

**Isolation, purification and characterization
of proteins from indoor strains of *Eurotium amstelodami*, *Eurotium rubrum* and
Eurotium herbariorum that are antigenic to humans**

Shari A. Levac, B.Sc.

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ABSTRACT

Eurotium species grow on damp building materials and studies have shown that human health can be affected through allergic and toxic reactions to these fungi. By measuring human allergens or antigens, exposure assessments can be performed to study allergic responses. The goal of this research was to identify *Eurotium amstelodami*, *Eurotium rubrum* and *Eurotium herbariorum* proteins that are antigenic to humans. A 48 and 50 kDa protein was recognized as antigenic through screening against human sera from atopic patients by ELISA and immunoblotting. Their characterization was accomplished by liquid chromatography tandem mass spectrometry (LC-MS/MS) and the 48 kDa protein was identified as an α -amylase and the 50 kDa was identified as a fructosyltransferase. The target proteins were purified by anion exchange chromatography and their antigenicity was confirmed by the production of polyclonal antibodies in rabbits. Cross-reactivity tests ensured that other fungi did not produce the antigenic proteins. Further testing on monoclonal antibodies will determine their ability to specifically and selectively detect *Eurotium* antigens to assess their exposure in the indoor environment.

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ABBREVIATIONS

1° Ab – Primary antibody.

2° Ab – Secondary antibody.

ABPA – Allergic bronchopulmonary aspergillosis

AP- Alkaline phosphatase.

CBB- Coomassie brilliant blue stain (Pierce Biotechnology Inc, Rockford, IL).

CBS- The Centraalbureau voor Schimmelcultures. Institute of the Royal Netherlands Academy of Arts and Sciences, Utrecht.

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

E. amstelodami- *Eurotium amstelodami*.

ELISA- Enzyme-linked immuno-sorbent assay.

HP – Hypersensitive pneumonitis

HpAb- human polyclonal antibody.

HRP- Horseradish peroxidase. In the presence of a hydrogen donor such as TMB (ELISA) the molecule is converted from a colourless solution to a blue solution quantified via OD spectroscopy.

IBT- Mycology Group. Technical Group, Denmark.

IgE – Immunoglobulin E

IgG – Immunoglobulin G

kDa- kilodalton (1000 Da).

LMW- Low molecular weight marker. Used in SDS gels to determine protein sizes (kDa).

mAb- monoclonal antibody.

MEA- Malt extract agar.

MS/MS – Tandem mass spectroscopy.

MVOC – Microbial volatile organic compound.

OD- optical density.

pAb- polyclonal antibody.

PMSF- phenylmethylsulphonyl fluoride.

PVDF membrane (immunoblot)- Polyvinylidene fluoride membrane.

RpAb- Rabbit polyclonal antibody.

VOC – Volatile organic compound.

1. INTRODUCTION

1.1 Fungi

According to fossil records, fungi have inhabited the earth for at least 559 million years. Fungi are eukaryotic, saprophytic organisms lacking chlorophyll. It is estimated that 1.5 million species exist of which only 100 000 have been identified (Deacon, 2006). They comprise approximately 25% of the earth's biomass (Chapman et al., 2003). These adaptable organisms have found niches in every part of every ecosystem. They are found growing in marine environments; decaying material; building materials; on paints and on jet fuel (Miller, 1992). Most fungi exist as multicellular filamentous colonies but some are unicellular such as yeasts.

The fungi that are discussed in this work are the teleomorphic ascomycetes. Spores can be asexual-- a product of mitosis, or sexual—a product of meiotic recombination (Malloch and Cain, 1972). There are five traditionally recognized phyla of true fungi (Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota and Glomeromycota). The reproductive structures for each group are distinct (Lutzoni et al., 2004). Ascomycetes (fig 1a) such as *Claviceps purpurea* and *Eurotium* species contain spores within a sac-like organ called the ascus. The ascus is an elongated cell containing the spores. Once the spores have matured, turgor pressure is built up until the tip of the cell bursts and ascospores are ejected. The ascospores of some species can be projected a distance of 0.01 to 50 cm (Kendrick, 1992). Basidiomycetes (fig 1b) such as smuts, mushrooms and

Wallemia sebi produce their spores on a basidium. The interior side of gills on a mushroom illustrate is an example of where the basidiospores are produced. After maturation, the basidiospores are delicately launched from the basidia and free-fall between the gills. Once they reach the open air below the cap, natural turbulence carries them away. Zygomycetes are mostly terrestrial and can be found on decaying plants and animals. The most common form of reproduction is asexual. The main difference between zygomycetes and ascomycetes is the evolution of the conidia from the sporangiospores found in zygomycetes (Cain, 1972). Chytridiomycetes are found as unicellular or filamentous forms found in aquatic or terrestrial habitats. They produce flagellated cells in their life cycle (Lutzoni et al., 2004). Glomeromycetes can be found in wet lands but are mostly found terrestrially. They form arbuscular mycorrhizas with more than 80% of all plant species.

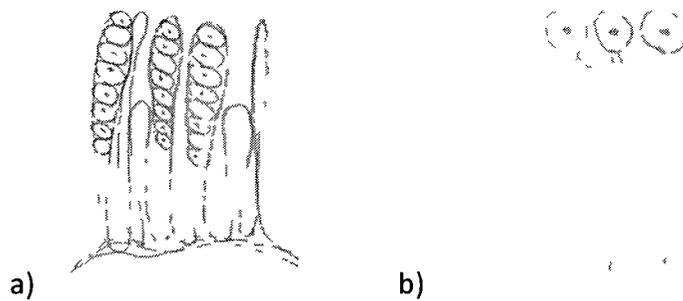


Figure 1: Reproductive structures of a) ascomycete and b) basidiomycete. Reproduced from Kendrick, 1992 and <http://biodidac.bio.uottawa.ca> (respectively).

Multicellular fungi typically grow as thread-like filaments called hyphae which intertwine to form a mycelium. In a suitable environment, this process begins when a spore germinates into a germ tube followed by growth into a hypha. Branching creates hyphae and this process continues until mycelium is formed (Watling, 2003). These cells are composed largely of water; the fresh weight of mycelia is approximately 85-90% water.

Fungi have cell walls comprised of strong flexible polysaccharides including chitin, N-acetyl glucosamine and various glucans. The glucans are attached to proteins, carbohydrates and lipids. The anamorphic ascomycetes have a triple helical 1, 3-D glucan in their cell walls. Many fungi are beneficial organisms used for the manufacturing of foods, beverages, cheeses antibiotics and anti-rejection drugs (Moore, 2001). Fungi are also known as agents of biodeterioration and biodegradation.

Fungi and bacteria are some of the primary decomposers of organic matter in most ecosystems. They are essential in nutrient cycling; the exchange of carbon and nitrogen as well as the release of elements such as calcium, hydrogen, iron, magnesium, oxygen, phosphorous, potassium, sulfur and zinc. However, their important environmental role is contrasted by their ability to cause infections, spoil foods and crops (Bauman, 2003) and the deterioration of damp building materials resulting in large economic losses (Miller et al., 2008; Pitt and Hocking, 2009). The acquisition of nutrients involves the secretion of enzymes into their environment to break down large molecules facilitating

their absorption. These enzymes are some of the major fungal allergens (Solomon et al., 1999).

1.2 Indoor fungi

The contamination of indoor air by fungi is often related to biodeterioration of building materials such as wallboard, paints, wood, insulation and wallpaper (Miller et al., 2008). A significantly higher number of propagules with a diameter smaller than spores are released from contaminated surfaces such as carpets and furniture via everyday activities. Part of the Prince Edward Island Infant Health Study found that approximately 30% of the airborne exposure to fungi was as intact spores, 30% as recognizable spore and mycelia fragments, and the remainder was to much smaller particles (Foto et al. 2005).

Fungal growth on substrates in the indoor environment relies on organic and inorganic nutrients; a sufficient amount of water and the appropriate temperature. The water activity (a_w) is defined as the ratio between the vapour pressure of the water in the material and the vapour pressure of pure water of the material and determines the water availability. The minimum a_w and optimal a_w for each fungus differs but most filamentous fungi cannot survive if the a_w is below 0.64. Xerophiles such as *Wallemia sebi* and *Eurotium* species favour condition of $a_w < 0.75$. a_w of 0.75 to 0.79 will allow the growth of moderately xerophilic species such as *Aspergillus versicolor*. Slightly

xerophilic fungi such as *Cladosporium* species and *Aspergillus fumigatus* will grow at a_w 0.8 to 0.9. When the $a_w > 0.9$ hydrophilic fungi such as *Stachybotrys chartarum* and *Chaetomium globosum* thrive (Flannigan and Miller, 2011). The most common fungi to colonize the indoor environment under moist conditions are *Aspergillus versicolor*, *Cladosporium sphaerospermum*, *Stachybotrys chartarum*, *Chaetomium globosum* and *Penicillium chrysogenum* (Miller et al., 2008; Flannigan and Miller, 2011).

1.3 Health effects related to indoor fungi

Unlike industrial or accidental exposure, low-level indoor exposures to chemicals or physical and biological hazards are very common. One of the most common environmental health issues many clinicians face are problems associated with indoor environments (Redlich et al., 1997). Population health can be greatly affected by the presence of fungi in the indoor environment which results from exposure to contaminants such as allergens, metabolites, triple helical β -1,3-D-glucan and volatile organic compounds (Horner and Miller, 2003; Rand et al., 2010, Miller et al., 2010). Studies have revealed that American homes with visibly contaminated surfaces also have poor indoor air quality and that the housing conditions are associated with exposure to the biological agents (Brunekref et al., 1989). Of approximately 14000 Canadian homes surveyed in a 1988 study conducted by Health Canada, the reported prevalence of mold was 32.4 %, moisture was 14.1% and flooding was 24.1%. Lower respiratory symptoms such as coughing, wheezing, asthma and chest illness were

approximately 50% higher in these damp homes. Upper respiratory and nonrespiratory symptoms were increased by 20-25% (Dales et al., 1991). Mold is associated with exacerbation of allergic rhinitis and allergic asthma in sensitized individuals but molds can also illicit inflammation via non allergenic mechanisms (Krieger et al., 2010).

Indoor air contaminants include volatile organic compounds (VOCs), combustion gases, bacteria and fungi. Many investigators have concluded that there is an association between damp, mold-contaminated buildings and adverse health effects of individuals occupying these dwellings (Zock et al., 2002; Mudarri et al., 2007). Reports from the European Community Respiratory Health Survey in addition to studies conducted across Canada indicated that the sensitization of molds is a risk factor for severe asthma in adults and that countries with a higher rate of asthma have a higher percentage of damp homes (Zock et al., 2002). Building and mold related dampness were associated with a 52% increase in upper respiratory symptoms, a 50% increase in coughing and a 44% increase in wheezing (Dales et al., 1991).

Biological particles can enter the body via the nose, mouth, conjunctiva epithelia, bronchi, alveoli and epidermis. Once introduced into the respiratory tract, the depths to which a bioaerosol can travel depends on its size, shape, density and reactivity (Rand et al., 2010; Miller et al., 2010). The majority of indoor fungal exposure is to particles with a diameter less than 2.5 μ m. These particles will deposit deep into the lung and resulting in exposures to toxins, allergens triple helical glucan. This form of glucan increases

results in pulmonary inflammatory responses in mice at doses that can occur in moldy buildings. At low doses, it induces the dectin-1 gene transcription and expression in proximal lung regions. Because fungi like the *Eurotium* species discussed here contain triple helical β -glucan fragments upregulate chemokines, interleukins, lymphotoxins and tumour necrosis factor leading to phagocytosis and cytokine production (Rand et al., 2010).

Although water-damaged homes have mold contamination many homes without visible water damage can become contaminated (Dales et al., 2010). A Health Canada review found an association between reported mold or dampness in homes and the prevalence of respiratory complaints (Dales et al., 2008). Everyday activities such as cooking and cleaning and the presence of pets and plants coupled with air-tight windows and doors (for energy savings) are conducive to fungal infestations (Chapman et al., 2003). Because energy efficient homes involve a lower rate of air exchange with outdoor air, there is a possibility that the sources of contamination are not diluted as quickly as they once were. Contaminated air conditioners and humidifiers can also circulate fungal materials. Because Canadians spend up to 90% of their time indoor the relationship between indoor and outdoor airborne fungal concentrations is of great importance (Health Canada, 2004).

Building related illness is a common problem and refers to non-specific complaints of headache, upper respiratory irritation, asthma-like symptoms, fatigue, rashes and gastrointestinal complaints (Hung et al., 2005). It is rarely attributed to a single specific exposure. It is nevertheless associated with a particular building and the duration of the exposure and occupant health determine the severity of the symptoms but once the occupant leaves the location, the symptoms generally subside (Hung et al., 2005). Building related illness is not to be confused for illnesses such as rhinitis, asthma and hypersensitive pneumonitis which can result from exposure to mold allergens.

Fungal metabolites play roles in diseases and poisoning of insects and animals (Gloer, 1995). Mycotoxins are low molecular weight (<1 kDa) compounds. The term is restricted to metabolites that pose health risks to humans and animals (Kendrick, 1992). They may be classified by their structure; the taxonomy of the producing fungi; the disease condition or depending upon the occupation of the individual studying the case. Exposure via inhalation, ingestion and dermal contact to mycotoxins may result in mycotoxicosis. Their health effects include rashes, nausea, compromised liver function and immunosuppression (Health Canada, 2004). Poor nutrition, age, sex, race and prior infections are all major determinants of mycotoxicosis (Jarvis & Miller, 2005). There is a great complexity of the relationship between mycotoxin exposure and health outcomes. Human cases of toxicosis have not been well characterized and cannot be associated with occupant exposure to mold-damaged buildings (NAS, 2004). It is believed that

mycotoxins are produced by fungi as interference for competition when limited amounts of nutrients are available. The production of the given metabolites limits the growth of competing species on the substrate. Each species produces a mixture that is species-specific (Nielsen, 2003) and characteristic of the environment the fungus has colonized (Jarvis and Miller, 2005).

Compounds other than mycotoxins such as structural polymers can cause respiratory illnesses. Beta 1, 3-D-glucan in triple helical form illicit immune responses in animals due to the activation of the dectin-1 receptor (Rand et al., 2010). The symptoms of exposure are dry cough; itchy skin; eye, nose and throat irritation, hoarseness and fatigue (Rand & Miller, 2011).

VOCs are also released during the active growth of fungi. Although their effects on human health is unclear (Horner and Miller, 2003) exposure to some VOCs is known to cause nasal irritation (NAS, 2000).

Exposure to fungal allergens can result in allergies. Approximately 20% of the population is atopic and can easily become sensitized by exposure to low concentrations, the remaining 80% of population requires exposure to a larger dose. Conditions such as chronic hypersensitive pneumonitis or pulmonary mycotoxicosis (farmer's lung) are often related to occupation (Kaukonen et al., 1994, 1996; Hjort et al.,

1986). Allergy or hypersensitivity can be described as an immune response against a foreign antigen that is exaggerated beyond the norm. Antibodies are the molecules capable of identifying the allergen and triggering the immune responses when antigens are introduced into the body. Monoclonal antibodies are produced by a single B cell (naturally occurring in a body) or a single clone of hybridoma cells (often produced in mice in a laboratory). The resulting monoclonal antibody is a pure, homogeneous antibody capable of recognizing a specific epitope. Polyclonal antibodies are derived from more than one B cell line. This mixture of polyclonal molecules are secreted against a specific antigen, and depending from which B cell line they originated, each one will be capable of recognizing a different epitope (Murphy et al., 2008; Bauman, 2003). Every individual is exposed to environmental allergens against which a normal immune response occurs comprised of IgG production but in allergic individuals, the response includes the production of both IgG and IgE. When the allergen subsequently enters the body, it binds to the active sites of the IgE on the surfaces of sensitized cells triggering an excessive activation of mast cells and basophils, resulting in a systemic inflammatory response that can lead to death. IgE molecules make up less than 1% of antibodies in the blood serum and act as signal molecules between specific and non-specific responses. They can trigger a rapid release of histamine responsible for inflammation (Murphy et al., 2008; Bauman, 2003). IgG are small and make up approximately 85% of blood serum antibodies; they have two paratopes and can easily leave the blood vessels to bind antigens before they enter the circulatory system (Bauman, 2003). The other 14% is composed of IgA, IgD and IgM antibodies.

1.3.1 ABPA (Allergic bronchopulmonary aspergillosis)

ABPA is a pulmonary disease that occurs when *A. fumigatus*, and sometimes, other *Aspergillus* species have colonized the lower respiratory tract after long term exposures of high concentrations of spores (Hung et al., 2005; Dillon et al., 2007). Individuals with cystic fibrosis are at great risk. *A. fumigatus* spores are small enough to be inhaled into the respiratory tract where they grow successfully in the mucous. Once the spores germinate, they produce gliotoxin triggering the production of macrophages and inflammation. In predisposed individuals such as cystic fibrosis patients, IgG as well as IgE antibodies are produced against surface antigens of *A. fumigatus* (Ellis and Day, 2011).

1.3.2 Allergic rhinitis and asthma

Atopic patients have elevated levels of IgE antibodies (Hung et al., 2005) and are therefore predisposed to allergic responses such as allergic rhinitis and asthma. Allergic rhinitis presents itself with symptoms such as runny nose, sneezing and congestion (Ellis and Day, 2011). Studies have shown that patients with allergic rhinitis exhibit an increase in inflammatory cells in the bronchial mucosa as well as bronchial hyperresponsiveness (Pawankar, 2004). Symptoms of asthma include wheezing, coughing, shortness of breath and chest tightening. It is a chronic inflammatory disease of the airways and is characterized by the recurrence of airflow obstruction and

bronchospasms. This is different than chronic obstructive pulmonary disease since the airway obstructions are usually reversible (Yawn, 2005). The inflammation is induced by the interaction of IgE antibodies with an allergen (Hung et al., 2005). Common household contaminants such as pets, dust mites, smoking and mold as well as socio-economic factors are known to affect respiratory health. Asthmatics with an underlying mold allergy living in damp environments and exposed to indoor fungi could have increased asthma symptoms (NAS 2000).

1.3.3 Hypersensitive pneumonia (HP)

Hypersensitive pneumonia is an immunological lung disorder caused by inflammation due to exposure to bacteria, fungi, low molecular weight compounds, chemicals or inorganic particulates (Hodgson and Flannigan, 2001; Hung et al., 2005). It is most often encountered in occupational settings but some building related exposures have been linked (Hung et al., 2005). HP often occurs 4 to 12 hours after heavy exposure to the particles and symptoms include cough, fever, chills, shortness of breath and body aches. The symptoms subside hours to days after exposure and are reversible if the exposure is not repeated. Chronic HP may cause fibrosis if the exposure is persistent (Hung et al., 2005).

1.3.4 Conjunctivitis

The symptoms of conjunctivitis include itchiness and redness of the eyes, tears, blurry vision and a discharge from the corners of the eyes as well as an increased sensitivity to light (Simoni et al., 2007). It is caused by the inflammation of the conjunctiva (the clear membrane) that covers the eyeball and the inside of the eyelid (Hung et al., 2005). Allergic conjunctivitis is not contagious and is triggered by exposure to plant pollen, grasses, animals or microbial agents.

1.4 *Eurotium* species

Eurotium species are found in the order Eurotiales which is comprised of 50 genera and 140 species including *Penicillium* and *Aspergillus*. They are extremely successful and are of great importance due to their production of antibiotics, mycotoxins, biodeterioration and food spoilage. *Eurotium* are primary colonizers capable of growth below a_w of 0.8. The low moisture requirement allows them to colonize areas where other fungi could not grow due to the minimal or intermittent moisture availability. The rapid growth of the large numbers of small dry spores makes them a significant contaminant with respect to indoor air (Abbott & Abbott, 2004).

Some species of *Aspergillus* have their teleomorphs (sexual reproductive stage) in the genus *Eurotium* (Kendrick, 1992; Pitt & Samson, 2007). *E. amstelodami*, *E. herbariorum*

and *E. rubrum* comprise the teleomorphic states of *Aspergillus glaucus*. These osmophilic *Eurotium* species have pseudoparenchyma – tightly interwoven hyphae, one cell thick, that superficially resemble plant tissue. They produce cleistothecia which contain the ascospores. *Eurotium amstelodami* ascospores (figure 2A) are hyaline, bright yellow, rough-walled with a furrow and two longitudinal ridges (Malloch and Cain, 1972).

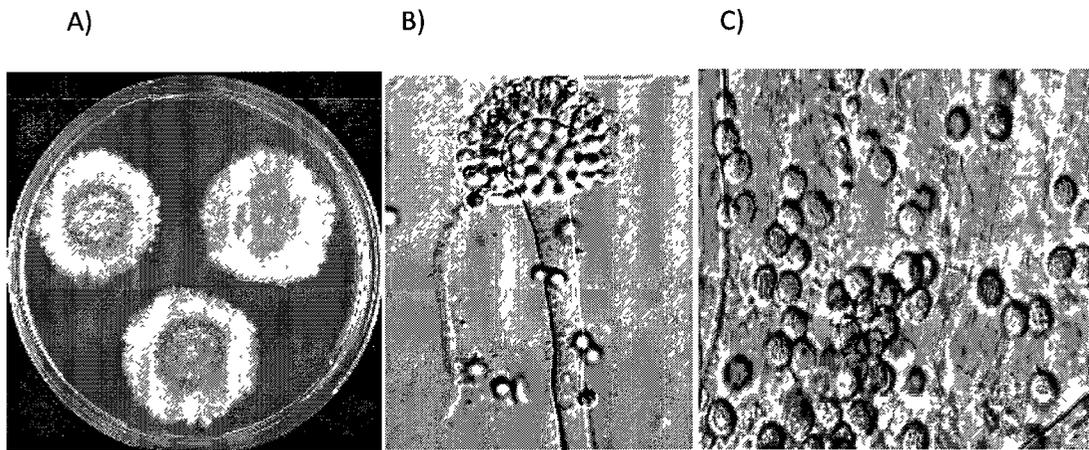


Figure 2: **A)** *E. amstelodami* on MEA. **B)** *E. amstelodami* conidiophores. **C)** *E. amstelodami* conidia. Used by permission of the CBS, The Netherlands.

Ascospores are 5 to 8 μm in diameter, globose and are only produced on media with elevated sugar concentrations (Pitt & Samson, 2007). *Eurotium rubrum* ascospores (figure 2B) have a distinct furrow and the hyphae become brick red as they age (Klick, 2002). *Eurotium herbariorum* will produce ascospores (figure 2C) after 19 days when grown on media of pH 3.8 containing glucose or fructose at a_w 0.74 and can grow in low oxygen concentrations (Pitt & Hocking, 2009). When grown on MEA (malt extract agar) the colony will be approximately 70 mm in 11 days at room temperature. They are odorless, appear velvety, and the colour changes from yellow-green to green-white to a feathery white at the very edges of the colony (Malloch & Cain, 1972).

In the natural environment, *Eurotium* species are found in soils and are often isolated from harvested hay (Roussel et al., 2010). In the built environment, *E. herbariorum*, *E. amstelodami* and *E. rubrum* are commonly found on mould-damaged, paper-faced gypsum wallboards, textiles, ceiling tiles and wood which have been previously wetted or subjected to periodic condensation (Flannigan & Miller, 2011; Miller et al., 2008). Salt tolerance is a critical trait for species found on wallboard and other building materials (Slack et al., 2009). Some *Eurotium* species have been isolated from moderately saline soils and waters, and, most recently *E. amstelodami*, *E. rubrum* and *E. herbariorum* have been isolated from the Dead Sea – an environment so saline that it was once believed to contain only prokaryotic organisms capable of adaptation to these extreme environments (Butinar et al., 2005). The characteristics of the strains found

in the Dead Sea did not differ from known species previously described from other environments but their ascospores were slightly larger (Butinar et al., 2005). A small portion (up to 15%) of the *E. amstelodami* and *E. herbariorum* spores grown in the Butinar et al. (2005) study remained viable at up to 30% NaCl whilst 50% of *E. rubrum* spores were capable of survival. In the same study, *E. amstelodami* was shown to grow at temperatures up to 37°C but, *E. rubrum* and *E. herbariorum* showed little to no growth.

Fungi can be found contaminating foods at all a_w values but spoilage occurs at $a_w < 0.9$. *Eurotium* are usually the first colonizers of improperly dried commodities and as they amass they increase the a_w allowing other species such as *Penicillium* to grow (Abdellana et al., 1999). They are often found on foods preserved with high concentrations of NaCl or sugar. Bakery products contaminated by xerophilic fungi such as *Eurotium* are usually contaminated during the post-baking cooling period since the baking process would destroy any previous contamination. *Eurotium* species produce a variety of secondary metabolites which cause oxidative rancidity in grains and nuts (Wheeler & Hocking, 1993).

1.5 Health issues related to *Eurotium* species

Fungi in general cause diseases such as infections, allergies and inflammation (Sorenson, 2001). Fungal proteins give allergic reactions within minutes of exposure (type I allergy) as well as histamine release. Mycotoxins and peptides released from spores after inhalation may cause mycotoxicosis in people living or working in infested buildings (Nielsen et al., 1999). Most superficial and invasive infections are opportunistic because the patients have a pre-existing condition or impaired immunity (Piecková, 2003).

Eurotium amstelodami is believed to cause farmer's lung – a common form of hypersensitive pneumonia (Kaukonen et al., 1994; Hjort et al., 1986) and may evolve into end-stage lung disease (Lacasse, 2003). It can result from repeated exposures to high concentrations or prolonged exposures to low concentration of inhaled allergens from mold-covered straw and hay to which the patient has previously been sensitized (Fenoglio, 2007; Radon, 2002; Roussel et al., 2010). Pig farmers, poultry farmers and greenhouse workers are at high risk for work related respiratory symptoms. In poultry and pig houses, the predominant fungi are *Eurotium* species (52.8% and 30.8% respectively). Due to the difference in conditions between animal houses and greenhouses, the *Eurotium* species only comprise 2.7% of fungi found in greenhouses (Radon, 2002). In the study done by Roussel et al. 2010, hay handled by farmers with hypersensitive pneumonia contained large amounts (up to 6.5×10^5 CFU/m³) of molds. The main species found on hay known to cause farmer's lung include *W. sebi*, *E. amstelodami* and *A. corymbifera* (Fenoglio, 2007). The differentiation of hypersensitive

pneumonia from other interstitial lung disease is difficult because it relies on non-specific clinical symptoms, chest x-rays, lymphocytic alveolitis on bronchoalveolar lavage and granulomatous reactions on lung biopsies (Reboux, 2001; Fenoglio 2007). The diagnostic value of serum precipitins is controversial due to the lack of sensitivity and specificity. Fenoglio (2007) demonstrated that relevant antigens help diagnose mold-induced hypersensitive pneumonia. *E. amstelodami* serum precipitations allowed the differentiation of patients with farmer's lung versus the control group in 8 of 11 cases (Reboux, 2001).

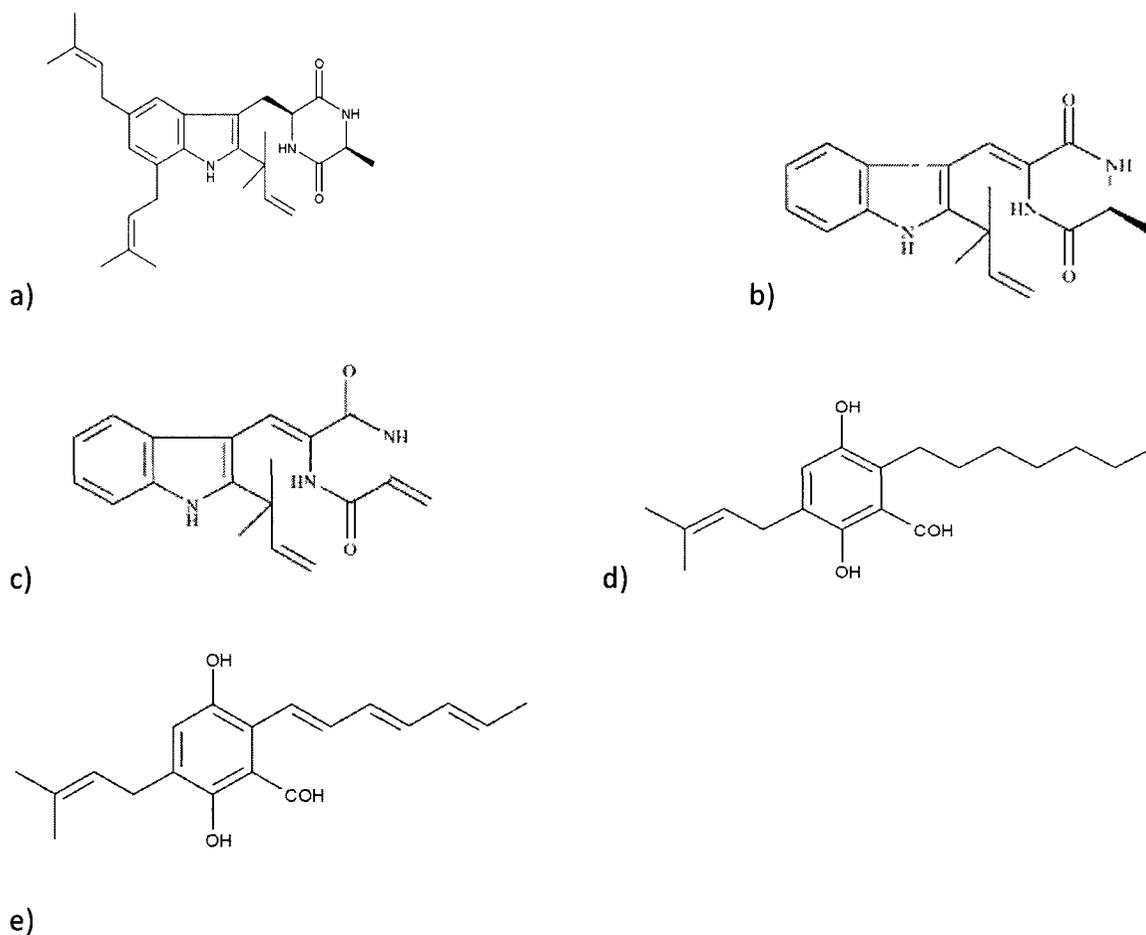


Figure 3: Metabolites of the *Eurotium* species studied: a) echinulin with the diketopiperazine backbone highlighted. b) neochinulin c) neochinulin B. d) flavoglaucin. e) auroglaucin (Slack et al. 2009).

Not including mushroom toxins, 350 to 400 fungal metabolites are considered toxic – the majority of which are 200 to 500 Daltons in size (Sorenson, 2001). The study of *Eurotium* species as indoor contaminants is important in relation to human health because several metabolites produced by these fungi are known to be toxic. *Eurotium* species produce compounds such as echinulin (figure 3a) and its derivatives (Casnati et

al., 1973); anthraquinones such as physcion; and benzoquinones such as flavoglaucin (figure 3d) and auroglaucin (figure 3e; Ashley et al., 1939). Echinulin (figure 3a) was first isolated from *Eurotium amstelodami* in 1964 and many related compounds such as neochinulin A and neochinulin B (figure 3 b, c respectively) have since been isolated each containing the diketopiperazine component (Gatti & Fuganti, 1979).

A 1989 study by Ali et al., revealed that when female mixed breed rabbits were injected intraperitoneally with 10 mg/kg BW of purified echinulin severe damage to the lungs occurred as seen by the thickening of alveolar walls, damage to the alveolar organization and the presence of large numbers of red blood cell aggregates. There was also evidence of significant liver damage as seen by elevated levels of plasma lactate dehydrogenase; glutamic-oxaloacetic and glutamic-pyruvic transaminases and cardiac derived isozymes (Ali et al., 1989). Echinulin was cytotoxic to HeLa cells at 100 mg/mL (Umeda et al., 1974) but was not genotoxic to hepatocytes isolated from ACI rat and C3H/HeN mice livers at 10^{-4} M in the hepatocytic primary culture/DNA repair test (Mori et al., 1984). There were several studies that determined echinulin to be the cause of feed refusal in swine at 8µg/g and mice refused water contaminated at levels of 90 µL/mL (Vesonder et al., 1988).

Another metabolite, flavoglaucin (figure 3 d) and its derivatives such as auroglaucin (figure 3e) have been detected in mycelial extracts of *Eurotium* species (Slack et al., 2009). These compounds have been reported as weakly cytotoxic to HeLa cells (Umeda

et al., 1974) and have shown inhibition of mitochondrial respiration; induction of mitochondrial swelling (Kawai et al., 1986) and the cause of liver damage in rabbits (Nazar et al., 1984).

Eurotium species contain the triple helical form of 1,3- β -D-glucan which is known to induce inflammation-associated genes responses (Rand et al., 2010). Low doses of curdlan (40 ng/kg lung) induced dectin-1 gene transcription and expression in proximal lung regions. Localization of dectin-1 mRNA transcript and expression along the respiratory bronchial epithelia further confirmed the important immunomodulatory function of the cells and the potential role they play in responses to fungal glucans (Rand et al., 2010).

1.6 Biological mechanisms associated with allergies

Once a person has been exposed to an antigen, sensitization may occur. When the allergen subsequently re-enters the body, it binds to the active sites of IgE on the surfaces of sensitized cells triggering an excessive activation of mast cells and basophils, resulting in a systemic inflammatory response (Gould et al., 2003). IgG and IgE molecules are involved in allergic reactions and both have heavy and light chains with variable and constant regions. IgE molecules are responsible for breathing difficulties, swelling, and anaphylactic shock. IgG molecules provide long-term resistance (Gould et al., 2003). IgG are small and make up approximately 85% of blood serum antibodies;

they have two binding sites (paratopes) and can easily leave the blood vessels to bind antigens before they enter the circulatory system. IgE molecules make up less than 1% of antibodies in the blood serum and act as signal molecules between specific and non-specific responses (Murphy et al., 2008; Bauman, 2003) and may reach over 10X the normal level in atopic individuals (Gould et al., 2003).

The FcεRI receptor on mast cells has a high affinity for IgE and is expressed at 200 000 molecules/cell in mast and basophils but at much lower concentrations in monocytes, platelets and eosinophils. The FcεRI:IgE complex has a very slow dissociation rate (K_a of 10^{10} M^{-1}) with a half life of 20 hrs and the IgE may remain on the mast cell for another 14 days (Gould et al., 2003).

Mast cell activations in the nose, lung, gut and skin will cause hay fever, asthma, reaction to foods and eczema, respectively. Anaphylaxis is caused by basophils—the IgE effector in blood (Gould et al., 2003). The activated cells undergo degranulation due to cytolysis caused by allergen stimulation resulting in early phase allergic reactions such as histamine, serotonin, lipid mediators, proteases, chemokines and cytokines. This immediate allergic response is usually followed by the late phase response. The T_H2 cytokines responsible for the initiation of the late phase provoke the migration of the eosinophils to the site of the allergen which in turn release proteins, free radicals and other compounds amplifying the inflammatory response (Murphy et al., 2008).

Environmental and genetic factors both play a role in the development of allergies. Some studies have shown that 40 to 80% of patients with allergic rhinitis or asthma had positive family histories compared to only 20% of individuals without allergic diseases (Ownby, 1990). However, an environment where children are not exposed to other siblings and thus lack exposure to infectious diseases at a young age often results in under-developed immune systems – a term described as the “hygiene effect” which makes the individual prone to allergic diseases later in life (Strachan, 1989).

1.7 Allergens and their diagnosis

Allergens are characterized by their biological function. They can be enzymes (Asp f1), enzyme inhibitors (wheat α -amylase inhibitor), ligand-binding proteins (cockroach Bla g 4) or structural proteins such as paramyosin. Allergens have a given nomenclature – the first three letters from the genus name, followed by the first letter of the species name and the number indicating the order of discovery (Chapman et al., 2000). For example, Asp f1 was the first allergen discovered from *Aspergillus fumigatus*.

Environmental allergens such as fungi, pollen, latex, dust mites and animal dander are derived from biological and chemical origins. Except for allergens from *Alternaria alternata* and the most widely studied fungus *Aspergillus fumigatus* (Horner et al., 1995), the characterization of most fungal allergens has been challenging for several reasons as discussed below. Numerous allergens including Alt a1 are capable of reacting

with an antigen that did not stimulate its production or with a similar antigenic site on a different protein. This cross-reactivity may be caused by structural similarities of the epitopes or due to homologous amino acids present in the reactive sites (Fedorov et al., 1997).

To demonstrate allergic sensitization, there are several *in vivo* and *in vitro* methods that measure the IgE antibody concentration in blood. The most common *in vivo* method is the skin prick test (SPT). It attempts to provoke a small controlled allergic response by adding a few drops of purified allergen onto a gently pricked skin surface. The resulting swelling is correlated to the inflammatory mediators active during an allergic response (Hung et al., 2005). Several limitations exist, especially for building related fungi. There is no standardization of the extracts and because they are manufactured by different companies, the protein compositions vary from batch to batch. Also, each company has its own growth and storage conditions (Hung et al., 2005; Simon-Nobbe et al., 2008). In a study where extracts of the same fungus produced by two different companies were tested via skin prick test, one extract resulted in 4% of patients having positive responses to *C. globosum* whereas the other company had a response in 7% of patients (Beezhold et al., 2008). The lack of standardization affects the specificity and sensitivity of the tests (Esch, 2004) and cross-reactivity between the available allergen extracts make it difficult to determine which fungus is responsible for the allergic reaction and whether it actually originated from the indoor environment (Hung et al., 2005). *In vitro*

methods include the radioallergosorbent test (RAST), ImmunoCAP, halogen immunoassay (HIA), ELISA (enzyme linked immunosorbent assay) and western blots. The RAST uses blood to determine allergic reactions. The suspected allergen is bound to an insoluble material and the test serum is added. A radiolabelled anti-human IgE is used as the tag and the amount of radioactivity is proportional to the IgE concentration in the serum. It is less sensitive and specific than the SPT (Hung et al., 2005). ImmunoCAP by Pharmacia quantitatively measures IgE in serum. It has a high binding capacity including to those IgE that are present at very low levels (Carrer et al. 2001). The halogen immunoassay can simultaneously visualize individual particles collected by air samplers together with expressed antigens following immunostaining with human IgE. It can detect allergens expressed by germinating fungal conidia (Green et al., 2006a). ELISA kits for mouse, cockroach, dust mites, cats, dogs, *A. alternata* and *A. fumigatus* allergens have been purified (Pate et al., 2005; Sporik et al., 1993). They have been widely used for environmental allergen detection due to their specificity, accuracy and high throughput (Chapman et al., 2001). Limitations include the characterization of only a few fungal allergens and many fungi share common antigens increasing the risk of cross reactivity (Chapman et al., 2001; Cramer 2011; Sporik et al., 1993). The use of monoclonal antibodies greatly reduces this risk. The very nature of their development allows for extreme specificity and they are usually epitope-specific (Green et al., 2006). Western blots rely on immunostaining with human sera to identify allergen sensitization. The presence of IgG antibodies in serum can be used because they are

considered an indirect marker for allergen exposure but not as a sign of disease (Douwes et al., 2003).

1.8 Detection of indoor fungi

Features used to characterize fungi include macromorphology, micromorphology, physiology, secondary metabolites, extracellular enzymes and DNA sequencing (Frisvad, 2007). The identification via morphology, growth rate and colour on one or more types of agar is widely used (Nielsen, 2003). Some such methods include tape sampling and agar slide methods and often, they are performed using air sampling and agar plates (Miller, 1992). Every one of these methods relies on media for growth then identification resulting in associated difficulties. Extensive knowledge of fungal taxonomy is needed to identify the colonies on the given agar. Often, taxonomic keys and a great deal of experience are needed to identify some species (Gutarowski et al., 2007). Only viable cells will grow on agar which may not necessarily be indicative of the concentration of allergenic proteins or mycotoxins present in the environment. The inherent problem remains that the different growth requirements of fungi commonly found indoor will necessitate different media. There is not one medium on which all fungi will grow (Flannigan et al., 2011). If 2% malt extract agar is chosen, and xerophilic fungi are present, they will most likely not appear since they will be outcompeted by less xerophilic fungi present in the sample, however, this does not mean that fungi such as *W. sebi* or *Eurotium* species are not present. Therefore, it must be known if the fungi

are xerophilic or halotolerant before sampling. If the wrong medium is chosen, it may result in false negatives/positives. The efficient detection of fungal propagules with an air sampler requires the operator to choose appropriately sized filters as fungal spores vary in size from 3-4 μm to 60 μm . Also, spore concentrations change over time. In houses, weekly variations of 1 or 2 orders have been demonstrated (Miller, 1992) so, the air sampling would have to be done at a time that is representative of normal conditions. These variables contribute to the difficulties in determining indoor fungal concentrations.

Quantitative measurements of fungi can be accomplished by the detection of 1,3- β -glucan concentrations since it is found in all fungal cell walls whether viable or not (Miller, 1992). Although this would give a definite amount of fungi present, it cannot give any kind of qualitative information such as which species fungi are present. Because the costs associated with molecular biology have decreased this method is becoming more popular in identification. However this approach is limited by the availability of data on properly identified reference strains (Nielsen, 2003).

Given that mixtures of metabolites are species specific, chemotaxonomy can be an important tool for identifying fungi. This method would avoid incorrect identification via microscopy or contamination of the cultures but, it requires analytical chemists with extensive knowledge of fungal metabolites and state of the art instrumentation (Nielsen, 2003). As is often the case, pure metabolite standards are not available for methods such as HPLC, MS and NMR (Daisey et al., 2003).

The culture-based methods for the identification of fungi are only capable of identifying viable cells, but, many allergic responses of fungi are to proteins found in mycelial extracts (Gorny, 2004), or spore fragments which are undetectable using agar plates. The most reliable method of detection remains antigen capture methods such as enzyme-linked immunosorbent assay (ELISA) which are capable of identifying the presence of antigens that may not be evident in other methods. These methods exploit the use of antibodies. The goal of industrial antibody production is to produce a high affinity, high titer sera. Antibodies have been manipulated to produce a wide range of useful tools—they can be used to locate an antigen at the cellular level or to isolate an individual antigen from a mixture and to determine the amount of antigen present by ELISA (Harlow & Lane, 1999). The extent to which they are selective and specific to its antigen is of great use in laboratories. If a sample contains fungal fragments or dead cells, the antibodies will still recognize the epitope and allow for a reaction to occur. This will allow the investigator to detect exactly the concentration of a given species.

Because antibodies recognize a small region of an entire antigen, there are occasions when the antibodies react with a related structure of another molecule, this is known as cross reactivity. This can be useful when finding related proteins but in the case of antigen detection, it is unwanted. This issue would be resolved by ensuring the use of monoclonal antibodies. There are five main factors that influence the performance on antibodies in immunochemical techniques: avidity of the antibody to the antigen; specificity; alteration of the structures during the technique; physical accessibility; and

the type of secondary reagents (Harlow & Lane, 1999). In ELISA techniques, the only concerns are that the antibodies produced are in fact specific and have a fair degree of avidity.

The traditional ELISA techniques are time-consuming and allow for only one antigen to be probed at a time. The Luminex Corporation has created a bead-based multiplexing technique by combining several technologies to allow for the detection of up to 100 different analytes per well. A microbead with radius of 5.6 μm containing combinations of two dyes allows for its detection. Each antibody can be bound to a set of beads of a specific mixture which can be identified by the detection system once the reactions are completed. This technique, combined with an appropriate amount of antibodies could perhaps be an easy solution to the fungi identification issues.

1.9 Project Aim

The goal of this project is to determine whether there is a fungal protein antigenic to humans common to *E. herbariorum*, *E. amstelodami* and *E. rubrum* and to subsequently identify and purify this protein followed by the production of polyclonal and monoclonal antibodies to be used for detecting the presence of these fungi in indoor environments.

REAGENTS

5X protein sample loading buffer – 15% sodium dodecyl sulfate (SDS, J.T. Baker, Phillipsburg, NJ); 50% glycerol (EDH, USA); 0.05% bromophenol blue (USB, Cleveland, OH); 5% β -mercaptoethanol (Sigma-Aldrich, Oakville, ON) and 30% 624mM Tris-HCl (Sigma-Aldrich, Oakville, ON), pH 6.8.

Ammonium bicarbonate- 100 mM in ultrapure water. (Sigma-Aldrich, Oakville, ON).

AP-conjugated anti-human IgG antibody- alkaline phosphatase (AP) conjugated mouse anti-human IgG (Sigma-Aldrich, St. Louis, MO). Diluted 1:10000 in 1% BSA-TBST buffer. 1° Ab for immunoblot.

AP-conjugated anti-rabbit IgG antibody – alkaline phosphatase (AP) conjugated goat anti-rabbit IgG (Sigma-Aldrich, Oakville, ON). Diluted 30 000x in 1% BSA-TBST.

BCIP developing solution- Liquid substrate system (BCIP/NBT 5-bromo-4-chloro-3-indolyl phosphate dipotassium/ nitrotetrazolium blue chloride) purple liquid for immunoblot membranes (Sigma-Aldrich, Oakville, ON).

Blocking solution- 1%(w/v) bovine serum albumin (BSA; Sigma-Aldrich, Oakville, ON) dissolved in TBST buffer (immunoblots) or PBST buffer (ELISA). pH 7.5.

Bradford dye reagent (protein concentration assay)- 150 μ L aliquot of Quick Start Bradford dye reagent (Bio-Rad, Hercules, CA) containing methanol and phosphoric acid.

Coating buffer- 50mM carbonate-bicarbonate buffer. pH 9.6. Promotes protein adhesion to ELISA plate. (Sigma-Aldrich, Oakville, ON).

Developing solution (silver staining)- 5 μ L formaldehyde (37% w/v) was added to 25 ml aliquot of the stock solution (6.25 g sodium carbonate made up to 250 mL with ultra pure H₂O) just prior to developing GE Healthcare, Piscataway, NJ).

DTT- Dithiothreitol. 20mM in sodium bicarbonate. (Sigma-Aldrich, Oakville, ON).

Ennatin medium containing 1% glycerol- 50 g maltose (Sigma-Aldrich, Oakville, ON); 8 g peptone (Difco, Lawrence, KS); 5 g yeast extract (Sigma-Aldrich, Oakville, ON); 0.75 g KH₂PO₄(Sigma-Aldrich, Oakville, ON); 0.5 g MgSO₄-7H₂O (J.T. Baker, Phillipsburg, NJ); 0.067 g CaCl₂-2H₂O (Difco, Lawrence, KS) and 20 g glycerol per L of ultrapure H₂O.

Equilibration buffer I- (20mL) contained 0.91g Trizma Base (Sigma- Aldrich, Oakville, ON), 14.12mL of 8.5M urea (Sigma- Aldrich, Oakville, ON), 0.4g SDS (J.T. Baker, Phillipsburg, NJ), 4mL glycerol (BDH, Toronto, ON) and 0.4g DTT (Sigma- Aldrich, Oakville, ON).

Equilibration buffer II- (20mL) Similar to equilibration buffer I, except 0.5g Iodoacetamide (Sigma- Aldrich, Oakville, ON) replaces the 0.4g DTT (Sigma- Aldrich, Oakville, ON).

Fixation solution- 10 mL ethanol, 2.5 mL glacial acetic acid made up to 25 mL with ddH₂O (GE Healthcare, Piscataway, NJ).

Gel filtration column- Sephacryl S-300-HR resin (Sigma-Aldrich, Oakville, ON)

Glycoprotein oxidizing solution- 250 mL 3% acetic acid was added to 2.5 g of oxidizing reagent (Pierce, Rockford, IL).

Glycoprotein reducing solution- 250 mL 3% acetic was added to 1.25 g of reducing solution (Pierce, Rockford, IL).

Glycoprotein horseradish peroxidase positive control- 0.5 mL of ultra pure H₂O was added to 1 mg horseradish peroxidase (Pierce, Rockford, IL). Diluted to 1 mg/mL with SDS-PAGE sample buffer.

Glycoprotein soybean trypsin inhibitor control- 0.5 mL of ddH₂O was added to 1 mg horseradish peroxidase (Pierce, Rockford, IL). Diluted to 1 mg/mL with SDS-PAGE sample buffer.

Glycoprotein stain- (Pierce, Rockford, IL).

HRP-conjugated goat anti-human IgG antibody- Horseradish peroxidase (HRP) conjugated goat anti-human IgG (Sigma-Aldrich, St. Louis, MO). Diluted 1:10 000 in 1% BSA-PBST buffer. 1^o Ab for ELISA.

Iodoacetamide- 100 mM in ammonium bicarbonate. (Bio-Rad, Hercules, CA).

Leammli running buffer- 15 g Tris (Sigma-Aldrich, Oakville, ON), 72 g glycine (Sigma-Aldrich, Oakville, ON), 10 g SDS (USB, Cleveland) made up to 1 L ultrapure H₂O.

Novex[®] IEF anode buffer- (Carlsbad, CA). Diluted 50X with ultrapure H₂O.

Novex[®] IEF cathode buffer- (Carlsbad, CA). Diluted 20X with ultrapure H₂O.

Novex® IEF sample buffer- (Carlsbad, CA). Diluted 2X with protein sample.

PBST buffer (Phosphate buffered saline with Tween 20)- 80 g NaCl, 26.8 g Na₂HPO₄-7H₂O, 2 g KCl, 2.4 g KH₂PO₄ and 1.0 g Tween 20 made up to 1 L with ultrapure H₂O. pH 7.4.

PMSF (phenylmethanesulphonyl fluoride) – A serine protease inhibitor (Sigma-Aldrich, Oakville, ON).

Protease inhibitor cocktail- contains 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64 and 1,10-phenanthroline. Broad specificity for the inhibition of serine, cysteine, aspartic and metallo-proteases (Sigma-Aldrich, Oakville, ON).

Protein sample loading buffer- 11.9% 0.5 M Tris (pH 6.8; Sigma-Aldrich), 23.8% glycerol, 1.9% SDS, 1.9% bromophenol blue (0.5%; USB). This was diluted 4X with protein sample.

Q-sepharose anion exchange resin- Q sepharose fast flow anion exchanger, wet bead size: 45-165µm pre-swollen in 20% ethanol. (GE Healthcare, Piscataway, NJ).

Ready gel IEF gel- pH 3-10.(BioRad, Hercules, CA).

SDS-PAGE gels (10 %)- Running gel: 1.7 mL 30% bis-acrylamide (Bio-Rad), 1.3 mL 1.5 M Tris (pH 8.8; Sigma- Aldrich), 0.05 mL 10% SDS (Bio-Rad), 0.05 mL 10% APS (USB), 0.005 mL TEMED (N, N, N', N'-tetramethylethylenediamine; USB) added to 2.0 mL ultrapure H₂O.

Stacking gel: 0.17 mL 30% bis-acrylamide (Bio-Rad), 0.13 mL 1.0 M Tris (pH 6.8; Sigma-Aldrich), 0.01 mL 10% SDS (Bio-Rad), 0.03 mL 10% APS (USB), 0.003 mL TEMED (USB) added to 0.68 mL ultrapure H₂O.

Sensitizing solution- 7.5 mL ethanol, 1.0 mL sodium thiosulphate (5% w/v), 1.7 g sodium acetate made up to 25 mL with ultrapure H₂O and 0.125 mL glutardialdehyde (25% w/v) added immediately before use. (GE Healthcare, Piscataway, NJ).

Silver solution- 2.5 ml of silver nitrate (2.5% w/v) made up to 25 mL with ultrapure H₂O. 10 µL of formaldehyde (37% w/v) added immediately before use. (GE Healthcare, Piscataway, NJ).

Stopping solution- 0.365 g of EDTA-Na₂·2H₂O made up to 25 mL ultrapure H₂O. (GE Healthcare, Piscataway, NJ).

TBST- 60.5 g Trizma base (Sigma-Aldrich, Oakville, ON), 87.6 g NaCl (BioShop), 5.6 g Tween 20 (BioShop) made up to 1L in ultrapure H₂O. pH 7.4.

TMB (ELISA) - 3, 3', 5, 5'- tetramethylbenzidine liquid substrate for ELISA (Sigma-Aldrich, Oakville, ON).

Towbin (transfer buffer)- 12 g Trizma base (Sigma-Aldrich, Oakville, ON), 57.6 g glycine (BioShop), 4 g SDS (USB), 200 mL ethanol made up to 1 L ultrapure H₂O.

Transfer membrane (immunoblot) - Hybond-PVDF membrane (GE Healthcare, Piscataway, NJ).

Tris buffer (50mM)- 6.057 g/L ddH₂O Trizma base (NH₂C(CH₂OH)₃), pH 7.5. (Sigma-Aldrich, Oakville, ON).

2. MATERIALS AND METHODS

2.1 Culture and spore production

All fungal strains were isolated from indoor air from a wide geographical area in Canada. These strains were isolated from Paracel Laboratories Ltd. and deposited in DAOM (National Mycological Herbarium, Department of Agriculture, Ottawa, ON.), CBS (Centraalbureau voor Schimmelcultures, Netherlands) and IBT (Denmark Technical University, Denmark). Each strain was transferred onto sterile 2% malt extract agar (containing 1% glycerol) slants and stored at 4°C. Each strain was grown and extracted as follows: To produce proteins, a slant was macerated in sterile water from which a 5% aliquot (v/v) was used to inoculate 2.8 Litre Fernbach flasks each containing 560 ml of a medium developed for the accumulation of proteins and peptides (Traber et al. 1989) containing 1% glycerol and demonstrated to be useful in allergen discovery (Wilson et al. 2009; Xu et al. 2007). The liquid cultures were put on a rotary shaker (3.81cm throw) at 220 rpm at 25 °C for 72 h in the dark. At the end of the incubation period, all cultures were filtered through cheese cloth using a Buchner funnel. The filtrate from each flask was collected and stored at 4 °C and processed within 24h. The mycelium was rinsed twice with water, wrapped in aluminum foil, frozen and then dried under vacuum.

Spores were produced on rice cultures using the method developed by Murad et al. (1993). 50 g Uncle Ben's™ converted long grain rice was placed in 500 mL wide-mouth Erlenmeyer flasks with 30 mL of distilled, de-ionized water and autoclaved for 30 min at

121°C. The rice cultures were inoculated and incubated at 25°C in the dark for 2.5 weeks, then the rice was air dried in a fumehood. The spores were carefully removed by gently rubbing the rice in a strainer allowing the spores to fall through into a container below. They were then stored at 4°C.

2.2 Extracellular protein extraction

Following the separation of cells from the filtrate, 200 mM NaCl; 10 ppt (0.007mM) protease inhibitor cocktail (Sigma) and 0.075ppt (0.43 mM) PMFS (Sigma) were added to the filtrate. The pH was adjusted to 9.0 and the filtrate was centrifuged at 21612 g for 20 min to remove lipids and cell debris. The supernatant (8L) was concentrated using the Vivaflow 200 with 10000 MWCO (Sartorius Stedim biotech, Germany) to 100 mL. To remove the salt, the growth media was replaced with 20 mM Tris base, pH 9.0. 100 mL of concentrated media was diluted to 1 L with 20 mM Tris base and concentrated down to 100 mL (10X media exchange). This was repeated 2 more times (for a total of 1000X media change).

2.3 Intracellular protein extraction

2 g of freeze-dried cells were added to 20 ml Tris buffer (50mM Tris with 0.05% Tween), mixed with a vortex and placed on ice for 10 min. The mixture was homogenized with a Polytron rotor-stator homogenizer at maximum speed for 5 X 3 min at 10 min intervals.

2.4 Protein extraction from spores

The identical method was used to extract protein from all spore samples. Fifty mg of spores were weighed with a Mettler 163 Analytical balance ($\pm 0.0001\text{g}$), placed in a vial with a polystyrene bead and milled for 30 minutes with a Spex-Certiprep mixer mill (model 5100, Metuchen, NJ). The fragmented spores were then suspended in 0.2 mL of PBST (pH 7.4), placed in an ice bath in a sonicator for 1 hr, followed by shaking at 4°C for 1h.

2.5 Protein concentration determination

The protein concentrations of all protein fractions (crude or purified) were determined with the Bradford assay. The protein sample was diluted 150 times in a microtitre plate to a total of $150\mu\text{L}$. $150\mu\text{L}$ of Quick Dye Bradford Reagent (Bio-Rad) was added and incubated at room temperature for 10 min. The OD was read at 595 nm with Molecular Device Spectramax 340PC plate reader (Sunnyvale, California) and data analyzed using the Softmax Pro software 4.8. The line equation from a BSA standard curve was used to calculate the protein concentration in the sample.

2.6 Protein molecular weight determinations

The molecular weights of the proteins were determined by comparing the R_f (the distance ratio between the protein and the loading buffer's bromophenol blue) of the

sample proteins to those of the LMW. One LMW marker (Amersham Biosciences) consisted of: 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 other, a pre-stained marker, (SeeBlue® by Invitrogen) consisted of myosin (250 kDa), phosphorylase (148 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa), insulin, β chain (4 kDa). The values of the LMW stated here are for the Tris-glycine buffer system that was used; the values may differ when used in different buffer systems.

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

Electrophoresis was performed using the mini VE Vertical Electrophoresis System placed in an Amersham Biosciences electrophoresis tank (GE Healthcare). The current was generated using a Power Pac 1000 gel electrophoresis power supply (Bio-Rad, Hercules, CA). The proteins present in each sample were separated on 10% acrylamide gels using the Laemmli running buffer system. Prior to electrophoresis, were mixed 4:1 (v/v) with the loading buffer and boiled for 10 min. For each gel, one lane had LMW applied as a standard. Once the samples were applied to the gel, electrophoresis was carried out at 200 V for 70 min following a preliminary step at 100V for 20 min, this allowed for the best resolution and maximum separation of the protein bands.

2.8 Silver staining

To determine the protein profile of each strain, the proteins were separated via SDS-PAGE (section 2.7) and silver stained as follows: The gels were removed from the casts and incubated in 25 mL of fixation solution for 30 min. The fixation was then removed and sensitization solution was added for 30 min. The gel was then rinsed with 50 mL of ddH₂O for 3 X 5 min. Silver solution was then added to the gel and incubated for 20 min. The gels were rinsed with 50 mL of ddH₂O for 2 X 1 min and the developing solution was added to the gels until the protein bands had reached the desired intensity. The reaction was stopped by replacing the developing solution with stopping solution. The gel was then scanned with a GS800 densitometer imaging system (Bio-Rad).

2.9 Antibodies used for initial screening

The primary testing via immunoblots and ELISA were performed using human sera from atopic patients provided by the research-clinical laboratory ProGene Ltd., Lenexa, KS. During clinical diagnostic testing, the sera had been screened using Pharmacia ImmunoCAP[®] reagents for responses to various fungi (Halsey et al., 2002). Once the tests were completed the identifiers were removed and the samples were provided to the Miller lab for research purposes. Table #1 in the appendix contains a complete list of sera utilized in this experiment.

2.10 Immunoblot

The proteins of interest, loaded at concentrations of 6 µg/ lane were tested against human sera for their antigenic properties. The initial screening was against sera from 40 atopic individuals and performed as follows: The PVDF membrane and blotting pads were incubated in Towbin buffer for 30 min prior to use to ensure equilibrium. After SDS-PAGE separation (section 2.7), the proteins were transferred to Hybond PVDF membrane (Amersham Biosciences) in an electro-transfer unit (Hoefer miniVE, Amersham Biosciences, UK). Sandwiched between six blotting pads and four sponges, the membrane was placed on the SDS-PAGE gel ensuring the corners were properly aligned. The bundle was then placed in the electro-transfer unit at a constant current of 400 mA for 30 min in an ice bath. Upon completion, the membrane was placed in 20 mL of blocking solution (1% BSA- TBST) and shaken for 1 h at room temperature. The membrane was then incubated in one of the 1^o antibodies (human sera, diluted 1:2000 in 1% BSA-TBST) for 1 h at room temperature, shaken on an Aros 160 orbital shaker at 60 RPM. The membrane was then washed 3 X 5 min with TBST and incubated in 2^o antibody (alkaline phosphatase- conjugated anti-human antibody diluted 1:10000 in 1% BSA-TBST; Sigma, St. Louis, MO) for 1 h at room temperature, shaken. The membrane was washed 3 X 5 min with TBST and 4-6 mL of BCIP developing solution was added. The proteins with immunological activity were stained purple.

2.11 Analysis of immunoblots

Each immunoblot was analyzed and tables created to score the reactivity of each protein and given a mark (1 to 3) based on intensity of reaction – the faint reactions were given a score of 1 and darkest reactions were scored as 3. The proteins that gave below-average responses were rejected. To avoid the likelihood of highly cross-reactive proteins, the proteins that reacted for every strain in every patient were excluded. The reactivity of each sera to the proteins in the 17 strains were scored based on their reactivity. For example, QC 3825 was found to react 7 times and for strain IBT 28248 it reacted weakly twice and strongly once ([2 weak bands (with a score of 1 each) + 1 strong band (score of 2)]* reaction with 7 strains out of 17) therefore the score was as follows: $[2(1) + 1(2)] * 7/17 = 28/17$ for a total score of 28 (denominator dropped to give final score). The proteins that scored the highest within the 38 to 50 kDa range were chosen for further testing against the best-responding sera via ELISA.

2.12 Enzyme-linked immunosorbent assay (ELISA) protocol

The protein samples were normalized (using the data obtained from the concentration tests) and assessed in triplicate for total antigenic protein against human sera. Nunc-Immuno MaxiSorp plates (M9410, Sigma) were used for all experiments and the protein concentrations were diluted to extinction.

The ELISA protocol performed was as follows: Nunc-Immuno MaxiSorp plates (M9410, Sigma) were coated overnight at 4°C using 50 mM carbonate-bicarbonate buffer pH 9.6 containing double dilutions from 1.0 µg protein/well to 0.0005 µg protein/well. The plate was washed 3 X 5 min with 150 µL PBST. The plate was then blocked with 100 µL 1% BSA/PBST for 1 h at room temperature. The plate was washed 3 X 5 min with 150 µL PBST and incubated with 100 µL of 1^o (human) antibody solution for 1 h at room temperature. The antibody solution consisted of antibody diluted in 1% BSA/PBST. The dilutions were as follows: QC 3352 = 1 : 4000, QC 3825 = 1: 16000, QC 3831 = 1: 2000 and QC 3501 = 1: 8000. The plate was washed 3 X 5 min with 150 µL PBST and incubated with 2^o Ab (HRP-conjugated goat anti-human IgG, γ-chain specific; A8419, Sigma) diluted 1:2000 in 1% BSA-PBST for 1 h at room temperature. The plate was washed 3 X 5 min with 150 µL PBST. The colour was developed by adding 100 µL of TMB solution and shaking at room temperature. After 10 min, the reaction was stopped by adding 50 µL of 0.5 M H₂SO₄. The plate was read in a microplate spectrophotometer at 450 nm (Max 340PC, Molecular Devices, CA).

2.13 Analysis of ELISA

Each ELISA response was then compared to the corresponding immunoblot response. The proteins that gave strong immunoblot responses (at 4 µg/lane) and high ELISA responses (at 0.0005 µg/well) were compared and the proteins of interest were found to be 48kDa and 50kDa (based on estimation from LMW).

2.14 Purification of antigen for antibody production

Prior to sending the protein for immunization of rabbits, the target protein was used to perform an immunoblot to ensure antigenic activity. The four immunizations of the 50 kDa protein and the initial immunization of the 48 kDa protein were performed with antigen removed from a SDS-PAGE gel. After concentration (section 2.2), the sample was run on SDS-PAGE (section 2.7) and stained with CBB overnight at 4°C. The gel was de-stained with ultrapure water. The target bands were removed using a sterile scalpel, freeze-dried and dissolved in 1 mL PBS. Each of the frozen samples were shipped to ImmunoPrecise for immunization of the corresponding test rabbit.

The subsequent immunizations of the 48 kDa protein were performed using protein purified as follows. The harvested culture filtrate was adjusted to 1 M NaCl, pH 9.0 and 0.05% fungal protease inhibitor cocktail was added. Once dissolved, the solution was centrifuged at 11 000 RPM for 20 min at 4°C. The supernatant was then filtered through cheesecloth to ensure all cell debris and lipids were removed. The culture filtrate was concentrated fifty times (50×) through a Vivaflow 200 concentrator with a Hydrosart 10,000 Da cut-off membrane at a speed of 10 mL/min for 12h to obtain the extracellular proteins. A buffer exchange (50 mM Tris-HCl buffer, pH 9.0) was performed on the protein by the above concentrator and the final sample of 200 mL was loaded to a 20 mL Q-sepharose fast flow column at the speed at 1mL/min. The protein in the column was eluted with an NaCl gradient concentration from 0 to 0.5 M with total volume of 600ml mixed by the AKTAprime FPLC system (Buffer A: 50mM Tris-HCl,

pH=9.0; Buffer B: 50mM Tris-HCl, pH=9.0 with 1M NaCl). Fractions of 10mL were collected and 50 μ l from each of the fractions were mixed with 10 μ l SDS-PAGE sample buffer for 10% SDS-PAGE electrophoresis. CBB staining of the gel was used to characterize the separated fractions. The fractions with the target proteins were then concentrated by Millipore Amicon ultra centrifuge tubes (MWCO 10kDa) to 0.5 mL. 50% v/v glycerol was then added to the concentrated samples to stock in -20°C for further applications. This optimized condition allowed for > 95% purity, eliminating the need for the gel filtration step since each step is associated with a significant loss of protein.

2.15 Gel filtration

The 48 kDa protein was not pure enough after the anion exchange column and was subsequently loaded onto a gel filtration column (separation based on size exclusion). This purification step consisted of packing a 96 X 1.6 cm column with Sephacryl S-300-HR resin (Sigma-Aldrich, Oakville, ON) and washing with 20 mM Tris buffer containing 200 mM NaCl (pH 7.5) at a flow rate of 1 mL/min. The partially purified protein (from the anion exchange column) was loaded onto the column using a 1 mL syringe at a flow rate of ~0.5 mL/min. The elution was performed with 20 mM Tris buffer containing 200 mM NaCl (pH 7.5) at a flow rate of 1 mL/min and the 10 mL fractions were collected using the fraction collector. The fractions were silver stained to determine which fractions contained the protein of interest. Those with the protein of interest were pooled and concentrated. Immunoblotting was performed to ensure the correct protein was selected.

2.16 Preparation of purified proteins for MS-MS analysis

All precautions (hair tied back, gloves (washed), lab coat) were taken to avoid keratin contamination. One mg of the purified protein to be sent for MS-MS analysis was run on an SDS gel (section 2.7) and stained with CBB overnight. It was de-stained using ddH₂O. The target protein was removed using a sterile scalpel, cut into 4 pieces and freeze-dried in a 2 mL tube. One hundred μ L of 20 mM DTT in 100 mM ammonium bicarbonate was added to the freeze-dried gel and incubated for 1 hr at 60°C. The DTT was removed and 100 μ L of 55 mM iodoacetamide in 100 mM ammonium bicarbonate was added and incubated at room temperature for 45 min in the dark. The iodoacetamide was removed and the gel was rinsed by vortex with 100 mM ammonium bicarbonate for 10 min. This rinse was repeated with acetonitrile. Five hundred μ L of acetonitrile was added and shaken at room temperature for 15 min. The liquid was removed and the step repeated with ammonium bicarbonate and then again with acetonitrile. The gel was freeze-dried for 15 min to remove all liquid. Twenty μ g of sequencing grade trypsin was dissolved in 1 mL of 50 mM ammonium bicarbonate, added to the sample and shaken overnight at room temperature. The solution was transferred into another tube and 50 μ L of 5% acetonitrile with 0.1% TFA (trifluoroacetic acid) was added to the gel and shaken at room temperature for 15 min. The liquid was removed and pooled in the other tube. This was repeated followed by the addition of 50 μ L of 50% acetonitrile with 0.1% TFA and shaken for 15 min, and the eluate was again pooled. The eluate was freeze-dried and sent to Health Canada for MS-MS analysis.

2.17 Antibody purification with protein G column

A 1 mL HiTrap™ Protein G HP column (GE Healthcare) was washed with 10 mL of PBS binding buffer at a rate of 1 mL/min. The rabbit serum was applied to the column at a flow rate of 1mL/min. The flow through was re-applied to the column two more times to ensure all antibodies were bound to the column. The column was washed with 20 mL of PBS buffer to ensure all unwanted components were removed from column prior to elution. To ensure the eluent did not contain protein, it was tested with Bradford reagent and compared to a blank. When the OD was similar to the blank, the elution of the protein was performed by adding 10 mL of 0.1 M glycine-HCl, pH 2.7. The antibody fractions were collected in tubes containing neutralization buffer (1.5M Tris, pH 8.8) to ensure a pH of 7.5. The column was washed with 20 mL of binding buffer and finally with 5 mL of 20% etOH for storage. The antibody fractions were concentrated using a microcentrifuge and the purity tested by running a gel and staining with CBB.

2.18 Isoelectric point (pI) determination

All precautions were taken to avoid SDS contamination. As such, the electrophoresis tank and gel caster were washed and rinsed numerous times with ddH₂O prior to use. The purified protein was mixed 1:1 with the Novex® IEF sample buffer (does not contain SDS). A standard was also mixed 1:1 with sample buffer. The standard contained: Cytochrome c (pI 9.6), Lentil lectin- 3 bands (8.2, 8.0 7.8), Human hemoglobin C (7.5), Human hemoglobin A (7.1), Equine myoglobin- 2 bands (7.0, 6.8), Human carbonic anhydrase (6.5), Bovine carbonic anhydrase (6.0), β-Lactoglobulin B (5.1) and

Phycocyanin- 3 bands (4.75, 4.65, 4.45). Ten mL of the Novex® anode running buffer (50X) was added to 490 mL ddH₂O, mixed thoroughly and added to the bottom of the electrophoresis tank. Ten mL of the Novex® cathode running buffer (10X) was mixed with 90 mL ddH₂O. The pre-cast IEF gel (Biorad) was placed in the caster and the cathode buffer was added to the upper chamber. One µL of IEF standard (Biorad) was loaded into the first well. One µL of the sample was loaded into each of the next 3 wells. The apparatus was attached to the power pac 1000 and programmed to run as follows: 100 V constant for 1 hr, followed by 200 V constant for 1 hr and 500 V constant for 30 min. The gel was then stained with CBB overnight and de-stained with ddH₂O. The pI of the protein was determined by comparing the R_f of the protein to that of the standard.

2.19 Glycoprotein determination

All samples were diluted to 1mg/mL with sample buffer and 20 µL each were used. The purified protein, HRP positive control, soybean trypsin inhibitor negative control, and LMW were run on SDS-PAGE. After electrophoresis, the gel was fixed by immersing in 100 mL of 50% methanol for 30 min. The gel was gently washed by agitating in 100 mL of 3% acetic acid for 10 min, drained and repeated. The gel was transferred to 25 mL of oxidizing solution and gently agitated for 15 min. It was washed with 100 mL of 3% acetic acid, drained and repeated 2 times. It was then transferred to 25 mL of Glycoprotein staining reagent and agitated for 15 min. Twenty-five mL of reducing solution was added to the gel and agitated for 5 min. The gel was rinsed three times

with 3% acetic acid followed by ddH₂O. The protein was compared to the controls to determine if it was glycosylated.

3. RESULTS

3.1 Culture and spore production, protein extraction and concentrations

3.1.1 *Eurotium amstelodami*, *E. herbariorum* and *E. rubrum* protein and spore production

Four strains were originally grown in a medium developed to optimize peptide and protein production. Glycerol was added to reduce the water activity. Fungal protein production was compared in two versions of the optimized media, one supplemented with sucrose and the other with maltose. Protein profiles and protein quantities were similar with each medium, and therefore the media without modification (maltose) was used for the remainder of the study. The 17 strains of *Eurotium* species were each obtained from the indoor environment across a large geographic area in Canada (accession number and related Paracel number can be found in appendix Table A1). Protein fractions extracted from the culture filtrate and cells was used for further analysis. The spores were obtained from rice preparations and the protein extracted.

3.1.2 Protein extraction from culture filtrate (extracellular), cells (intracellular) and spores

First, the extracellular protein was extracted from the culture filtrate using an acetone protein precipitation method and a method using a the Cole Parmer Masterflex L/S concentrator equipped with a Sartorius Stedim 10 000 Da cut-off Vivaflow 200 membrane. The culture IBT 28305 was used to determine that the concentrator

method yielded more useful proteins (figure 4). The concentrator method was used for the remainder of the culture production. IBT 28305 was chosen since it produced large amounts of 48 and 50 kDa proteins. The intracellular protein was extracted from the macerated cells using 50 mM Tris buffer (figure 5). The spores were fragmented and the proteins extracted using 50 mM Tris buffer with 1% (1g/ 100 mL buffer) SDS (figure 6).

3.1.2.1 Time of growth vs. protein production

The culture IBT 28305 was used to determine which cell density allowed for the largest amount of useful proteins (figure 4).

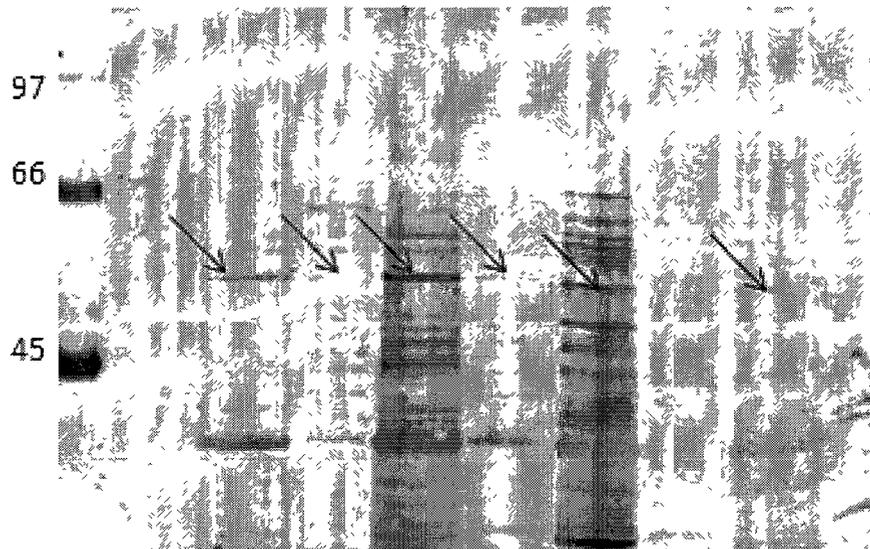


Figure 4: CBB stain of culture grown to various cell densities and comparing acetone precipitation vs. concentrator method.

15µg/lane of crude protein

lane 1: marker: LMW-SDS marker from GE Healthcare

lane 2: 30% cell density, acetone method. lane 3: 30% cell density, concentrator.

lane 4: 50% cell density, acetone method. lane 5: 50% cell density, concentrator.

lane 6: 60% cell density, acetone method. lane 7: 60% cell density, concentrator.

lane 8: 80% cell density, acetone method. lane 9: 80% cell density, concentrator.

lane 10: flow through from concentrator.

arrows indicate the 50 kDa target protein.

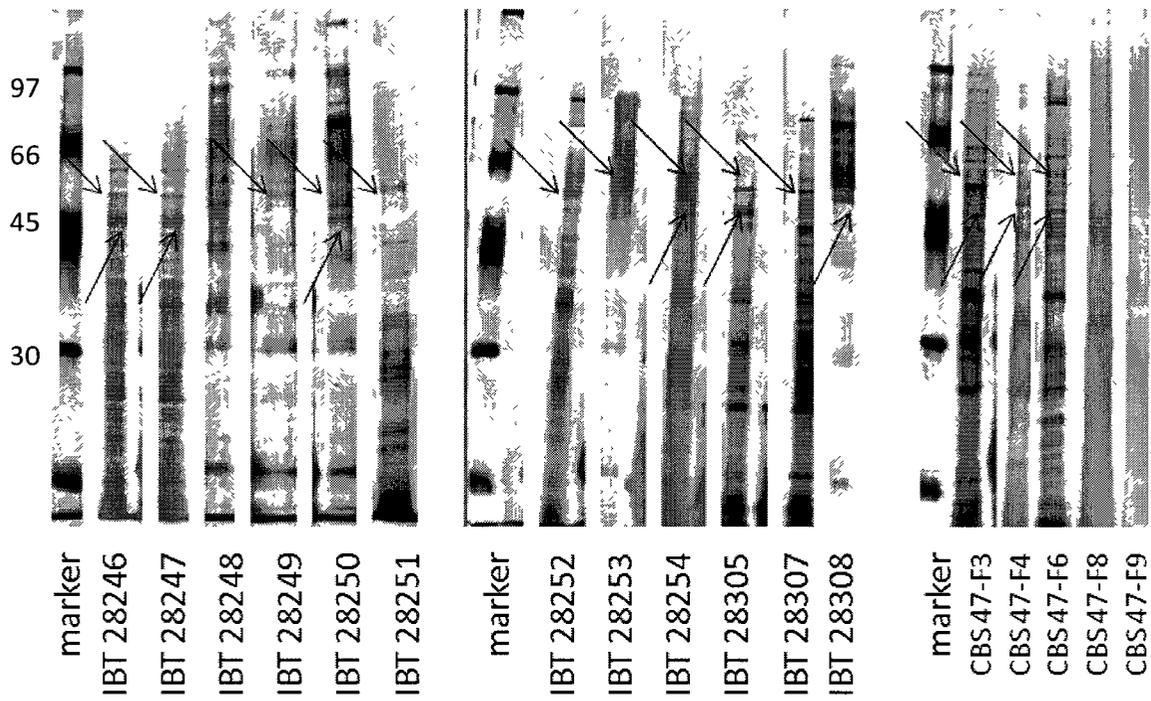


Figure 5: Silver staining of the intracellular proteins of the 17 *Eurotium* strains.

6 µg/lane of crude protein

marker: LMW-SDS marker

arrows indicate the 48 and 50 kDa proteins.

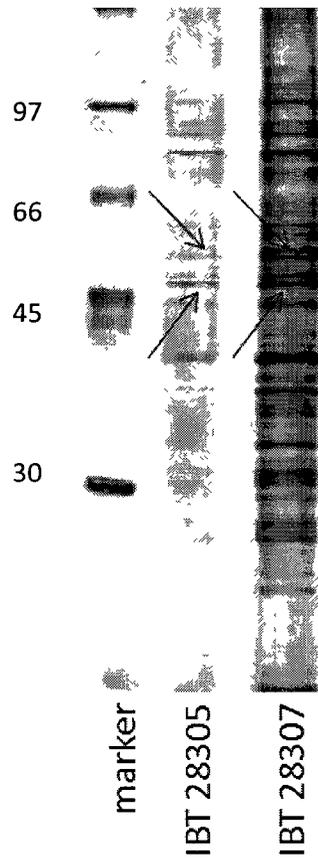


Figure 6: Silver stain of the proteins extracted from the spores of *Eurotium amstelodami* strains IBT 28305 and IBT 28307.

5µg/lane of crude protein

marker: LMW-SDS marker from GE Healthcare

arrows indicate the 48 and 50 kDa proteins.

3.1.3 Protein concentration

The acetone precipitation method for extracellular protein yielded an average of 250 mg of crude protein/8L media. The concentrator method yielded an average of 375-500 mg/8L media, all concentrations determined with the Bradford assay. The average amount of intracellular protein extracted from 1 g of cells was 15 mg (0.15 % m/m). The mean mass of total protein extracted from 1 mg spores was 9.8 µg (0.0009.8 % m/m).

3.2 *Eurotium amstelodami*, *E. herbariorum* and *E. rubrum* human antigen screening using extracellular protein

3.2.1 Initial antigen screening with western blots

Immunoblotting was used as the preliminary screening method for the detection of antigen responses to the *Eurotium* species. The 17 strains of *Eurotium* were tested against the HpAbs (human polyclonal antibodies). The list of human sera can be found in Appendix Table A1.2. Several proteins were observed to be antigenic to humans from the atopic patient sera (figures 7 to 12). Proteins extracted using the concentrator method were analyzed.

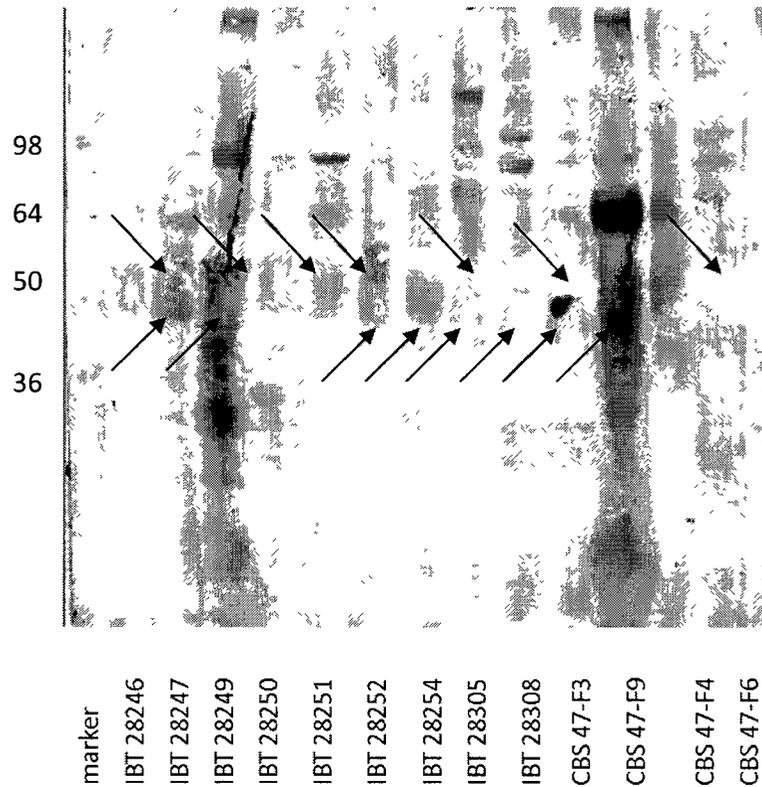


Figure 7: Immunoblot comparing the response of HpAb QC 3825 to different *Eurotium* strains

10µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard. Invitrogen.

arrows indicate the 48 and 50 kDa proteins

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

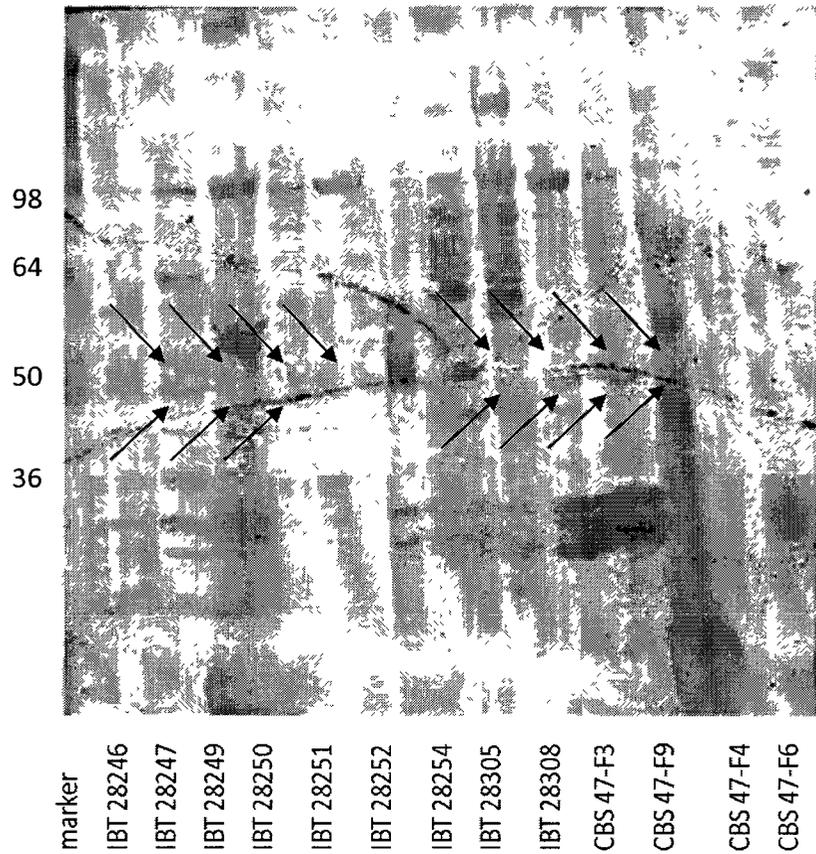


Figure 8: Immunoblot comparing the response of HpAb QC 3494 to different *Eurotium* strains

10µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard.

arrows indicate the 48 and 50 kDa proteins

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

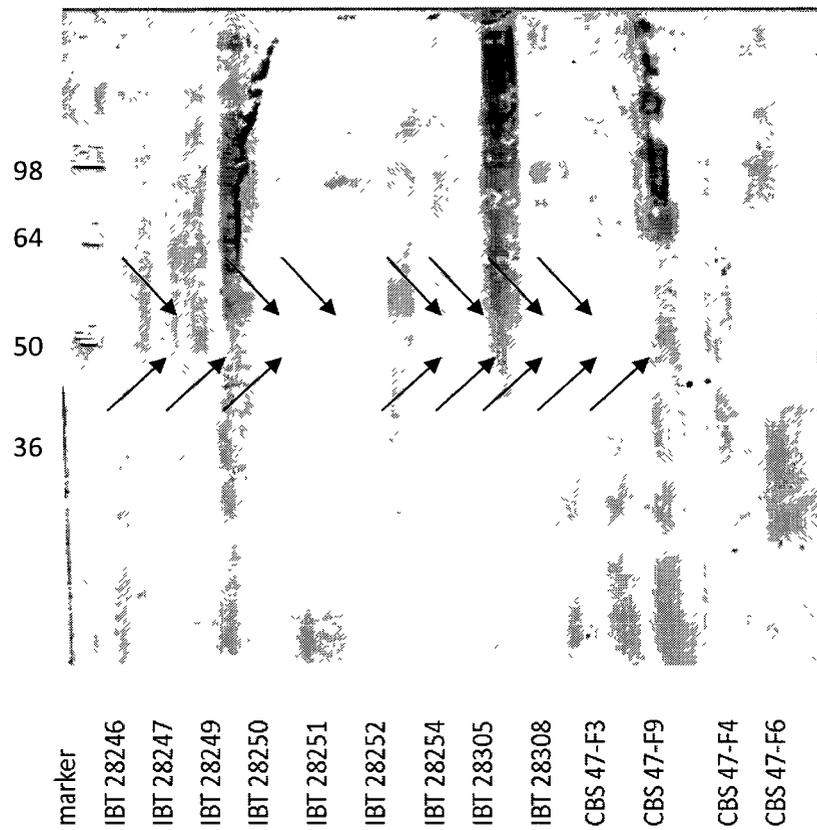


Figure 9: Immunoblot comparing the response of HpAb QC 3510 to different *Eurotium* strains

10µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard.

arrows indicate the 48 and 50 kDa proteins

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

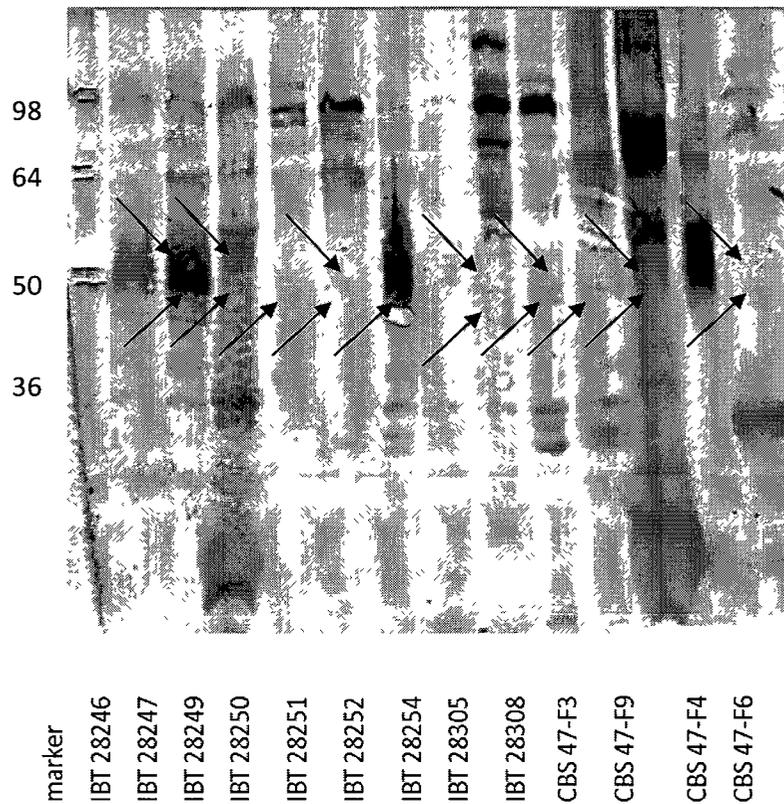


Figure 10: Immunoblot comparing the response of HpAb QC 3831 to different *Eurotium* species strains

10µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard.

arrows indicate the 48 and 50 kDa proteins

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

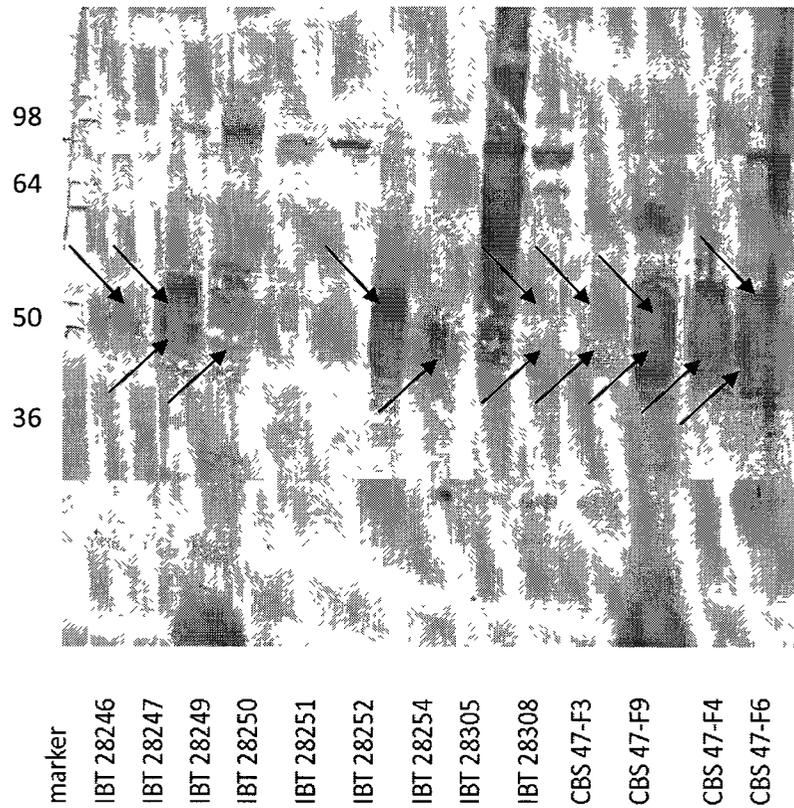


Figure 11: Immunoblot comparing the response of HpAb QC 3835 to different *Eurotium* strains

10µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard.

arrows indicate the 48 and 50 kDa proteins

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

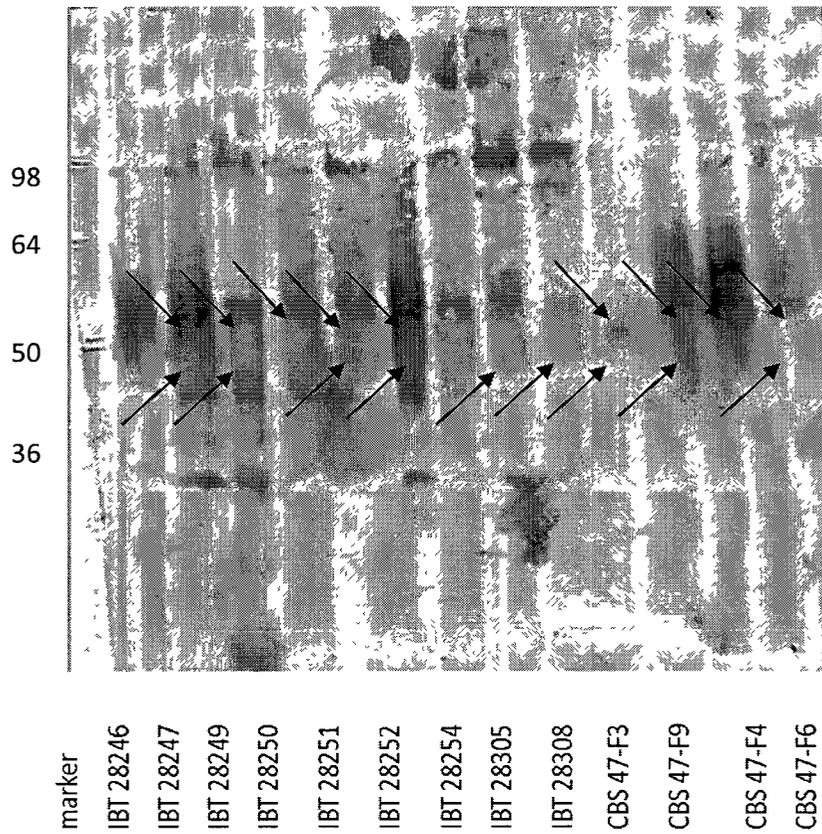


Figure 12: Immunoblot comparing the response of HpAb QC 3845 to different *Eurotium* species strains

10µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard.

arrows indicate the 48 and 50 kDa proteins

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

3.2.2 Antigen screening with ELISA

ELISA was used to test the response of the most reactive HpAbs determined with immunoblotting, against the antigenic proteins from the 17 strains. The determination of the best dilutions of antigen and antibody were determined using a checkerboard ELISA platform. The concept of the checkerboard ELISA is to have decreasing amounts of antigen from top to bottom and decreasing amounts of antibody from left to right. The total response for each strain against the HpAbs was determined (figure 13). The best combination of antibody and antigen concentrations was used. From the checkerboard ELISA, it was determined that the best amount of antigen to be tested was 1.5 µg/well, with the HpAbs diluted 1:5000. ELISA showed that there was a strong difference in responses between each HpAb to each of the different *Eurotium* strains (figure 14). The ELISA and immunoblot responses of each strain tested against the 12 different sera were combined on a three-dimensional graph (figure 15). ELISA responses are on the x axis; the immunoblot responses, graded on a scale of 1 to 3, are on the y axis; the 12 individual human sera are on the z axis.

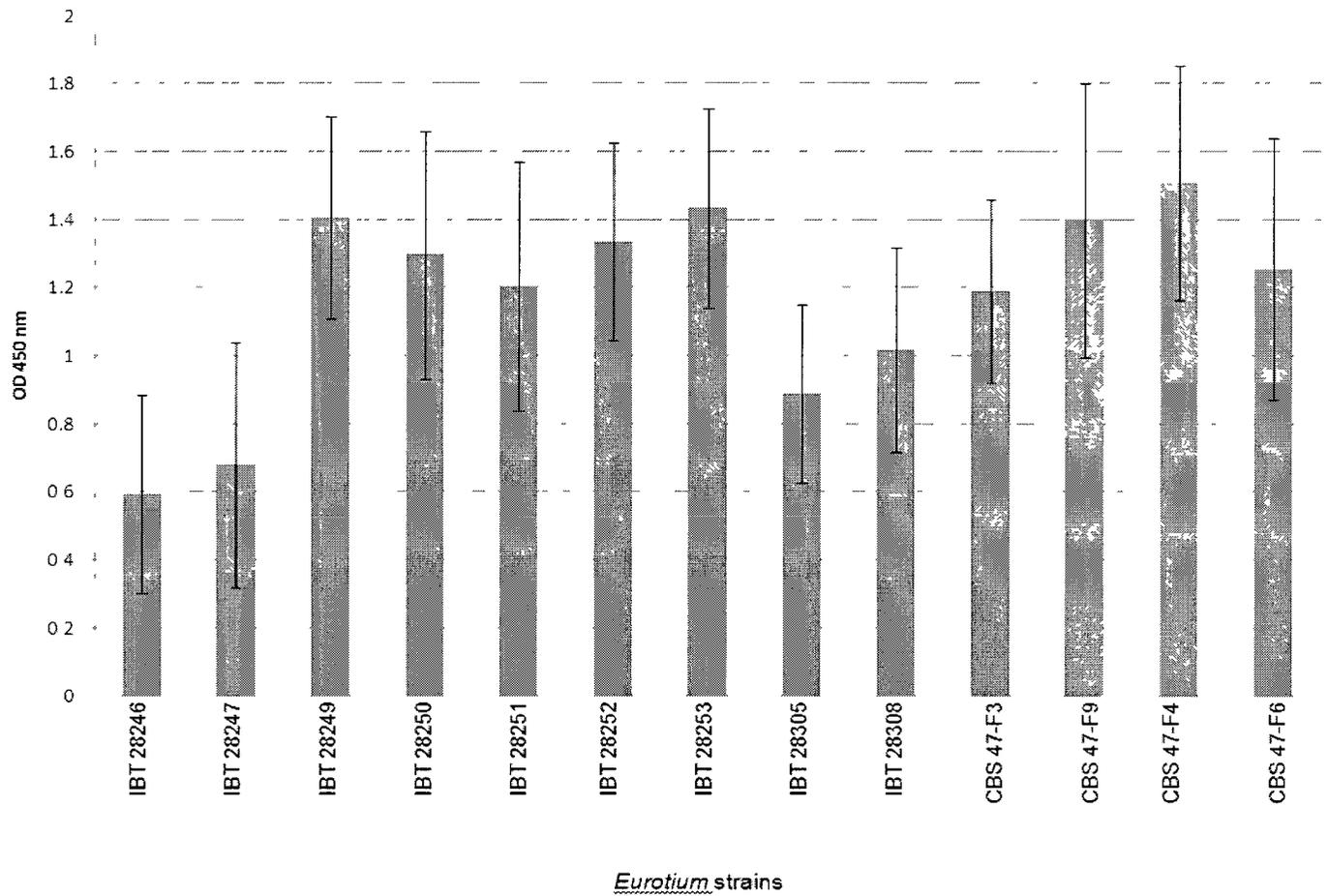


Figure 13: Average ELISA response of all *Eurotium* species culture extracts against 7 HpAbs. Tests were performed in triplicate. Error bars represent the standard deviations.

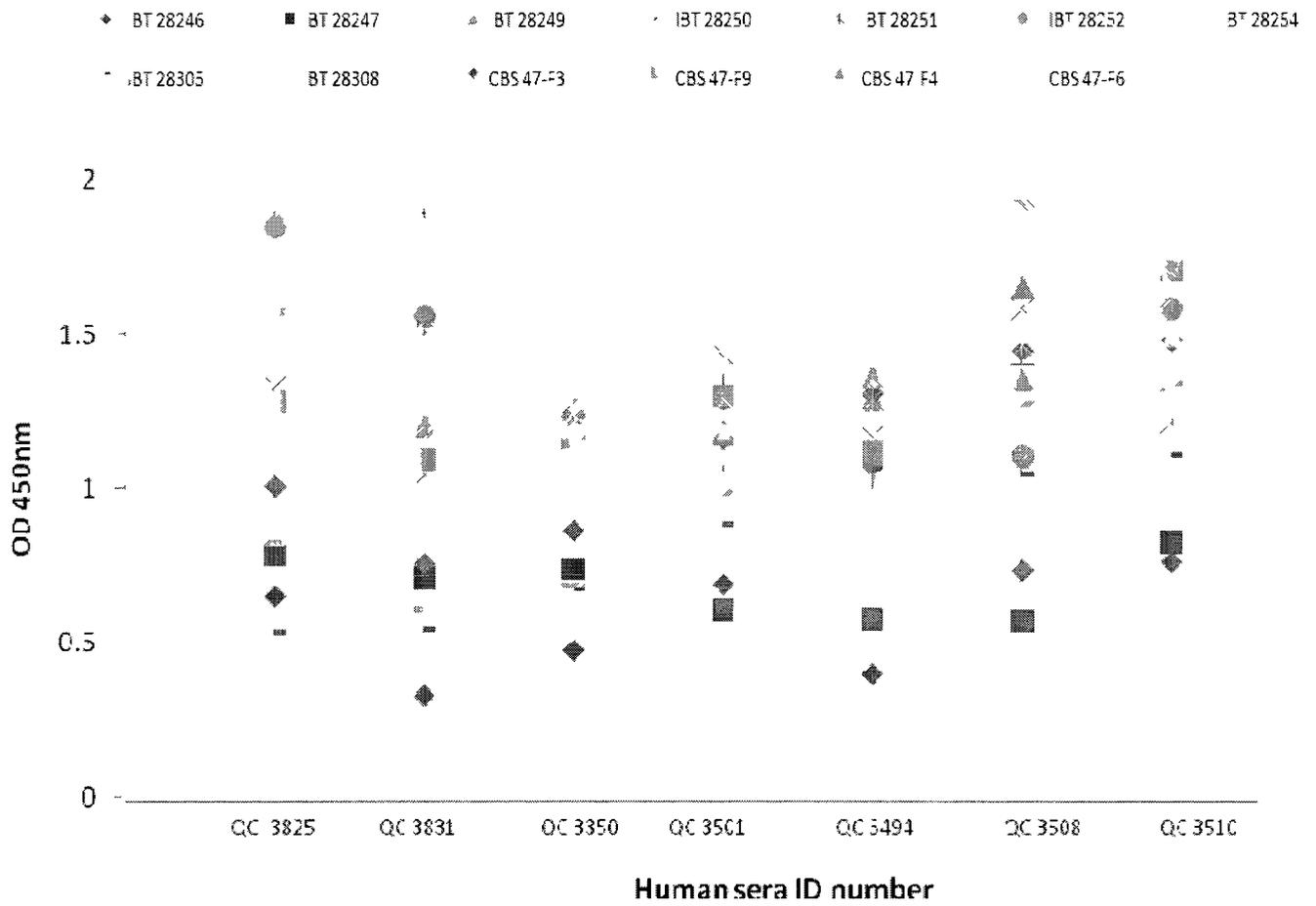


Figure 14: Average ELISA response of all *Eurotium* species culture extracts against 7 HpAbs. Tests performed in triplicate.

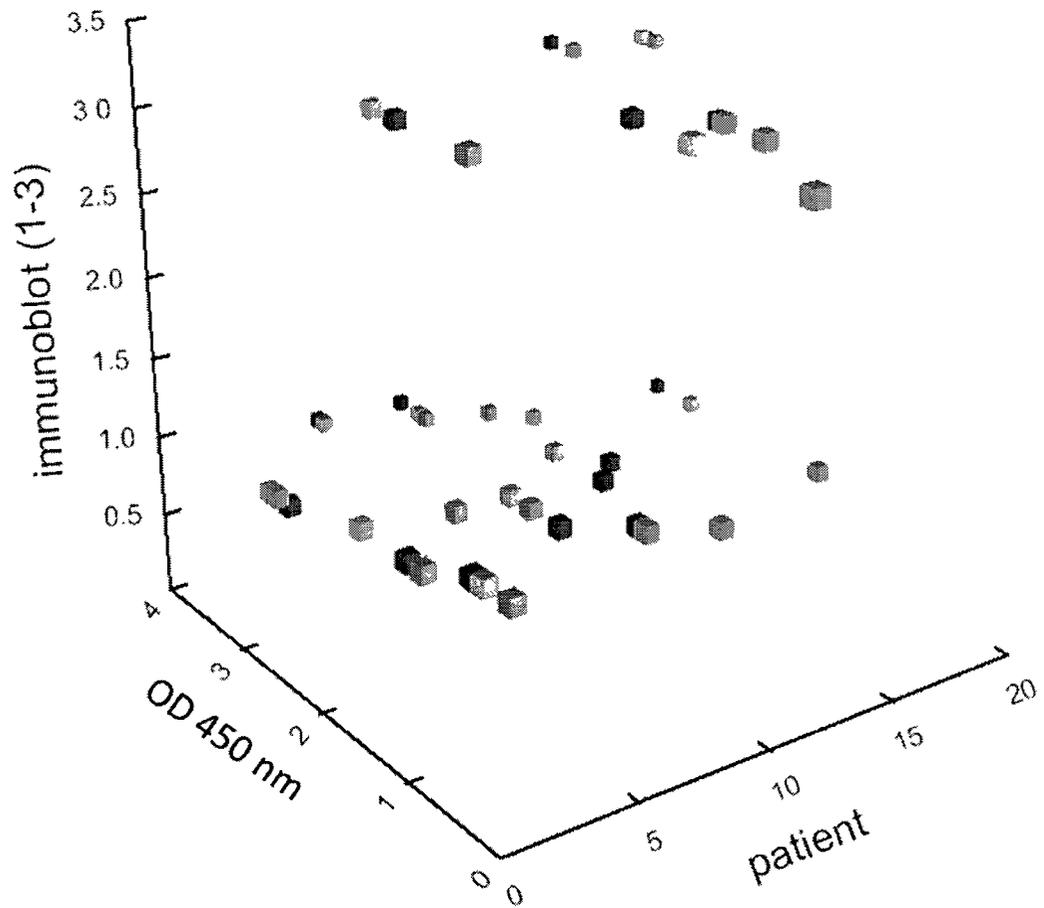


Figure 15: A three-dimensional graph containing ELISA (OD values; X axis) and immunoblot (Y axis) response of *E. amstelodami*, *E. rubrum* and *E. herbariorum* strains against 18 individual human sera (Z axis)

3.3 Selection of antigenic proteins, detection in cells and spores and cross-reactivity testing

3.3.1 Selecting *Eurotium* target proteins

Each immunoblot was analyzed and the reactivity of each serum to the given extracellular proteins was scored and a table was created (not shown). From this table, the sera with average responses were removed creating a refined table with data set for extracellular protein that reacted very strongly or very weakly (table 1). The data was then analyzed for the best possible target proteins (table 2). The responses were given values of 1 (weak) to 3 (strong) based on the intensity of the response as judged by eye. According to these results, two proteins, 48 and 50 kDa were chosen for further investigation. The absence of a serum in the table means that it did not react to either the 48 or 50 kDa proteins. Many proteins responded to the human sera (polyclonal antibodies). Of the numerous HpAbs that responded to the target proteins, the four best responding sera were used to perform ELISA of protein extracts to extinction for 3 cultures (figure 17). A silver stain of the 17 strains was used to visualize the total proteins produced (figure 16). Although in varying amounts for each strain, the presence of the 48 kDa protein was observed in 10 strains, while the 50 kDa protein can be seen in 13 strains. The HpAbs QC 3825 and QC 3831 reacted most strongly to the two target proteins, on both the immunoblots and the ELISA assays. For the remainder of the research, these sera were used as the primary antibodies for further screening

and purification of the 48 and 50 kDa proteins. Some HpAb responses against the strain IBT 28305 are shown in fig. 18.

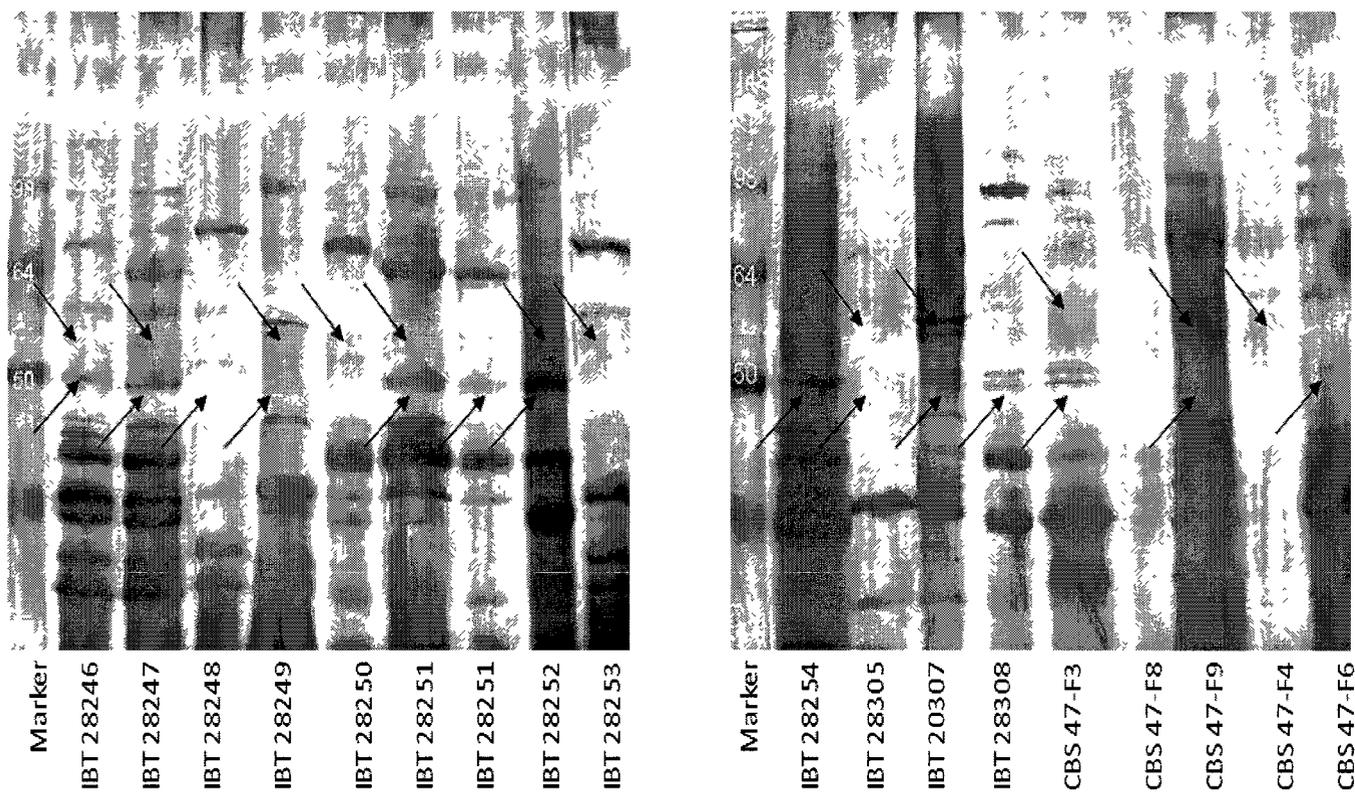


Figure 16: Silver stain comparing the amount of the 48 and 50 kDa proteins produced in extracellular extracts in the 17 strains of *Eurotium* species.

6 µg protein loaded per well.

marker: SeeBlue® Plus2 Pre-Stained Standard

arrows indicate the 48 and 50 kDa proteins

Table 1: Summary of immunoblot responses of some *Eurotium* proteins to a selection of HpAbs. 1 is a weak response, 3 is a strong response.

IBT 28246

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58									
50									
48									
40									
39									
38							1		
30		1						3	

IBT 28247

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58									
50				1					
48									3
40									
39									
38									
30									3

IBT 28248

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58							3	3	
50		1							3
48							1		
40									
39									
38							1		
30									

IBT 28249

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58				3					3
50			3						3
48				1					
40				1					
39									
38									
30					3				

IBT 28250

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58							1		
50									
48				1			1		
40									
39									
38									
30									

IBT 28252

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58									
50									
48				1					
40									
39									
38		1							
30									3

IBT 28254

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58							1		3
50							1		3
48				1					
40				1					
39				1					
38									
30									

IBT 28305

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58									
50				1					3
48				1				1	3
40				1					
39									
38									3
30									

IBT 28307

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58		1							1
50									1
48									1
40									
39									
38									
30									

IBT 28308

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60	3								
58									
50						1			
48									
40									
39									
38									
30									1

CBS 47-F3

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58						1			
50									
48									
40									
39									
38									
30						1			

CBS 47-F4

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58		1							1
50									
48									1
40									
39									
38									3
30									3

CBS 47-F6

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58									
50	1								
48									
40									
39									
38									
30						3			

CBS 47-F8

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58			1						
50									
48				1			3		
40									
39									
38									
30									

CBS 47-F9

MW (kDa)	3342	3351	3353	3493	3494	3501	3825	3830	3831
60									
58									
50									
48						1			
40						1			
39									
38									
30									

Table 2: Immunoblot responses of *Eurotium* target proteins to a selection of HpAbs. 1 is a weak response, 3 is a strong response.

IBT 28247

MW (kDa)	QC3354	QC3830
50	1	
48		3

IBT 28248

MW (kDa)	QC3351	QC3825	QC3831	QC3850
50	1		3	
48		1		1

IBT 28249

MW (kDa)	QC3353	QC3831
50	3	3
48	1	

IBT 28250

MW (kDa)	QC3353	QC3510	QC3835	QC3850
48	1	1	1	1

IBT 28252

MW (kDa)	QC3353
48	1

IBT 28253

MW (kDa)	QC3350	QC3490
48	1	1

IBT 28254

MW (kDa)	QC3825	QC3831
50	1	3

IBT 28305

MW (kDa)	QC3352	QC3354	QC3493	QC3825	QC3831	QC3841	QC3851
50			1	3		3	
48	3	1	1	1	3		1

IBT 28307

MW (kDa)	QC3346	QC3490	QC3825	QC3831
50	1		1	1
48			1	1

IBT 28308

MW (kDa)	QC3494
50	1

CBS 47-F6

MW (kDa)	QC3346
48	1

CBS 47-F9

MW (kDa)	QC3352	QC3501
50	1	
48		1

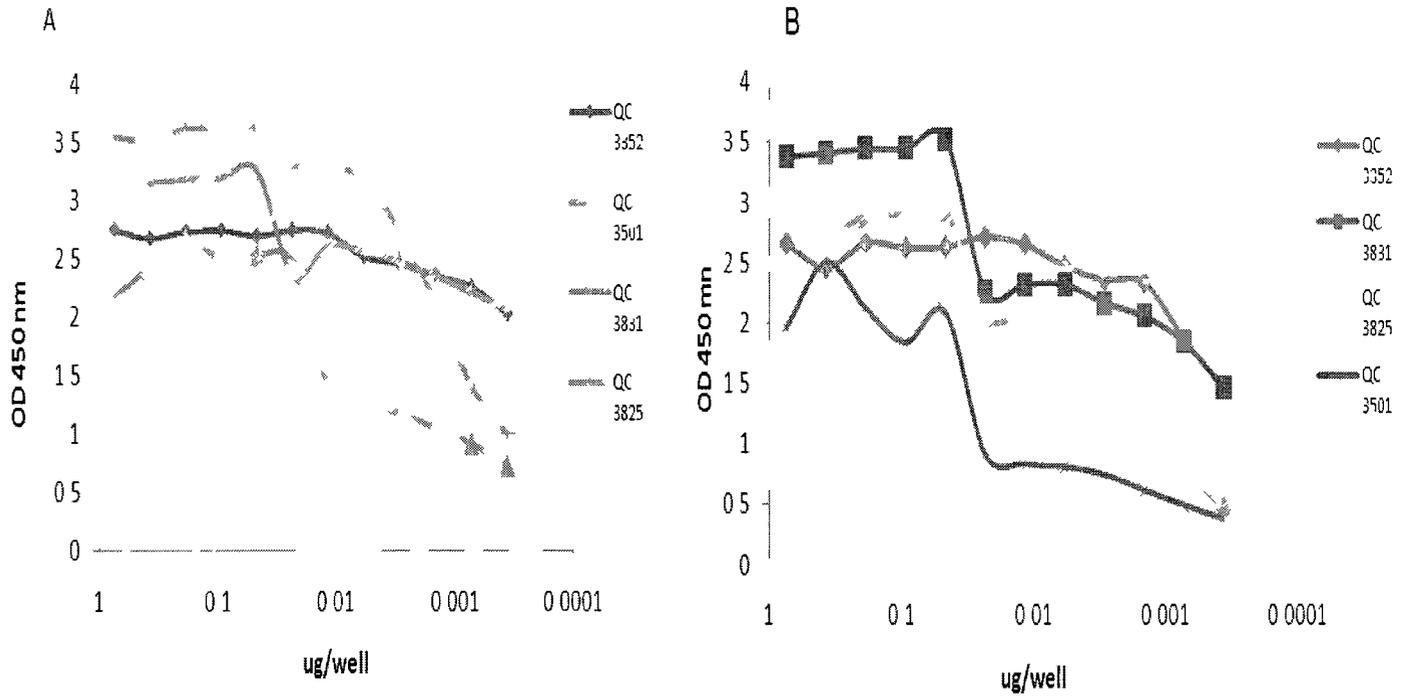


Figure 17 ELISA to extinction of *Eurotium amstelodami* strains A) IBT 28307 and B) IBT 28305 against human sera QC 3352, 3501, 3825 and 3831

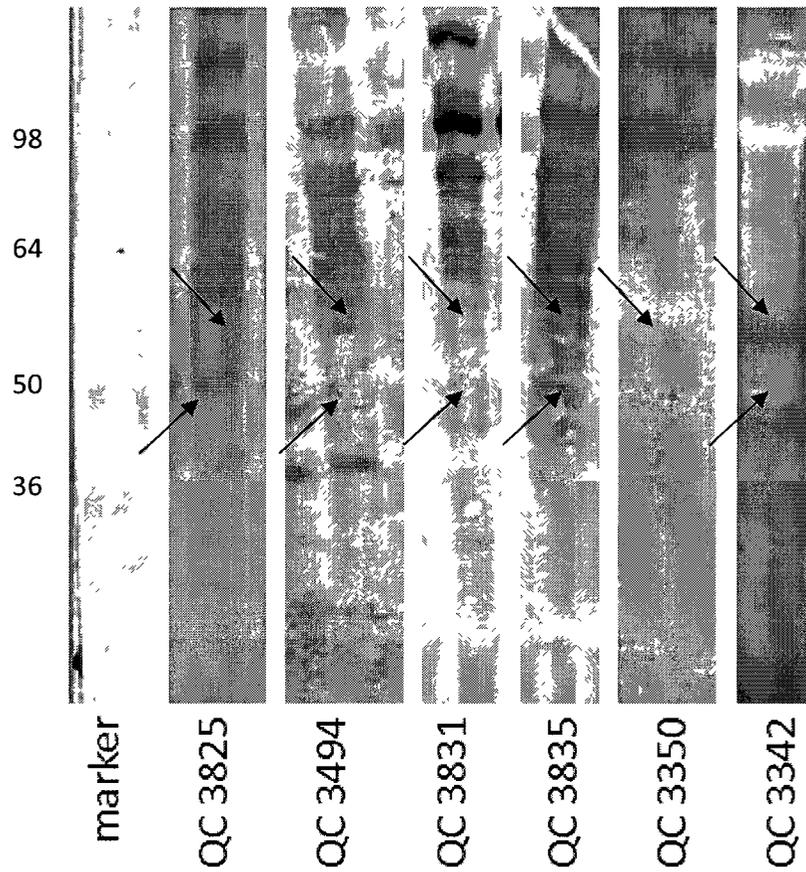


Figure 18: Immunoblot comparing the responses of several human sera to *Eurotium amstelodami* strain IBT 28305.

10µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard.

arrows indicate the 48 and 50 kDa proteins

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

3.3.2 Verifying the presence of the target antigenic proteins in the cells and spores

The presence of the target (48 and 50 kDa) proteins in mycelia and spores were confirmed, as seen in the silver stain (figure 4 mycelia, figure 5 spores) before proceeding with further isolation and purification. These proteins were confirmed as the target proteins. Immunoblots of the intracellular protein (figure 19) and the spores (figure 20) were performed to confirm the antigenicity with the sera that showed positive responses in the testing with culture extracts. The two target proteins demonstrated antigenicity in both the cells and the spores.

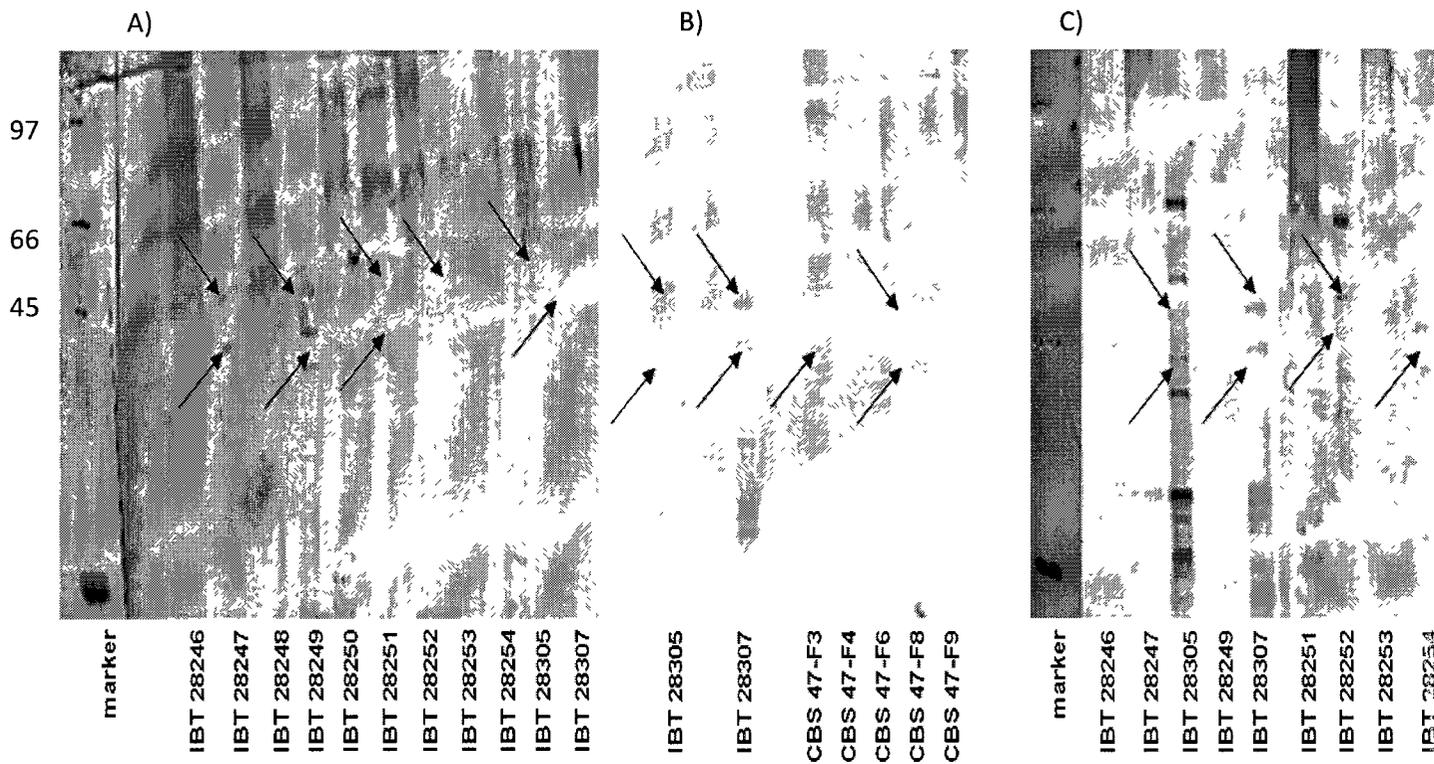


Figure 19: Immunoblot comparing the responses of the intracellular proteins of several *Eurotium* strains against HpAbs QC 3342 (A), QC 2797 (B) and QC 3346 (C).

10µg/lane of crude protein

marker: LMW-SDS marker from GE Healthcare

arrows indicate the 48 and 50 kDa proteins

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

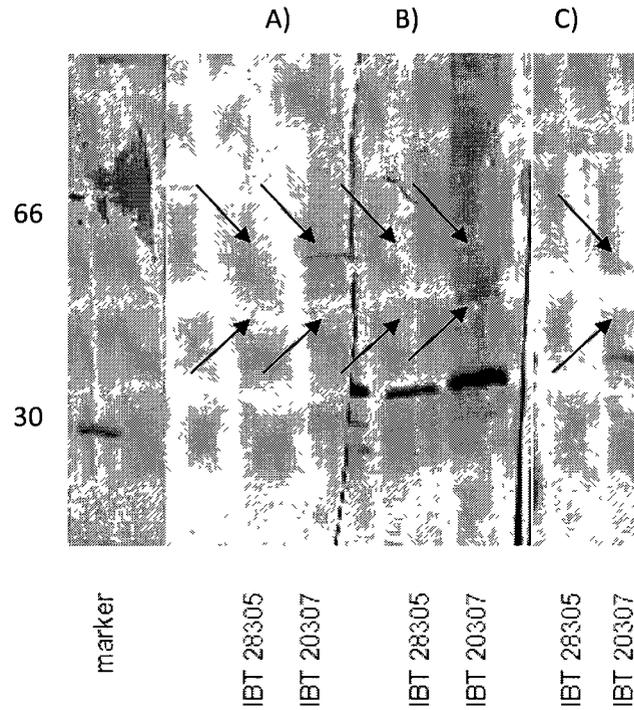


Figure 20: Immunoblot comparing the responses of the proteins extracted from *Eurotium amstelodami* strains IBT 28305 and 28307 spores against HpAbs QC 3831 (A), QC 3825 (B) and QC 3352 (C)

10µg/lane of crude protein

marker: LMW-SDS marker from GE Healthcare

arrows indicate the 48 and 50 kDa proteins

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

3.3.3 Cross reactivity of other fungal species to the target proteins

The possibility of cross reactivity of the target proteins to other fungal species had to be assessed prior to antibody production and purification. The crude protein samples obtained with the concentrator method of several species were examined by immunoblot (figure 20) and compared to the *Eurotium amstelodami* IBT 28305. A silver stain of the protein extracts (figure 21) was performed to compare the proteins of similar size produced by the other species.

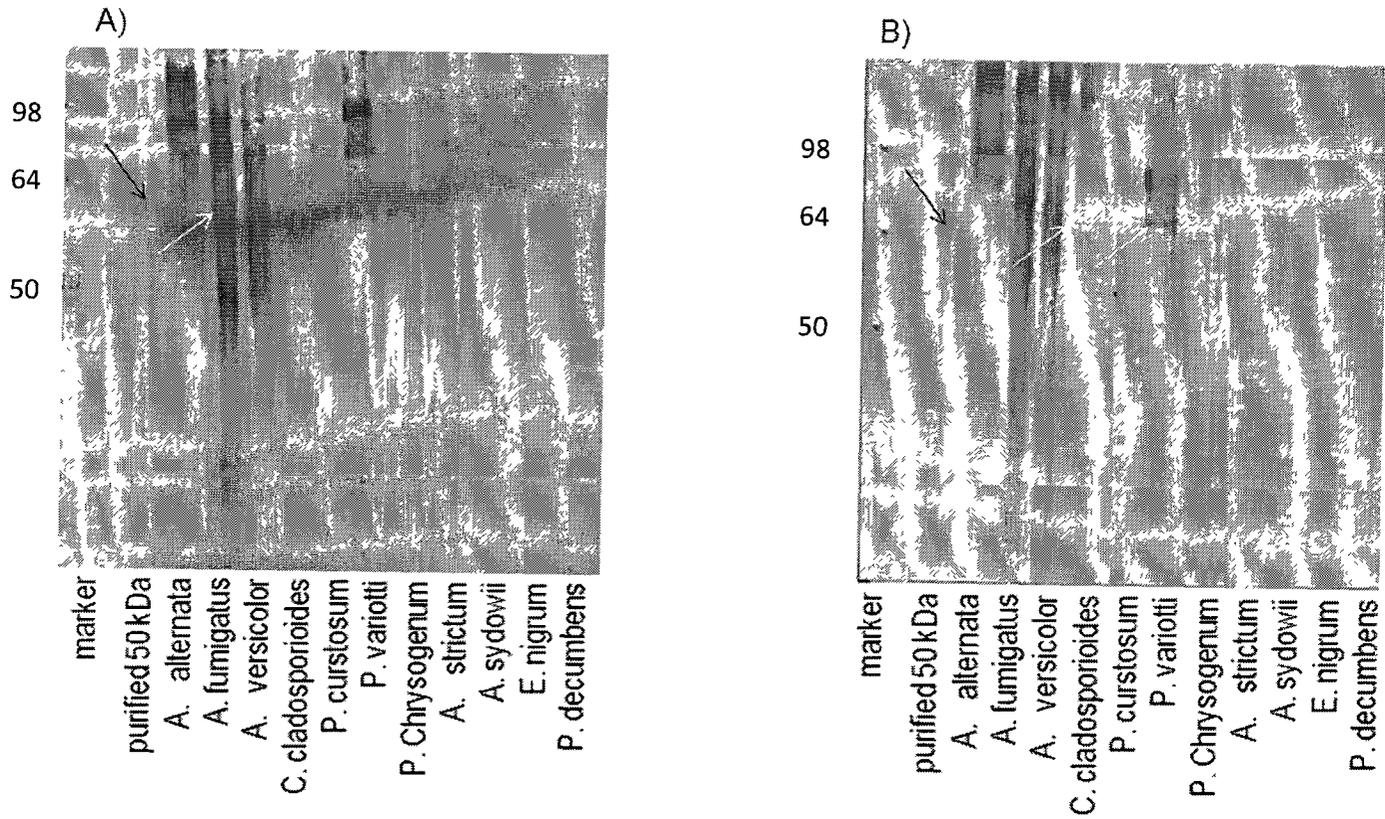


Figure 21: Immunoblot comparing the response of *Eurotium amstelodami* strain IBT 28305 and other fungi to HpAbs A) QC 3825 and B) QC 3831.

10µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard.

black arrows indicate the target protein.

white arrows indicate other proteins of similar size that elicited a response.

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

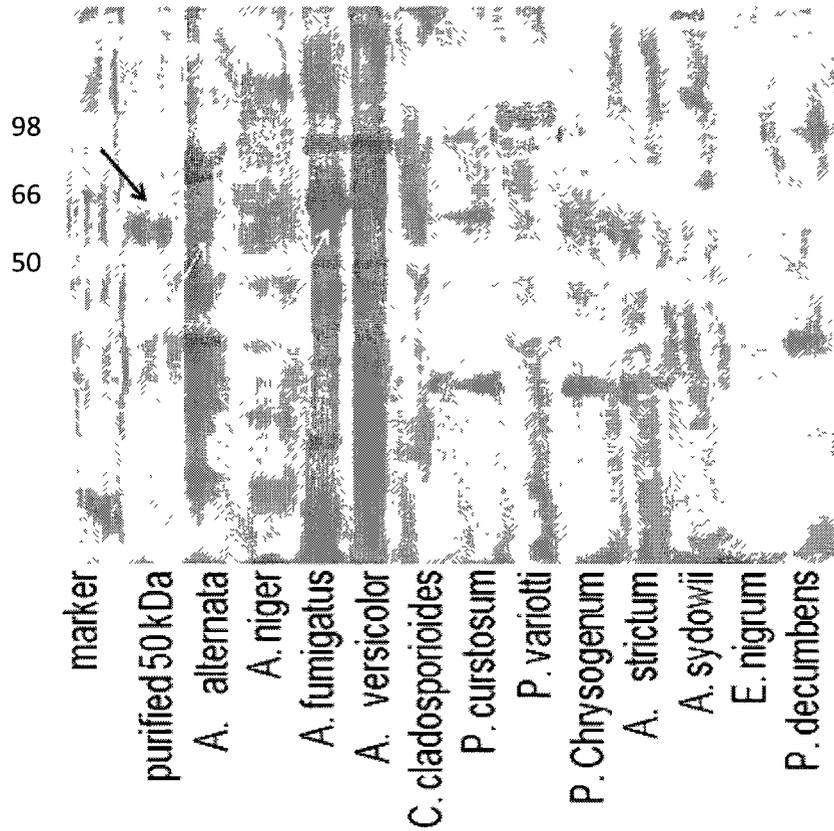


Figure 22: Silver stain of several fungi that have similar weight proteins to the target proteins of *Eurotium* species.

6 µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard.

black arrows indicate the 50 kDa protein present in *Eurotium amstelodami* IBT 28305.

white arrows indicate proteins of similar size in other fungi.

3.4 Purification of *Eurotium amstelodami* target proteins

3.4.1 Ion exchange chromatography

The crude protein obtained using the concentrator produced the target proteins in larger amounts (figure 4). The crude protein sample from *E. amstelodami* IBT 28305 was further purified using an anion exchange chromatography column. The fractions were silver stained (figure 23).

The fractions containing the target proteins were combined and concentrated. Their reactivity was tested by immunoblot. The total amount of protein obtained from the columns at pH 7.5 and 8 were 0.2 mg/ 8L batch of 48 kDa and 0.9 mg/ 8L batch of 50 kDa. The target proteins were not purified to an extent that was useful. This was repeated numerous times and the results were consistent. The 50 kDa protein became the protein of choice since it was produced in greater abundance.

To determine the appropriate conditions for the best purification of the target proteins the procedure was performed using a gradient elution (0 – 50%, 450 mL) of increasing 1M NaCl-Tris buffer pH 8 (figure 24) and pH 9. The cultures were grown until green—a sign of secondary metabolite production and usually not conducive to protein production (figure 25). The fractions containing the target proteins were combined and concentrated. Their reactivity was tested by immunoblot (figure 26). The total

amount of protein obtained from the column at pH 9 was 8 mg/ 8L batch of 50 kDa. It was determined that the best target protein production was obtained with green cultures and anion exchange column of pH 9. The following purification steps were performed with the anion exchange column pH 9 and the cultures were left to grow an additional 12 hours to allow optimal target protein production. The degree of purity (95%) and recovery of the 50 kDa protein was sufficient for monoclonal antibody production (table 3) and did not require any further purification steps.

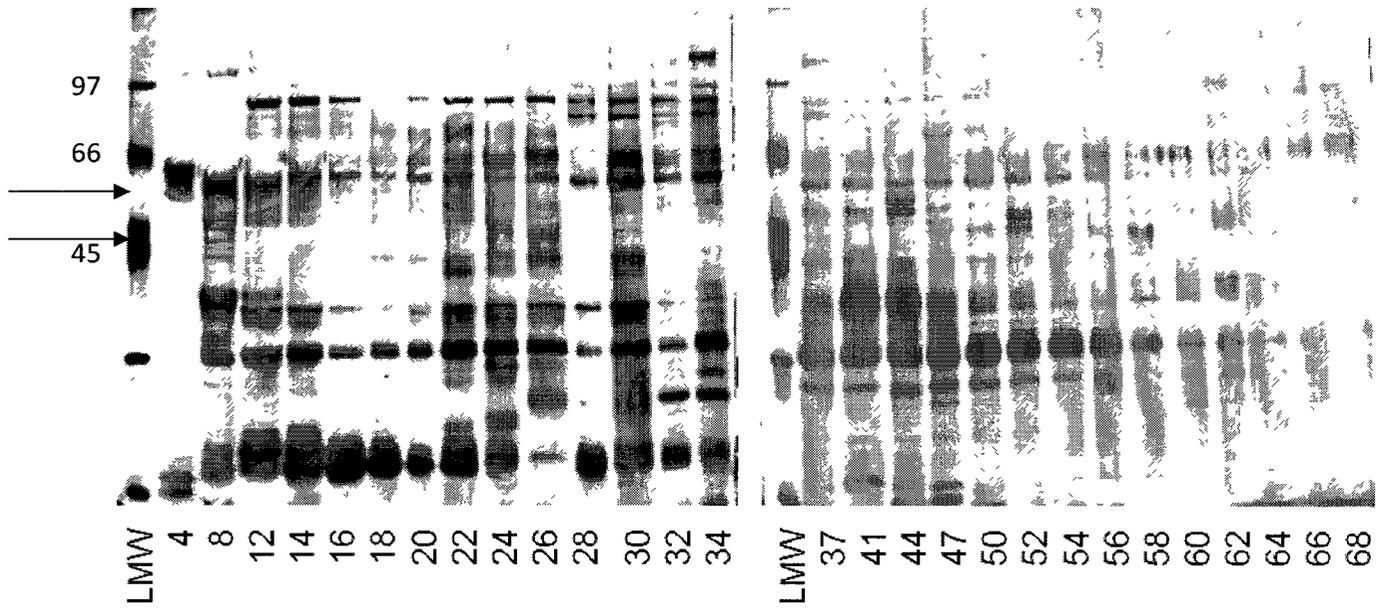


Figure 23: Silver stain of the different fractions eluted from anion exchange column pH 7.5

6 μg /lane of crude protein

marker: LMW-SDS marker

fractions 18-34 comprise the 48 kDa protein.

fractions 37-52 comprise the 50 kDa protein.

the arrows indicate where the 48 and 50 kDa protein are located.

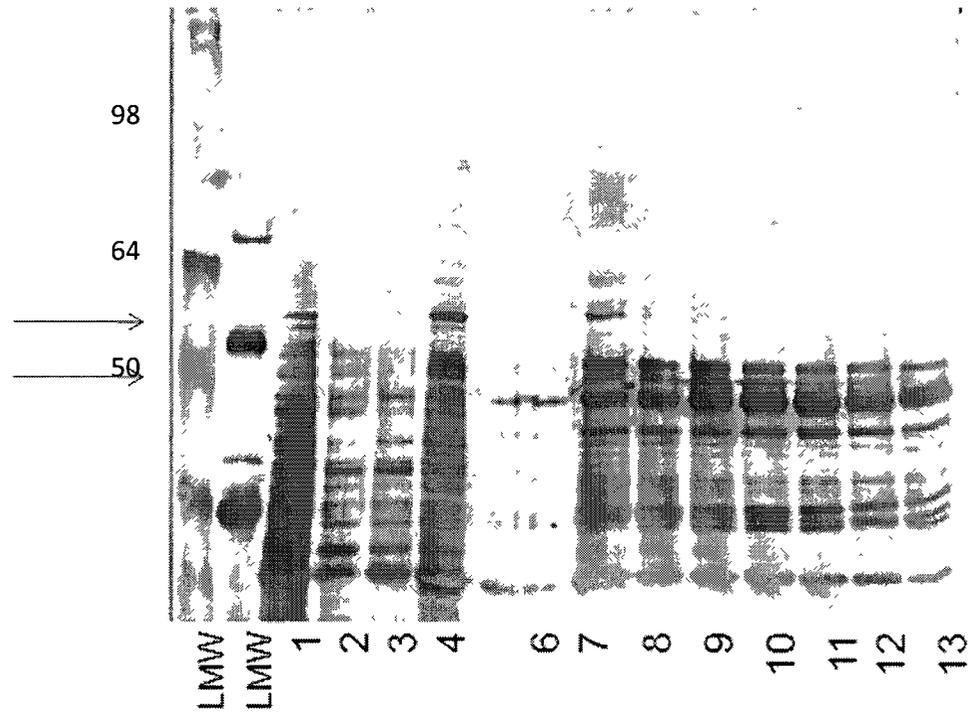


Figure 24: Silver stain of the different fractions eluted from anion exchange column pH 8.

6 μ g/lane of crude protein

lane 1: SeeBlue[®] Plus2 Pre-Stained Standard

lane 2: LMW-SDS marker

fractions 1-4 comprise the 48 kDa protein

fractions 7-13 comprise the 50 kDa protein

the arrows indicate where the 48 and 50 kDa protein are located.

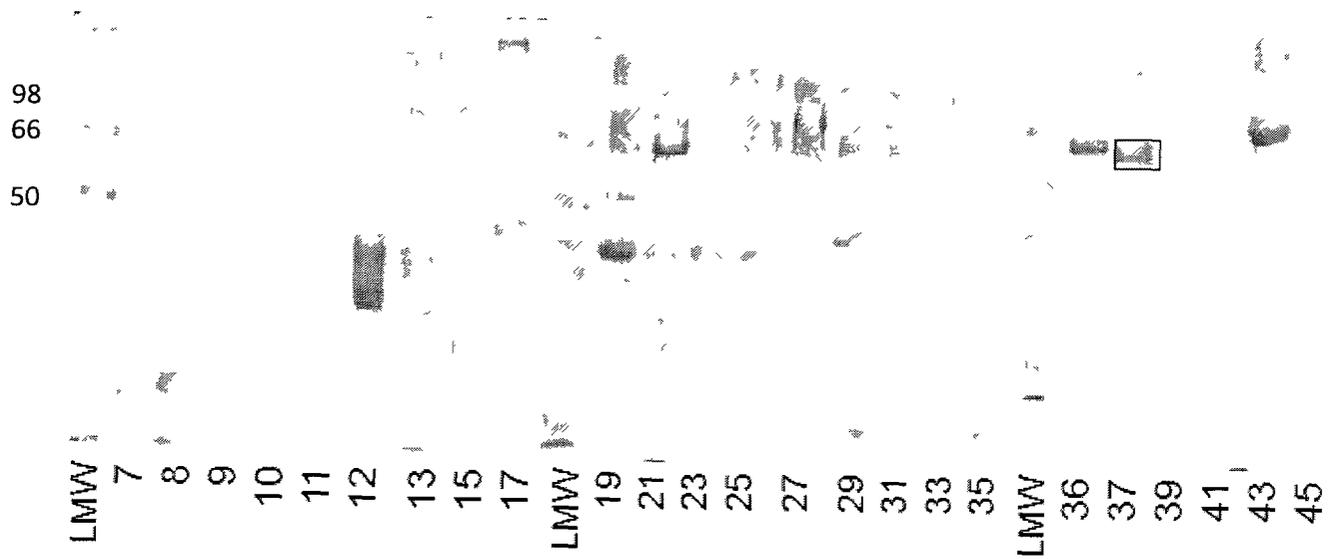


Figure 25: CBB stain of the different fractions eluted from anion exchange column pH 9 with cultures grown until green.

8 μ g/lane of crude protein

marker: SeeBlue[®] Plus2 Pre-Stained Standard.

The 50 kDa protein fraction is highlighted in red.

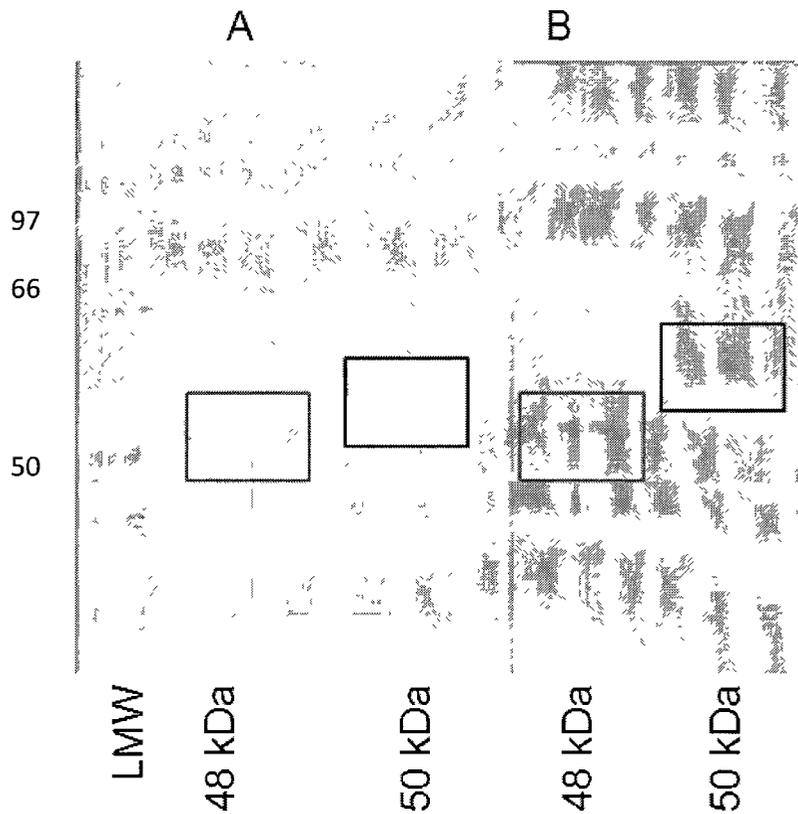


Figure 26: Immunoblot comparing the response of the purified 48 kDa and 50 kDa proteins from *Eurotium amstelodami* strain IBT 28305 against human sera A) QC 3831 and B) 3825.

10µg/lane of crude protein

marker: LMW-SDS marker from GE Healthcare

The 48 kDa and 50 kDa proteins are highlighted.

1° Ab: HpAb diluted 1: 4 000 with 1% BSA/TBST

2° Ab: AP-anti-human IgG diluted 1: 10 000 with 1% BSA/TBST

Table 3: Protein recovery from each purification step for *Eurotium amstelodami*.

Purification step	Total protein (mg)	48kDa protein (mg)	Yield (%)	50 kDa protein (mg)	Yield (%)
concentrator (per 8L batch)	450				
ion exchange chromatography	450	74.3	74.3	27.5	6.1
gel filtration chromatography	74.25	11	11	3.25	4.4

3.5 Polyclonal antibody production

3.5.1 Analysis of the polyclonal antibodies from test bleeds

A test bleed of each rabbit was performed 17 days after the initial spore injections. The response of the target proteins and spore proteins to the RpAbs was determined by immunoblot (figure 27).

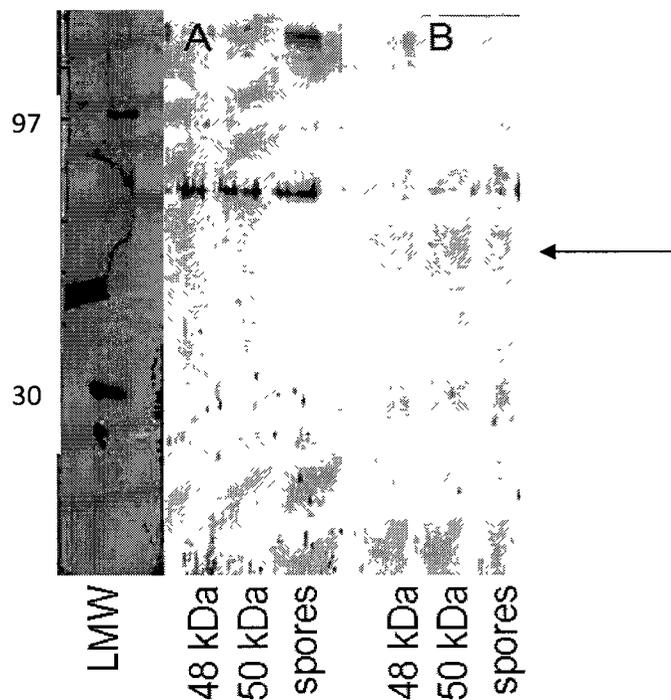


Figure 27: Immunoblot comparing the response of the purified 48 kDa, 50 kDa protein and spores from *Eurotium amstelodami* strain IBT 28305 against test bleed of A) rabbit #130 and B) rabbit #290.

10µg/lane of crude protein

marker: LMW-SDS marker from GE Healthcare

the 48 kDa and 50 kDa proteins are indicated by the arrow

1° Ab: RpAb diluted 1: 250 with 1% BSA/TBST

2° Ab: AP-anti-rabbit IgG diluted 1: 10 000 with 1% BSA/TBST

3.5.2 Purification of the polyclonal antibody

After the production bleed, the polyclonal antibodies were purified using a protein G column. The purity of each antibody was determined by electrophoresis followed by CBB staining (figure 28). The only proteins present are the heavy and light chains confirming the purity of the antibodies. Figure 29 shows the progression of the antibody responses from sera obtained prior to the initial spore injection, test bleed and production bleed. The response becomes greater with each boost. The performance of each antibody was evaluated using ELISA (figure 30).

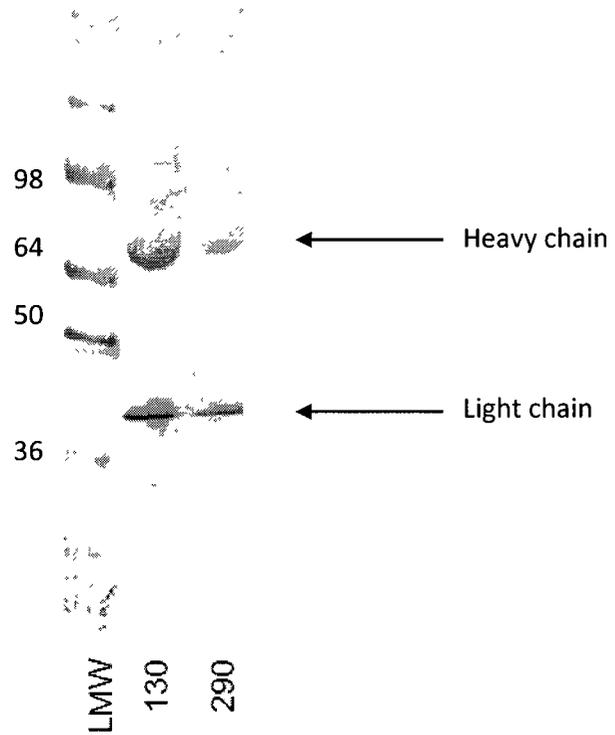


Figure 28: CBB stained gel of the rabbits #130 and #290 polyclonal antibodies after purification using a protein G column.

1.0 μg /lane of purified antibody

marker: SeeBlue[®] Plus2 Pre-Stained Standard

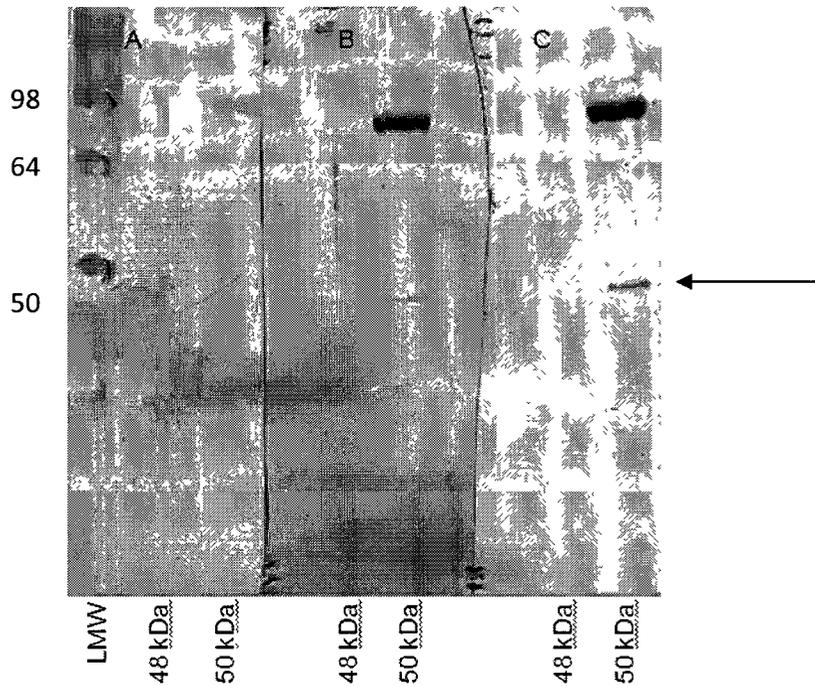


Figure 29: Immunoblot comparing the response of the purified 48 kDa and 50 kDa proteins from *Eurotium amstelodami* strain IBT 28305 against A) pre-immunization B) test bleed and C) production bleed of rabbit #290.

10µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard

the arrow indicates the 50 kDa protein

A) 1° Ab: RpAb diluted 1: 250 with 1% BSA/TBST

B) 1° Ab: RpAb diluted 1: 500 with 1% BSA/TBST

C) 1° Ab: RpAb diluted 1: 25 000 with 1% BSA/TBST

2° Ab: AP-anti-rabbit IgG diluted 1: 10 000 with 1% BSA/TBST

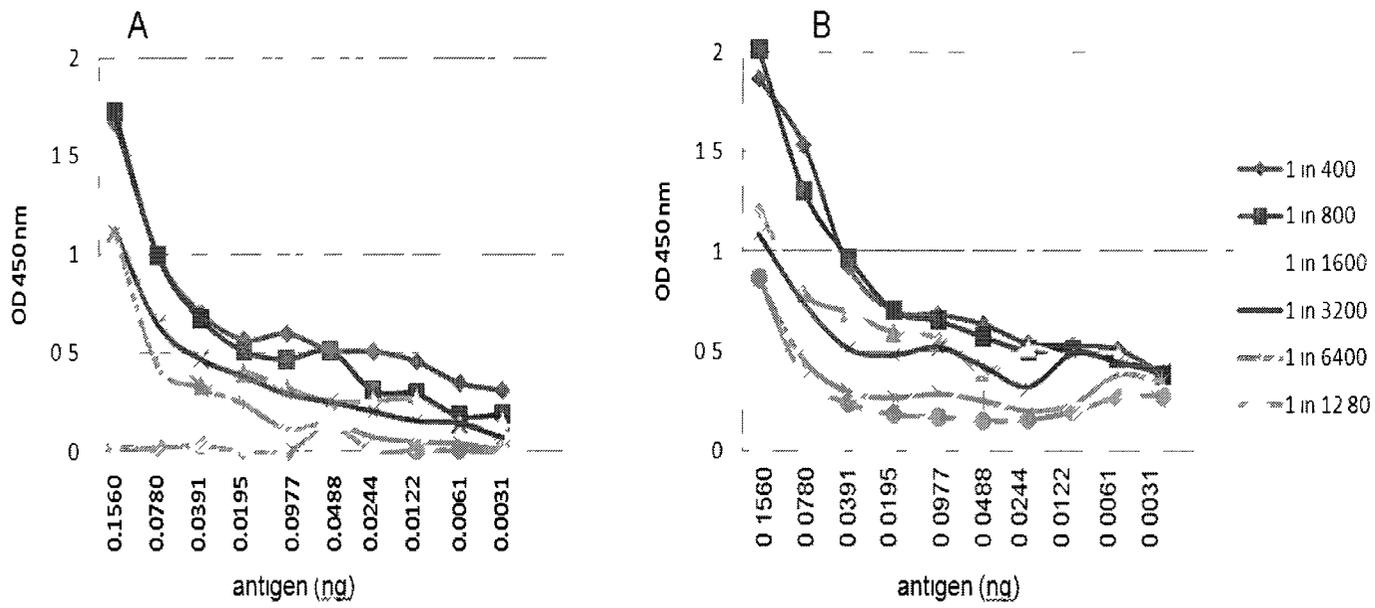


Figure 30: ELISA results demonstrating the activity of the RpAb #130 (A) and RpAb #290 (B) against the 50 kDa purified protein.

3.5.3 Cross reactivity testing with the RpAbs

Cross reactivity (RpAb #290) testing by immunoblots of the target protein against other fungi revealed that the other species had limited responses to the RpAb in comparison to *Eurotium amstelodami* (figure 31). The cross reactivity of the spores was also assessed using ELISA. The *Eurotium amstelodami* displayed a much higher response to the RpAb #290 than the other fungi (figure 32). The target proteins from the culture filtrate, the spores, and the purified extracts reacted against the rabbit #290 polyclonal antibodies (figure 33). These immunoblots and ELISA results confirm that the 50 kDa target protein is appropriate for further study.

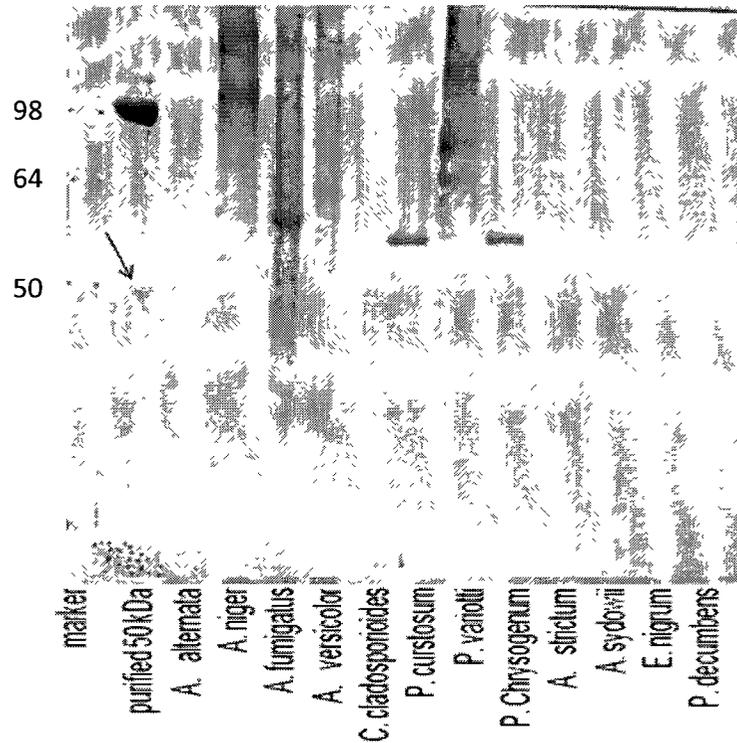


Figure 31: Immunoblot comparing the response of *Eurotium amstelodami* strain IBT 28305 and other fungi against rabbit pAb #290

10µg/lane of crude protein

marker: LMW-SDS marker from GE Healthcare

black arrow indicates the target 50 kDa protein.

yellow arrows indicate proteins of similar size in other fungi.

1° Ab: RpAb diluted 1: 25 000 with 1% BSA/TBST

2° Ab: AP-anti-rabbit IgG diluted 1: 30 000 with 1% BSA/TBST

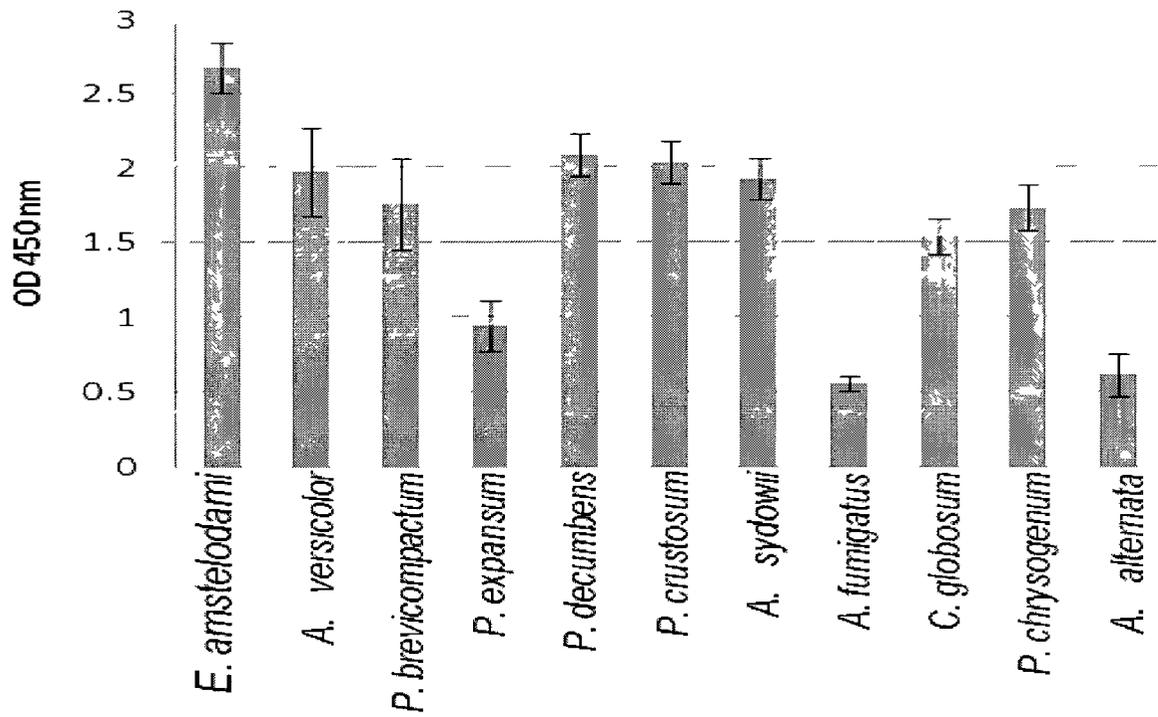


Figure 32: ELISA results demonstrating the response of spore proteins of *E. amstelodami* and different fungi against the rabbit #290 polyclonal antibody. Each test was performed in triplicate.

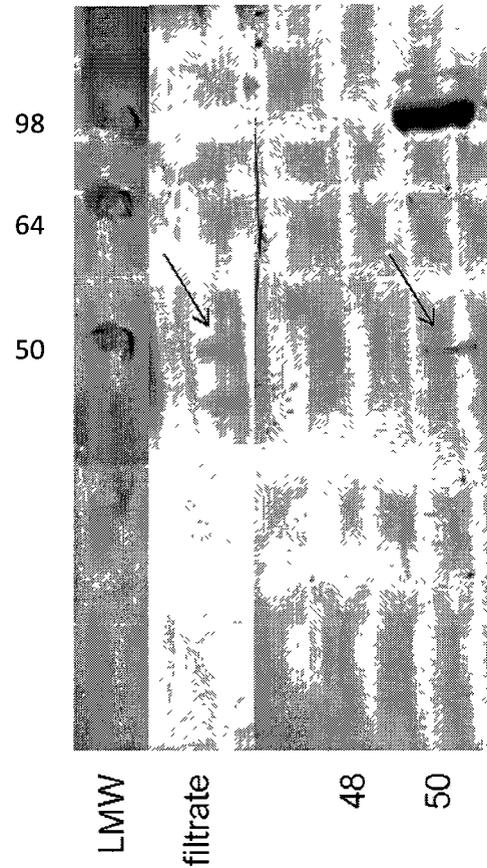


Figure 33: Immunoblots demonstrating the antigenicity of the 50 kDa protein using the RpAb 290.

10µg/lane of crude protein

marker: SeeBlue® Plus2 Pre-Stained Standard

Black arrow indicates the target 50 kDa protein.

1° Ab: RpAb diluted 1: 25 000 with 1% BSA/TBST

2° Ab: AP-anti-rabbit IgG diluted 1: 30 000 with 1% BSA/TBST

3.6 Characterization of the target antigens

3.6.1 MS/MS

The 48 and 50 kDa proteins were cut from a CBB gel and sent to Health Canada for MS/MS analysis. The liquid chromatography tandem mass spectroscopy (LC-MS/MS) was performed using a Linear Trap Quadrupole Fourier Transform (LTQ-FT). The resulting peptide sequences were BLAST (Basic Local Alignment Search Tool) compared to other sequenced proteins in the database. The peptide sequences comparisons determined that the 48 kDa protein is an α -amylase and the 50 kDa protein is a fructosyltransferase. The spectra for each target protein is displayed in figure 34.

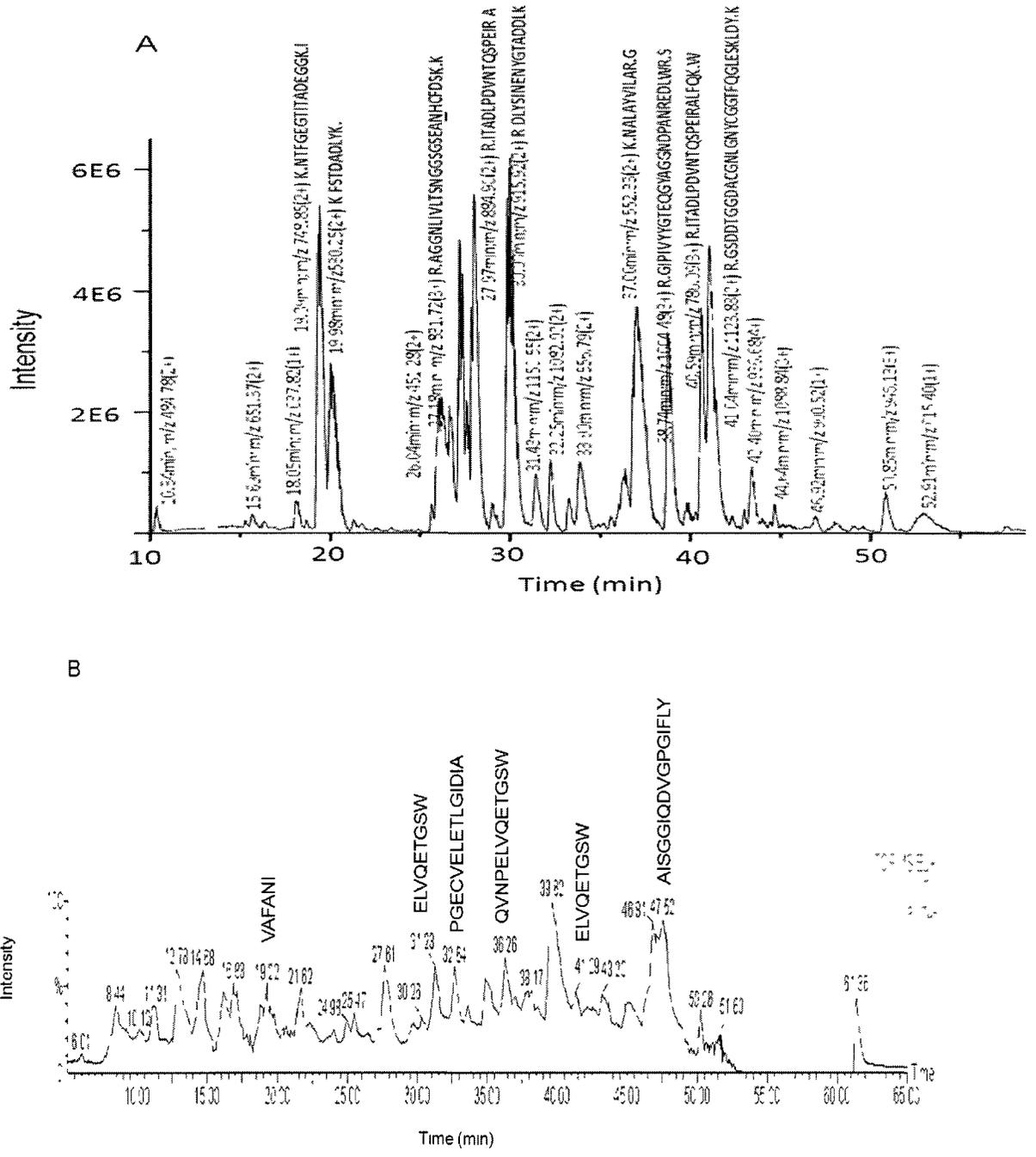


Figure 34: Alignment of the LTQ-FT spectra for A) the 48 and B) the 50 kDa proteins.

3.6.2 Isoelectric point (pI)

Isoelectric focusing was used to determine the pI of the 50 kDa protein (figure 35). The pH was calculated by comparing the R_f of the standards and of the target protein. The calculated pH was determined to be 5.82 indicating that the 50 kDa protein is acidic.

3.6.3 Glycoprotein assay

A glycoprotein stain was used to determine whether the 50 kDa protein was glycosylated. The target protein was deglycosylated using a TFMS (trifluoromethanesulphonic acid) treatment. One gel was stained with CBB, a second gel was stained with the glycoprotein staining kit (figure 36) and then compared for evidence of glycosylation.

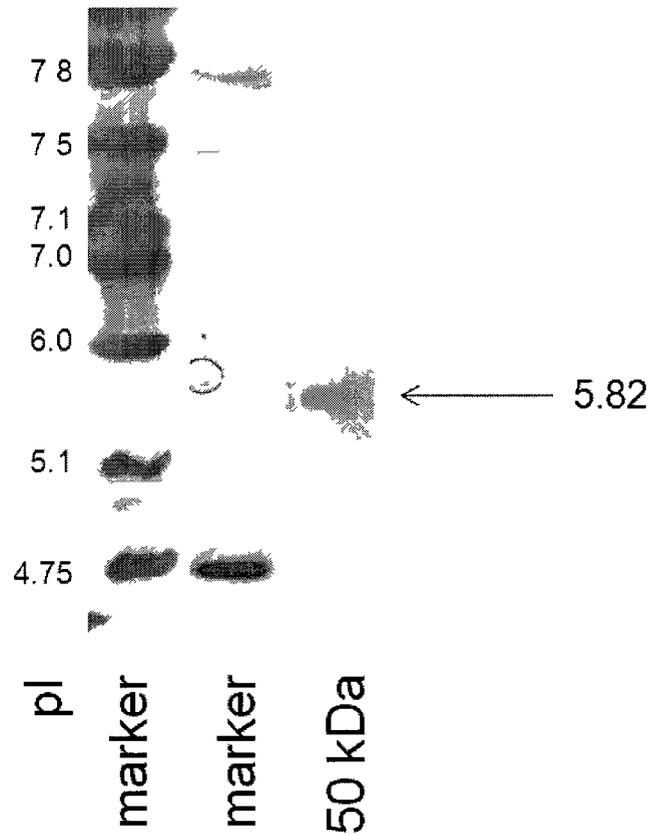


Figure 35: CBB stained isoelectric focusing strip (pH 4.45 to 9.6), arrow indicates a pI of 5.82 for the *E. amstelodami* 50 kDa protein.

5µg/lane of protein.

marker: IEF Standard, Bio-Rad. From bottom: phycocyanin, β-lactoglobulin B, bovine carbonic anhydrase, human carbonic anhydrase, equine myoglobin, human hemoglobin A, human hemoglobin C, lentil lectin,.

Black arrow indicates the target 50 kDa protein.

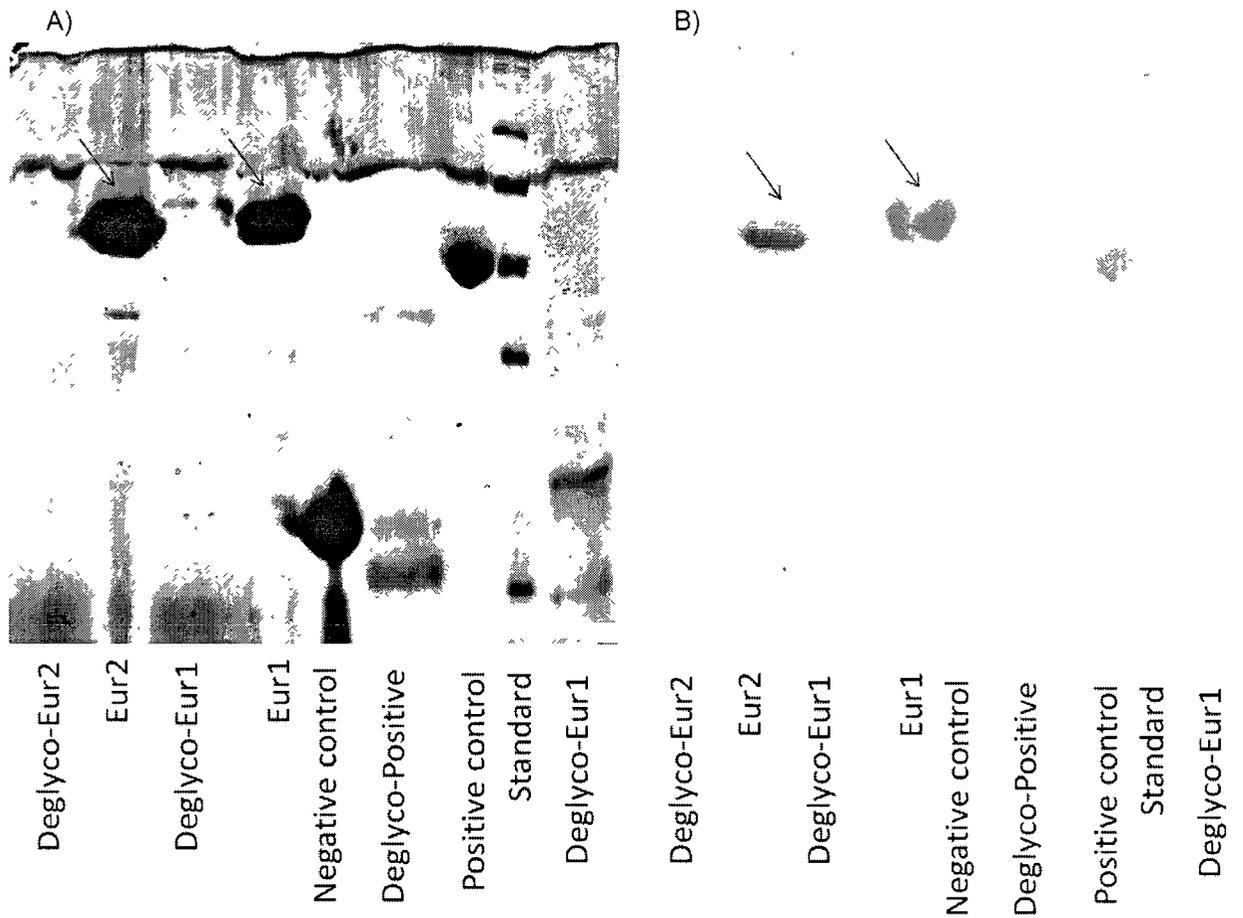


Figure 36: CBB stained gel (A) and glycoprotein stain (B) demonstrating that the 50 kDa target protein is glycosylated.

5 μ g/lane of protein

marker: SeeBlue[®] Plus2 Pre-Stained Standard

black arrows indicate the target 50 kDa protein.

yellow arrows indicate absence due to deglycosylation.

negative control: soybean trypsin inhibitor

positive control: horseradish peroxidase

4. DISCUSSION

Under the correct conditions, the built environment can be colonized by a large number of fungal species. These often vary by geographic location and climate. The indoor environment is conducive to fungal growth due to the temperature and readily available nutrients, it is the available moisture that becomes the controlling factor for mold growth (NAS, 2004).

Indoor air contamination such as VOCs, particulate matter and biologicals negatively affect health. Exposures resulting from biologicals include allergens from insects, dust mites, animals, pollen, fungal spores and mycelium. Exposure to allergens compounds can lead to allergy and the exacerbation of asthma in allergen-sensitive individuals and other allergic responses (Health Canada, 2004). In the United States, the incidence of asthma and allergy has increased steadily from 1964 to 2000 (CDC, 2003). Exposure to mold and dampness is associated with increased asthma and upper respiratory disease and the removal of mold and eliminating moisture problems is known to have public health benefit (Krieger et al. 2010; WHO 2009). The contribution that fungi make to asthma in Canada is difficult to reliably determine because allergens from fungi that grow in buildings have not been studied. This means that exposure cannot be measured to determine exposure-outcome (NAS 2004).

Exposure assessment to fungal allergens has been difficult by recurrent identification of numerous species- and genus-specific, but minor allergens (Crameri, 2011; Soeria-Atmadja 2010). The Miller group has focused on studies on proteins found on the surfaces of spores, spore- and hyphal fragments, present in strains of the target fungi collected over a wide geographic area and screened with a large collection of human sera (e.g. Liang et al., 2011; Wilson et al. 2009; Xu et al. 2007). These have proved to comprise a diverse array of excreted proteins. The dominant allergens of *S. chartarum* comprise a secretory, alkaline, Mg-dependent exodeoxyribonuclease which is attached to a larger protein of unknown function (Shi et al. 2011). The dominant allergen of the indoor clade of *P. chrysogenum* is a 52 kDa excreted glucoamylase (Luo et al. 2010). The allergen of *A. versicolor* is a secreted, subtilisin-like serine protease with 6 IgE- and 7 IgG binding epitopes (Shi & Miller 2011). The dominant allergen of *C. globosum* is a 47 kDa chitonase (Provost et al. 2011).

Asthma is a common disease in Canada. Between 1998 and 2009 the cases of asthma in children increased from 10.7% to 13.4% and in adults from 7.5% to 8.4%. These numbers represent 2.3 million affected Canadians (Health Canada 2004; Statistics Canada, 2009). It is evident that controlling the presence of fungi in indoor environments is becoming an important role in reducing the risk of asthma and allergies. The four main sources attributed to mold growth in the indoor environment include leaks in building structures, condensation, plumbing leaks that are left unrepaired and

mold growth on bathroom and kitchen surfaces or garbages (Dales, et al., 2008). Interventions that can remediate these issues include the use of eavestroughs to direct water away from the building, repairing faulty plumbing immediately, and the use of dehumidifiers and adequate ventilation (Prezant et al., 2008).

In North America the most common indoor fungi include species of *Aspergillus*, *Penicillium* (and their teleomorphs), and *Stachybotrys* (Flannigan et al., 2011; Prezant et al. 2008). Commercial detection kits relying on allergens exist for only a small number of fungi (*A. fumigatus*, *S. chartarum*, *A. versicolor* and *A. alternata*). There are over 100 known indoor fungal allergens registered with the International Union of Immunological Societies Allergen Nomenclature Sub-committee and the WHO (www.allergen.org, 2011) but the actual number may be much higher. With the large number of indoor fungi and their corresponding allergens, a much larger amount of these kits are necessary to accurately detect exposure levels. Currently most exposure assessments are provided using culturable fungi but an expert panel of the Institute of Medicine, National Academy of Sciences recommended the development of a standardized method for assessing exposure to fungal allergens as opposed to culturable fungi (NAS 2000). The process of isolating human allergens useful for this purpose is complicated by the fact that different strains of fungi often produce many antigens. Strains from different geographic locations or those growing on different substrates may produce different antigens. Therefore, an antigenic protein to humans that is common in strains over a large geographic location is required for a successful screening method.

Although a wide variety of fungi can grow in the indoor environment, the three *Eurotium* species studied are common on mold-damaged paper-faced gypsum wall boards, ceiling tiles, insulation and textiles (Flannigan & Miller, 2011; Miller et al., 2008). The presence of *Eurotium* species indoor should be further investigated since the inhalation of their spores and hyphae fragments has been known to elicit IgE-mediated responses as well as non-allergenic responses (Sorenson, 1999; Nielsen et al., 1999; Miller et al. 2010; Rand et al. 2011). The methods involving the production of antibodies that were developed in this research are expected to be useful for the assessment of *Eurotium* species in the indoor environment.

Conditions that need to be carefully monitored when isolating fungal antigens include temperature, oxygen availability, growth time and nutrients. Studies performed by Xu et al., 2007 compared several media for high protein yields which led to the choice of medium used in this experiment. Because *Eurotium* species are known to be xerophilic, the medium was adjusted with 1% glycerol (w/v) to promote a preferred growth environment by lowering the a_w .

The first step of this experiment consisted of screening the *Eurotium* species proteins from the culture filtrate extracts against HpAbs by immunoblotting and ELISA methods. The immunoblotting allowed for the visual identification of the antigenic proteins while

the ELISA results would determine the reactivity of the *Eurotium* species proteins to the HpAbs.

To optimize the detection signals for the ELISA, preliminary (checkerboard) tests were performed to identify the appropriate concentration of antigen and antibodies. This would allow for a maximum signal while maintaining a low background. Non-specific binding can occur if too much antibody is used leading to an increase in background levels. Using too small of an antibody concentration can cause signal reduction resulting from un-reacted antigen epitopes.

All of the *Eurotium* strains had antigenic responses to the HpAbs with ELISA testing. Some strains (IBT 28246 and IBT 28247) had lower responses to the HpAbs must most had comparable reactivity (figure 12). There were several variations observed in the average responses of each HpAb to the different strains (figure 13). While most were very similar (OD values ranging from 0.5 to 2) there were 3 sera (QC 3493, 3501 and 3510) that had responses of only 0.5 to 1.25. These variations can be explained by the fact that the responses are proportional to the *Eurotium* exposure levels of the individual. The titre of the antibodies in each individual would be associated with the length of exposure and how much time had elapsed between the exposure and the drawing of blood. The patients with the lowest ELISA responses were either not recently exposed to the allergens or were only exposed for a short amount of time resulting in a decreased titre of corresponding antibodies. Conversely, the patients

with high ELISA responses (QC 3508, 3825 and 3831) would have been exposed to higher levels of antigens over a longer period of time or would have most recently been exposed.

The immunoblot analysis (figures 6 to 11) displayed a similar outcome to the ELISA results. A similar pattern of variation was observed, however, with this technique the molecular mass of the antigenic proteins could be determined. The human sera reacted to many proteins from each *Eurotium* strain. Each immunoblot was analyzed and the various proteins that demonstrated an antigenic response were noted. A brief summary of these proteins are noted in table 1. Some proteins (30 and 95 kDa) were present in over 75% of the strains and reacted with over 66% of the sera tested. Some proteins (20 kDa) were only present in a few strains and responded to only a few sera. The smaller proteins were excluded since they are often highly conserved and would increase the possibility of cross-reactivity. The larger 95 kDa protein—although highly reactive on immunoblots was also excluded. The target protein should be an excreted protein and 95 kDa proteins may not be easily excreted due to possible modifications. The 48 kDa protein was present in 53% of the strains and the 50 kDa protein was present in 76% of the strains. They both showed a response to a large portion of the HpAbs and were therefore considered a good choice for antibody production. The intensity of the reactions on the immunoblots were rated on a scale of 1 (weak signal) to 3 (strong signal). When each strain was compared, the majority reacted to approximately 50% of the HpAbs. However, strains CBS 47-F3, CBS 47-F8, CBS 47-F9,

CBS 47-F4 and CBS 47-F6 reacted with less than 16% of the HpAbs. These strains correspond to *E. herbariorum* and *E. rubrum*. According to these results it would appear that more patients exhibit allergenic responses to *E. amstelodami*. Previous studies (Slack et al., 2009) have shown that *E. herbariorum* and *E. rubrum* are more frequently found in indoor environments than *E. amstelodami*. One possible explanation for these results may be that the growth conditions were not optimized for *E. herbariorum* and *E. rubrum* during the protein extract production which is an important factor in ensuring reproducible protein profiles (Esch, 2003). Figure 3 demonstrates the effect of growth time and extraction methods of the target protein production for the *Eurotium amstelodami* strain IBT 28305. It can be seen that there is a large difference between using the concentrator and acetone precipitation methods. The amount of time allowed for growth is also an important factor in the production of the target proteins. These conditions were not tested for the optimization of the *E. herbariorum* or *E. rubrum*. Another explanation may be that the titre of those antibodies in some of the patients may have declined. Strain IBT 28305 was the most responsive in the immunoblot tests with 12/36 human sera tested reacting to the 50 kDa protein and 11/36 human sera tested reacting to the 48 kDa protein. It also had moderate responses in the ELISA results.

To visualize the combined ELISA and immunoblots results more easily, a 3-dimensional graph (figure 15) was created. It consists of ELISA responses of the culture filtrates of 14 strains of *Eurotium* species (X axis), immunoblot responses (graded on a scale of 1 to 3)

to the 50 kDa protein (Y axis) and 18 individual human sera (Z axis). The different colours on the graph indicate where these combinations overlap. The patient responses fall within three categories. The first corresponds to patients that are allergic to several fungi represented by the combinations of strain/antibody with uniformly low immunoblot responses and varied (weak to high) ELISA responses. The second category combines the patients that are allergic to *Eurotium* species as well as to other fungi represented by the combination of strain/antibody with variable (weak to highly reactive to target protein) immunoblot responses and varied ELISA (weak to high) responses as well. The third category corresponds to the patients that are mostly allergic to *Eurotium* species represented by strain/antibody combinations that have relatively high responses in immunoblots to the target protein as well as high ELISA responses.

Because the spores, spore fragments and mycelium usually carry antigenic proteins the presence of the 48 and 50 kDa proteins was confirmed in spores (figure 5) and hyphae (figure 4) and their responses to a selection of HpAbs (figure 19, mycelia; figure 20, spores) were also confirmed prior to continuing onto the next step. The target (48 and 50 kDa) proteins were present in the spores and mycelia although to a lower extent when compared to the extracellular proteins. A plausible explanation for this may be that the allergenic proteins are mainly exported out of the cells into the culture filtrate. For this reason, the liquid culture as opposed to spore extracts or mycelia extracts was used for further protein production, purification and antibody production.

The target proteins were extracted from the liquid culture using two different techniques: acetone precipitation and a concentrator equipped with a 10 000 Da cut-off membrane. The acetone precipitation method allows the precipitation of the proteins from the solution due to altered solubility. Water-based buffers have large dielectric constants which stabilize the proteins resulting in the favored dissolution of the protein. Organic solvents such as acetone have a small dielectric constant which discourages the dissolution of the proteins. As such, when an organic solvent such is added to a water-based buffer, the solubility of the protein is decreased allowing for aggregation and precipitation of the proteins (Rosenberg, 2005). Acetone is widely used for this process due to its ease of availability, purity, low cost and in most cases, it does not denature the protein. This process was performed using ice cold acetone to avoid protein denaturation and involved two steps. The first step resulted in a 50% acetone mixture which removed the carbohydrates, lipids and remaining cell debris. The second step resulted in a 75% acetone mixture which allowed for the protein to precipitate. Although a small amount of protein is likely to precipitate from the 50% acetone mixture, yields are usually relatively high (Xu et al., 2007; Wilson et al., 2009).

The second method used for the protein extraction from culture filtrate included the use of a concentrator with a 10 000 Da cut-off membrane. The culture filtrate was concentrated approximately 50X resulting in a more manageable sample size with minimal protein loss. When the two methods were compared, the concentrator

method was more effective in producing larger quantities of the target proteins (figure 3). It was then the method of choice for the remainder of the production process. The 50 kDa protein represented approximately 6% of the total protein yield (table 3).

Prior to further purification steps, the cross reactivity of the target proteins was assessed using HpAbs and several other fungi. Only 2 fungi showed a response to HpAbs within the same molecular weight as the target proteins (figure 21, yellow arrows). As a result, a silver stain was performed to determine if the cross reacting proteins were indeed the same molecular weight and it was determined that they were slightly larger than the target proteins (figure 22 yellow arrows). These results allowed for the purification step to take place.

The first step of protein purification most frequently used is anion exchange chromatography. This technique is simple, rapid and effective in separating proteins based on their net charge. The stationary phase was a beaded, cross-linked agarose with a bound charged group $(-\text{OH}-\text{CH}_2\text{CHOHCH}_2\text{OCH}_2\text{CHOHCH}_2\text{N}^+(\text{CH}_3)_3$. This positively charged stationary phase interacted with the negatively charged amino groups present in the proteins. The elution of the column was achieved by addition of NaCl. As the anion, NaCl had a strong affinity for the column thereby competing with the already bound proteins facilitating their elution. The weaker the net negative charge a protein had, the easier it was to elude from the column. At pH 7.5, fractions 22 to 28 contained

the 48 kDa protein while fractions 37 to 58 contained the 50 kDa protein (figure 23). This indicated that the 50 kDa protein was more negatively charged than the 48 kDa protein since a higher concentration of NaCl was needed to elute it. At pH 9, fraction 37 contained the 50 kDa protein and the 48 kDa protein was negligible (figure 25). In the early stages of this experiment, it was determined that the 48 kDa protein was produced in larger quantities when the culture was removed at 48 hours growth and its production declined the longer the culture was allowed to grow. At this step, it was noted that the 48 kDa protein resulted in a higher yield (16%) but at a much lower purity (25%) compared to the 50 kDa protein which yielded 6% with a purity >90%. The culture seen in figure 25 was allowed to grow to 72 hours since it was optimal for the larger quantity of 50 kDa protein. Also, it should be noted that at pH 9, the 50 kDa protein was eluted in one fraction as opposed to the ion exchange performed at pH 7.5 (7 fractions). There may be several explanations including the efficiency of the column or perhaps due to different growth conditions there were different modifications resulting in a different affinity to the column.

After this purification step, the protein fractions (from column at pH 7.5) were pooled and concentrated. The concentrated protein (from pH 7.5) and fraction 37 (from column pH 9) were tested to confirm their activity by immunoblot (figure 26). Because the 50 kDa protein yield was acceptable and the purity was greater than 90%, it was used for antibody production.

The RpAbs were produced by injecting two rabbits with *E. amstelodami* spores, 1 boost with proteins removed from CBB stained gels and 2 subsequent boosts with the purified proteins. The injection of spores was performed to show that the antigenic protein is present in the spores. The test bleed was tested by immunoblot (figure 27) against spore extracts and culture filtrate extracts to confirm the presence of the antigenic target protein. The proteins were present in both extracts however the spore extract had an extremely weak response, most likely due to the low concentration of target protein. The 48 kDa protein was boosted in the rabbit identified as #130 and the 50 kDa protein was boosted in the rabbit identified as #290.

The test bleeds after the second boost of the antigens and the terminal bleed were tested by immunoblot (figure 29) and it was demonstrated that the response to the target antigen had greatly increased. The dilution of the pre-immunization bleed was only 1:250 and showed no response whereas the test bleed had a dilution of 1:500 which showed a response and the greatest response was for the terminal bleed which was diluted 1:25 000. This confirmed that the 50 kDa protein was antigenic to rabbits as well as to humans. Because the response to the target antigen increased with every boost of purified protein it confirmed that the protein was produced by the fungi and not some artifact of growth, isolation or other method. This established the fact that the 50 kDa protein could be a useful biomarker for the presence of *Eurotium* species in the indoor environment and that the RpAb produced by rabbit #290 could be used as a capture antibody if minimal cross reactivity occurred.

The activity of the purified RpAbs was first verified by ELISA (figure 30). Both RpAbs reacted proportionally at every dilution tested however the RpAb from rabbit #290 appeared to give higher OD values. The finding that the 48 kDa protein was difficult to isolate came after the rabbits had been boosted therefore the remainder of the experiments was performed using only the RpAb from rabbit #290 but RpAb from rabbit #130 was included in the testing until this point due to time constraints. The amount of time required to purify the protein overlapped the antibody production since we were given several weeks between every boost to obtain enough purified antigenic protein. The RpAb from rabbit #290 was then tested for cross reactivity against other fungi by immunoblot (figure 31) and by ELISA (figure 32). The immunoblot reactivity of the RpAbs (figure 31) showed a much stronger response in the 50 kDa range than did the HpAbs (figure 21) against the target antigen and lower responses against cross reactivity in the same 50 kDa range. This is expected since human patients (HpAbs) are more likely to have been exposed to several indoor fungi and will therefore show a greater amount of cross reactivity to multiple fungi. The RpAbs did cross react with proteins in the same molecular weight range produced by *A. fumigatus*, *P. crustosum* and *A. sydowii*. This could be explained by the fact that the rabbits were firstly injected with spores that contain proteins likely to be highly conserved among other species allowing for the cross reactivity to occur. As well, it is possible that the proteins contain epitopes similar to those found on the antigenic protein which are recognized by the antibodies. The cross reactivity was also tested with ELISA (figure 32) which showed that several other fungi responded however the OD values were much lower than that produced by

E. amstelodami but when verified on immunoblot, there were no reactions associated in the same molecular range. It is also necessary to note that ELISA OD values are a log based response. A value of 2.7 for *E. amstelodami* is almost 10X greater than a value of 2.0 for *P. crustosum*. The ELISA and immunoblot results demonstrated that the RpAb could potentially be used as a marker for the identification of *Eurotium* species in the indoor environment.

Several experiments were performed to characterize the 48 and 50 kDa proteins. These included an Edman degradation and mass spectrometry. The Edman degradation of the 50 kDa protein was unable to be performed since the N-terminus was blocked, and unable to be degraded. This is common in fungi and over ten kinds of blocking groups have been found including acetyl, formyl and pyroglutamyl (Huang et al., 2002). Mass spectrometry is widely used in the analysis of proteins because it is quick, accurate and is reasonably priced. The target antigens were digested using the protease trypsin and the resulting peptides were analyzed by liquid chromatography tandem mass spectrometry (LC- MS/MS) using a Linear Trap Quadrupole Fourier Transform (LTQ-FT) at Health Canada. The peptide sequences were queried using NCBI/ BLAST. The 48 kDa protein was determined to be an α -amylase and the 50 kDa protein was deemed to be a fructosyltransferase.

The α -amylase hydrolyses the α -1-4-glucosidic bonds of starch, glycogen and related polysaccharides. The α -amylase produced by *A. oryzae* is now considered an important allergen responsible for “bakers’ lung” (Baur et al., 1998).

BLAST database search of the de novo peptides sequences from the 50 kDa protein indicated it is highly homologous to fructosyltransferase (XP_002561396.1 and CAB89083.1). Alignment of these characterized peptides with their homologous proteins showed a highly conserved score and confirmed the accuracy of LC-MS/MS characterization.

The fructosyltransferase is part of the glycosyltransferase family of enzymes. In fungi, glycosyltransferases are involved in the generation of 1,3- β -glucan present in the cell walls (Utsugi et al., 2002) which is known to illicit inflammatory responses (Horner and Miller, 2003; Rand et al., 2010).

Fructosyltransferase occurs in various bacterial, fungal and plant species. In plants they are synthesized as storage carbohydrates. In bacteria fructans are produced as part of the exopolysaccharide. However, little is known about the physiological significance of fructans in fungi. A fructosyltransferase-producing trisaccharide has been reported for *A. niger*, *F. oxysporum*, *P. chrysogenum* and *A. sydowii*. Heyer and Wendenburg (2001) purified a fructosyltransferase from *A. sydowii* spores with a molecular mass of 55 kDa whose N terminus was blocked. This might explain the cross reactivity between the

RpAb and *A. sydowii* exhibited in the immunoblot and ELISA responses. Even though a few fructosyltransferases have been isolated from several fungi there are still none reported as allergens by the Allergen Nomenclature Sub-Committee of the WHO (www.allergen.org ; 2011) and only one reported (Lol p FT) from the International Union of Immunological Societies (www.allergome.org ; 2011) however, that allergen is found in pollen from rye grass.

A glycoprotein assay was also performed on the 50 kDa protein. It was determined that the protein is in fact glycosylated (figure 36). This is not unusual since glycosylation is often required for the proper folding, function and activity of proteins (De Pourcq et al., 2010). The pI of the protein was determined to be 5.82 (figure 35) which is acidic. This supports the fact that the 50 kDa protein was eluted in the later fractions using the anion chromatography technique. When the pH of the column (9.0) is much higher than the pI of the protein (5.82) the amino acids will have a larger net negative charge which increases the affinity of the protein to the column resulting in a later elution time.

In summary, two proteins with molecular weights of 48 and 50 kDa were isolated to different extents from *E. amstelodami*. The 48 kDa protein was not isolated in any great amount while there were nearly 10 mg of 50 kDa protein isolated. Both proteins were antigenic to humans and rabbits. The 48 kDa protein is an α -amylase. The 50 kDa protein is a fructosyltransferase and is glycosylated with an acidic pI (5.82). Both the 48 and 50 kDa proteins were present in spores, mycelium and culture filtrate.

Rabbit polyclonal antibodies were successfully produced. These outcomes combined with the relatively low levels of cross reactivity and the fact that the target protein is present in the majority of strains tested make the 50 kDa antigen a potentially useful biomarker in the detection of *Eurotium* species in the indoor environment.

Future research is required to further characterize the protein. In the present study, IgG antibodies were investigated. Similar experiments studying IgE antibody responses to the target antigen would be required since IgE antibodies are associated with allergic reactions (Hung et al., 2005; Day and Ellis, 2011).

Another future step would involve the production of monoclonal antibodies using hybridoma cells. This would result in antibodies capable of recognizing one single epitope on the antigenic protein allowing for extreme specificity. The specificity of the monoclonal antibodies in combination with the already produced polyclonal antibodies are required for the capture ELISA used in determining the presence of *Eurotium* from dust samples collected from indoor environments. There is currently a need for a rapid and accurate method to determine the human exposure to indoor fungi (NAS, 2004). ELISA is a method capable of satisfying this need and the future work of this project would allow for the development of such a tool.

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6. APPENDIX

Table A1.1 – List of the different *Eurotium spp.* strains

species	deposited accession number	parcel number	location	sampling material
<i>amstelodami</i>	IBT 28246	J0695-10	Ottawa	indoor
<i>amstelodami</i>	IBT28247	J9761-4	Ottawa	outdoor
<i>amstelodami</i>	IBT28248	J0653-5	Ottawa	indoor
<i>amstelodami</i>	IBT28249	J0897-1	Calgary	outdoor
<i>amstelodami</i>	IBT28250	J9529-13	Ottawa	outdoor
<i>amstelodami</i>	IBT28251	J9672-13	Ottawa	indoor
<i>amstelodami</i>	IBT28252	J9893-17	Ottawa	indoor
<i>amstelodami</i>	IBT28253	J1587-9	Ottawa	outdoor
<i>amstelodami</i>	IBT28254	J0656-38	Ottawa	indoor
<i>amstelodami</i>	IBT28305	J9858-1	Ottawa	indoor
<i>amstelodami</i>	IBT28307	J9508-1	Ottawa	outdoor
<i>amstelodami</i>	IBT28308	J1029-7	Carp, ON	indoor
<i>herbariorum</i>	CBS 47-F3	J9571-1	Calgary	indoor
<i>herbariorum</i>	CBS 47-F8	J9683-2d	Ottawa	indoor
<i>herbariorum</i>	CBS 47-F9	J9862-3m	Calgary	indoor
<i>rubrum</i>	CBS 47-F4	J0976-2d	Ottawa	indoor
<i>rubrum</i>	CBS 47-F6	J0879-5	London, ON	indoor

Table A1.2 – List of the identification number for the human sera (HpAbs)

QC 2797	QC 3490	QC 3835
QC 2798	QC 3493	QC 3836
QC 3342	QC 3494	QC 3837
QC 3346	QC 3496	QC 3839
QC 3347	QC 3501	QC 3841
QC 3349	QC 3508	QC 3845
QC 3350	QC 3510	QC 3848
QC 3351	QC 3825	QC 3849
QC 3352	QC 3828	QC 3850
QC 3353	QC 3830	QC 3851
QC 3354	QC 3831	QC 3852