known, one could theorize which other fungi may also be present based on studies of indoor fungal populations under specific environmental conditions (Flannigan et al. 2001).

P. chrysogenum was studied in the present research for several reasons: (1) it is a very common indoor fungus, (2) it is a good representative of fungi with moderate water activity, (3) many studies have implicated it as a agent of allergy and asthma, and (4) few allergens have yet to be described for the species. In order to satisfy these goals, a series of experiments were performed to identify a protein antigenic to humans, and that was representative to a majority of the clade 4 strains found across Canada.

1.14 - Project aim

The goals of this research are to determine whether there is a fungal protein antigenic to humans, that is representative to all indoor strains (clade 4) of *P. chrysogenum*. If there is indeed a common antigen, the subsequent aim of the project is to purify the protein, verify its antigenicity in a goat-model, and then produce specific and sensitive monoclonal antibodies.

1.15 - Anticipated results

Previous research in the Miller laboratory has shown that other indoor species of fungi (*S. chartarum* and *A. versicolor*) produce human antigens that are common to a wide geographic representation of strains, and one would anticipate that similarly, this should be the case for *P. chrysogenum*. Established purification methods should allow the extraction of very pure antigen samples that can be used for antibody production and as allergen standards. Inoculation of laboratory test goats should allow verification that the purified antigen is indeed eliciting an immunogenic response and present in natural materials, and is not simply an artifact of one of the extraction procedures. The mouse monoclonal antibodies to be produced should have sensitivity and selectivity to the antigen. However, selectivity or cross-reactivity may present some issues due to the fact that *P. chrysogenum* is closely related to a number of other fungi common in buildings (Samson and Frisvad 2004).

1.16 - Techniques to assess fungal allergens

The techniques that will be utilized in this study have been shown to be useful by the Miller Laboratory as well as by other groups (Xu et al. 2007, Shen et al. 1999). The primary methods to be used in the initial screening, antigen selection process, and verification of activities will be assessed with immunoblotting, and enzyme-linked immunosorbent assays (ELISA). Purification of the antigens will be performed with a variety of techniques that have proven to be reliable and effective. Solvent and salt precipitation steps will be utilized for initial purification of the crude protein culture filtrates. After these initial protein extractions, the sample will be purified with various chromatography methods. The effectiveness of ion exchange, hydrophobic and size exclusion (gel filtration) will be assessed. Polyclonal goat antibodies will be produced by Cedarlane Laboratories Ltd. and the monoclonal mouse antibodies will be produced by Immuno Precise Antibodies Ltd. The antibodies will be assessed again with immunoblotting and ELISA.

REAGENTS

5X protein sample loading buffer - 15% SDS, 50% glycerol (EDH, USA), 0.05% bromophenol blue (USB), 5% β -mercaptoethanol (Sigma, St. Louis, MO) and 30% 624 mM Tris/HCl, pH 6.8.

$(\text{NH}_3)_2\text{SO}_4$ – ammonium sulfate, generally used for protein precipitations

AP-conjugated anti-human IgG antibody - alkaline phosphatase (AP) conjugated mouse anti-human IgG (Sigma, St. Louis, MO). Diluted 2000x in 1% BSA/TBST buffer for 1 h unless stated otherwise.

BCIP developing solution - Liquid substrate system (BCIP/NBT 5-Bromo-4-chloro-3-indolyl phosphate dipotassium/nitrotetrazolium blue chloride) for membranes (Sigma, St. Louis, MO). Typically 5 mL of solution added directly to membrane.

Blocking solution (immunoblot) - 1% (w/v) bovine serum albumin (BSA; Sigma) dissolved in TBST buffer (1 g of BSA dissolved in 100 mL of TBST, mixing for 1 h).

Coating buffer - Buffer promoting protein adhesion to ELISA plate. 50 mM carbonate-bicarbonate pH 9.6.

Developing solution – This solution was made up from 0.625 g sodium carbonate and made up to 25 mL with ddH₂O. A 5 µL aliquot of formaldehyde (37% w/v) was added just prior to developing, similar to the previous step.

Dialysis tubing - Spectra/Por 1 Membrane, Spectrum; molecular weight cut off 6-8 kDa, 40 ± 2 x 25.5 mm (Biolynx).

Enneatin media – culture media specific for protein production. The media contains 50 g maltose (Sigma, M5885), 8 g peptone (Difco, 211677), 5 g yeast extract (Sigma, Y0375), 0.75 g KH₂PO₂, 0.5 g MgSO₄·7H₂O and 0.067 g CaCl₂·2H₂O (Difco, P0662) per L of ultrapure de-ionized water.

Fixation solution - Fixation solution was composed of 7.5 mL ethanol, 2.5 mL glacial acetic acid and then made up to 25 mL in de-ionized distilled water.

Laemmli running buffer system (running buffer) - Tris, 15 g, glycine (Sigma St. Louis, MO) 72 g, SDS (USB, Cleveland), 10 g dissolved up to 1 L in ultra-pure water, Laemmli, 1970.

Q-sepharose anion exchange resin - Q Sepharose Fast Flow Anion exchanger, wet bead size: 45-165 µm (pre-swollen in 20% ethanol).

PBST buffer – Phosphate buffered saline, with Tween-20. PBST was made by dissolving 80 g NaCl, 26.8 g Na₂HPO₄·7H₂O, 2 g KCl, 2.4 g KH₂PO₄ and 1.0 g Tween 20 in 700 mL deionized water. The pH was adjusted to 7.4 with 1 M HCl and the solution was diluted 1 L with deionized water.

Phenol solution - Lowry assay reagents were prepared with 50 g Na₂CO₃ was dissolved in 400 mL ultrapure water, 0.5 g potassium sodium tartrate in 50 mL water and 0.25 g of CuSO₄ in 50 mL water. One mL of phenol reagent (Sigma, St.Louis, MO) was diluted by adding 8 mL ultrapure water.

SDS-PAGE gels (10%) - The 10% acrylamide gel was made by mixing 2.0 mL, 1.7 mL of 30% acrylamide (Bio-Rad), 1.3 mL of 1.5 M Tris/HCl buffer, pH 8.8, 50 µL 10% SDS, 50 µL of 10% ammonium persulfate (APS; USB) and 5 µL TEMED(N,N,N',N'-Tetramethylethylenediamine; USB). The stacking gel was made by mixing 1.4 mL deionized water, 0.33 mL 30% acrylamide mix, 0.25 mL 1.0 M Tris/HCl buffer (pH 6.8), 20 µL 10% ammonium per sulfate, 20 µL 10% APS and 2 µL of TEMED.

Sensitizing solution – This solution was made up of 7.5 mL ethanol, 1 mL sodium thiosulphate (5%w/v), 1.7 g sodium acetate, 125 µL glutardialdehyde (25%w/v) and then made up to 25 mL with de-ionized distilled water. The glutardialdehyde was added to the sensitizing solution directly prior to transfer to the gel.

Silver solution - This solution is made up of 2.5 mL of silver nitrate solution (2.5% w/v) and made up to 25 mL with ddH₂O. Just prior to adding the silver solution to the gel, 10 µL of formaldehyde (37% w/v) was added to the silver solution.

Stopping solution - This solution was made up from 0.365 g of EDTA-Na₂·2H₂O and made up to 25 mL in ddH₂O.

TBST buffer – This buffer was made by dissolving 60.5g Trizma base, 87.6 g NaCl, 5.6 g of Tween 20 (0.05%) in 600 mL deionized water, adjusted to pH 7.4 with 1 M HCl and then made up to 1 L with ddH₂O.

TMB developing solution (ELISA) - 3,3',5,5' tetramethybenzidine liquid substrate for ELISA (Sigma).

Towbin buffer (transfer buffer)- 12g Trizma base, 57.6 g glycine, 4 g SDS in 200 mL ethanol to 1 L with deionized water

Transfer membrane - Hybond-PVDF membrane (Amersham Biosciences), membrane used for protein transfer in immunoblotting.

Tris buffer – Buffer with stable conditions suitable for protein stability. 50mM Tris/HCL buffer, pH7.5 (6.057 g/L ddH₂O Trizma base ((NH₂C(CH₂OH)₃), acidified to pH 7.5 with 1 M HCl).

Ultrapure water – distilled, de-ionized water

MATERIALS AND METHODS

2.1 Culture production

All of the fungal strains were isolated from indoor air or materials from across Canada by Paracel Laboratories Ltd. (Ottawa, ON). The strains were identified by Dr. J. David Miller and most were confirmed by DAOM (Ottawa, ON; De La Campa et al. 2007). DAOM is the internationally recognized acronym for the National Mycological Herbarium, which holds over 300 000 specimens (both numbered and accessioned and major exsiccatae holdings) at the Department of Agriculture, located in Ottawa, Ontario. Each strain was grown and extracted with the same procedures described below. A complete list of all strains can be found in the tables 7 and 11 (appendix). Each strain was transferred onto sterile 2% malt extract agar (Difco: 214530, Lawrence, KS) slants or 2% malt extract agar plates and stored at 5°C.

A slant was macerated in sterile ultrapure water from which a 5% (v/v) aliquot was used to inoculate 2.8 L Fernbach flasks containing 560 mL of Enniatin medium or a 2.0 L Erlenmeyer flasks containing 400 mL. Enniatin medium is designed for high yields of peptides and proteins (Traber et al. 1989). The cultures were put on a rotary shaker at 220 rpm for 72 h in the dark at 25°C. All cultures were filtered through cheese cloth, separating the culture filtrate from the cells. The culture filtrate was pooled and stored at 5°C for later protein extraction. The mycelium was rinsed twice with ultrapure water, wrapped in aluminum foil and frozen. Once the cells were frozen, they were freeze-dried.

Spores were produced on rice cultures using the method developed by Murad et al. (1993). Uncle Ben's™ converted long grain rice (50 g) was placed in a 500 mL wide-mouth Erlenmeyer flasks with 30 mL of ultrapure water. The flasks were autoclaved for 30 min. The rice cultures were inoculated and then incubated at 25°C in the dark for 3 weeks and then air dried in an oven at 30°C. The spores were carefully removed from the rice grains with a spatula and stored in vials at -20°C.

2.2 - Extracellular protein extraction

The first extraction steps were designed to remove as much non-protein material as possible. Initially, one volume of ice-cold acetone (Caledon, ON) was slowly mixed with one equivalent volume of culture filtrate, resulting in a 50% acetone solution. The 50% acetone/culture filtrate was kept at -20°C overnight to allow precipitation. The non-protein material was removed by centrifugation (Beckman Coulter Avanti J-E) at 17, 400 x g for 5 min at -8°C. The non-protein material was precipitated and discarded, and then the supernatant was again mixed at a 1:1 ratio with more ice-cold acetone. This 75% v/v acetone/culture filtrate solution was again left at -20°C overnight to allow protein precipitation. The filtrate solution was centrifuged for 5 min as above. The protein precipitate was removed and left for 30 min in a fume hood to allow the remaining acetone to evaporate. The pooled protein was then dissolved in 50 mM Tris/HCl, pH 7.5 buffer (Tris-buffer, Sigma, T-1503), ensuring protein stability.

Since the total protein extract from the 75% acetone precipitation comprised many different proteins, a further salt precipitation was used to facilitate the isolation of target

proteins. A salt precipitation of increasing ammonium sulphate $(\text{NH}_3)_2\text{SO}_4$ (AS) fractions had been found to be useful in similar work in the laboratory. Sufficient AS was slowly dissolved in the solution until reaching 50% (w/w) saturation. AS solution was stirred in an ice bath for 1 h to ensure the salt was completely dissolved. This was left overnight at 4°C. The solution was then centrifuged for 10 min at 17,400 x g at 4°C. The pellet was separated from the 50% AS solution supernatant and dissolved in Tris-buffer. Dialysis tubing (molecular weight cut off 6-8 kDa) was utilized to de-salt the protein fraction, followed by concentration with 10 kDa cut-off Amicon Ultra centrifugal filter device (Millipore corp., MA). Further AS was slowly added to bring the 50% AS /culture filtrate solution to 70% saturation. The 50-70% AS culture filtrate solution was centrifuged for 10 min as previous and the pellet was dissolved in Tris buffer, de-salted and concentrated as above.

2.3 – Protein extraction from spores

All spore protein samples were extracted identically. One mg of harvested spores were weighed with a Mettler 163 analytical balance (± 0.02 mg) and then placed in a vial with a polystyrene bead. The vial with the spores was then milled for 30 minutes with a Spex-Certiprep mixer mill (model 5100, Metuchen, NJ). The crushed spore fragments were then dissolved in 0.1 mL of PBST for 2 h, while shaking at 4°C. Previous experiments had shown PBST to have the highest protein recovery for *P. chrysogenum* spores.

2.4 – Protein concentration determination

All protein fractions produced in the extraction procedures and purification experiments had the protein concentration determined prior to proceeding to the next test. Depending on the suspected concentration of each fraction based upon optical density values measured at 280 nm, the protein concentration was determined with the appropriate method.

The Lowry protein assay was utilized when the protein concentration of samples was relatively high. An aliquot (0.2 mL of sample diluted in ultrapure water) and 0.2 mL folin mixture was mixed well and left at room temperature for 10 min, followed by adding 0.4 mL diluted phenol solution. This sample was then placed in a 55°C water bath for 5 min, followed by immediate cooling on ice. The OD was measured at 650 nm (Lowry et al. 1951).

When the protein concentration was $> 20 \mu\text{g/mL}$, the bicinchoninic (BCA) method was used to determine the protein concentration with the Micro BCATM Protein Assay Kit (23232; Pierce, Rockford, IL), conducted according to the manufacturer's instructions. A 250 μL aliquot of working reagent was placed into a small tube, 250 μL of sample (diluted appropriately in ultrapure water) and then vortexed until homogenous. The tube was covered and placed in a 60°C water bath for 1 h and then quickly placed on ice. The OD was measured at 562 nm. A standard curve was used to determine the protein concentration.

2.5 - Enzyme linked immuno sorbent assay (ELISA) protocol

Once the protein content of each sample was determined with one of the protein concentration protocols, samples were normalized and assessed for total antigenic activity with ELISA experiments. Each protein concentration was replicated 3x.

The protocol for ELISA was performed as follows. Nunc-Immuno MaxiSorp plates were used in all experiments (M9410, Sigma-Aldrich). Each 100 μ L protein sample was diluted with coating buffer, then added to each plate well. For each well, 100 μ L [200 μ g] of protein was present. The plate was then incubated on a microplate shaker overnight at 4°C. Subsequent manipulations were done at room temperature. The plate was washed 3 x 5 min with 150 μ L PBST between incubations. The coated plate was blocked with 100 μ L of 2.0% BSA/PBST buffer (blocking buffer) for 1 h, washed as above and incubated for 1 h with 100 μ L of the primary antibody. The primary antibodies were diluted 1000x in blocking buffer unless stated otherwise. Human sera polyclonal antibodies (HpAbs) were used in the initial screening experiments, diluted 1000x. The plate was then incubated with 100 μ L of alkaline phosphatase (AP) conjugated anti-human IgG (γ -chain specific, Sigma, A-31A7) diluted 2000x in 1% BSA/PBST for 1 h. The colour was developed by adding 100 μ L of TMB developing solution, shaking for 10 min, and the colour development reaction was stopped by adding 50 μ L of 0.5M H₂SO₄. The plate was read in a microplate spectrophotometer at 450 nm (Max340PC, Molecular Devices, CA).

2.6 - Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity and molecular weight of the target protein fractions from each test were estimated using SDS-PAGE. The proteins were separated by SDS-PAGE (8.5 x 8 cm vertical slab gels) with 10% acrylamide gels using the Laemmli running buffer system. Before application to the gel, protein samples were mixed 4:1 (v/v) with a 5 x loading buffer, boiled for 5 min and then immediately placed on ice to cool. Electrophoresis was performed using a mini VE Vertical Electrophoresis System in an Amersham Biosciences electrophoresis tank (GE Healthcare) connected to a Power Pac 1000 gel electrophoresis power supply (Bio-Rad, Hercules, CA). Electrophoresis was carried out at 100 V for 20 min followed by 200 V for 50 min, or until the bromophenol blue dye reached 1 cm from the bottom of the gel. The gel was stained with GelCode Blue Stain (CBB stain; Rockford, IL). In the cases where protein concentrations were too low for CBB stain detection (≈ 50 ng), silver staining was utilized.

2.7 – Immunoblotting

Immunoblot analysis can detect one or more antigenic proteins in a mixture for which antibodies are present. This method was utilized in the initial screening process to determine the presence of proteins common to most strains, as well as possible cross-reactivity with other fungi.

The proteins were separated by SDS-PAGE (Section 2.5) and were transferred to a Hybond-PVDF membrane (Amersham Biosciences) with an electro-transfer unit (Hoefer

miniVE, Amersham Biosciences, UK). The transfer membrane and blotting pads were equilibrated in Towbin buffer for 30 min prior to the transfer. The transfer membrane was sandwiched with the SDS-PAGE gel between six blotting pads and four sponge pieces. The electro-transfer was carried out at a constant current of 400 mA for 30 min in an ice bath. After the transfer, the membrane was removed carefully from the transfer kit and incubated in 25 mL of blocking solution. After the 1 h incubation period, the blocking solution was removed and the membrane was washed for 3x5 min in TBST-buffer.

The membranes were then incubated in one of the primary antibodies overnight, shaking, at 4°C. The primary antibodies that were utilized in the initial human antigen assessment and purification optimization experiments were the human sera polyclonal antibodies (Section 3), and all the sera were diluted 2000 x unless stated otherwise. The membrane was then washed 3x5 min with TBST buffer and incubated with the alkaline phosphatase (AP)-conjugated anti-human secondary antibody diluted 2000 x in 1% BSA/TBST buffer, shaking at RT for 1 h. The membrane was then washed 3x5 min with TBST buffer. The proteins with immunological activity were stained by incubating the membrane in 5 mL of BCIP developing solution. In the HpAb sera experiments conducted examining the possible cross-reactivity of the target proteins to other fungi, the 75% acetone protein samples were compared.

All immunoblots testing the activity of the goat pAb (GpAb) were diluted 1000 x unless stated otherwise in the results. The corresponding secondary antibody was an alkaline

phosphatase (AP) conjugated rabbit anti-goat IgG (Sigma, St. Louis, MO) diluted 2000 x in 1% BSA/TBST buffer, shaking at room temperature for 1 h. The GpAb sera were initially determined to have a very high background. This effect was reduced with IgG purification steps, generating immunoblots that had significantly reduced background intensity.

Immunoblot experiments of the monoclonal antibody supernatants activity were tested with 500, 1000, and 200 x dilutions. The dilutions for figures 31, 32, and 33 were 2000, 500 and 1000 x, respectively. The secondary antibody used was an AP-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) diluted 2500 x in 1% BSA/TBST buffer, shaking at room temperature for 1 h.

2.8 - Antibodies

Immunoblots were done with three sources of antibodies. The first included human sera from atopic patients provided by ProGene Ltd., a large research-clinical laboratory in Lenexa, KS. These sera had been screened using Pharmacia ImmunoCAP® reagents for response to various fungi during clinical diagnostic testing (Halsey et al. 2002). After the tests were completed, the samples were stripped of identifiers and provided to the Miller lab for research purposes. A complete list of the human sera utilized in these experiments can be found in table 8 of the appendix. The human sera samples were diluted 2000x in 1% BSA/TBST unless stated otherwise in the results.

The GpAb were produced at Cedarlane Laboratories Ltd in Burlington, Ontario. Initially, each goat was injected with 5 mg of spores from *P. chrysogenum* DAOM strain 234 056, followed 23 days later by an injection of 1 mg of each protein cut from CBB stained electrophoresis gels (Section 2.9). The resulting GpAbs, from each stage of the inoculation and the terminal bleeds, were used in this project. A comparison of the performance of the GpAbs through the various stages was also performed in the present work.

To initiate the production of mAb producing hybridoma cells, mice were injected with 1 mg of the 52 kDa target protein from gel filtration. The pure protein produced in the Miller laboratory was sent to Immuno-Precise Antibodies Ltd (Victoria, BC) where the mice were inoculated and the initial testing of the mAb supernatant lines were performed. The results from mAb supernatant testing can be found in results table 6.1. Several dilutions were assessed for the mice cell supernatant antibodies, usually 1000x and 1500x. The dilution for figures 31-34 was 750x, tested against 1.75 µg of the target protein and 200 µg of culture filtrate, 75% acetone protein fraction for each fungal strain.

2.9 – IgG antibody purification

A protein G column (BioLynx Inc., Brockville, ON) was used to remove unwanted material from the GpAb sera. The first step of the antibody purification involved adding ammonium sulfate (AS) to the goat serum until attaining a 50% saturation level. This was done by slowly mixing 1 mL of 100% AS saturated ultrapure water with 1 mL of GpAb serum and leaving the resulting solution on a shaker over night at 4°C. The next

day the 50% saturated AS/GpAb serum solution was centrifuged at $17\,400 \times g$ for 10 min, removing the lipids and other unwanted material. The supernatant was discarded and the pellet was dissolved in 1 mL of PBS buffer. The AS remnants (de-salted) were removed with ultrafiltration (10 kDa cut-off). After sufficient desalting, the antibody solution was brought back to a 1 mL volume with Tris-buffer.

A protein G column (Sigma, St. Louis, MO) was washed with 10 column volumes of PBS binding buffer before applying the 50% AS/GpAb solution to the column. After washing the column, the antibody sample from the previous step was added to the column and incubated on a microshaker for 2 h at room temperature. Prior to antibody elution, 50 μ L of 1.5 M Tris/HCL buffer pH 8.8 (which lowers the pH of elution buffer to 7.0) was added to each of the collection test tubes. The IgG antibodies were eluted with 0.1 M glycine-HCL, pH 2.7, and collected in 1 mL/test tube fractions.

2.10 - Identification of protein content using CBB stained SDS-PAGE gels

CBB-stained gels were performed to visualize proteins and molecular weight determination. Aliquots of between 100-300 μ g of protein samples were added to each well, depending on the sample composition. When analyzing 75% acetone precipitate fungal samples, 200 μ g of protein was added to each well, unless stated otherwise in the results. Gels were typically run at 100 V for 20 min followed by 200 V for 50 min as explained in Section 2.5. The gel was stained with 25 mL of GelCode Blue Stain and incubated overnight, and then washed with ultrapure water for 30 min the following day. A protein marker was always run in a separate lane for molecular weight estimation.

2.11 – Antigen band removal from CBB-stained polyacrylamide gels

Target protein bands were removed from the CBB-stained polyacrylamide gels for the purpose of immunizing the polyclonal antibody production goats. Once the target protein bands were visualized with CBB stain, they were removed with a sterile scalpel. The target protein bands were then immersed in 40% methanol/ultrapure water to de-stain the gel target protein bands. After removing the CBB stain, the target protein gel bands were freeze-dried and stored at -20°C until required for GpAb production. The protein bands from several gels were combined for the required 1 mg of target protein for GpAb production.

2.12 - Identification of protein content using silver stained SDS-PAGE gels

The silver staining of SDS-PAGE gels was performed to determine the total protein content and purity of samples when CBB staining would not provide the required sensitivity. Samples were loaded and run on SDS-PAGE in the exact fashion as explained with CBB staining.

The gels were removed from their casts and stained as follows. The gel was immediately submerged in 25 mL of fixation solution for 30 min. After the half hour, the fixation solution was removed and the sensitizing solution directly added. The gel was incubated in the sensitizing solution for 30 min. After removing the sensitizing solution, the gel was washed 3x5 min with ultrapure water. Silver solution was then added on the gel for 20 min. Following the incubation, the silver solution was removed and the gel washed

for 2x1 min with ultrapure water. Developing solution was then added and left on the gel until the protein bands developed to a desired intensity, approximately 5-10 min. After the gel had developed as required, the developing solution was removed and stopping solution was added and allowed to stand for 10 min. After the solution had been left for 10 min, the stained gel was washed with ultrapure water and then scanned with a GS800 densitometer imaging system (Bio-Rad).

2.13 - Protein molecular weight determinations

The native molecular weights of the proteins were estimated from a calibration plot. A low molecular weight calibration kit for SDS electrophoresis (Amersham Biosciences) was used as molecular weight markers with the molecular weight range between 14-97 kDa. The proteins that composed the molecular weight marker were as follows: phosphorylase B (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lactoalbumin (14.4 kDa). The gel electrophoresis was performed as detailed above (Section 2.5).

2.14 - Ion exchange chromatography

The first step of the target protein purification process was anion exchange chromatography. Previous studies have shown that this is an effective method to separate samples based on protein charge. Anion exchange chromatography was performed using a Q-Sepharose system and placed in a 1 x 17 cm glass column.

Experiments to optimize the NaCl elution concentration are summarized in results section 4.2. The anion exchange column was washed with 50 mM Tris-HCl buffer, pH 7.5 until the UV optical density at 280nm was consistently below 0.04. Approximately 50-80 mg of protein from the 50-70% AS sample was loaded at a flow rate 1 mL/min. The column was washed with Tris buffer until the UV optical density was again negligible. The protein content on the column was eluted with increasingly concentrated NaCl-Tris buffer solutions. Fractions were collected at 0.6 M NaCl-Tris buffer, 0.7 M NaCl-Tris buffer, 0.8 M NaCl- Tris buffer and 1.0 M NaCl-Tris buffer. The target *P. chrysogenum* protein fraction was collected at 0.8 M NaCl-Tris buffer at 1 mL/min in a 5 mL/tube fraction with a fraction collector (RediFrac collector, Amersham Bioscience) in 10 mL test tubes. Each fraction was desalted and assessed for target protein activity with immunoblotting, and the purity was examined with silver stained SDS-PAGE gels.

The column was washed and regenerated with Tris-buffer until the OD at 280 nm of the wash was zero. If the concentrated NaCl-Tris solution did not sufficiently clean the column, other measures were adopted. In this case, 2 mL of 1.0 M NaOH was run through the column, followed by a 50 mL wash of ultrapure water. This process was repeated with 75% ethanol followed again by a 50 mL wash with ultrapure water. In order to sufficiently remove unwanted protein from the column, this process was repeated several times.

2.15 - Gel filtration chromatography

The protein fractions from the anion exchange column had removed most of the protein content from the target protein samples, thus allowing the use of size exclusion

chromatography (gel filtration). Once the ion-exchange samples contained 5 or fewer major protein bands detectable with silver stain SDS-gels, gel filtration was appropriate to separate these individual proteins based upon molecular weight. Sephacryl S-200-HR (Sigma-Aldrich) was chosen as an intermediate separation gel. The resin was slowly loaded onto a Sepharose column (96 x 1.6 cm) and allowed to settle for 24 h at 4°C. A solution consisting of 0.15 M NaCl -Tris buffer was allowed to flow through the column at 0.4 mL/min for ≥ 12 h to prepare the column. A 0.5 mL concentrated aliquot of protein fraction sample (approximately 1.5 mg protein) was slowly loaded onto the Sepharose column using the 0.15 M NaCl-Tris buffer at 0.4 mL/min. After loading, two additional 1 mL portions of buffer were added to load the proteins on the column. The 0.15 M NaCl-Tris buffer was run through the column at flow rate 0.4 mL/min. Fractions were collected with a fraction collector at 1 mL/tube in 10 mL test tubes. The proteins present in each fraction were identified with immunoblotting and silver stained SDS-PAGE gels.

RESULTS

3 – *P. chrysogenum* human antigen screening

3.1 - Initial antigen screening with ELISA

Eighteen strains of *P. chrysogenum* were initially screened for antigenic response to HpAbs. The *P. chrysogenum* DAOM numbers and human serum identification numbers can be found in appendix tables 8 and 9. ELISA was the method utilized to perform the mass screening of total activity of each strain in relation to each human polyclonal antibodies (HpAb). Initially a series dilution of a moderate performing HpAb determined a dilution of 1000x allowed a fair comparison between each HpAb and each *P. chrysogenum* strain. Each well contained 200 µg of total protein. The data from this series of tests are displayed in figures 6-9. For clarity, a selection of the *P. chrysogenum* strains and the HpAbs examined are shown in the figures 4 and 5. The raw data used for these figures can be found in tables 10 to 12 (appendix).

Figure 4 - ELISA response of *P. chrysogenum* strains against HpAbs. DAOM numbers and symbols are given across the top. HpAbs numbers are designated at the bottom (n = 18).

Figure 5 - Average ELISA response for each *P. chrysogenum* strain against all HpAbs. Standard deviations are indicated for each sample (n = 108).

The initial screening process with ELISA displayed a range of total responses for each strain. The human sera also had varied responses to the different strains of *P. chrysogenum* examined. There were statistically significant differences in the response of some of the HpAbs to the strains (figure 5). Some of the HpAbs that responded to many proteins with immunoblotting, had a lower response with ELISA, whereas, some of the HpAbs that predominantly responded to the target proteins had higher ELISA responses.

3.2 – Initial screening of each *P. chrysogenum* strains with SDS-PAGE and immunoblotting

P. chrysogenum strains were tested for antigenic response to the HpAbs. This was done to determine whether there were common proteins antigenic to allergic patient sera produced by all 17 strains of *P. chrysogenum*. The immunoblot figures below represent a wide geographic and genetic array of *P. chrysogenum* strains (see Table 8). The first set of immunoblots was generated to determine whether the HpAb collection displayed similar activity patterns to each strain. The *P. chrysogenum* samples tested were the 75% acetone protein fractions, 200 µg protein in each lane.

Figure 6 - Immunoblot comparing the a selection of HpAbs to DAOM 234 056. Each lane had 30 μ g of protein.

Figure 7 - Immunoblot comparing the response of HpAbs to *P. chrysogenum* strains.

The primary HpAbs are designated across the top and were diluted 1000x in blocking solution. Each lane contained 200 µg of protein.

1 – DAOM 234056

2 – DAOM 234054

Figure 8 - Immunoblot comparing the response of the designated HpAbs to the DAOM 234 054.

Figure 9 - Immunoblot comparing the response of the designated HpAbs to DAOM 234 054.

After the initial target protein screening immunoblot experiments, proteins were identified as suitable for further investigation. A 52 kDa fungal protein that was recognized by all HpAbs tested was observed in all of the *P. chrysogenum* strains. Another fungal protein that responded to most of the HpAbs and was present in all of the strains was a 40 kDa extracellular protein. Although varying in intensity, both of these proteins were observed to respond to all of the HpAbs. The 52 kDa and 40 kDa arrows in figures 6-8 indicate these human antigens. Some of the HpAbs only showed responses to the 40 and 52 kDa proteins, but had ELISA values that were as strong or stronger than other HpAbs that responded to many proteins (table 3).

3.3 – Comparison and analysis of activity between various strains of *P. chrysogenum*

The next series of experiments involved determining whether the 52 and 40 kDa proteins were found in all available indoor strains of *P. chrysogenum*. This set of experiments was also intended to determine which strains produce the strongest response to the 52 kDa and 40 kDa target proteins. Immunoblots were utilized to compare the activity between the different strains. A selection of the most reactive HpAbs was used in this series of experiments. Images of the comparison immunoblots are found in figures 10 to 14. The proteins that had activity to the HpAbs are displayed in table 3. The HpAbs and *P. chrysogenum* strains shown were a selection that was studied in the most detail. They represent a cross section of both the HpAbs and fungi. The arrows and labels indicate the target proteins and previously reported allergens. Pen ch 20, Pen ch 13, and Pen ch 18 have molecular weights of 68, 34 and 32 kDa, respectively.

Table 3 - *P. chrysogenum* proteins with activity to HpAbs.

DAOM 155 627

Protein MW	QC 1288	QC 1290	QC 1303	QC 1307	QC 2397	QC 2398
97					97	
68 (Pen ch 20)	68	68	68	68	68	68
60			60	60	60	
52	52	52	52	52	52	52
40	40	40	40	40	40	40
34 (Pen ch 13)						
32 (Pen ch 18)					32	
16 (Pen ch 33)					16	

DAOM 155 628

Protein MW	QC 1288	QC 1290	QC 1303	QC 1307	QC 2397	QC 2398
97						
68 (Pen ch 20)			68	68	68	
60						
52	52		52	52	52	52
40			40		40	40
34 (Pen ch 13)			34			
32 (Pen ch 18)						
16 (Pen ch 33)						

DAOM 215 337

Protein MW	QC 1288	QC 1290	QC 1303	QC 1307	QC 2397	QC 2398
97						
68 (Pen ch 20)		68			68	68
60					60	
52						52
40						40
34 (Pen ch 13)			34			
32 (Pen ch 18)		32	32		32	
16 (Pen ch 33)					16	

Table 3 continued - *P. chrysogenum* proteins with activity to HpAbs.

DAOM 234 052

Protein MW	QC 1288	QC 1290	QC 1303	QC 1307	QC 2397	QC 2398
97					97	
68 (Pen ch 20)	68	68	68	68	68	68
60			60	60	60	
52	52	52	52	52	52	52
40	40	40	40	40	40	40
34 (Pen ch 13)						
32 (Pen ch 18)					32	
16 (Pen ch 33)					16	

DAOM 234 054

Protein MW	QC 1288	QC 1290	QC 1303	QC 1307	QC 2397	QC 2398
97					97	97
68 (Pen ch 20)	68		68	68	68	68
60	60				60	
52	52	52	52	52	52	52
40	40	40	40	40	40	40
34 (Pen ch 13)			34			
32 (Pen ch 18)					32	32
16 (Pen ch 33)						16

DAOM 234 056

Protein MW	QC 1288	QC 1290	QC 1303	QC 1307	QC 2397	QC 2398
97					97	97
68 (Pen ch 20)			68	68	68	68
60	60					60
52	52	52	52	52	52	52
40		40	40	40	40	40
34 (Pen ch 13)			34	34		34
32 (Pen ch 18)					32	
16 (Pen ch 33)						

Table 3 continued - *P. chrysogenum* proteins with activity to HpAbs.

DAOM 234 057

Protein MW	QC 1288	QC 1290	QC 1303	QC 1307	QC 2397	QC 2398
97					97	
68 (Pen ch 20)				68	68	
60					60	60
52		52	52	52	52	52
40			40	40	40	40
34 (Pen ch 13)						
32 (Pen ch 18)					32	32
16 (Pen ch 33)						

DAOM 234 059

Protein MW	QC 1288	QC 1290	QC 1303	QC 1307	QC 2397	QC 2398
97					97	97
68 (Pen ch 20)		68		68	68	68
60				60		
52	52	52	52	52	52	52
40	40	40		40	40	40
34 (Pen ch 13)						34
32 (Pen ch 18)						
16 (Pen ch 33)

Figure 10 - Immunoblot comparing *P. chrysogenum* DAOM strains reacting to HpAb QC 2398. DAOM identification number is indicated at the bottom of each lane.

Figure 11 - Immunoblot of *P. chrysogenum* strains against HpAb QC2397.

Figure 12 - Immunoblot of *P. chrysogenum* strains against HpAb QC2398.

Figure 13 - Immunoblot of *P. chrysogenum* DAOM strains against HpAb QC1303.

Figure 14 - Immunoblot of *P. chrysogenum* DAOM strains against HpAbs QC 1307 (A) and QC 1290 (B).

All of the *P. chrysogenum* strains were immunoblotted against three of the HpAbs that were found to have the strongest responses with ELISA experiments. The common target proteins, 52 and 40 kDa were present in all of the different chemo/genotypes of *P. chrysogenum*. The HpAbs were tested against the remaining strains to confirm suitable target proteins. The remaining HpAbs reacted to the target proteins, however, some these antibodies had a weaker response when compared to the top-three performers. The overall best performing HpAb was QC2397. This HpAb had the strongest response to two potential target proteins. Replication of the tests above determined that HpAb QC2397 was the most suitable primary antibody for further screening and purification research. QC2398 and QC1307 were also utilized in further experimentation to confirm QC2397 results. Antigenic responses were also noticed for proteins with molecular weights of 68, 34 and 32 kDa and possibly Pen ch 20, 13 and 18. DAOM 215 336 and 215337 appeared to have very distinct antigenic proteins with molecular weights approximately 34 and 32 kDa, the proposed molecular weights for Pen ch 13 and 18.

All of the *P. chrysogenum* strains produced the two target proteins, although, in variable yields. DAOM strain 234056 had the highest yields and was selected for studies on isolation and purification of target protein. This strain is also a member of the clade 4 haplotypes for the species, in accord with previous research. This strain was also selected to be the best strain to produce polyclonal antibodies in goats.

3.4 – Cross-reactivity testing of candidate target proteins

Before moving forward with the purification of the 52 and 40 kDa target proteins, the possibility of cross-reactivity with other fungi was investigated. Immunoblotting experiments were performed examining the responses of each of the other fungi compared to *P. chrysogenum*. The protein concentrations were all normalized (Figure 15). Similar to the previous immunoblots, each 200 µg sample from the various species tested was the 75% acetone protein fraction.

The initial cross-reactivity testing showed the other fungi did not react with the *P. chrysogenum* target antigens. *S. chartarum* and *A. versicolor* cross reacted with the HpAbs, but not to the *P. chrysogenum* target proteins. Silver stain analysis of the protein extract from the culture filtrate showed that *S. chartarum* and *A. versicolor* proteins reacting had molecular weights different from the 52 kDa target antigen (figure 16).

Figure 15 - Immunoblot comparing *P. chrysogenum* to other fungi with HpAb QC2397.

Figure 16 - Silver stained SDS-PAGE gel comparing the molecular weights of proteins present in the culture filtrates of fungi. The 52 target protein is indicated by the black arrows in lanes 2 and 3. The larger protein from *S. chartarum* is indicated in lane 4 (red arrow).

4 – Polyclonal antibody production

4.1 – Goat polyclonal antibody production

Two goats were initially injected with 5 mg of *P. chrysogenum* 234056 spores. After 21 days, one goat was injected with 1 mg of 52 kDa target protein and the other goat with 1 mg of 40 kDa target protein. The protein samples were injected in the goats prior to the gel filtration experimental progress. The purified samples were 0.08 M NaCl fractions from the anion exchange column that were run on SDS-PAGE gels, and then stained with CBB stain. Once the target protein was visualized, the bands were cut out of the SDS-PAGE gel, de-stained, and freeze-dried.

4.2 – Analysis polyclonal antibodies from goat test-bleeds

The goat serum polyclonal antibodies (GpAb) response from the spore boosted and antigen boosted test bleeds were compared against *P. chrysogenum* spore and protein samples. Immunoblots were utilized to examine the response of each GpAb to *P. chrysogenum* protein and spore samples. Each of the GpAbs was tested against both *P. chrysogenum* spores and 50-70% AS protein samples (DAOM 234 056). The following images compare the responses of each boost stage.

Figure 17 - Immunoblots comparing the 52 kDa target protein boost (lanes 1, 2) and the spore boost (lanes 3, 4) of the goat. Each lane had 200 µg of protein. The spore extract and 50-70% AS protein fraction are from DAOM 234 056. A response to the 52 kDa target protein (black arrows) and the decreased response to other antigens are indicated (red arrows).

The spore boosted GpAbs (lanes 1 and 2) had a response to the 52 kDa target protein, indicated by the arrows in figure 17. The second, 52 kDa target protein boosted GpAb had a reduced response to the large matrix of proteins present in the spore samples and 50-70% AS fractions, but maintained a consistent response to the targeted proteins. The increased specificity was especially clear with the response to several antigens in the spore samples. The intensity of the response to the target protein was increased, whereas there was a decrease in the response to the other proteins (figure 15; target protein, black arrows). The response to Pen ch13 and 18 (figure 17; red arrows) were prominent after the spore boost, but the response could not be visualized after the second target protein boost (figure 17; lanes 3 and 4).

The immunoblots that were done for this initial testing of the GpAbs were found to have high-background signals when developed. To enhance these images, an antibody purification procedure was performed on the sera. After the IgG antibodies were purified with a protein-G column, there was significantly less background noise with the immunoblots. An image of the immunoblot using the purified GpAb is shown in figure 18. The second figure contrasts the specificity of the GpAb to the HpAb utilized in the initial target protein screening experiments. Other *Penicillium* species were included immunoblots (figures 20-22) to investigate possible cross-reactivity to the target proteins.

Figure 18 - Immunoblot of 52 kDa target protein boosted GpAb against the extracellular and spore fungal protein.

Figure 19 - Immunoblot of HpAb QC2397 against the extracellular protein content from fungi.

When figures 18 and 19 were compared, there was increased specificity of the boosted GpAb to the target proteins. The HpAb had more than 10 distinct bands reacting to the *P. chrysogenum* extracellular protein extract, whereas, the purified GpAb reacted to 5 bands in the same protein extract. The reaction to the targeted proteins was also much stronger in comparison to the other minor bands with the GpAb than with the HpAb. Also, the proteins that cross-reacted with other *Penicillium* species when examined with the HpAb were not apparent when developed with the purified GpAb.

The following images are immunoblots further examining possible cross-reactivity of the GpAbs to other fungi.

Figure 20 – Immunoblot comparing cross-reactivity of the 52 kDa target protein boosted GpAb to fungi protein extracts.

52 —→
kDa

40 —→
kDa

234 056 spore
5457 spore
P. crustosum 245 045
P. brevicompactum
234 049 P. variotii 235 357
234 056; 50-70% AS
S. brevicaulis 5869-3
A. alternata 27.1

Figure 21 - Immunoblot comparing the cross-reactivity of the 40 kDa target protein boosted GpAb to various fungi.

Figure 22 - Immunoblot comparing fungi reactions to purified target protein boosted GpAb. The target *P. chrysogenum* protein (black arrow, lane 7) and smaller *P. crustosum* protein (red arrow, lane 8) are indicated.

Most of the other fungi did not react to the 52 kDa target protein boosted GpAbs. *P. crustosum* DAOM 245045 had a response to a protein close to the 52 kDa target protein size when tested with both purified GpAbs. Silver staining and further immunoblotting demonstrated that this protein had a smaller molecular weight than the 52 kDa target protein (figure 38, figure 22, respectively).

The GpAb response to the spores, more specifically, the presence of the target protein in the spores, showed that the target protein does elicit an immune response. When the initial spore boosted GpAb was compared to the target protein boosted GpAb, there was a consistent response to the target protein and a decreased response to the matrix of other proteins present in the spores. The results from the target protein boosted GpAb experiments showed that the 52 kDa target protein had a strong response to the boosted GpAbs and was a suitable target for further study.

5 – Purification of target proteins from *P. chrysogenum*

The first purification step was performed prior to examining the response of the protein content from the different strains of *P. chrysogenum* (chapter 3). This first step was an acetone protein precipitation. This step involved performing a 1:1 v/v protein precipitation with ice-cold acetone. After centrifugation, the pellet was removed and the supernatant had a subsequent 1:1 ice-cold acetone precipitation performed (75% v/v acetone/supernatant). The first 1:1 acetone pellet was tested for immunoblot activity and was found not to exhibit any response to the HpAbs. The 75% acetone/supernatant solution had activity to a multitude of proteins. All subsequent purification steps were performed with the 75% acetone protein fraction. The 75% acetone fraction from an 8L culture yielded 400 mg of protein.

5.1 – Ammonium sulfate precipitation proteins

A series of ammonium sulfate (AS) protein precipitations were performed on the 75% acetone protein fraction. The AS fractions were as follows: 0-30%, 30-50%, 50-70% and 70-80% AS saturation and the corresponding images are shown on the following pages.

Figure 23 - A CBB protein stain comparing the total protein content of each *P. chrysogenum* AS fractions. The 52 kDa target protein was most concentrated in the 50-70% AS fraction (arrow; lane 3). The 40 kDa target protein can be visualized as well (arrow; lane 5).

Figure 24 - An Immunoblot comparing the activity of each *P. chrysogenum* AS precipitation fraction. The primary antibody was HpAb QC2397.

After reviewing both the CBB protein stain and the corresponding immunoblot of each AS fraction, the 50-70% AS fraction was found to contain most of the target proteins. This fraction also exhibited the strongest signal when examined with immunoblotting, and thus was determined to be the most appropriate sample to continue the purification process.

5.2 – Ion exchange

Anion exchange chromatography was utilized to further purify the target proteins. Similar to AS precipitation, a series of NaCl-Tris buffer concentrations were examined. The results of the anion exchange chromatography experiments are displayed in figures 25-26.

Figure 25 - Silver stained SDS-PAGE gel of each eluted fraction from the anion exchange chromatography. The target antigen was observed in the 0.08 NaCl fraction (lane 4; arrow), and the 40 kDa target protein was visible in the flow through (lane 8; arrow).

Figure 26 - Immunoblot of each elution fraction from anion exchange chromatography using HpAb QC2397 as the primary Ab, diluted 2000x. The 52 and 40 kDa target protein are indicated with arrows (lanes 4 and 7, respectively).

The silver staining and immunoblotting of the 50-70% AS protein fraction showed that the 52 kDa target protein had eluted at a NaCl-Tris buffer concentration of 0.08 M. This sample was considerably more pure than the previous AS protein sample. The 50-70% AS sample injected into the anion column had 14 bands that were detectable with silver staining, and after the target protein fraction was eluted, only five protein bands were detectable. The 52 kDa protein represented more than 75% of the total protein content in this fraction. The anion exchange partially purified 52 kDa target protein sample was utilized for the GpAb boost.

The 40 kDa target protein passed through the anion exchange column in the initial 0.0 M NaCl-Tris buffer wash. The wash contained more than 10 detectable proteins when visualized with silver staining.

5.3 – Gel filtration

The 0.08 M NaCl anion exchange partially purified 52 kDa target protein fraction was further purified with gel filtration (size exclusion chromatography). The concentrated and desalted 0.08 M NaCl anion exchange sample was eluted at a flow rate of 1 mL min⁻¹. The results from this experiment are found in Figures 27-29.

Figure 27 - Graph of the optical density measured at 280nm for each fraction eluted from the gel filtration column at a flow rate of 1 mL min⁻¹.

Figure 28 - CBB stain of each protein fraction eluted from the gel filtration column. The target protein is indicated by the arrows in lane 1 and 3.

Figure 29 - Silver stain target protein fraction eluted from the gel filtration column. The target protein is indicated by the arrows.

The purity of the 52 kDa target protein had improved to over 95% after the gel filtration purification experiments. When the gel filtration sample was silver stained (Figure 29) only the 52 kDa target protein was visible. The activity of the protein sample was confirmed with immunoblotting using the target protein boosted GpAb. The protein shown to be purifiable and was adequate for use as a standard, for amino-acid sequencing, or for monoclonal antibody production.

Table 4 - Recovery of 52 kDa target protein through each purification step.

purification step	total protein (mg)	target protein (mg)	yield (%)
acetone precipitation	300	8.2	100
ammonium sulphate precipitation	68	7	85
ion exchange	5.2	1.3	16
gel filtration	0.8	0.8	9.8

6 – Monoclonal antibodies

Prior to generating the mAb hybridoma cell lines, cell supernatants were produced, collected and initially tested with ELISA and dot-blot for activity. A complete list of the results is also presented the table 14 (appendix).

Figure 30 - ELISA results for cell supernatants. Responses are to IgG Abs, except the cell supernatants with stars (*), which are IgM responses.

After examining the results, the top 20 cell supernatants were tested against 52 kDa target protein with immunoblotting.

Figure 31 - Immunoblot of cell supernatants reacting to 1 μ g 52 kDa target protein. The arrows indicate the target protein reacting to cell supernatant antibodies.

Figure 32 - Immunoblot of cell supernatants against 1 μ g 52 kDa target protein. The arrows indicate the target protein reacting to cell supernatant antibodies.

Based on the results of the initial cell supernatant immunoblotting, the top 10 cell supernatant Abs were studied in further detail (Figure 33). Again, immunoblotting was utilized to characterize the activity of these antibodies. For this series of tests, a spore sample was also included alongside the 52 kDa target protein. This ensured that the mouse cell supernatants were reacting to the target protein similar to how it would be found in the natural environment.

Figure 33 - Immunoblot comparison of each cell supernatant antibodies. Each antibody was tested against both the 52 kDa target protein (lane 1) and DAOM 234 056 spore samples (lane 2). 15 μ g of pure 52 kDa target protein and 35 μ g of spore proteins were added to each well. The 52 kDa target protein response in the purified and spore samples are indicated by the arrows.

6.2 – ELISA characterization of selected monoclonal cell supernatants

Based on the previous immunoblotting results, several of the strongest performing cell supernatants were tested with ELISA against the 52 kDa *P. chrysogenum* target protein. The data below compares testing performed upon the top performing cell supernatants. Also included were selected cell supernatants that did not perform well in the initial immunoblotting, but had very high ELISA responses. These cell supernatants were included to eliminate the chance that an initially high reported responding antibody was not ignored.

Table 5 - ELISA response of 1 µg of 52 kDa *P. chrysogenum* target protein to a series dilution of the top performing cell supernatants.

mAb	Dilution of mAbs			
	1x	10x	25x	50x
2F9	3.589	2.861	2.301	1.002
4H2	3.285	1.3816	1.735	1.081
8H1	2.721	N/A	0.616	0.209
5G8	2.258	N/A	1.278	0.638
3E8	0.503	0.4527	0.575	0.686

The top three cell supernatants from this ELISA testing allowed the choices to be narrowed to the top three performing antibodies. The smaller selection for further ELISA analysis included cell supernatants 2F9, 4H2 and 8H1. Cell supernatant 7H7 was also included in this new group. The sensitivity of this new set of antibodies was further

tested by diluting the 52 kDa target protein. Some of the ELISA responses could not be included (N/A) because the background response was masking the target protein activity, effectively making the OD values zero.

Table 6 - ELISA response to 0.33 µg of 52 kDa *P. chrysogenum* target protein to the of the top four performing cell supernatants.

mAb	Dilution of mAbs					
	1x	10x	25x	50x	100x	200x
2F9	3.381	3.439	1.255	0.940	0.469	0.301
4H2	0.964	0.574	0.188	0.116	0.038	0.323
7H7	3.411	3.447	2.700	1.501	0.735	0.404
8H1	0.816	0.229	0.301	0.108	0.113	0.163

6.3 – Immunoblot comparison of top four cell supernatants

The final decision of which cell supernatants were selected for monoclonal producing hybridoma cells was based on a combination of the ELISA data and the following immunoblot cross-reactivity experiments. Cell supernatants 2F9, 4H2, 7H7 and 8H1 were tested for possible cross-reactivity against selected fungi. The images of these immunoblots are displayed in the following pages.

Figure 34 - Immunoblot examining the cross-reactivity of cell supernatant 7H7.

Figure 35 - Immunoblot examination of cross-reactivity of the cell supernatant 2F9.

Figure 36 - Immunoblot examining cross-reactivity of cell supernatant 8H1.

Figure 37 - Immunoblot examining cross-reactivity of cell supernatant 4H2. The red arrow indicates a reaction from the CBS 277 067 strain.

The previous set of experiments suggested that there could possibly be cross-reactivity of the *P. chrysogenum* 52 kDa target protein with the fungi species *P. crustosum* 245045. This situation was investigated further to determine if there was indeed cross-reactivity occurring with an extremely similar protein or perhaps with a different protein entirely. A silver stained SDS-PAGE gel was run to determine if the possibly cross-reacting protein in *P. crustosum* had a different molecular weight than the 52 kDa *P. chrysogenum* target protein (figure 38). The protein that was thought to possibly be cross-reacting with the cell supernatants in Figures 31 to 34 was found to have a slightly lower molecular weight when tested with SDS-PAGE silver staining. This protein is approximately 48 kDa in size (Figure 38).

Overall, none of the fungi tested for cross reactivity have reacted to the 52 kDa target protein. The following table shows all of the fungi examined for cross reactivity, and which antibody was used for the assessment (table 7).

Figure 38 - Silver stain gel comparing the size of the pure 52 kDa *P. chrysogenum* target protein to proteins present *P. crustosum*. Fungi protein samples applied were 75% acetone fractions. The DAOM numbers for *P. chrysogenum* and *P. crustosum* were 234 056 and 245 45. The 52 kDa protein is indicated with the arrows in lanes 2 and 5. The 48 kDa protein from *P. crustosum* is indicated by the red arrow in late 4.

Table 7 - List of fungi examined for cross reactivity and the antibody used for testing.

fungi	antibody tested
<i>A. alternata</i> 27.1	GpAb
<i>A. niger</i> 5869.6	HpAb, GpAb
<i>A. versicolor</i> 235 361	HpAb, GpAb
<i>A. penicilloides</i> 5053.25	GpAb
<i>C. cladosporioides</i> 48.1	GpAb
<i>E. nigrums</i> 51-2	GpAb, mAb
<i>P. brevicompactum</i> 234 049	GpAb, mAb
<i>P. variotii</i> 235 357	GpAb, mAb
<i>P. corylophilum</i> 5585.1	HpAb, GpAb
<i>P. crustosum</i> 245 045	GpAb, mAb
<i>P. decumbens</i> 5566.8	mAb
<i>P. funiculosum</i> L6321-3	mAb
<i>P. spinulosum</i> 5053.1	HpAb, GpAb
<i>P. thomii</i> L6322-54	GpAb, mAb
<i>S. brevicaulis</i> 5869.3	GpAb
<i>S. chartarum</i> 235 364	HpAb, GpAb
<i>W. sebi</i> 5755	GpAb

DISCUSSION

Asthma and allergies have become an increasingly significant public health issue in developed countries. The numbers of individuals experiencing the affects of asthma and allergy have doubled in that same time period, with larger increases experienced by children. The magnitude of these increases suggests that changes to diagnosis or clinical description can not be the sole explanation (Rand 2002). In the United States alone, data from 1964 to 2000 showed steadily increasing incidence of asthma and allergy. More than 14.2 million persons or 7.2% of the U.S. population were suffering from asthma in 2000, with statistically significant increases in all age, race, gender and ethnic groups (CDC 2000).

There are several proposed explanations for the increasing rates of asthma, however, the answer is most likely a combination of factors. Increasing proportions of Western populations are now overweight or obese, with the largest increases found in youth. Statistical comparisons of individuals with weight problems or low levels of physical activity were found to have higher levels of asthma incidence. Other hypotheses incorporate the “hygiene hypothesis”, that is, studies have shown that having older and greater numbers of siblings protects children from developing asthma. The proposed explanation for this theory is that children with several older siblings actually benefit from being exposed to infection early in life. Children with older siblings or early attendance in a day care were found to have a higher incidence of asthmatic symptoms by the age of two, but were less likely to show the same symptoms by 11-13 years of age (Ball et al. 2000). This agrees with the present theories of the development of the

immune system in the early stages of life. Early exposure to infection has been shown to induce the development of T_H1 immune response over the reciprocal T_H2 response. T_H2 lymphocytes produce interleukins, which are involved in the body's defense against parasites and allergic inflammation. The mechanisms involved in the later include IgE production, mast cell differentiation, and eosinophil growth, migration, and activation. The production of interferons to defend against viruses and bacteria are performed by T_H1 lymphocytes. Some have put forward the idea of treating asthma and allergies through modulating the T_H1 and T_H2 balance (Salvi et al. 2001). Recent studies have begun to question the T_H1/T_H2 dichotomy, suggesting that the situation is not that simple. They have noted that in some instances some T_H1 interferons are involved in up-regulating some T_H2 responses. At this point, the exact mechanisms involved in allergy and asthma have yet to be elucidated (Ngoc et al. 2005).

Indoor environmental exposure to antigenic compounds often leads to the onset and exasperation of asthma and allergies (IOM 2000; Health Canada 2004). Contaminants of indoor air negatively affecting health include ambient and non-ambient chemicals, fine and course particulate matter, and various types of biological elements. There are many biologically derived allergens found in the indoor environment, dust-mites, cockroach, animal dander, plant allergens and fungal spores and hyphae fragments are some of the more common allergens.

Commercial fungal detection exists for all of these groups of allergens, however, with the large number of indoor fungi and their corresponding sets of allergens, information is still

required to accurately measure exposure levels. Without methods of making accurate, quantitative measurements of indoor fungi, assessing population exposure and the correlations to asthmatic and allergenic disease are difficult. As of 2007, over 70 indoor fungi allergens have been accepted by the IUIS Allergen Nomenclature Sub-committee and WHO, however, the actual number that exists is significantly higher. One element that has been often over-sighted in fungal allergen research is the incidence of each allergen to the species as a whole. That is, often different strains will produce many human antigens, with strains from different geographic regions or substrates producing different proteins. The key to assessing exposure to a particular fungal allergen is discovering a protein antigenic to humans that is common to strains over a large geographic area.

In North America and Europe the most common indoor fungi include species from the genres *Alternaria*, *Aspergillus*, *Penicillium*, *Cladosporium* and to a lesser extent *Stachybotrys* (Green et al. 2006; Flannigan et al. 2001). Presently, there are not any accepted allergens and corresponding antibodies that could be used as a diagnostic tool representative of an entire species. Until a number of these theorized allergens are discovered, specific species exposure will have to continue to be assessed with colony plating and identification or crude immuno-detection methods which are quantitatively ineffective.

The first steps of this experimentation involved screening the *P. chrysogenum* culture filtrate protein extracts reactions to HpAbs with ELISA and immunoblotting. The goal

from these experiments was to determine the HpAbs reaction to the *P. chrysogenum* extracts with ELISA, and to visually identify reacting proteins common to all available strains and HpAbs with immunoblotting.

All of the strains tested with ELISA responded to most, and in a majority of the cases, all of the HpAbs. There were a small portion of the strains that had a low response to many of the HpAbs. The response for each DAOM strain tested was more or less in the same order, although variations were observed between HpAbs. Statistical variance testing by Dr. J.D. Miller confirmed that the values found in the some of the HpAbs did have different reaction levels. Figure 4 displays this observation, showing the large differences HpAbs response to the various proteins. There were also statistical differences in the reactions of the DAOM strains, as well as some that appear to respond in a similar fashion. The important fact was that the responses appeared to form two separate groups, some reacting primarily to the 40 and 52 kDa proteins, and the other group of patients responded to many proteins. This means that some patients were reacting to the indoor *P. chrysogenum* chemotype while others to *Penicillium* strains.

Understanding and developing an idea as to why these results would be observed is complex. One hypothesis as to why this would occur is that the response is proportional to the exposure characteristics of the individual. The titre of immune response in each individual would be associated with the time and length of exposure to *P. chrysogenum*. The patients with the lowest ELISA responses were not recently exposed to the allergens and thus, were exhibiting a decreasing titre of the responding antibodies. Also, since the

strains produce many antigenic proteins, and many of the antigens are not inclusive to all strains or are present in varying concentrations, the patients would display a large range of responses to the strains analyzed. The HpAb (QC2397) utilized for a majority of the immunoblotting experiments was probably taken from an individual who had recent and continued exposure and thus a very high titre to the antibodies. Also, it appears that some of the strains do indeed produce higher levels of antigens, and certainly different combinations of these proteins (table 3).

The immunoblotting analysis of the reacting proteins of each strain to the collection of patient HpAbs displayed similar patterns of variation, however, with this technique, the individual proteins antigenic to humans could be observed. The human sera reacted to many proteins in each *P. chrysogenum* strain. Figures 6 through 14 illustrate the many responding proteins. The major responding proteins were also examined in table 3.

Almost all of the HpAbs and DAOM strains responded to both the 52 and 40 kDa proteins. Pen ch 20, the 68 kDa allergen was the next most responsive protein, however, it responded to less than half of the HpAbs and strains. The allergens Pen ch 13 and 18 were observed to have strong responses in some patients and strains, however, this response was significantly lower or absent in some instances, indicating that this allergen is not common to the species, or even the relevant clade. Also, since Pen ch 13 and 18 appear in very strong and very weak or absent in some patients, it appears that these human antigens have a much shorter immune response compared to the target proteins in the present research. Since these allergens are proteases, which are common in indoor fungi, cross-reactivity has also been shown to be a problem (Shen et al. 1996). This

observation also shows the importance of identifying an allergen that is common to a wide geographic array of the strains. The allergens Pen ch 13 and 18 would not be very useful in assessing *P. chrysogenum* exposure in North America.

The immunoblots show that nearly all clade 4 strains of *P. chrysogenum* from across Canada produced a 40 and 52 kDa protein, which is a human antigen (figures 10 -14). The two strongest responding HpAbs both had 17 of the 18 DAOM strains responding to both the 52 and 40 kDa proteins. This is a large contrast from the response of the same HpAbs to Pen ch 13 and 18. Less than half of the strains had a detectable response to these allergens when immunoblotted against the same HpAbs. Pen ch 13 and 18 both appear to have a strong response in humans, however, this was only observed in the DAOM strains 215 336 and 215 337 in the present research. Figures 11 and 12 illustrate this response to Pen ch 13 and 18. This observation emphasizes the importance of discovering human antigens with a very strong response but also present in most of the indoor strains of the fungi.

Previous studies have identified various metabolites and toxins produced by clade 4 strains of *P. chrysogenum*. Since these combinations of toxins produced by each strain appear to be unique, one could not use them as a marker for detecting the presence of *P. chrysogenum* in all cases (De La Campa et al. 2007; Scott et al. 2004). The protein antigen in this research appears to be consistent to the clade 4 strains, and thus would be considerably more useful as a biomarker for the presence of *P. chrysogenum* in indoor environment.

Perhaps the most important step in isolating human antigens from fungi is the growth conditions of cultures. Many studies have shown that the growth conditions of the culture often do not produce the same proteins as found in nature. The proteins that are antigenic to humans, which this research is based, are found naturally in spores, spore fragments and mycelium. Growing spore cultures and extracting the required amount of protein is not usually a viable method of antigen isolation, leaving liquid cultures as the best alternative. Without great care, liquid culture protein production can exhibit large variations. The temperature, oxygen content, growth time and nutrients available in the medium are critical factors that must be replicated in each culture to ensure consistent replicable protein yield (Esch 2003; Bush and Yunginger 1989).

As with the isolation of human antigens from *S. chartarum* and *A. versicolor* the extracellular proteins had the strongest responses to the HpAbs. Protein samples from the mycelium examined with immunoblotting showed that there were not obvious candidate proteins antigenic to humans in the strains. Also, the protein content was more than two-fold higher in the culture filtrate than mycelium, and was much simpler to isolate the target proteins. Thus, since the mycelium proteins did not present an obvious common target protein, and the protein content was much lower than the culture filtrate, the mycelial proteins were not examined further in the present research. The proteins which were human antigens isolated from *S. chartarum* and *A. versicolor* were found to be exported outside the cell, and thus found at higher concentrations in the culture filtrate. If

this is the case with the *P. chrysogenum* target proteins, it would explain why recovery is better in the culture filtrate as opposed to the mycelium.

The purification of 52 kDa target protein involved three main steps, protein precipitation reactions, ion exchange chromatography, and size exclusion chromatography. The protein content was extracted from the culture filtrate with acetone and ammonium sulphate protein precipitations.

An acetone precipitation involves extracting protein out of solution by altering the dielectric constant. More specifically, water based solutions or buffers have large dielectric constants, which stabilize themselves and the protein molecules, favoring the dissolution of the protein. In contrast, an organic solvent such as acetone, has a small dielectric constant, and thus discourages the dispersion of protein in solution. Therefore, effectively lowering the dielectric constant with the organic solvent acetone also decreases protein solubility. Acetone is an effective method for this due to the relative low cost, purity, availability and generally it does not inhibit or destroy protein. In this research, two acetone precipitation fractions were collected, yielding large quantities of protein. The initial 50% acetone precipitation precipitated a small quantity of protein, and a large portion of the precipitate being carbohydrate and other non-protein material. The 75% acetone precipitation fraction had a very high protein yield, over 50 mg L⁻¹ liquid culture, a result consistent with literature (Xu et al. 2007). Previous experiments have found that acetone precipitations coupled with ultrafiltration have high yield recoveries (Jiang et al. 2004).

The protein recovery had a high yield in the first purification step; however, the 52 kDa target protein represented only a small percentage of the total protein content. The target protein represented less than 3 % of the total protein content in the 75% acetone fraction. The 75% acetone fraction protein content and response to HpAbs is shown in figure 23 and 24.

Before moving forward to purification applications such as chromatography, a method was required that would separate the proteins into less complicated fractions, without inducing major losses of the target antigen. AS protein precipitation or more specifically, AS fractionation is an excellent method to divide a complex protein sample into several manageable fractions. The solubility of proteins in solution depends on many factors, one being the salt concentration of the solution. At low AS concentrations, the various charged groups on the proteins become stabilized, thus enhancing the solubility of the material in solution. Depending on the characteristics of each individual protein and its associated charged side-groups and domains, salt can be added until the maximum solubility is achieved, at which point, additional AS will reduce the amount of water available for the proteins to dissolve. Once this is achieved, there will not be sufficient water available to ensure solubility of the protein and thus it will precipitate or be “*salted-out*” of solution. This method can also be performed in a reverse manner, whereby, all of the protein is salted-out in an initially large AS addition, and then fractionated by re-dissolving portions of the total protein. In the present research, AS precipitation was used to precipitate the total protein recovery from the acetone

precipitation into several fractions. The solution was separated into a 0-30%, 30-50%, 50-70% and 70-80% AS saturation fractions. After desalting procedures, each of the fractions was examined for content and activity with Silver-stained SDS-PAGE gels and immunoblotting. The overwhelming majority of the target protein was precipitated in the 50-70% AS fraction (Figure 23-24). After desalting with ultrafiltration, the target protein had increased to approximately 10 % of the protein content (table 4). Each of the fractions, including the target fraction, not only contained considerably fewer proteins that could be visualized with silver staining, but also separated many of the proteins responding to the HpAb. The 50-70 AS fraction contained 11 proteins that could be visualized with silver staining, with five of those responding to the HpAb. Figure 24 shows that the 50-70% AS fraction did contain a majority of the protein responding to the pAbs though ($\approx \frac{1}{2}$ of the responding proteins from the culture), perhaps suggesting that there is some commonality in the chemistry of these antigenic proteins. The 52 kDa target protein response was the strongest responding protein in this fraction.

The AS fractionation of the 75% acetone culture filtrate solution separated the target protein into a more manageable fraction, allowing purification steps to then be performed. The approximate percentage of the 50-70% AS fraction protein content represented by the target protein had improved to over 10% compared to the 75% acetone fraction, where it was less than 3% (table 4).

Ion exchange chromatography is one of the most frequently used purification steps in protein purification, and previously had been found as an effective method in the Miller

laboratory (Xu et al. 2007). This method is rapid, simple and effective in separating proteins based on their net charge. This separation involves manipulating the interactions of proteins with an immobilized solid matrix. In this research, anion exchange chromatography was utilized, that is, the negatively charge amino acid groups on the proteins interact with the positively charged matrix. The immobilized matrix was a macroporus, beaded, cross-linked agarose with the bound charged group $(-O-CH_2CHOHCH_2OCH_2CHOHCH_2N^+(CH_3)_3)$, a strong exchanger, or more specifically, it has a high extent of ionization with pH alterations. In ion exchange chromatography, elution may be accomplished with two methods, pH or ionic manipulation. Ionic manipulation was utilized in the present research, where the proteins that were bound to the column matrix were eluted through the addition of NaCl. NaCl works as an anion donator, its strong affinity for the matrix competing with the proteins bound to the column. A 0.08 M NaCl buffer effectively eluted the target protein, indicating that the negative charges on the novel protein must be minimal. After this procedure, the target protein fraction was nearly purified. This elution had a total of 5 proteins, with the 52 kDa target protein representing over 25 % of the total fraction content. Silver staining of each elution fraction from the anion exchange column shows this data, with arrows indicating the target proteins (figure 25, 26). At this point of the research, purification of the 40 kDa target protein was discontinued. This protein did not bind to the anion exchange resin, and thus was excluded from future purification experiments (figure 25, 26; arrow). The success of this purification step allowed the sample to be purified with size exclusion chromatography.

In the early stages of the process, hydrophobic chromatography was attempted with little success. The target protein mostly passed through the column accruing substantial losses. Many reasons could exist for this occurrence, however, the most likely explanation is that the non-physiological pH of the column had denatured the target proteins. The results from these experiments determined that this step could be avoided and size exclusion chromatography would be utilized as the final step in the purification process. The 5 proteins contained in this sample were 15, 30, 52, 66, and 100 kDa, with only the 52 kDa target protein responding to the HpAb and the GpAb. The size exclusion chromatography experiments were very successful, purifying the target protein to over 95% (table 4). When the gel filtrations target fraction was silver stained on SDS-page gels, only the 52 kDa human antigen was visualized in the fraction. With the protein now being purified, it was then used as a standard and to boost mAb production in lab mice.

The goat pAbs were made immunizing laboratory goats with *P. chrysogenum* spores and then boosting with a partially purified 52 kDa target protein extract. The reason for proceeding with boosting the goats prior to completing the purification experiments was time constraints. The boosting of the goats required more than 60 days, and thus, the progress could be streamlined by boosting the goats with target protein being physically removed from stained gels, and then being injected into the spore boosted goats. The isolated protein was tested for purity and activity prior to being injected in the spore boosted goats.

Immunization of the goats was performed for three reasons. The most important reason was to show that the protein was present in spores. The second was to demonstrate that the target proteins were indeed produced by the fungi and not an artifact of one of the isolation procedures or other methods. Also, fungal spores and mycelium contain large numbers of proteins, and it is known that the distribution of these in laboratory media compared to the occurrence in nature can exhibit large variation (Xu et al. 2007). This also demonstrated that the protein chosen to be examined was antigenic to goats, as well as humans. The next stage of the GpAb boosting was the injection of the 52 kDa target protein one month later and then comparing this test bleed to the previous. This portion of the experimentation ensured that the pAb could be used in the process of further isolation of proteins, and as a possible capture antibody. The titre of the responses to the mixture of *P. chrysogenum* spore proteins had decreased or even disappeared, and the response to the target protein had an increased response. The response to Pen ch 13 and 18 had strong responses to the spore boosted GpAbs, but decreased in intensity when developed with the 52 kDa target protein boosted GpAbs (figure 17, red arrows). The increased specificity and continued sensitivity to the 52 kDa target proteins were evident in the final goat immunization (figure 17, black arrows). Several other strains of *P. chrysogenum* from across Canada were also tested for response against the GpAb to ensure that this target protein was common to indoor strains, similar to the HpAbs (Figure 19). The strains from various culture collections responded similarly and thus could be potentially used as a marker for identifying exposure to *P. chrysogenum* in the indoor environment. Establishing these facts was essential to moving the research

forward; ensuring that the novel protein originally investigated remained the best candidate for the continuation of the experimentation.

Aside from confirming the hypothesis of the target protein being an antigen, the GpAb were also produced to possibly be incorporated in detection methods. After the GpAb had been purified, the immunoblot images produced were observed to be more specific to the target protein and also did not cross react with as many fungi as the HpAbs (figures 17 and 19). When the HpAbs were used to screen the 50-70% AS sample from *P. chrysogenum*, many of the responding proteins appeared to cross-react with some of the other fungi (figure 18-22). The patient sera used in the primary screening could have been exposed to many indoor fungi, and thus developing cross-reactivity conclusion from the HpAbs was preliminary (figure 15). The patient responses to *S. chartarum* extracts illustrate this statement. *S. chartarum* is relatively unrelated species, and the fact that it is also a species encountered in buildings, it was hoped that the atopic patients (HpAbs) had been exposed to many fungi, including the two mentioned. This hypothesis regarding the relevance of those early cross-reactivity experiments were confirmed with the GpAbs, which did not react to *S. chartarum* extracts. The protein that appeared to be cross-reacting with the HpAb was found to be more than 54 kDa in size with silver staining (figure 16). The GpAbs did show what appeared to be some possible cross-reactivity with some similar *Penicillium* and *Aspergillus* species (figure 21). The proteins that were reacting from other fungi differed greatly in molecular weight from the 52 kDa target protein. This was an acceptable result because the GpAbs were boosted initially with spores, thus one would expect there to be numerous proteins responding. Cross-reactivity

experiments also removed studies of the 40 kDa target protein from the present research. *P. corylophilum* and *P. brevicompactum* all responded to the 40 kDa target protein, but did not react to the 52 kDa target protein (figure 21). *P. crustosum* however, did appear to respond to the 52 and 40 kDa proteins. When run beside a *P. chrysogenum* on gel electrophoresis, the *P. crustosum* protein was approximately 48 kDa, smaller than the 52 kDa target protein (figure 38). Fungal exposure studies in Mexico City found *P. chrysogenum* to be 10-15 more frequent in asthmatics homes than *P. crustosum* (Rosas et al. 1997). The Wallaceburg study of fungi in 369 homes also recorded similar results. *P. chrysogenum* was found to be the third most frequently observed indoor fungi, whereas *P. crustosum* was determined to be rare, the 38 out of a total of 55 observed species (Flannigan et al. 2001). Thus, cross-reactivity of *P. crustosum* and the *P. chrysogenum* target protein data should not impede the progress of future antibody detection strategies.

The information gathered from the GpAb studies allowed the research to proceed to the production of monoclonal antibodies (mAbs). One of the primary goals of this research was to develop one or more mAbs to a common protein(s) antigenic to humans. Since mAbs only recognize a single epitope on specific proteins, producing these antibodies is the best method for having a tool to detect *P. chrysogenum* exposure with great sensitivity and selectivity.

Hybridoma cells were developed to produce monoclonal antibodies to the 52 kDa target protein. Since the mice used were only exposed to the target protein, monoclonal cell lines would be complementary to epitopes found only on the 52 kDa protein, thus, the

antibodies would theoretically have enhanced specificity. Many cell lines were initially produced and screened for their response to both spores and target protein. Many immunoblotting and ELISA experiments were undertaken in the present research to establish which supernatant cell lines elicited the most sensitive and most specific response to the target protein. One of the issues in assessing the cell supernatant Abs is that they were not very concentrated at this stage of the production, and thus determining sensitivity proved difficult. This did not impede determining which supernatant cell lines had the strongest response to the target protein, but lead to some concerning results with the cross-reactivity screening. The cross-reactivity screening was performed with immunoblotting rather than ELISA. The target protein was responding to the supernatant cell lines, however, many other proteins were also displaying a response. This non-specific response from the supernatant cell lines may be non-discriminate binding, due to the high concentration of the antibodies utilized for these experiments. Between 9 and 11 proteins from the *P. chrysogenum* spores responded to the cell supernatant for each of the final four cell lines (figure 34-37). For this reason, the cross-reactivity investigations on the target protein were primarily performed to give an indication which fungi may pose a problem when the final mAbs are received and tested.

The top performing supernatant cell lines from the ELISA and immunoblot tests were screening against 7 fungi that were either closely related to *P. chrysogenum* or had exhibited possible cross-reactivity with the GpAbs. *P. thomii*, *P. crustosum*, *P. variotii*, and *P. brevicompactum* had varying degrees of responses to the supernatant cell lines. Of these four fungi, *P. crustosum* posed the most concern. The other three species were

showing a response, but to proteins that were considerably different in molecular weight than the target protein. *P. crustosum* however, appeared to be responding to the supernatant cell lines with a protein of the same molecular weight as the target protein. As done before with the GpAbs, both were run side-by-side on SDS-PAGE and then silver stained. This silver stained gel showed that the *P. crustosum* protein that was reacting to the supernatant cell lines was approximately 4 kDa smaller in molecular weight than the target protein (figure 38). Thus, this was a different protein responding, most likely because of the poor sensitivity of the supernatant cell lines at this early stage of their production.

An Edman degradation amino acid sequence of the 52 kDa target protein was done Sick Kids Hospital in Toronto, Ontario. The amino acid sequence was analyzed with the NCBI program Protein-Protein BLAST (blastp). The N-terminal 52 kDa target protein amino acid sequence matched a *P. chrysogenum* protein (Sugimoto et al. 2004) in the database with over 95% certainty. Since the protein was also from the same species, it was concluded that this was indeed the identity of the 52 kDa target protein. Sugimoto et al. (2004) expressed this protein from *P. chrysogenum* CBS strain 277067. This protein was identified through cloning techniques in aluminum-tolerant mutants of *P. chrysogenum* CBS 277067. This protein was one that was found to be expressed in the cDNAs of Al-tolerant mutants. The authors suggested that this glucoamylase protein was important in Al-tolerance mechanisms (Sugimoto et al. 2004). This was an important piece of literature because it showed that others have identified this protein in *P.*

chrysogenum, and it reacted to the cell supernatant antibodies produced in this research (figure 37, red arrow).

Surprisingly, the 52 kDa glucoamylase in this research had not been previously proposed as an allergen in literature. There are currently five *P. chrysogenum* allergens that have been accepted by the International Union of Immunological Societies (IUIS) – Allergen Nomenclature Sub-Committee. The molecular weight and identity of these allergens are a 68 kDa N-acetyl-glucosaminidase, a 34 kDa alkaline serine protease, a 32 kDa vacuolar serine protease, and a 16 kDa protein of undetermined function. When compared to the 52 kDa target protein, it is evident that these proteins are quite different in size and function. The IUIS and WHO allergen database (www.allergen.org 2007) provides a publicly accessible database of known fungal allergens. The data available was reviewed to determine if there were any other similar fungal allergens previously proposed. Since most of the fungi that are associated with the indoor environment belong to the group Ascomycota, these allergens were reviewed in the most detail. There were one allergen in this database which had a similar size and function, a 53 kDa TAKA-amylase A protein from *Aspergillus oryzae*.

The amino acid sequence of the 52 kDa target protein was first shown in the 2004 article by Sugimoto et al. where the authors were searching for the genes and proteins responsible for aluminum (Al) tolerance in *P. chrysogenum* strains. The glucoamylase from *P. chrysogenum* found to have a 60% sequence identity with an amylase allergen form *Aspergillus oryzae* (AJ304803; Sugimoto et al. 2004). Since this sequence

alignment was not similar and generally a domesticated fungus (Pitt 2000), cross reactivity should not be encountered when using the *P. chrysogenum* detection antibodies generated in the present research.

A. oryzae is a fungus that is usually found in the soil, but is now generally associated with the production of Soya sauce and some other food products. Proteins produced by this fungus have also been synthetically introduced in other operations in the food industry. This protein from *A. oryzae* is widely available commercially as it is added to flour as a baking enzyme in mills and bakeries (Baur et al. 1994; Baur and Posch 1998). This enzyme, α -amylase, is now considered an important allergen, thought to be one of the primary inducers of the condition “bakers’ lung”. In the baking industry, it is used for its carbohydrate cleaving activity on flour, standardizing baking conditions. One study in Germany showed that 23% of bakers suffering from work-place related rhinitis and/or bronchial asthma were sensitized to the α -amylase (Baur et al. 1989). Inhalation studies have shown α -amylase to induce significant nasal or bronchial reactions in sensitized workers at concentrations of < 1mg/mL. The 1994 study by Baur et al. examined the sera of 89 bakers for sensitivity to this enzyme. In the group of individuals that were experiencing symptoms of allergy or asthma, 48% tested positive for exposure to the enzyme with allergosorbent test (EAST) testing, and 35% of that same group tested positive with the skin prick test.

Two other protein cloned by Sugimoto et al. 2004 which had sequence identities similar to the *P. chrysogenum* glucoamylase were proteins from *A. fumigatus* (AF284645) and an

enolase from *P. citrinum* (AF254643). Both of these proteins have different functions and are dissimilar in size (47 kDa) when compared to the target protein (Sugimoto et al. 2004).

Ravi-Kumar et al. (2007) discovered an amylase allergen produced by *Aspergillus niger*, similar in size and function to the target protein in this work. The 53 kDa amylase was secreted by *A. niger* cultures when grown in a starch medium. Antibodies against the precursor *A. niger* 53 kDa enzyme were responsive to Asp o 21, however, this protein is not officially recognized as an allergen at this date (Ravi-Kumar et al. 2007). The accepted allergens produced by *A. niger* are Asp n 14, a 105 kDa β -xylosidase 105; Asp n 18, a 34 kDa vacuolar serine protease; and Asp n 25, a 66-100 kDa 3-phytase B (www.allergen.org 2007).

The absence of quantitative exposure methods for specific fungi in the indoor environment has lead to considerable confusion. The scientific community generally can agree that the presence of some fungi in the indoor environment causes health complications such as asthma and allergies. In-vivo and in-vitro studies have shown immune response to fungal allergens, and large exposure studies have shown that indoor exposure does increase the incidence of asthma and allergies (Health Canada 2004; IOM 2004; US EPA 2003). Without methods of determining which species are responsible for negative health conditions, agencies such as the US EPA avoid the problem by recommending complete remediation of any fungal presence in the indoor environment (US EPA 2003). However, in many cases, indoor fungi colonize areas that may not be

easily noticed such as basements, beneath carpets and the insides of walls. Also, without identifying the proteins responsible for a suspected allergy, measuring one's personal exposure with immunological methods can not be done, and thus making clinical assessment difficult and vague. Aside from the suspected health effects, indoor fungi can cause structural damage and leave a building foul smelling and aesthetically displeasing (Portnoy et al. 2004).

The IUIS and WHO have developed a system of nomenclature for allergens, creating a system that eliminates naming confusion and disorder. The naming system labels purified allergens by using the first three letters of the genus followed by the first letter of the species, and a numeral for their order of isolation. Designations of allergen genes or proteins are acceptable, using Roman and Arabic numerals, respectively. In order to have an allergen registered the taxonomic, immunochemical and physiochemical characteristics must be submitted. Also, the author must show that the allergen has prevalence of IgE reactivity above 5%, with a minimum of five patients responding (www.allergen.org 2007).

The human antigen discovered in this research does meet most of the prerequisites explained above. However, future research would be required to further characterize the protein, allowing it to satisfy all of the required information. In the present research IgG antibodies were investigated and characterized. Similar experiments studying IgE antibodies response to the target protein would have to be performed, as well as further

characterization of the IgG response. A proposed name for this allergen could be Pen ch 52.

Once the research of the Pen ch 52 is completed it could be immediately used in exposure studies. There is currently a need within the regulatory community for a rapid and accurate method to determine the indoor fungi populations and human exposure to the corresponding allergens. The purified allergen and monoclonal antibodies could be rapidly utilized as a immunodetection device, satisfying the requirements of health agencies such as Health Canada, EPA, CEPA, CDC, NIH, and CMHC. If researchers had kits to many allergenic indoor fungi, similar to the one proposed for *P. chrysogenum*, large studies could be performed investigating connections between fungal allergen presence and allergy and asthma. In the Western world, up to 50% of the residences suffer from dampness and fungi problems and up to 10% of the population testing positive to fungi with skin prick tests (Verhoeff and Burge 1997). A six-year study in Espoo (Finland), it was found that the children living in homes with moldy odours had a >100% increased risk of developing asthma (Jaakkola et al. 2005). A cross-sectional study of 18 different European countries found that mold exposure was associated with current asthma symptoms and bronchial responsiveness (Zock et al. 2002). Increased severity of asthma symptoms and episodes has also been statistically linked to fungi sensitivity. Other allergens such as pollens and cats did not have the same correlation (Zureik et al. 2002). Considering these facts, one can now realize that indoor fungal contamination is a much more serious problem than an unpleasant odours and sporadic cases of rhinitis.

REFERENCES

1. <http://www.allergen.org/Allergen.aspx>. Accessed July, 2007.
2. <http://www.allergome.org>. Accessed July, 2007.
3. Aalberse RC. *Structural biology of Allergens*. Journal of Allergy and Clinical Immunology. 106, 228-238. 2000
4. Aas K, Leegaard J, Aukrust L, Grimmer O. *Immediate Type Hypersensitivity to Common Molds*. Allergy. 35, 431-451. 1980.
5. Alonso A, Mouchián K, Albónico JF, Rodríguez SM, Irañeta SG. *Hypersensitivity pneumonitis induced by Penicillium notatum antigens in guinea pigs*. Journal of Investigational Allergology and Clinical Immunology. 8, 52-57, 1998.
6. Alonso A, Rodríguez SK, Rodríguez SM, Mouchián K, Albónico JF, Irañeta SG. *Interstitial pneumonitis induced in guinea pigs by the antigens of Phizopus nigrans*. Journal of Investigative Allergology and Clinical Immunology. 7, 103-109. 1997.
7. Ball TM, Castro-Rodriguez JA, Griffiths KA, Holdberg CJ, Martinez FD, Wright AL. *Siblings, day-care attendance, and the risk of asthma and wheezing during childhood*. New England Journal of Medicine. 343, 538-643. 2000.
8. Baur X, Chen Z, Sander I. *Isolation and denomination of an important allergen in baking additives: α -amylase from Aspergillus oryzae (Asp o II)*. Clinical and Experimental Allergy. 24, 465-470. 1994.
9. Baur X, Degens PO, Sander I. *Baker's asthma : Still among the most frequent occupational respiratory disorders*. Journal of Allergy and Clinical Immunology. 102(6:1), 984-997. 1998.
10. Baur X, Posch A. *Characterized allergens causing bakers' asthma: Review article*. Allergy. 53, 562-566, 1998.
11. Baur X, Czuppon AB. *Allergic reaction after eating α -amylase (Asp o 2)-containing bread. A case report*. Allergy. 50, 85-87, 1995.
12. Bisht V, Kukreja N, Singh BP. *Current status of fungal allergens*. Indian Journal of Allergy, Asthma and Immunology. 17(1), 9-19. 2003.

13. Bornehag C.-G, Sundell J, Sigsgaard, T. *Dampness in buildings and health (DBH): Report from an ongoing epidemiological investigation on the association between indoor environmental factors and health effects among children in Sweden*. Indoor Air 14 (s7), 59–66. 2004.
14. Brunekreef B, Dockery DW, Speizer FE, Ware JH, Spengler JD, Ferris BG. *Home dampness and respiratory morbidity in children*. American Review of Respiratory Disease. 140, 1363-1367. 1989.
15. Burnett, J. Fungal populations and species. 2003. Oxford, UK, Oxford University Press.
16. Bush RK, Portnoy JM, Saxon A, Wood RA. *The medical effects of mold exposure*. Journal of Allergy and Clinical Immunology. 117(2), 326-333, 2006.
17. Bush RK, and Portnoy JM. *The role and abatement of fungal allergens in allergic diseases*. Journal of Allergy and Clinical Immunology, 107(3), 430-442. 2001
18. Bush RK and Yunginger JW. *Standardization of fungal allergens*. Clinical reviews in allergy 5 (1), 3-21. 1987.
19. Carter SE, Platts-Mills TA. *Searching for the cause of the increase in asthma*. Curr Opin Pediatr. 10, 594-599. 1998.
20. CDC, 2003. *Asthma Prevalence, Health Care Use and Mortality, 2000-2001*. National Center for Health Statistics.
21. Chang JCS, Foarde KK, Vanosdell DW. *Growth evaluation of fungi on ceiling tiles*. Atmosph Environ. 29, 2331-2337. 1995.
22. Chou H, Lai HY, Tam MF, Chou MY, Wang SR, Han SH et al. *cDNA cloning, biological and immunological characterization of the alkaline serine protease major allergen from Penicillium chrysogenum*. International Archives of Allergy and Immunology. 127, 15-26. 2002.
23. Chung YJ, Coates NH, Viana ME, Copeland L, Vesper SJ, Selgrade MK, Ward MDW. *Dose-dependent allergic responses to an extract of Penicillium chrysogenum in BALB/c mice*. Toxicology. 209(1), 77-89. Apr 2005.
24. CHMC. 1993. *The clean air guide: how to identify and correct indoor air problems in your home*. Ottawa: Canada Mortgage and Housing Corporation. 32p.

25. Cramer R, Weichel M, Flückiger S, Glaser A, Rhyner C. *Fungal Allergies: A Yet Unsolved Problem*. Chemical Immunology and Allergies. 91, 121-131. 2006.
26. Cooley JD, Wong WC, Jumper CA, Straus DC. *Fungi and the indoor environment: Their impact on human health*. Advances in Applied Microbiology. 55, 1-30. 2004.
27. Cooley JD, Wong WC, Jumper CA, Huston JC, Williams HJ, Schwab CJ, Straus DC. *An animal model for allergic penicilliosis induced by the intranasal instillation of viable *Penicillium chrysogenum* conidia*. Thorax. 55, 489-496. 2000.
28. Cooley, JD, Wong, WC, Jumper, CA, Straus, DC. *Correlation between the prevalence of certain fungi and sick building syndrome*. Occupational and Environmental Medicine. 55: 579-584. 1998.
29. Cuijpers CE, Swaen GMH, Wesseling G, Strumans F, Wouters EFM. *Adverse effects of the indoor environment on respiratory health in primary school children*. Environmental Research. 68, 11-23. 1995
30. Daisey J. M. , Angell W. J., Apte M. G. *Indoor air quality, ventilation and health symptoms in schools: an analysis of existing information*. Indoor Air 13 (1), 53-64. 2003
31. Dales RE, Choi B, Chen Y, Tang M. *Influence of family income on hospital visits for asthma among Canadian school children*. Thorax. 57, 513-517. 2002.
32. Dales R, Miller J. *Residential fungal contamination and health : microbial cohabitants as covariates*. Environmental Health Perspectives. 107(suppl), 481-483. 1999.
33. Dales R, Miller JD, White J, Dulberg C, Lazarovits AI. *Influence of residential fungal contamination on peripheral blood lymphocyte populations in children*. Archives of Environmental Health, 53(3), 190-195. 1998.
34. Dales RE, Miller JD, McMullen E. *Indoor air quality and health: validity and determinants of reported home dampness and molds*. International Journal of Epidemiology. Feb:26(1), 120-125. 1997.
35. Dales RE, Burnett R, Zwanenburg H. *Adverse health effects among adults exposed to home dampness and molds*. American Review of Respiratory Disease. 143, 505-509. 1991a.

36. Dales RE, Zwanenburg H, Burnett R, Franklin CA. *Respiratory health effects of home dampness and molds among Canadian children*. American Journal of Epidemiology. 134, 196-203. 1991b.
37. Dekker C, Dales R, Bartlett S, Zwanenburg H. *Childhood asthma and the indoor environment*. Chest. 100, 222-226. 1991.
38. De La Campa R, Seifert K, Miller JD. *Toxins from strains of Penicillium chrysogenum isolated from buildings and other sources*. Mycopathologia, 163(3):161-168. 2007.
39. Denizel T. *A catalogue of potentially biodeteriogenic fungi held in the culture collections of CBS CMI and QM*. Int Biodet Bull. 10, 3-23. 1974.
40. Esch RE. *Manufacturing and standardizing fungal allergen products*. Journal of Allergy and Clinical Immunology. 113(2), 210-215. 2005.
41. Fanger, P.O. *What is IAQ?* Indoor Air. 16, 328-334. 2006.
42. Fogelmark BJ, Thorn J, Rylander R. *Inhalation of (1->3)- β -D-Glucan causes airway eosinophilia*. Mediators of Inflammation. 10, 13-19. 2001.
43. Flannigan, B., R.A. Samson, and J.D. Miller. *Microorganisms in home and indoor work environments: diversity, health impacts, investigation and control*. London, UK: Taylor & Francis. 2001
44. Foto M. Vrijmoed LLP, Miller JD, Ruest K, Lawton M, Dales RE. *A comparison of airborne ergosterol, glucan and Air-O-Cell data in relation to physical assessments of mold damage and some other parameters*. Indoor Air, 15, 257-266. 2005.
45. Frisvad JC, Samson RA. *Polyphasic taxonomy of Penicillium subgenus Penicillium a guide to identification of food and airborne terverticillate Penicillia and their mycotoxins. Penicillium subgenus Penicillium: new taxonomic schemes, mycotoxins and other extrolites*. Studies in Mycology. 49, 1-173. 2004.
46. Garrett MH, Raymetn PR, Hooper MA, Abramson MJ, Hooper BM. *Indoor airborne fungal spores, house dampness and associations with environmental factors and respiratory health in children*. Clinical & Experimental Allergy. April 28(4), 459-467. 1998.
47. Garrett MH, Rayment PR, Hooper MA, Abramson MJ, Hooper BM. *Indoor airborne fungal spores, house dampness and associations with environmental factors and respiratory health in children*. Clinical and Experimental Allergy. 28, 459-467. 1998.

48. Górný RL, Reponen KT, Willeke D, Schmechel E, Robine M, Boissier M, Grinshpun. *Fungal fragments as indoor air contaminants*. Applied and Environmental Microbiology. 68, 3522-3531. 2002.
49. Grant C, Hunter CA, Flannigan B, Bravery AF. *The moisture requirements of molds isolated from domestic dwellings*. Int Biot. 25, 259-284. 1989.
50. Green BJ, Tovey ER, Sercombe JK, Blachere FM, Beezhold DH, Schmechel D. *Airborne fungal fragments and allergenicity*. Medical Mycology. 44, S245-S255. 2006.
51. Green BJ, Sercombe JK, Tovey ER. *Fungal fragments and undocumented conidia function as new aeroallergen sources*. Journal of Allergy and Clinical Immunology. 115(5), 1043-1048. 2005a.
52. Green BJ, Schmechel D, Sercombe JK, Tovey ER. *Enumeration and detection of aerosolized Aspergillus fumigatus and Penicillium chrysogenum conidia and hyphae using a novel double staining immunostaining technique*. Journal of Immunological Methods. 307, 127-134. 2005b.
53. Hawksworth DL and Rossman AY. *Where Are All the Undescribed Fungi?* Phytopathology. 87(9), 888-891. 1997.
54. Hessel PA, Klaver J, Michawlschuk D, McGhan S, Carson MM, Melvin D. *The epidemiology of childhood asthma in Red Deer and Medicine Hat, Alberta*. Canadian Respiratory Journal. 8, 139-146. 2001.
55. Health Canada. 2004. *Fungal Contamination in Public Buildings: Health Effects and Investigation Methods*. Ottawa: Health Canada. 47p.
56. Horner, W E. *The damp building effect: understanding needed, not more debate*. Annals of Allergy, Asthma and Immunology, 94(2), 213-215(3), 2005.
57. Horner WE, Helbling A, Salvaggio JE, Lehrer SB. *Fungal Allergens*. Clinical Microbiology Reviews. Apr. 8(2), 161-179. 1995.
58. Hu FB, Persky V, Flay BR, Richardson J. *An epidemiological study of asthma prevalence and related factors among young adults*. Journal of Asthma. 34, 67-76. 1997.
59. Immonen J, Meklin T, Taskinen T, Nevalainen A, Korppi M. *Skin-prick test findings in students from moisture- and mold-damaged schools: A 3-year follow-up study*. Pediatric Allergy and Immunology. 12, 87-94. 2001.

60. Insitute of Medicine (IOM), Committee on Damp Indoor Spaces and Health. *Damp Indoor Spaces and Health*. Washington, DC: National Acedemy Press; 2004:276.
61. Insitute of Medicine (IOM), Committee on the Assessment of Asthma and Indoor Air, Division of Health Promotion and Disease Prevention, Insitute of Medicine. *Cleaning the Air: Asthma and Indoor Air Exposures*. Washington, DC: National Acedemy Press. 2000.
62. Jaakkola JJK, Hwang B-F, Jaakkola N. *Home Dampness and Molds, Parental Atopy, and Asthma in Childhood: A Six-Year Population-Based Cohort Study*. Environmental Health Perspectives. 113(3), 357-361. 2005.
63. Jiang L, He L, Fountoulakis M. *Comparison of protein precipitation methods for sample preperation prior to proteomic analysis*. Journal of Chromatography A. 1023, 317-320. 2004.
64. Fifth Kingdom, The, 3nd ed., Bryce Kendrick, Mycologue Publications 2001, Sydney, BC.
65. Kilpelainen M, Terho EO, Helenius H, Koskenvuo M. *Home dampness, current allergic diseases, and respiratory infections among young adults*. Thorax. 56, 462-467. 2001.
66. Kodzius R.; Rhyner C.; Konthur Z.; Buczek D.; Lehrach H.; Walter G.; Cramer R. *Rapid Identification of Allergen-Encoding cDNA Clones by Phage Display and High-Density Arrays*. Combinatorial Chemistry & High Throughput Screening. 6, 147-154. 2003.
67. Koskinen OM, Husman TM, Meklin TM, Nevalainen. *The relationship between moisture or mold observations in house and the state of health of their occupants*. European Respiratory Journal. 14, 1363-1367. 1999
68. Korppi M, Laitinen S, Taskinen T, Reiman M, Nevalainen A, Husman T. *Mold-specific immunoglobulin G antibodies in a child population*. Pediatr Allergy Immunol. 14, 371-377. 2003.
69. Kurup VP, Shen HD, Vijay H. *Immunobiology of fungal allergens*. International Archives of Allergy and Immunology. Nov 129(3), 181-188. 2002.
70. Kurup VP, Banerjee B. *Fungal allergens and peptide epitopes*. Peptides. 21, 589-599. 2000.
71. Lacey J and Cook B. *Fungal and actinomycete spores as pollutants of the workplace and occupational allergens*. Annals of Occupational Hygiene. 32, pp 515-533, 1988.

72. Li CS, Hsu LY. *Home dampness and childhood respiratory symptoms in a subtropical climate*. Archives of Environmental Health. 51, 42-46. 1996.
73. Licorish K, Noverly HS, Kozak P, Fairshter RD, Wilson AF. *Role of Alternaria and Penicillium spores in the pathogenesis of asthma*. The Journal of allergy and clinical immunology. Dec 76(6)8. 19-25. 1985.
74. Madigan MT, Martinko JM, Parker J. *Brock Biology of Microorganisms: 9th Edition*. 2000. Prentice Hall, Upper Saddle River, NJ, USA.
75. Malone DC, Lawson KA, Smith Dh, Arrighi HM, Battista C. *A cost of illness study of allergic rhinitis in the United States*. Journal of Allergy and Clinical Immunology. 99, 22-27. 1997.
76. Mannino DM, Homa Dm, Pertowski CA, Ashizawa A, Nixon L, Johnson CA *et al*. *Surveillance for asthma – United States, 1960-1995*. MMWR Morb Mortal Weekly Rep. 47, 1-28, 1998.
77. Masoli M, Fabian D, Holt S, Beasley R. *The global burden of asthma: executive summary of the GINA Dissemination Committee Report*. Allergy. 59(5), 469-447. 2004.
78. Metcalf SW, Orloff KG. *Biomarkers of exposure in community settings*. J Toxicol and Environ Health A. 67, 715-726. 2004.
79. Miller JD and Dales RE. *Residential Fungal Contamination and Health: Microbial Cohabitants as Covariates*. Environmental Health Perspectives. 107(3), pp. 481-483. 1999.
80. Miller JD. *Fungi as Contaminants in the Indoor Air*. Atmospheric Environment. 26A (12), pp. 2163-2172. 1992.
81. Miller JD, Duandzic R, Frescura A-M. *Indoor- and Outdoor-Derived Contaminants in Urban and Rural Homes in Ottawa, Ontario, Canada*. Journal of Air and Waste Management Association. 57, 297-302. 2007.
82. Miller JD, Day JD. *Indoor mold exposure: epidemiology, consequences and immunotherapy*. Canadian Journal of Allergy & Clinical Immunology 2, 25-32. 1997.
83. Mudarri D, Fisk WJ. *Public health and economic impact of dampness and mold*. Indoor Air. 17, 226-235. 2007.

84. NAS. *Clearing the air: asthma and indoor air exposures*. National Academy of Sciences Institute of Medicine, Division of Health Promotion and Disease Prevention. Washington DC: National Academy Press, 2000.
85. National Institute of Health. *Report of the NIAID task force on immunology and allergy*. Bethesda (MD): US Department of Health and Human Services, NIH; NIH publication No. 91-2414. 1997.
86. Ngoc LP, Gold DR, Tzianabos AO, Weiss ST, Celedón JC. *Cytokines, allergy, and asthma*. *Current Opinions in Allergy and Clinical Immunology*. 5, 161-166. 2005.
87. Pate AD, Hamilton RG, Ashley PJ, Zeldin DC, Halsey JF. Proficiency testing of allergen measurements in residential dust. *Journal of Allergy and Clinical Immunology*. 116(4), 844-850. 2005.
88. Pellizzari ED, Clayton CA, Rodes CE, Mason RE, Piper, Fort, B, Pfeifer G, Lynam D. *Particulate matter and manganese exposures in Toronto, Canada*. *Atmospheric Environment*. 33, 721-734, 1999.
89. Pirhonen I, Nevalaninen A, Husman T, Pekkanen J. *Home dampness, molds, and their influence on respiratory infections and symptoms in adults in Finland*. *European Respiratory Journal*. 9, 2618-2622. 1996.
90. Pitt, JI, Miscamble BF. *Water relations of Aspergillus flavus and closely related species*. *Journal of Food Protection*. 58, 86-90. 1995.
91. Pitt, J.I. *A Laboratory guide to common Penicillium species*. 2000. North Ryde, NSW, Food Science Australia.
92. Pitt J.I. 1980. *The genus Penicillium and its teleomorphic states. Eupenicillium and Talaromyces*. New York: Academic Press. 634p.
93. Portnoy JM, Barnes CS, Kennedy K. *Sampling for indoor fungi*. *Journal of Allergy and Clinical Immunology*. 113(2). 189-198. 2004.
94. Peden DB and Bush RK. *Advances in environmental and occupational disorders 2006*. *Journal of Allergy and Clinical Immunology*. 119(5) pp 1127-1132. 2007.
95. Ravi-Kumar K, Venkatesh KS, Umesh-Kumar S. *The 53-kDa proteolytic product of precursor starch-hydrolyzing enzyme of Aspergillus niger has Taka-amylase-like activity*. *Applied Microbiology and Biotechnology*. 74(5), 1011-1015. 2007.

96. Reed SC. *Asthma in the United States: Burden and Current Theories*. Environmental Health Perspectives. 110(4), 557-560. 2002.
97. Repace JL. *Indoor air pollution*. Environment International 8 (1-6), 21-36. 1982.
98. Rosas I, Calderón C, Matinez L, Lacey J. *Indoor and outdoor airborne fungal propagule concentrations in Mexico City*. Aerobiologia, 13, 23-30.
99. Rydjord B, Hetland G, Wiker HG. *Immunoglobulin G Antibodies Against Environmental Molds in a Norwegian Healthy Population Shows a Bimodal Distribution for Aspergillus versicolor*. Scandinavian Journal of Immunology. 62, 281-288. 2005.
100. Salvi SS, Babu KS, Holgate ST. *Is asthma really due to a polarized T cell response toward a helper T cell type 2 phenotype?* American Journal of Respiratory and Critical Care Medicine. 164, 1343-1346. 2001.
101. Samson, R.A. and Frisvad J.C. *Penicillium subgenus Penicillium: new taxonomic schemes, mycotoxins and other extrolites*. 2004. Utrecht, The Netherlands, Centraalbureau voor Schimmelcultures
102. Sander I, Baur X. *Evidence for continuous B-cell epitopes on α -amylase of Aspergillus oryzae (Asp o 2)*. Journal of Allergy and Clinical Immunology. 93, 265. 1994.
103. Schwab C. J., Cooley J. D., Jumper C. J., Graham S. C., Straus D. C. *Allergic inflammation induced by a Penicillium chrysogenum conidia-associated allergen extract in a murine model*. Allergy 59 (7), 758–765. 2004.
104. Schwab CJ, Cooley JD, Brasel T, Jumper CA, Graham SC, Straus DC: *Characterization of Exposure to Low Levels of Viable Penicillium chrysogenum Conidia and Allergic Sensitization Induced by a Protease Allergen Extract from Viable P. chrysogenum Conidia in Mice*. International Archives of Allergy & Immunology. 130(3) pp.200-208, 2003.
105. Scott J, Untereiner WA, Wong B, Straus NA, Malloch D. *Genotypic variation in Penicillium chrysogenum from indoor environments*. Mycologia. 96(5), 1095-1105. 2004.
106. Shen HD, Choo KB, Wang SR, Lin WL, Chang ZN, Han SH. *Immunoblot analysis of components of Penicillium notatum recognized by human IgE antibodies*. Journal of Allergy and Clinical Immunology. 88(5), 802-807. 1991

107. Shen H-D, Lin WL, Tsai J-J, Liaw S-F, Han S-H. *Allergenic components in three different species of Penicillium: cross-reactivity among major allergens.* Clinical & Experimental Allergy. Clinical and Experimental Allergy. 26, 444-451. 1996.
108. Shen H-D, Chou H, Tam MF, Chang C-Y, Lai H-Y, Wang S-R. *Molecular and immunological characterization of Pen ch 18, the vacuolar serine protease major allergen of Penicillium chrysogenum.* Allergy. 58, pp. 993-1002. 2003.
109. Shen H-D, Lin W-L, Tam MF, Wang S-R, Tzean S-S, Huang M-H, Han S-H. *Characterization of allergens from Penicillium oxalicum and P. notatum by immunoblotting and N-terminal amino acid sequence analysis.* Clinical and Experimental Allergy. 29, 642-651. 1999.
110. Shen H-D, Lin W-L, Liaw S-F, Tam MF, Han S-H. *Characterization of the 33-kilodalton major allergen of Penicillium citrinum by using MoAbs and N-terminal amino acid sequencing.* Clinical and Experimental Allergy. 27, 79-86. 1997.
111. Simoni M, Lombardi E, Berti G, Rusconi F, La Grutta S, Piffer S, Petronio MG, Galassi C, Forastiere F, Viegi G, the SIDRIA-2 Collaborative Group. *Mold/dampness exposure at home is associated with respiratory disorders in Italian children and adolescents: the SIDRIA-2 Study.* Occupational Environmental Medicine. 62, pp 616-622. 2007.
112. Strachan DP, Carey IM. *Home environment and severe asthma in adolescence: a population based case control study.* British Medical Journal. 311, 1053-1056. 1995.
113. Sugimoto M, Saiki Y, Zhang D, Kawai F. *Cloning and characterization of preferentially expressed genes in an aluminum-tolerant mutant derived from Penicillium chrysogenum IFO4626.* FEMS Microbiology Letters. 230, 137-142. 2004.
114. Tarlo SM, Fradkin A, Tobin RS. *Skin testing with extracts of fungal species derived from the homes of allergy clinic patients in Toronto, Canada.* Clinical Allergy. 18, 45-52. 1988.
115. Taskinen TM, Laitinen S, Nevalainen A et al.. *Immunoglobulin G antibodies to molds in school-children from moisture problem schools.* Allergy. 57, 9-16. 2002.
116. US EPA. <http://www.epa.gov/eogaptil/module3/diameter/diameter.thm>. Accessed Sept 12, 2007.

117. US EPA Office of Air and Radiation, (6609J) IED. *A brief guide to mold, moisture, and your home*. Washington, DC: Environmental Protection Agency; 2003. EPA publication no. 402-K-02-003.
118. Verhoeff AP, van Strien RT, van Wijnen JH, Brunekreef B. *Damp housing and childhood respiratory symptoms: the role of sensitization of dust mites and molds*. *American Journal of Epidemiology*. 141, 103-110. 1995.
119. Wang JM, Densi M, Fournier M, Laviolette M. *Experimental allergic bronchopulmonary aspergillosis in the mouse: immunological and histological features*. *Scandinavian Journal of Immunology*. 39, 19-26. 1994.
120. Weichel M, Glaser AG, Ballmer-Weber BK. *Wheat and maize thioredoxins: A novel cross-reactive cereal allergen family related to bakers' asthma*. *Journal of Allergy and Clinical Immunology*. 117(3), 676-681, 2006.
121. Weiss KB and Sullivan SD. *The health economics of asthma and rhinitis. I. Assessing the economic impact*. *Current reviews of allergy and clinical immunology*. 107(1), 3-8. 2001.
122. Weiss KB, Gergen PJ, Hodgson TA. *An economic evaluation of asthma in the United States*. *New England Journal of Medicine*. 326, 862-866. 1992.
123. Williamson IJ, Martin CJ, McGill G, Monie RDH, Fennerty AG. *Damp Housing and asthma: a case controlled study*. *Thorax*. 52: 229-234. 1997.
124. Wilson SC, Straus DC. *The presence of fungi associated with sick building syndrome in North American zoological institutions*. *Journal of Zoo and Wildlife Medicine*: Vol. 33, No. 4 pp. 322–327. 2002.
125. World Health Organization.
<http://www.who.int/mediacentre/factsheets/fs307/en/index.html>. Accessed July 2007.
126. WHO Moulds and Moisture, Local Authorities: health and environment briefing #42, 2004 ,WHO Indoor Air Quality Collaborating Centre, Bonn, Germany.
127. Verhoeff AP, and Burge HA. *Health risk assessment of fungi in home environments*. *Annals of Allergy, Asthma & Immunology*. 78, 544-556. 1997
128. Xu J-P, Belisle D, Miller JD. *Purification and characterization of a protein from Stachybotrys chartarum antigenic in humans*. *Medical Mycology*. In print. 2007.

129. Yu C-J, Chen Y-M, Su S-N, Forouhar F, Lee S-H, Chow L-P. *Molecular and Immunological characterization and IgE epitope mapping of Pen n 18, a major allergen of Penicillium notatum*. Journal of Biochemistry. 363, 707-715. 2002.
130. Zacharasiewicz A, Zidek T, Haidinger G, Waldhör, Suess G, Vutuc C. *Indoor factors and their association to respiratory symptoms suggestive of asthma in Austrian children aged 6-9 years*. Wiener Klinische Wochenschrift. 111, 882-886. 1999.
131. Zhao, W. *Studies on human antigens of Aspergillus versicolor*. Masters Thesis. Carleton University, Ottawa, Ontario, Canada. 92p. 2006.
132. Zock J-P, Jarvis D, Luczynska C, Sunyer JS, Burney P. *Housing characteristics, reported mold exposure, and asthma in the European Community Respiratory Health Survey*. Journal of Allergy and Clinical Immunology. 110(2). 285-292. 2002.
133. Zureik M, Neukirch N, Leynaert B, Liard R, Bousquet J, Neukirch F. *Sensitisation to airborne moulds and severity of asthma: cross sectional study from European Community respiratory health survey*. British Medical Journal. 225: 411, 24 August. 2002.

APPENDIX

Table 8 - List of DAOM *P. chrysogenum* strains.

DAOM strain	Source	Material
155 627		
155 628		
212 031		
215 336		
215 337		
234 051	Saskatoon, SK	rcs-indoor
234 052	Ottawa, ON	pipe-wrap
234 053	Ottawa, ON	rcs-indoor
234 054	Calgary, AB	dust
234 056	Calgary, AB	wallboard
234 057	Deseronto, ON	dust
234 058	Saskatoon, SK	rcs-indoor
234 059	Surry, BC	
234 064	Ottawa, ON	
234 065	Ottawa, ON	
234 066	Sudbury, ON	
234 067	Ottawa, ON	rcs-indoor

Table 9 - List of human sera positive for fungi antibodies (ImmunoCAP).

Sera I.D.	Sera I.D.
QC1287	QC1504
QC1288	QC1505
QC1289	QC1506
QC1290	QC1668
QC1291	QC2122
QC1292	QC2123
QC1293	QC2124
QC1294	QC2125
QC1295	QC2126
QC1296	QC2127
QC1297	QC2128
QC1303	QC2129
QC1304	QC2130
QC1307	QC2131
QC1310	QC2132
QC1500	QC2133
QC1501	QC2134
QC1502	QC2397
QC1503	QC2398

Table 10 - ELISA O.D. values @450 nm for *P. chrysogenum* strains against human sera QC1287-QC1303.

	QC1287	QC1288	QC1289	QC1290	QC1291	QC1292	QC1293	QC1294	QC1295	QC1296	QC1297	QC1303
155 627	2.57295	3.055	3.30335	3.34775	0.71595	2.82075	0.8329	0.45425	0.7734	0.99515	1.285	3.37945
155 628	1.45735	3.0887	2.13855	3.18485	0.04305	0.71785	0.3606	0.09085	0.4727	1.08115	0.4636	3.37945
212 031	0.76735	2.0678	1.08755	3.34775	0.18845	0.47305	0.2849	0.20785	0.7407	0.52745	0.4284	3.37945
215 336	0.63935	1.957	1.89225	1.51495	-0.02005	0.33865	-0.0229	-0.11675	0.0179	0.37155	0.1719	3.37945
215 337	2.3934	2.10785	2.1473	1.8526	1.92505	2.344	2.12285	1.83625	1.51195	1.48465	0.9699	2.32665
234 051	0.7118	1.4359	1.9053	0.432	-0.8276	0.4984	0.3117	-0.1143	-0.7988	0.4768	0.4416	2.2429
234 052	3.49595	3.35425	2.71765	1.8975	1.28835	0.8448	1.5072	0.33075	2.1876	1.11065	-0.8686	2.866
234 053	0.363	2.2958	2.4634	0.7508	-0.7118	1.0389	0.1268	-0.0041	0.1928	0.746	0.5962	3.0001
234 054	3.63435	2.77325	3.57525	2.5035	2.20215	1.4808	2.6217	0.56915	3.522	1.62345	0.1606	3.7492
234 056	3.71145	3.80005	3.57525	3.2331	1.93155	2.2149	2.8587	0.69985	3.2255	2.21035	-0.022	3.7492
234 057	3.58005	2.82305	2.17315	2.2965	1.39195	1.4518	1.5799	0.44815	1.8251	1.17945	-1.0636	3.6166
234 058	3.71145	3.60525	2.76145	2.3888	2.12785	1.6689	2.292	0.59775	2.9646	1.62185	-0.4556	3.7492
234 059	3.33765	3.3688	2.5641	2.87465	-0.2314	-0.16615	2.9204	0.229	0.27265	0.08135	2.3307	3.2312
234 064	-0.71	2.73425	2.0024	1.7152	0.02935	2.0682	0.46605	0.04925	0.62715	1.62265	0.892	2.28375
234 065	2.6892	2.73425	2.2588	1.8526	1.34725	2.3237	1.54365	0.93945	1.66135	2.03805	1.4203	2.23025
234 066	2.7036	2.65505	2.2647	1.8526	1.77975	2.344	2.03385	0.81515	1.95105	1.71525	1.8485	2.01435
234 067	1.7334	1.9383	2.4319	0.9236	-0.0899	0.932	0.3966	0.046	-0.1757	0.7675	0.5273	2.3614

Table 11 - ELISA O.D. values @450 nm for *P. chrysogenum* strains against atopic human sera QC1304-QC2122.

	QC1304	QC1307	QC1310	QC1500	QC1501	QC1502	QC1503	QC1504	QC1505	QC1506	QC1668	QC2122
155 627	3.3377	3.3688	2.4330	3.1754	-0.1061	0.0536	1.2565	0.3992	0.1172	0.3704	1.5418	2.9390
155 628	3.3377	3.3688	2.3239	2.7453	-0.1775	-0.0115	1.3426	0.2725	0.5519	0.2936	-0.0925	3.2090
212 031	3.3377	3.3688	2.9044	3.2915	0.0188	0.7214	3.4158	1.8510	0.8336	0.8629	2.9037	3.2655
215 336	3.3377	3.3688	2.3507	1.7219	-0.2319	-0.1487	1.1217	0.2097	-0.0916	0.1292	0.7909	3.2655
215 337	2.7906	2.7762	1.7879	2.7697	0.6480	3.0729	3.0740	0.9106	1.0635	1.0250	2.3092	3.0535
234 051	2.8112	2.8614	2.5039	2.9279	-0.0542	0.2419	1.9450	0.6641	0.9486	0.4493	0.5569	2.5918
234 052	1.9557	1.6216	2.1098	2.8163	-0.3853	0.2288	2.9067	1.0101	0.3758	0.7036	0.7762	2.9583
234 053	3.1125	3.0656	2.7747	3.1125	0.0406	0.1976	2.2364	0.4318	0.8598	-0.0003	0.4885	3.1730
234 054	2.2220	2.0727	2.1257	2.8613	-0.0552	0.8093	2.6831	1.8254	1.9891	1.5807	1.3285	2.9960
234 056	1.6636	1.8873	2.1257	2.8613	-0.5038	0.1630	2.9728	0.7328	0.5041	0.6705	0.7654	2.9960
234 057	2.1835	2.1573	2.1257	2.7260	-0.5991	0.1688	2.4847	0.6579	0.3396	0.5555	0.7801	2.8105
234 058	2.2220	2.1820	2.1257	2.8613	-0.2550	0.3164	2.9728	1.4708	0.7179	0.8752	0.8818	2.9824
234 059	3.3377	3.3688	2.5641	2.8747	-0.2314	-0.1662	2.9204	0.2290	0.2727	0.0813	2.3307	3.2312
234 064	2.9025	2.4515	2.9693	2.9666	-0.0549	1.2726	1.9863	-1.7714	1.1826	0.5765	-0.8158	2.4813
234 065	3.0907	2.6390	2.9360	3.3073	0.9536	2.0089	2.6803	1.3891	2.4602	0.7456	2.2576	2.8896
234 066	2.2994	2.4788	2.7705	2.7189	0.5847	2.3515	2.1781	1.7566	2.2146	1.4322	2.1817	2.7688
234 067	3.1095	3.1075	2.5528	2.9926	0.0608	0.3464	1.9286	0.7889	0.7784	0.3366	0.3734	2.7403

Table 12 - ELISA O.D. values @450 nm for *P. chrysogenum* strains against human sera QC2123-QC2134.

	QC2123	QC2124	QC2125	QC2126	QC2127	QC2128	QC2129	QC2130	QC2131	QC2132	QC2133	QC2134
155 627	2.5888	3.0374	3.32665	2.83525	3.32375	2.48055	3.11265	3.0728	1.77595	3.0735	3.26775	3.2833
155 628	2.5888	3.0715	3.32665	2.83525	1.41345	0.79575	3.11265	3.0728	0.95015	2.2226	2.94845	0.7243
212 031	2.5888	2.8638	3.32665	2.83525	1.49445	1.65325	2.73055	2.164	0.83665	1.927	2.91655	0.9856
215 336	2.5888	3.2325	3.32665	2.83525	1.32435	0.66475	2.38505	2.5363	0.43025	2.0909	3.26775	0.6956
215 337	1.86275	1.99785	3.07285	2.0296	1.91095	0.641	1.58425	1.7562	0.11765	1.37315	2.0442	1.74935
234 051	2.4537	2.3353	3.2449	2.778	1.0125	0.6609	1.7557	2.6445	0.3159	1.4898	2.1559	0.6328
234 052	1.93555	2.20375	2.97125	1.9507	1.60045	1.4258	2.09185	2.2484	0.80795	1.70395	2.2707	1.65325
234 053	2.5795	2.9035	3.3943	2.7012	1.2177	0.2785	1.2956	2.3693	0.409	2.1257	1.7508	0.827
234 054	2.16445	2.41685	3.09195	2.1124	2.37195	2.3348	2.59985	2.44	2.04815	2.01325	2.5657	2.59485
234 056	2.07425	2.39885	3.15305	1.9913	1.84525	1.1486	1.98305	1.7507	0.91755	1.69205	2.2922	1.62615
234 057	1.86275	1.99785	3.07285	2.0296	1.91095	0.641	1.58425	1.7562	0.11765	1.37315	2.0442	1.74935
234 058	2.20245	2.08635	3.19245	2.1124	1.79865	1.2829	2.21175	2.5991	1.08295	2.21485	2.4859	2.35455
234 059	2.5888	3.2036	3.32665	2.83525	1.99285	2.25185	3.11265	3.0372	1.01655	2.3252	3.18665	2.1975
234 064	2.16445	2.41685	3.09195	2.1124	2.37195	2.3348	2.59985	2.44	2.04815	2.01325	2.5657	2.59485
234 065	2.20245	2.08635	3.19245	2.1124	1.79865	1.2829	2.21175	2.5991	1.08295	2.21485	2.4859	2.35455
234 066	1.93555	2.20375	2.97125	1.9507	1.60045	1.4258	2.09185	2.2484	0.80795	1.70395	2.2707	1.65325
234 067	2.1744	2.3374	3.3896	2.7234	1.1075	1.064	0.5947	2.2081	0.6039	1.5754	1.7985	1.5969

Legend:

Serum ID	Serum ID	Serum ID	Serum ID
1 QC2398	11 QC2133	21 QC2127	31 QC1291
2 QC2397	12 QC1310	22 QC2134	32 QC1504
3 QC2125	13 QC1289	23 QC1292	33 QC1502
4 QC1303	14 QC2126	24 QC2128	34 QC1506
5 QC2122	15 QC1503	25 QC1293	35 QC1297
6 QC1500	16 QC2130	26 QC1295	36 QC1294
7 QC1304	17 QC2123	27 QC1296	37 QC1501
8 QC1307	18 QC2129	28 QC1668	
9 QC1288	19 QC1287	29 QC2131	
10 QC2124	20 QC1290	30 QC1505	

Figure 39 - The average response for each HpAb to all of the DAOM strains of *P. chrysogenum*.

Table 13 - List of fungi tested for cross-reactivity.

Species	Identification number
<i>P. spinulosum</i>	5053
<i>P. corylophilum</i>	5585
<i>S. chartarum</i>	44
<i>A. versicolor</i>	2-3a
<i>A. niger</i>	5869-6
<i>A. fumigatus</i>	5053
<i>P. crustosum</i>	245045
<i>A. alternate</i>	30.1
<i>S. brevicaulis</i>	5854
<i>P. brevicompactum</i>	234049
<i>E. harbariorum</i>	55.3
<i>P. variotii</i>	8424-8m
<i>P. thomii</i>	L6322-4
<i>P. funiculosum</i>	L6321-3
<i>P. decumbens</i>	5566.8
<i>E. nigrum</i>	162.2

Table 14 - ELISA results of each ascite performed by Immuno-Precise Antibodies Ltd.

Clone	52kDa target protein response	Testing on HT antigen	Isotype
2F9	2.269	0.068	IgG
*3B5	2.405	0.089	IgM
3E 8	0.514	0.076	IgG
3F8	0.468	0.083	IgG
*4B6	2.311	0.067	IgM
*4H2	2.529	0.075	IgG
5D9	2.420	0.078	IgM
*5G8	2.699	0.08	IgG
6F3	2.574	0.068	IgM
6F5	1.072	0.066	IgG
6G2	0.490	0.071	IgG
7A2	2.034	0.068	IgM
7C11	2.369	0.079	IgM
7F4	2.851	0.062	IgG
7H3	2.258	0.06	IgM
7H7	2.712	0.068	IgG
8H1	2.200	0.065	IgG
8H12	2.320	0.077	IgM
9C12	1.675	0.054	IgM
9F7	2.335	0.059	IgM
9F8	1.926	0.059	IgM
9H11	1.949	0.061	IgM
<ul style="list-style-type: none"> • also tested positive by dot blot • HT- human transferrin used as negative control 			