Isolation, purification and characterization of a protein from indoor strains of *Wallemia sebi* that is antigenic to humans

by

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ABSTRACT

The purpose of this project is to isolate and characterize a human allergen from the moderate xerophile *Wallemia sebi*. This obscure fungus turns out to be very common in some homes all over the world. It is known that it causes allergic responses in sensitized individuals, but until now, the identity of the major allergenic protein was unknown. The second purpose is to develop antibodies for its detection in house dust. The quantification of human allergen (antigen) levels can be helpful in studying allergic responses and performing exposure assessments. Human sera obtained from atopic patients showing fungal sensitization were screened against crude *W.sebi* extracellular protein extracts by Western Immunoblot and Enzyme-linked immunosorbant assays (ELISA). Based on its immunogenic response to human polyclonal antibodies, a 47kDa dimer was recognized as antigenic and chosen as the target protein for this work. This protein was purified by fast protein liquid chromatography (FPLC) and further characterized by liquid chromatography tandem mass spectrometry (LC-MS/MS), isoelectric focusing, and by a glycoprotein assay. The antigenicity of the 47kDa protein was confirmed by its ability to produce polyclonal antibodies in rabbits. Capture ELISA and Western immunoblot with the purified 47kDa protein, the *Wallemia* arthrospore protein extracts and the arthrospore-spiked fine dust confirmed that the polyclonal antibody was capable of detecting the targeted *W. sebi* antigen. Cross-reactivity Capture ELISA tests using spore protein extracts from various indoor fungi showed that the rabbit polyclonal antibodies produced were specific to the *W. sebi* target protein. Subsequent research will involve producing monoclonal antibodies to the selected *W.sebi* antigen to develop an immunoassay for *W.sebi* detection in the indoor environment.

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ABBREVIATIONS

1° Ab – Primary antibody.
2° Ab – Secondary antibody.
AP- Alkaline phosphatase.
APC – Antigen presenting cell
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.
CFU – Colony forming units
CYA - Czapek Yeast Autolysate agar
DAOM- Department of Agriculture, Ottawa, Mycology, Ottawa, ON.
ELISA- Enzyme-linked immuno-sorbent assay.
ERMI - Environmental Relative Moldiness Index
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.
HRV – Heat resistant ventilation
IAQ – Indoor air quality
IBT- Mycology Group. Technical Group, Denmark.
ITS – internal transcribed spacer
IgE – Immunoglobulin E
IgG – Immunoglobulin G
IgM – Immunoglobulin M
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.
PCR – Polymerized chain reaction
PMSF- phenylmethylsulphonyl fluoride.
PVDF membrane (immunoblot)- Polyvinylidene fluoride membrane.
RAST - radioallergosorbent test
RBA – Rose Bengal agar
RpAb- Rabbit polyclonal antibody.
SDS – Sodium dodecyl sulfate
VOC – Volatile organic compound

1. INTRODUCTION

1.1 Indoor Air Quality

Indoor air quality (IAQ) can have an impact on human health, especially in countries such as Canada, where people spend the majority of their time indoors (Leech et al., 2002). This potentially translates into greater personal exposures to common indoor pollutants such as combustion by-products, off-gassing emissions and biological agents (Dales et al., 2008). Ventilation rates in Canadian homes have declined from the post war period and ca. 25% have less ventilation than recommended (Johnson & Miller, 2012). Unless there is mechanical ventilation (i.e. HRV, Heat Recovery Ventilation), the amount of fresh air entering in buildings can be inadequate depending on occupancy (Johnson & Miller, 2012). Dampness and inadequate ventilation can lead to the build-up of biological and chemical contaminants, which are often generated by sources within the built environment (Flannigan, 1992). Apart from floods, there are 4 major sources of mould growth in residences: leaks in building fabric, condensation, unattended plumbing leaks and household mould (e.g., mould growth on kitchen and bathroom surfaces, hidden food spills, garbage, defrost pans (Dales et al., 2008). This results in the production of aerosols containing several bioactive agents such as spores, allergens, low molecular weight compounds, triple helical (1,3)-beta-D-glucans and volatile organic compounds (Miller et al.,2007); (Miller et al.,2010). Inhalation exposure to whole spores, spore-fragments, and mycelial fragments may play a role in adverse health outcomes (WHO, 2009). Children exposed to moulds in homes have an increased risk of developing asthma and allergic rhinitis (Jaakkola & Hwang, 2005), (Jaakkola et

al.,2010). In adults, occupancy of water-damaged buildings and indoor dampness has been associated with the onset and exacerbation of respiratory conditions (Cox-Ganser et al.,2005); (Dales et al., 2008); (NIOSH, 2012); (WHO, 2009).

1.2 Fungi and the Built Environment

It has been estimated that between 10-30% of North American homes have sufficient mould contamination to elevate the risk of respiratory disease (Jarvis & Miller, 2005). Most moulds are saprophytic, meaning they derive their carbon nutrients from dead or decaying organic matter (Deacon, 2006). Given their marked degree of metabolic versatility and flexibility, saprophitic moulds can use a broad range of organic substrates for growth (Carlile et al.,2001). During growth, the mycelium adheres to substrates and secretes enzymes to degrade various biopolymers such as starch, cellulose, and lignin into simpler substances that can be reabsorbed by the hyphae (Deacon, 2006). Common carbon sources in the built environment include starchy adhesives often used with wallpapers, cellulosic substrates such as ceiling tiles, paper-faced gypsum wallboard and textiles, keratin in skin, and manufactured wood products. Fungal growth is regulated by several environmental conditions, such as temperature, pH, nutrient availability and moisture, the latter being of critical importance. Water activity (a_w) is a measure of the amount of water within a substrate, and each microorganism has a minimum and optimum a_w for growth. The a_w value is based on a scale of 0 to 1.0, with pure water having a water activity of 1.0. In general, indoor moulds are capable of growing on substrates with 0.7-0.8 a_w, and some at even less than 0.65. Hydrophilic moulds are species suited for growth at high water availability (minimum a_w >0.90),

whereas xerophilic species dominate under lower water activity values (minimum a_w <0.80) (McGregor et al.,2008). When a material becomes significantly wetted so that its water activity exceeds 0.90, it becomes susceptible to immediate colonization by all categories of moulds (tertiary colonizers may act as primary invaders) (Flannigan, 1992); (Flannigan & Miller, 2011). When conditions conductive to mould growth are present indoors and are not controlled, moulds can proliferate and colonize virtually any substrate (McGregor et al.,2008). Fungal contamination of building materials can result in the accumulation of spores, spore and hyphal fragments and dusts from mould-damaged substrates in air and settled dust (Foto et al.,2004).

Fungi typically have complex life cycles that can consist of asexual (anamorphic) and sexual reproductive stages (Carlile et al.,2001). Both these stages involve the production of spores, reproductive structures that are well adapted for airborne dispersal. Differences in sporulation exist between species; *Penicillium* species can produce 10^{12} spores within a few days, whereas other fungi produce many fewer spores (Miller, 1992). Spores of some species of fungi that damage building materials survive long periods under dry, adverse conditions. When there is sufficient moisture, spores may produce germ tubes that will differentiate into microscopic branching filaments called hyphae (Deacon, 2006). A colony consists of hyphae that intertwine and form visible masses called mycelium (Carlile et al.,2001).

1.2.1 Exposure to Fungal Aerosols and Health

Inhalation exposure to moulds and their aerosols can result in infectious, inflammatory and allergic responses (Health Canada, 2007); (WHO, 2009); (NIOSH, 2012). When inhaled, the efficiency of particle deposition in specific regions of the respiratory tract

strongly depends on the particle's aerodynamic diameter and the breathing flow rate. Contrary to pollen grains which are most often spherical and can reach up to 100μm in diameter (Pleasants et al.,2001), fungal spores are much smaller and vary in shape. Foto et al. (2004)have reported that *Penicillium citrinum* spores can have a mean surface area of 10.6 μm^2 ± 2.79 while *Aspergillus sydowii, Cladosporium cladosporiodies,* and *Stachybortys chartarum* spores can have respective surface areas of 20.4μm^2 ± 5.70, 56.88 μm^2 ± 26.6 and 75.0μm^2 ± 17.7. The locations where spores and fungal fragments deposit along the respiratory tract can cause variable respiratory reactions (Reponen, 1995). Inhaled spores are often >10 μm in size and tend to deposit in the nasopharynx region thus causing adverse effects at the ocular and/or nasal regions (Horner, 1995). However, in mouldy buildings, the majority of fungal metabolites, glucan and allergens are present as fine particles <0.5 μm (Salares et al.,2009). These particles are efficiently deposited along respiratory bronchiolar and alveolar surfaces, (Cho et al.,2005); (Miller et al.,2010).

Allergic responses are the most common and most clearly understood health effects associated with inhalation exposure to fungi (Miller et al.,2010). At least 112 fungal genera, belonging to the Ascomycota, Basiomycota and Deuteromycota phyla have been reported to produce allergens that cause allergic reactions in individuals (Li & Yang, 2004). Both spores and fungal fragments are allergen sources, however a large proportion of fungal allergens may be present in smaller fragments that can penetrate deeper into the lungs (Fluckiger et al.,2000), (Miller et al.,2010). Studies by Fluckiger et al. (2000) demonstrated an absence of correlation between indoor allergen concentrations and a number of viable spores in the built environment, and concluded

that cultivation methods can underestimate allergen load. Sensitivity to fungal allergens may develop in atopic (allergy-prone) individuals following low-level mould exposure, or in individuals that are not genetically predisposed to develop allergies after chronic and/or high level exposures (Takahashi, 1997). Expert panels from the United States National Academy of Science have found a clear association between fungal exposure in damp spaces and upper respiratory symptoms (nasal congestion, sneezing, running itching nose, and throat irritation), lower respiratory tract symptoms (coughing and wheezing), and exacerbation of asthma symptoms in mould-sensitized asthmatics (National Academy of Sciences, 2000), (National Academy Sciences, 2004). While exposure to mould in damp homes has been a suspected cause to the development of asthma, there is currently insufficient evidence to determine whether or not such an association exists (National Academy Sciences, 2004). The lack of standardized methods for assessing exposure to fungal allergens remains a significant impediment to outlining the relationship between fungi and asthma (National Academy of Sciences, 2000). Accurate detection of fungal allergens would facilitate development of causality for building related illnesses (Shi et al.,2011).

1.3 Allergy

There are four types of hypersensitivity reactions and these are classified on the basis of clinical presentation and underlying immune mechanism involved (Horner et al.,1995). Type I hypersensitivity is an immediate response that is mediated by Immunoglobulin (Ig)E antibody-triggering of mast cells or basophils (Horner et al.,1995) (Janeway et al.,2001). Cytotoxic or Type II hypersensitivity involves the reaction that

ensues when cell surface or matrix antigens are recognized by and form complexes with specific IgG or IgM class antibodies, ultimately resulting in cell lysis (Janeway et al.,2001). Type III immune complex-induced hypersensitivity is triggered by aggregated immune complexes and involves phagocytosis by neutrophils, which results in vasculitis (Horner et al.,1995), (Janeway et al.,2001). Finally, Type IV hypersensitivity is mediated by antigen-specific effector T cells that release various lymphokines upon encountering an antigen (Horner et al.,1995). Type I hypersensitivity responses such as allergic asthma and allergic rhinitis are the most common medical problems associated with inhalation exposure to fungi (Horner et al.,1995), (Janeway et al.,2001), but hypersensitivity pneumonitis, which comprises a mixture of immune response types can also occur in response to high allergen exposure levels (Janeway et al.,2001). These conditions are discussed in more detail below.

1.3.1 Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis (HP) or extrinsic allergic alveolitis is a lung disorder caused by repeated inhalation and sensitization to a type of organic dust, including microbial agents, animal proteins and low molecular weight compounds (Hung et al.,2005). HP is common in agricultural and occupational settings and is often generically named after its causative agent or related occupation. For example, HP caused by exposure to mouldy hay is often called farmer's lung (Horner et al.,1995). The disease is mediated by a combination of immune complex (Type III) and T lymphocyte-mediated (Type IV) hypersensitivity reactions that result in inflammation of the alveoli (Horner et al.,1995). Symptoms include fever, productive cough, chills, fatigue and body aches and can develop 4 to 6 hours following exposure but resolve

once the exposure to the offending agent has been terminated (Horner et al.,1995), (Hung et al.,2005). Irreversible pulmonary fibrosis can occur if exposure is persistent (Hung et al.,2005).

1.3.2 Allergic Rhinitis

Individuals with allergic sensitization to fungi will most often exhibit symptoms of rhinitis which develop due to inflammation of the nasal mucosa and include intense itching and sneezing, nasal discharge, irritation of the nose and congestion (Hung et al.,2005). While allergic rhinitis is not a life threatening condition, it has a high global prevalence of of 10% to 25% (Aberg et al.,1996), (Ozdoganoglu & Songu, 2012) and symptoms can cause debilitating conditions that reduce the quality of life and incur economic costs as high as $5.9 billion annually (Ray et al.,1999).

Being widespread in both indoor and outdoor environments, fungal spores are important causative agents in allergic rhinitis (Ellis & Day, 2011). Furthermore, spores are relatively small in size compared to pollen and therefore are readily deposited in the respiratory track. A large study involving 3371 patients found that 25.8% of subjects diagnosed with rhinitis were sensitized to fungi (Boulet et al.,1997). Furthermore, of the individuals diagnosed as having both asthma and rhinitis, 27.4% were sensitized to moulds (Boulet et al.,1997).

1.3.3 Asthma

Asthma is a chronic inflammatory respiratory disorder characterized by airway inflammation and hyperirritability of the bronchial mucosa. Symptoms include coughing, chest tightness, shortness of breath and wheezing (Hung et al.,2005). The onset of asthma can occur in atopic individuals who have been exposed and sensitized to

allergic agents. There are more than 250 airway-sensitizing agents known to cause atopic asthma via IgE-mediated mechanisms (van Kampen et al.,2000). Numerous species of mould have been shown to exacerbate asthma (Hung et al.,2005). Asthma affects approximately 2.2 million adult Canadians and more than 485,000 children ages 4 to 11 (Public Health Agency of Canada, 2007). The disease continues to be a leading cause for hospitalization (Public Health Agency of Canada, 2007). Since the best preventative measure for asthma symptoms is allergen avoidance, accurate environmental analysis of fungal allergens is critical (Hung et al.,2005).

1.4 Biological mechanisms of sensitization and allergy

An antigen refers to any molecule capable of binding specifically to an antibody (Janeway et al.,2001). While not all antigens are capable of eliciting immune responses, those that are immunogenic, (typically most foreign proteins), can stimulate antibody production (Janeway et al.,2001) (Huby et al.,2000). Intrinsically innocuous antigens that can trigger allergic responses, or hypersensitivity, upon repeated exposure are termed allergens (Janeway et al.,2001). Allergies can only develop in sensitized individuals -patients that have developed a prior immunization to an offending allergen (Janeway et al.,2001). It is estimated that close to 40% of the population worldwide is sensitized to foreign proteins in the environment (Pawankar et al.,2011). Atopy is the exaggerated tendency to mount IgE-mediated immune responses to innocuous environmental agents (Janeway et al.,2001). Individual susceptibility is complex, being affected by both environmental and genetic factors (Huby et al.,2000). Atopic individuals have elevated serum IgE levels (Horner et al.,1995).

Allergic sensitization is induced when specific IgE molecules are produced by B cells in response to an initial allergen provocation and distributed systemically (Janeway et al.,2001). Antigen presenting cells (APCs) are highly specialized cells that are widely distributed at portals of entry throughout the body (i.e. under the skin, under the epithelial surfaces of the airways and in the submucosa of the gut) (Janeway et al.,2001). These can be dendritic cells, macrophages and B cells. When an allergen (i.e offending protein) is encountered for the first time, it is engulfed and processed by APCs. The resulting peptide fragments are presented at the surfaces of APCs in association with class II major histocompatibility complex (MHC) molecules (Janeway et al.,2001). Antigen presenting cells then migrate to regional lymph nodes to interact with naïve CD4 T cells (T helper cells, T_H0). APC signalling via B7 molecules and binding of T-cell antigen specific receptors and CD4 co-receptor to peptide:MHC complexes stimulates T_H0 cell proliferation and differentiation into T effector cells (Janeway et al.,2001). Cytokine expression will favour either T_H1 or T_H2 differentiation. For instance, interleukin-12 (IL-12) and IL-4 production will favour T_H1 and T_H2, respectively. Low dose exposure to allergens can preferentially stimulate T_H2 cell differentiation over T_H1 cells (Janeway et al.,2001). T_H2 cells produce IL-4 which will inhibit T_H1 differentiation and also act as a growth factor for B lymphocytes (Janeway et al.,2001). B cells are located in the lymph nodes draining the sites of allergen entry and local regions where the allergic reaction occurs (Janeway et al.,2001). IL-5 signals B cells to differentiate into antibody producing and secreting plasma cell, while IL-4 and IL-13, along with co-stimulatory interaction between the T cell membrane-CD40 ligand and B-cell surface molecule CD40, drive class switching of B cells to IgE production

(Janeway et al.,2001). Anti-allergen IgE molecules are produced and via their constant (Fc) region bind to their high affinity IgE receptor (FcεRI) found on tissue mast cell and blood basophils (Janeway et al.,2001). Once captive, these bound IgE molecules can survive for several weeks and the individual is sensitised against the challenging allergen (Janeway et al.,2001).

Upon re-exposure to an allergen, divalent binding to captive IgE molecules causes membrane FcεRI to cross-link, thereby activating mast cells and basophils and triggering the release of early and late phase immune mediators (Janeway et al.,2001) Primary, preformed mediators such as histamine cause immediate vasodilation, increased permeability, smooth muscle contraction (responsible for bronchoconstriction and bronchospasm) and promotes glandular secretions (Janeway et al.,2001), A late phase response is triggered hours later by the synthesis and influx of inflammatory mediators such as prostaglandins, proteases, leukotrienes, monocytes, eosinophils cytokines and chemokines (Janeway et al.,2001).

1.5 Characteristics of allergens

1.5.1 B and T cell epitopes

Although no general consensus sequences exist, B and T cell epitopes are important contributors to a protein's allergenicity (Huby et al.,2000). B cell epitopes are 3-dimensional peptide units found on a protein's surface that act as antigenic determinants, enabling its recognition by B cells and its binding by immunoglobulins E (Janeway et al.,2001). B epitopes can either be linear or conformational, i.e. encompassing a tertiary structure composed of discontinuous sequences of amino

acids (Toda et al.,2011). Because FcεRI crosslinking requires divalent IgE binding, the provoking allergen must contain at least two B cell epitopes (Huby et al.,2000). This translates into a minimal amino acid sequence of 30 residues (2X15 amino acids) and a lower size limit of 3kDa (Huby et al.,2000). Most allergens however, contain several B cell epitopes; for instance, peanut allergen Ara h 1 is known to have at least 23 linear epitopes, arranged in two 3-dimensional clusters on the surface of the protein (Huby et al.,2000). Such conformational epitopes have also been shown to be important in maintaining a protein's immunogenicity (Huby et al.,2000). For certain proteins displaying conformational epitopes, elimination of intermolecular disulfide bonds and resulting polypeptide unfolding can reduce allergenic potency by rendering proteins more labile to gastric digestion and decreasing IgE binding abilities (Toda et al.,2011).

T cell epitopes are fragmented peptide sequences resulting from the intracellular processing of the antigen by APCs (Janeway et al.,2001). T cells recognize peptide:MHC class II complexes via T cell antigen receptors which grant specificity for a particular T epitope (Huby et al.,2000). In order for an allergic reaction to ensue, an antigen must therefore contain T epitopes capable of selectively polarizing T_H2 immune responses (Huby et al.,2000). It is not known however whether the potential to favour T_H2 differentiation is a property intrinsic to the allergen.

While the presence of B and T cell epitopes in a protein is paramount to its intrinsic allergenicity, other characteristics are believed to be involved (Huby et al.,2000). These include: resistance to proteolysis, enzymatic activity and glycolysis (Huby et al.,2000).

1.5.2 Resistance to proteolisis

It appears that relative stability in simulated gastric fluid (SGF) can correlate to allergenicity (Astwood et al.,1996). Astwood et al. (1996) evaluated the stability of common food allergens via the gastric tract in a simple model of gastric digestion and found that important allergens were stable in SGF, whereas non allergenic food proteins were digested in minutes. These findings suggest that gastric stability would allow orally-administered proteins to persist in the gastric tract for an amount of time sufficient to cause an immune response (Astwood et al.,1996), (Huby et al.,2000). Interestingly, gastric stability may also be a predictor of allergenicity for non-orally administered proteins (Hilton et al.,1994), (Huby et al.,2000). This is because SGF may reflect the stability in other acidic milieus such as the proteolytic fluid of APCs, which would affect antigen-processing and T cell responses (Vijh et al.,1998).

1.5.3 Enzymatic activity

Most proteins that are enzymatically active are capable of eliciting biological responses. Therefore, one might argue that to prevent biological disruptions, organisms are more prone to mount immune responses against foreign enzymes than non-enzymatic foreign proteins, and that the ability to do such would confer a selective advantage (Huby et al.,2000). Many clinically relevant allergens are known to be biologically active (Sehgal et al.,2005). Several fungal-derived enzymes used in the baking industry as dough additives have been shown to cause IgE-mediated sensitization (Sander et al.,1998). Alpha-amylase produced by *Aspergillus oryzae* is the main allergen present in

commercial preparations and this enzyme is considered to play a causative role baker's asthma and other work-place related allergic respiratory symptoms (Sander et al.,1998). Other enzymatically active fungal allergens include aldehyde dehydrogenases from *Alternaria alternata* and enolases from *Cladosporium herbarum* that can cause allergic rhinitis (Sehgal et al.,2005).

Enzymatic functions vary greatly between allergens and although speculated, it is not known whether general biological activity (i.e. irrespective of function), may render a protein more stable and enhance its allergenicity (Huby et al.,2000). Some allergens however, have biological activities that are directly pro-allergenic, notably protease enzymes (Sehgal et al.,2005). Proteolitically-active enzymes have been shown to facilitate allergen delivery across the bronchial epithelium by disrupting the tight junctions between epithelial cells, activating protease-activator receptor-2 (PAR-2) and producing thymic stromal lymphopoietin (TSLP), a cytokine involved in T_H2 cell recruitment and the stimulation of proinflammatory cytokine production (Matsumara, 2012). One of the most studied protease allergens, Der p 1, the major house dust mite *Dermatophagoides pteronyssinus* allergen may also promote an enhanced immune response by cleaving CD23, the low affinity IgE receptor present on B cells (Hewitt et al.,1995). In addition to producing more soluble CD23 fragments that stimulate increased IgE production, CD23 cleavage also results in the abolishment of an important inhibitory feedback mechanism that normally limits IgE synthesis (Hewitt et al.,1995).

1.5.4 Glycosylation status

Glycosylation refers to the cotranslational or posttranslational modification that occurs naturally in eukaryotic cells by which one or more glycan moieties (oligosaccharide

chains) are covalently added to asparagine (N-linked) or serine/threonine (O-linked) amino acids in the polypeptide chain (Huby et al.,2000). Many allergens are known to be glycosylated proteins (glycoproteins), including major cat allergen Fel d 1 (Counsell et al.,1996), peanut allergen Ara h 1 and Bermuda grass pollen allergen Cyn d 44 (Altmann, 2007). By altering physicochemical properties such as solubility, hydrophobicity and/or electrical charge, the presence of carbohydrate chains within protein allergens is thought to influence their allergenicity and immunogenicity (Huby et al.,2000). More specifically, the presence of glycosyl groups modifies B and T cell epitope identity, which in turn is thought to affect antigen capture via mannose receptors present on APCs and subsequent processing (Sallusto et al.,1995), (Huby et al.,2000).

1.6 Wallemia sebi

Wallemia sebi has been a common contaminant of foods that are sweet (cakes, dates, jams, fruits), salty (fish, meat, peanuts), and/or dry (cereal, bread, rice) (Samson et al. 2011). Taxonomically, *Wallemia* has been positioned within the Basidiomycota based on its dolipore-type hyphal septum, which is surrounded by parenthosomes or septal pore caps (Moore, 1986), (Zalar et al.,2005). The genus is comprised of three distinct species: *W. sebi, W. muriae, W. ichthyophaga* (Zalar et al.,2005). Rather than being halophilic as previously described by mycologists, *Wallemia* is xerophilic because its growth is independent of the solute used to increase the osmotic pressure of the media (Pitt & Hocking, 2009). Interestingly, xerotolerance is rarely observed among the Basidiomycetes. Studies have attempted to clarify the phylogeny and taxonomy of *Wallemia.* Phylogenic analyses of nuclear small subunit ribosomal DNA (SSU rDNA) and sequence data of the rDNA internal transcribed spacer regions 1 and 2 (ITS) rDNA,

including 5.8S rDNA also support the relationship of *Wallemia* with the Basidiomycota (Matheny et al.,2006); (Zalar et al.,2005). Zalar et al. have proposed a fourth Basidiomycetous class – Wallemiomycetes covering an order Wallemiales, to account for *Wallemia's* unique morphology, evolution and xerotolerance (Zalar et al.,2005). More recently, Padamsee et al. (2012) identified 93 putative osmotic stress proteins that suggested that High Osmolarity Glycerol pathway to be the mechanistic basis of its xerotolerance. They suggest that *Wallemia* is the earliest diverging lineage of Agaricomycotina.

1.6.1 Wallemia and Sporulation

Wallemia displays other unique characteristics. The mode of conidiogenesis is distinct from other members of the phylum. The fungus produces arthrospores – "conidia" that arise from hyphal fragmentation – in long chains on simple fertile hyphae (Figure 1) (Hill, 1974). The hyphal development is basauxic because the conidiogenous cells grow from a basal point (Zalar et al.,2005). During hyphal elongation, a cylindrical structure forms above a constriction in the branch. The cylinder septates, thereby creating two segments that further subdivide to produce a row of four cylindrical cells. These cells rapidely differentiate into small spherical brown spinose arthrospores (Pitt & Hocking, 2009) .

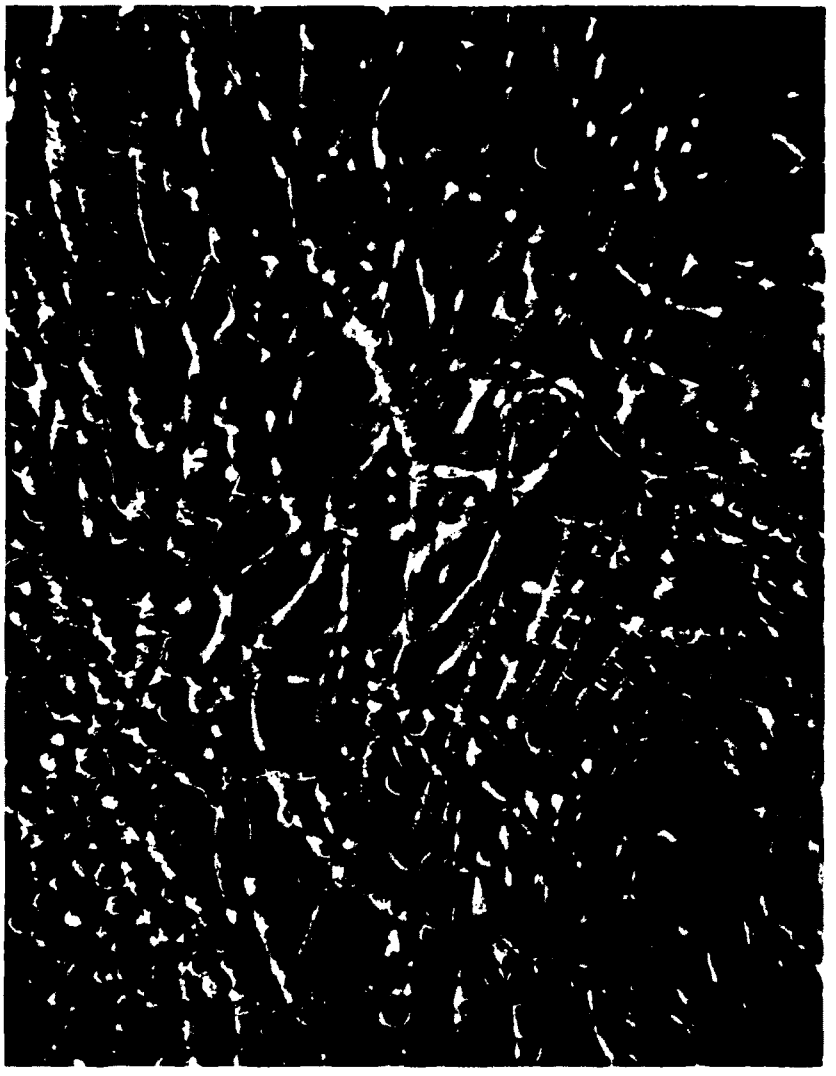

Figure 1. *W. sebi* arthrospores. Used with permission, Dr. R. Samson, CBS the Netherlands.

1.6.2 Anatomy of Wallemia

Of the 3 currently accepted species of *Wallemia*, *W. sebi* has the smallest arthroconidia, having a diameter of 1.5-2.5 µm in size, in comparison with *W. muriae and W. ichthyophaga* which have arthroconidia of 2.5-3.0µm in diameter and 3.5-5.0µm in diameter, respectively (Pitt & Hocking, 2009). *W. sebi* is capable of sporulating profusely with incredible speed – within 1 to 2 days of germination when grown on

media with a a_w between 0.997-0.91, and within 5 days on media of 0.85 a_w (Pitt & Hocking, 2009). ,Neither *W. muriae* nor *W. ichthyophaga* is able to grow on high water activity agar, such as malt extract agar (MEA) and Czapek Yeast Autolysate agar (CYA) but *W. sebi* is capable of growth over a wide range of water activities, from 0.69 to 0.997; its optimal water activity is 0.94 (Pitt & Hocking, 1977). *Wallemia* has a slow growth rate in culture (Zalar et al.,2005) and on CYA and MEA, colonies appear uniformly brown or orange brown, are 1-6 mm in diameter and are plane or crateriform and velutinous (Figure 2) (Gravensen et al.,1994); (Pitt & Hocking, 2009).

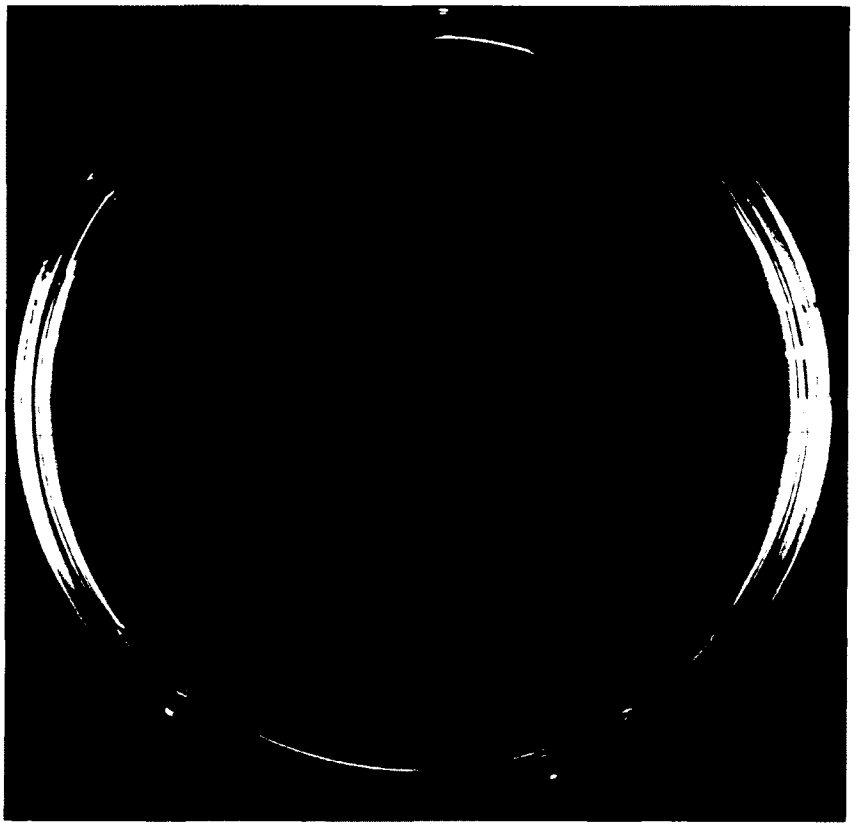

Figure 2. *W. sebi* colonies as grown on MEA. Used with permission, Dr. R. Samson, CBS the Netherlands.

1.6.3 Wallemia Genome

Being osmotolerant, *Wallemia* species have been found to colonize diverse habitats. *Wallemia* is known to undergo various morphological changes to adapt to different environments (Kuncic et al.,2010). For instance, under conditions of high salinity, osmoadaptations such as decreased hyphal compartment length and increased cell wall thickness have been observed in the fungus (Kuncic et al.,2010). Specific genes involved in these adaptations have not been fully elucidated. Padamsee et al. (2012) investigated the existence of osmoregulatory proteins in *W. sebi* and observed a high number of transporters thought to play a role in the fungus' ability to thrive in harsh environments. The authors noted two of three significant gene expansion families that may contribute to *Wallemia*'s osmotolerance - the AA_trans family, which could be involved in transporting small solutes across the membrane, and Dabb, which is known to be upregulated by plant species in response to high salinity (Padamsee et al.,2012)

1.6.4 Wallemia and Health

Many xerophilic species are not readily recovered with standard fungal media which has a water activity close to 1.00 ((Dillon et al.,1999). This effect resulted in the underestimation of Wallemia in the environment, and its role as an allergen was not considered. After the late 1970s, Japanese and some European researchers using low water activity media were able to show that xerophilic fungi including *W. sebi,* were abundant in settled dust (Lustgraaf, 1978) and air (Lustgraaf, 1978), (Sakamoto et al.,1989), (Takahashi, 1997) collected in homes. There are now many studies on its occurrence in settled dust in most temperate areas. For example, it has been reported

in house dust in Japan (Sakamoto et al.,1989), Canada, USA, (Amend et al.,2010); (Miller & Day, 1997); (Nonnenmann et al.,2012), and Europe (Amend et al.,2010).

W. sebi is known to cause allergies in humans and is suspected of causing bronchiolar asthma (Sakamoto et al.,1989). Sakamoto et al. studied the allergenic activity of *W. sebi* using mycelial extracts (Sakamoto et al.,1989). Skin prick tests on asthmatic individuals showed that 5.4% of subjects displayed positive immediate skin reactivity, a reaction indicative of immediate-type hypersensitivity to *W. sebi* (Sakamoto et al.,1989). These results were in concordance with those observed with skin prick tests performed using *Aspergillus fumigatus* extracts (Sakamoto et al.,1989). Furthermore, in vitro radioallergosorbent tests (RAST) also found that 18.9% of test subjects were positive, indicating that these patients exhibited IgE antibody to *W. sebi* (Sakamoto et al.,1989). RAST inhibition results also showed cross-allergenicity between *W. sebi* and *A. fumigatus* but the lack of a perfect cross-relationship suggests that each species contains distinct allergenic determinants (Sakamoto et al.,1989). Sensitization to *W. sebi* was also reported in a large scale population study of German children aged 3 to 14 (Kolossa-Gehring et al.,2007). Of the 1538 children studied, a proportion of 0.2% displayed allergic sensitisation against *W. sebi* arthrospores (Kolossa-Gehring et al.,2007).

Exposure to Wallemia may also result in subcutaneous infection due to the action of toxic metabolites (Wood et al.,1990). The metabolic profile of *W. sebi* is not well known and only a few metabolites have been characterized. Sesquiterpene mycotoxins such as walleminol (Figure 3, I), a colourless crystal having a melting point of 128-130°C and the molecular formula $C_{15}H_{24}O_2$, and walleminone (Figure 3, II), an oil from which

walleminol can also be obtained (Frank et al.,1999). Walleminol has an LD_{50} of 40µg/ml for brine shrimp and a minimum inhibitory dose of 50µg/ml for rat liver cells (Wood et al.,1990), (Pitt & Hocking, 2009). *In vivo* toxicity has not been defined in model animal systems and therefore the significance for human health is unknown. Other metabolites that have been isolated include tryptophol (Figure 3, III), a tryptophan metabolite (Wood et al.,1990), wallemia A (Figure 3, IV), a pyrrol pigment (Badar et al.,1972), and UCA1064-B (V), an anti-tumour antibiotic (Takahashi, 1993).

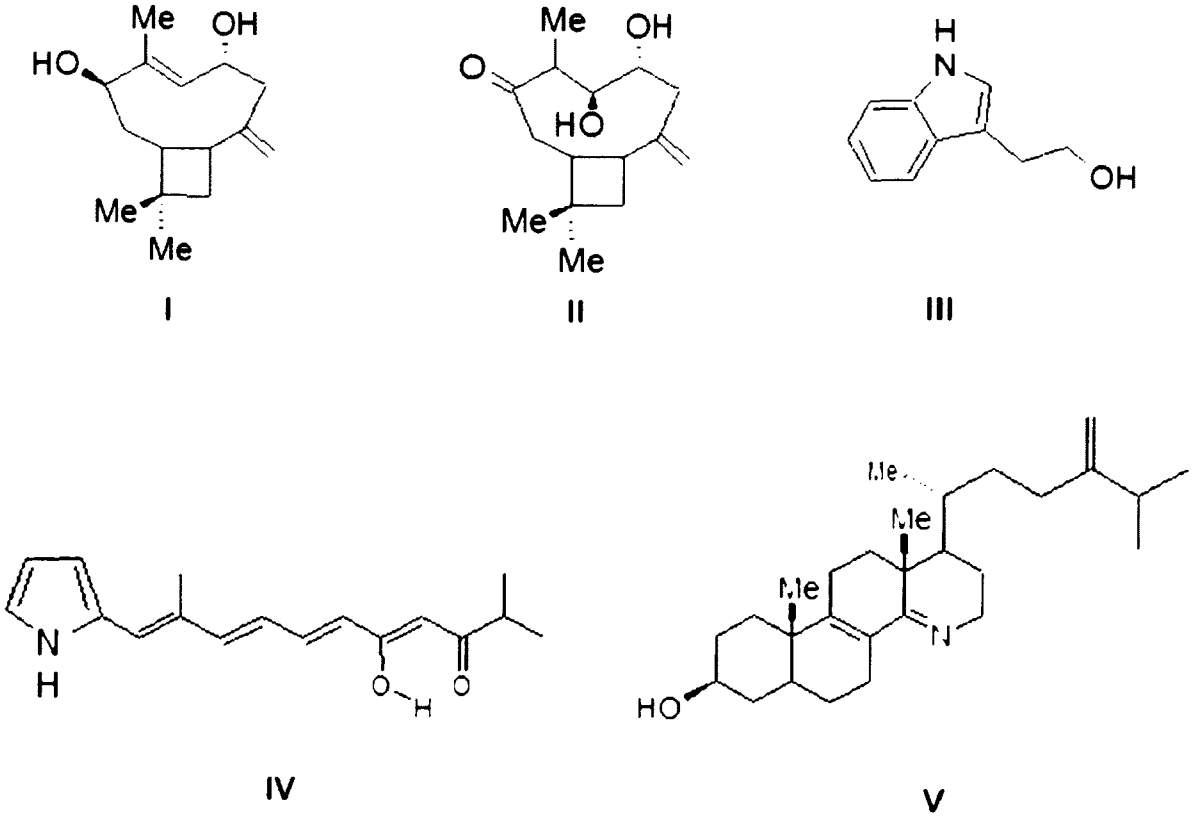

Figure 3. *W. sebi* secondary metabolites.

W. sebi has also been found to be amongst the most common fungi in some agricultural settings. This species has been isolated from straw, wood chip bedding and dry hay (Lappalainen et al.,1998). Heavy microbial exposures often occur in these environments with reported concentrations of culturable microorganisms ranging between 10 to 10^{10} colony forming units (cfu)/m^3 (Hanhela et al.,1995). Farmer's lung (hypersensitivity pneumonitis) has been described among agricultural workers, and most importantly dairy farmers who can be exposed to high spore concentrations during their daily activities. A study by Lappalainen et al. (1998) found elevated levels of serum IgG antibodies against *W. sebi* in Finnish farmers, indicating that these workers are exposed to *W. sebi* in their work environment to a greater extent than what has been previously reported.

1.7 Assessment Methods for Fungi

Quantitative and qualitative assessment methods can be useful in identifying mould growth which may cause adverse health effects (Prezant et al.,2008). Prompt remediation of indoor dampness and mould has been shown to decrease health risks (Krieger et al.,2010). Different sampling techniques and approaches exist for assessing the presence, location and amount of indoor mould and each has its unique characteristics that confer advantages and disadvantages. There is no standard method for enumerating fungal aerosols and therefore a dose-response relationship has yet to be established between inhalation exposure to fungal spores/fragments and respiratory illness (Cho et al.,2005).

1.7.1 Traditional Methods: Culture-Based and Non-Culture-Based Methods

Various methods and sampling equipment can be used to assess the occurrence and prevalence of mould in ambient air and dust. Traditionally, culture-based methods (i.e. growth of fungal propagules on culture media) and non-culture based methods (i.e microscopic examination) have been the most common techniques employed to identify and enumerate mould particles (Pitkaranta et al., 2008). Today, there is a growing trend towards molecular based fungal enumeration methods (Pitkaranta et al.,2008). Recent studies have shown that a large percentage of pathogenic and allergenic agents are found on fine particle fragments that are not always detectible with cultivation and microscopic approaches (Frohlich-Nowoisky et al., 2009). Also, most molecular-based methods are more representative of longer term exposure as they provide more than a snapshot of the sampled environment. Cognizant authorities agree that culture based methods have no value in determining risk to health (Health Canada, 2007); (NIOSH, 2012).

1.7.1.2 Culture-Based Methods

In culture-based methods, microorganisms are collected from a volume of air, mass of dust, or surface area and onto an agar sample which is incubated a 25°C (Dillon & Heinsohn, 1996). The fungal propagules present in the sample are allowed to grow for 2-7 days (Dillon & Heinsohn, 1996). Resulting colonies are enumerated and the total number of spore and/or hyphal fragments capable of producing a colony is expressed as the amount of colony forming units (CFU) per sample. Representative cultures are then transferred to an agar-containing petri dish and allowed to mature for identification to the species level via light microscopic examination (Miller et al., 2000).

An increasing number of studies are supporting that fungal components that contribute to the burden of respiratory disease, such as mycotoxins, allergens and (1-3)-β-D-glucans, can linger in the built environment irrespective of the viability of the moulds that produce them (Miller et al.,2000), (Cho et al.,2005), (Foto et al.,2005). Therefore, the most evident limitation of methods based on the enumeration of culturable fungi is that they fail to detect the non-viable fungal propagules in sampled environments (Dillon et al.,1999). It has been estimated that only less than 10% of sampled microbial components are culturable (Alvarez et al.,1995). There are additional factors that also affect the viability of culturable fungal spores and thus, there is little correlation between species that are the most readily cultured in media versus the species that have the most important environmental presence (Miller et al.,2000). Culture-based methods can misrepresent air spora due to variable rates of survival between fungal species, both in the environment and on the culture medium (Flannigan, 1997). Species such as *Aspergillus* and *Penicillium* produce and release spores that can survive for over 12 years, whereas other species such as *Stachybotry atra* have a more limited viability (Miller, 1992); (Hung et al.,2005). Furthermore, in mixed cultures, limited nutrients can result in the overgrowth of faster growing species or growth inhibition of slower growing species (Flannigan, 1997). The growth of some species on culture media may also inhibit other species. *Trichoderma* species, for example, may produce antifungal agents that can be detrimental to the viability of other species (Dillon et al.,1999). Other species may be under-represented in culture due to their limited growth on the selected sample medium. A variety of growth media can be used; Rose Bengal Agar (RBA) and Malt Extract Agar (MEA) are most commonly used as they are suitable for culturing a

wide spectrum of fungi. However, because different fungal species have different growth requirements, these types of agar will not be able to support the growth of every microorganism (Miller et al.,2000). Some more xerophilic species, like *W. sebi* may not be readily recovered with commonly used agar, and different media types with lower water content like Dichloran glycerol 18% agar (DG18) may better support their growth (Dillon et al.,1999).

While culture-based methods have little if any quantitative value in relation to fungal contamination, their strength lies in their ability to provide qualitative information about the types of species present in an environment (Flannigan, 1997). The qualitative advantage of culture-based methods was demonstrated in a study conducted by Miller et al. (2000), during which the authors collected culturable samples in 15 apartments with visible fungal contamination and 15 apartments without visible contamination. The authors found that while there was no difference in the average fungal colony forming unit values per m^3 of air between the apartments with visible fungal contamination and those without, the type of species identified in the mould-contaminated residences were significantly different than those found in the pooled outdoor air sample.

1.7.1.3 Non-Culture Based Methods

In non-culture based methods, microorganisms and fragments thereof are enumerated regardless of their viability. For this reason, this type of assessment is often referred to as total spore sampling. The most common approach is carried out with a Spore Trap, a sampling cassette which contains a slide coated adhesive surface to capture fungal propagules A vacuum pump draws air into the cassette, and particles collect onto the

adhesive surface by impaction (Dillon & Heinsohn, 1996), (Saldanha et al.,2008). The sample slide is subsequently stained and counted by microscopy (Dillon et al.,1999). The lactophenol cotton blue (LPCB) wet mount preparation is the most widely used staining technique for examining fungi by bright light microscopy (Leck, 1999). The phenol component in the stain preparation kills living microorganisms, the lactic acid acts as a preservative for the fungal structures, and the cotton blue agent stains the chitin in the fungal cell walls (Leck, 1999). In addition to being relatively inexpensive and having a fast analysis turn-around time, an advantage of the spore trap method is that it allows a relatively high percentage of spores to be captured from the environment (Foto et al.,2005). However, total airborne mould concentrations assessed by spore trap have shown to not always correlate to area of visible mould contamination in a home (Foto et al.,2005) and qualitative measurements may prove to be more useful indicator of a mould problem (Miller et al.,2000), (Gots et al.,2003). Incidentally, the major drawback to spore trap sampling, is that it provides little taxonomic information on the species collected from the environment (Miller et al.,2000), (Lin et al.,2011). Identification is often limited to the genus or group level for organisms like *Aspergillus* and *Penicillium* that the lack of distinctive features on their spores, and thus cannot be identified to the species level by microscopic observation of spores alone (Foto et al.,2005), (Lin et al.,2011). Fluorescent stains like acridine orange for epifluorencent microscopic examination are more sensitive; however some fungal fragments fail to absorb the dye, while others have dark pigments that mask it (Dillon et al.,1999).

1.7.2 Molecular-Based Methods of Mould Assessment

1.7.2.1 MSQPCR and the Use of DNA as Surrogates for Mould Exposure

The MSQPCR, or Mould Specific Quantitative Polymerized Chain Reaction (qPCR), is a DNA amplification technique that uses species-specific primers (DNA fragments) for the detection of a vast array of fungal species in an environment (Lin et al.,2011). This technology has been patented by the U.S. Environmental Protection Agency (EPA) and is currently being used in research to analyze fungal DNA contained in filter-based air and dust samples collected in homes (Vesper et al., 2007). Vesper et al. (2005) have developed a large variety of species-specific primers capable of identifying a vast number of moulds . This method offers a standardized method for the identification and enumeration of a large number of indoor fungi (Vesper et al., 2007). The incorporation of a fluorescent probe into an amplified gene product adds a quantitative element to the process; the amount of fluorescence is intended to be directly proportional to the number of cells for each specific mould (Vesper et al., 2005), (Lin et al., 2011).

Sample results obtained by DNA analysis are then compared to the ERMI, the Environmental Relative Moldiness Index (ERMI), which is an index or scale of moldiness which is intended to grade the mould burden within a home (Vesper et al., 2007). The ERMI is based on the prevalence of certain "indicator" species, and has no units. The index is divided into quartiles, the first or lowest quartile indicate the homes with the lowest mould burden, whereas the homes in the fourth or highest quartile (above 5) indicates the homes with the greatest mould burden (Vesper et al., 2007), (Lin et al. ,2011). Therefore a higher ERMI value corresponds to a greater likelihood of water-damage and mould growth (Lin et al., 2011).

PCR-based methods are useful in their ability to identify a large number of fungal species, some of which are potentially pathogenic and allergenic but only present in an environment at a low concentration (Vesper et al., 2005), (Frohlich-Nowoisky et al.,2009). With conventional sampling, air sampling times are usually short (<5min) to avoid culture overgrowth in agar plates or masking in spore traps (Meklin et al., 2007). Conversely, with DNA-based methods of assessment, longer sampling times are achievable (8h or longer), thereby increasing the limit of detection (Meklin et al., 2007). Frohlich-Nowoisk et al. (2009) used a high-volume dichotomous sampler to sample a total air volume of 3000m^3 and to separate coarse (particles with aerodynamic diameter >3 μm) from fine respirable particles (<3 μm) fractions. DNA analysis revealed that over 1000 fungal species were present in one sample and that more fungi known to be allergenic and pathogenic for humans were found in the respirable fraction. Additionally, this study found the indoor ratio of Basidiomycota to Ascomycota to be much higher than previously assumed. Similarly, Lignell et al. (2008) found that microbial concentrations in qPCR were several orders of magnitude higher than those recovered by culturing. These authors also demonstrated that the qPCR method is sensitive enough to reveal significant differences in microbial concentrations in house dust collected from moisture damaged and non-moisture damaged homes (Lignell et al.,2008). Another potential benefit of PCR for quantitative assessments of indoor fungi is its objectivity (Vesper et al., 2007).

While qPCR is gaining popularity in environmental studies (Haughland et al., 2004); (Vesper et al., 2007), one significant drawback to results obtained is that databases are lacking for comparing quantities of DNA in different environments and situations.

Another disadvantage is the high specificity of analysis: only fungi targeted with the specific gene sequences used are detected; other fungi, including potentially pathogenic species, with variant sequences do not (Flannigan & Miller, 2011). Any organism that differs in the target sequence will fail to be reported (Dillon et al. ,2007). It has been estimated that while only 100,000 fungal species have been identified, more than 1.5 million are estimated to exist (Deacon, 2006). Recently, studies have combined qPCR with pyrosequencing to evaluate the population of fungi in sets of dust samples collected from homes (Nonnenmann et al., 2012). Fungal tag-encoded flexible (FLX) amplicon pyrosequencing (fTEFAP) offers the possibility of detecting "unexpected" microbial species within environmental samples (Nonnenmann et al., 2012). This method uses universal primers to target specific 18S ribosomal DNA sequences to amplify extracted genomic material and provides a relative estimate of the fungi in the sample, often at the species level (Nonnenmann et al., 2012).

An inherent difficulty with all PCR-based technology is that the overall success is largely dependent on the extent to which the DNA is effectively isolated and purified (Keswanie et al., 2005). Each purification step results in the loss of some microbial DNA, making it impossible to achieve the same DNA recovery among all species (Keswanie et al., 2005). Some environmental samples may require additional purification due to the presence of PCR inhibitors. Examples of these compounds include humic and fulvic acids in soil, organic polyphosphates in fungi which are tightly associated with the microbial fraction of soil, and plant acidic polysaccharides (Keswanie et al., 2005). Such factors can significantly reduce the accuracy of the PCR quantification and thereby diminish the value of the ERMI in predicting mould burden and disease (Dillon

et al., 2007). Finally, studies by Dillon et al. (2007) have demonstrated the failure of MSQPCR to correlate with other methods of mould assessment such as structural fungal biomass, viable spore and total spore counts, thereby rendering it unreliable as a measure of exposure assessment (Meklin et al., 2004.

1.7.2.2 (1-3)-β-D-Glucans as Surrogates for Mould Exposure

As a major structural component found in fungal cell walls, the polysaccharide (1-3)-β-D-glucan is considered an indicator of fungal biomass (Bonlokke et al., 2006), (Iossifova et al., 2008). Glucan commonly accumulates in settled dust from Canadian homes (Salares et al., 2009). Typical airborne (1-3)-β-D-glucan concentrations range between 0.04 to 20.55 ng/m^3 (Foto et al.,2004), (Salares et al.,2009). Some studies have reported (1-3)-β-D-glucan levels as high as 100 ng/m^3 (Rylander et al.,1992), (Yao et al.,2009). There is evidence that the majority of the fungal glucan load is present of fungal particles smaller than 1 μm (Foto et al., 2004). Rand et al. (2010) studied inflammation-associated gene responses induced by curdlan, a linear (1-3)-β-D glucan, in the mouse model and found that low-doses of curdlan (40 ng/curdlan/kg lung wt) were capable of inducing *dectin-1* transcription and expression in the proximal lung regions. Glucans are thought to play an important role in respiratory disease by stimulating the *dectin-1* glucan receptor on the cellular surfaces of monocytes, neutrophiles, mast cells, dentritic cells, and alveolar macrophages, thus eliciting non-allergenic inflammatory responses (Rand et al., 2010). Furthermore, their experiments demonstrated *dectin-1* localization on alveolar macrophages, alveolar type II cells, and non-ciliated, respiratory bronchiolar epithelia (Rand et al., 2010). Experimental studies in which human subjects were exposed to curdlan have demonstrated that inhalation

exposures can elicit nasal and airway inflammatory reactions (Bonlokke et al., 2006). An association is also suspected to exist between fungal glucans and subjective symptoms like fatigue headache respiratory symptoms and decreased lung function (Iossifova et al., 2008).

Several studies have looked at glucan concentrations in air and dust samples for quantitative assessments of microbial contamination (Iossifova et al.,2008). The most commonly used analytical method is a *Limulus* amoebocyte lysate (LAL) assay, which is an aqueous extract of blood cells from the horseshoe crab (Aketagawa et al., 1993). It is based on the ability of (1-3)-β-D-glucans to activate Limulus (horseshoe crab) coagulation factor G (Aketagawa et al.,1993). A chromogenic substance is used in conjunction with the lysate preparation and a colour change can be observed upon enzyme activation by (1-3)-β-D-glucan (Aketagawa et al., 1993), (Douwes et al., 1996). The intensity of colour, which is proportional to enzyme activation, can be measured by spectrophotometer and the glucan content can be determined by comparison to a standard curve (Pickering et al., 2005). This method is highly sensitive and can detect as little as picogram levels of glucan in collected air and dust samples (Foto et al.,2004). One of the main disadvantages of this analytical method is that endotoxins are also known to trigger the LAL enzymatic cascade (via Factor C rather than Factor G). This cross-reactivity can yield false positive results (Foto et al., 2004). Researchers have attempted to address this issue by either pre-treating samples to 0.5M NaOH, which is detrimental to endotoxins, or developing modified assays that are glucan-specific and devoid of Factor C (Foto et al.,2004). Additional drawbacks of the LAL assay are the high cost and the variability of commercial horseshoe crab preparations (Sander et

al.,2008). An alternative analytical method was developed by Douwes et al. (1996) developed for quantifying the glucan content in collected air or dust samples. This method is an inhibition enzyme-linked immunosorbent assay (ELISA) in which rabbit antibodies are produced against (1-3)-β-D-glucan. This test measures the extent at which the (1-3)-β-D-glucan in the sample can disrupt the binding of the rabbit anti-glucan antibodies to (1-3)-β-D-glucan bound to the microtitre plate. Glucan quantification is determined by labeling the rabbit antibodies with an enzyme-linked anti-rabbit antibody (detection antibody) (Douwes et al., 1996). While this method has been employed in a variety of environmental and occupational settings, this assay is much less sensitive than the LAL in detecting glucan (Douwes et al., 1996), (Foto et al., 2004). This can be a significant issue, particularly for quantification in airborne dust samples, since fungal glucan levels in air are often less than 3 ng/m^3 (40 ng mL^{-1}) (Foto et al., 2004). Therefore increased sensitivity (lower limit of detection) is required (Foto et al., 2004). More recently, a monoclonal antibody-based two-site enzyme immunoassay was developed to estimate levels of airborne 1-3)-β-D-glucan (Sander et al., 2008). This assay is believed to be more cost effective, sensitive and amenable to standardization than the other glucan quantification methods (Sander et al., 2008). However, the factor G based methods determine the form of glucan that activates the dectin receptor in humans, thus these methods provide more health information (Rand et al.,2010); (Cherid et al., 2011).

Although glucan-based approaches are highly sensitive and less time consuming than cultivation or direct microscopic examination, there are a number of important limitations

(Iossifova et al.,2008). One disadvantage is that it cannot provide any qualitative data on the types of fungal species present within an environment.

1.7.2.3 Fungal allergen measurement

Immunoassays that are based on characteristic proteins capable of eliciting an allergic response in sensitized individuals appear promising in fungal exposure assessments. This form of assessment method is usually developed as a capture ELISA (see Figure 4 for a schematic overview) and is based on either polyclonal (pAb) or monoclonal antibodies (mAb) (Shi et al., 2011). Monoclonal antibodies are produced from a single lymphocyte B-cell type making them highly specific to a given antigenic site or epitope on the surface of an antigen. In contrast, polyclonal antibodies, which represent a mixture of antibodies, are produced by several B cell lines, and recognize a variety of different antigenic sites on the same macromolecule (Figure 5) (Lipman et al., 2005). Despite their higher cost of production, monoclonal antibodies are often preferred because they are more specific to the target and have less chances of cross-reacting non-target agents (i.e. from different species) than do polyclonal antibodies (Lipman et al., 2005).

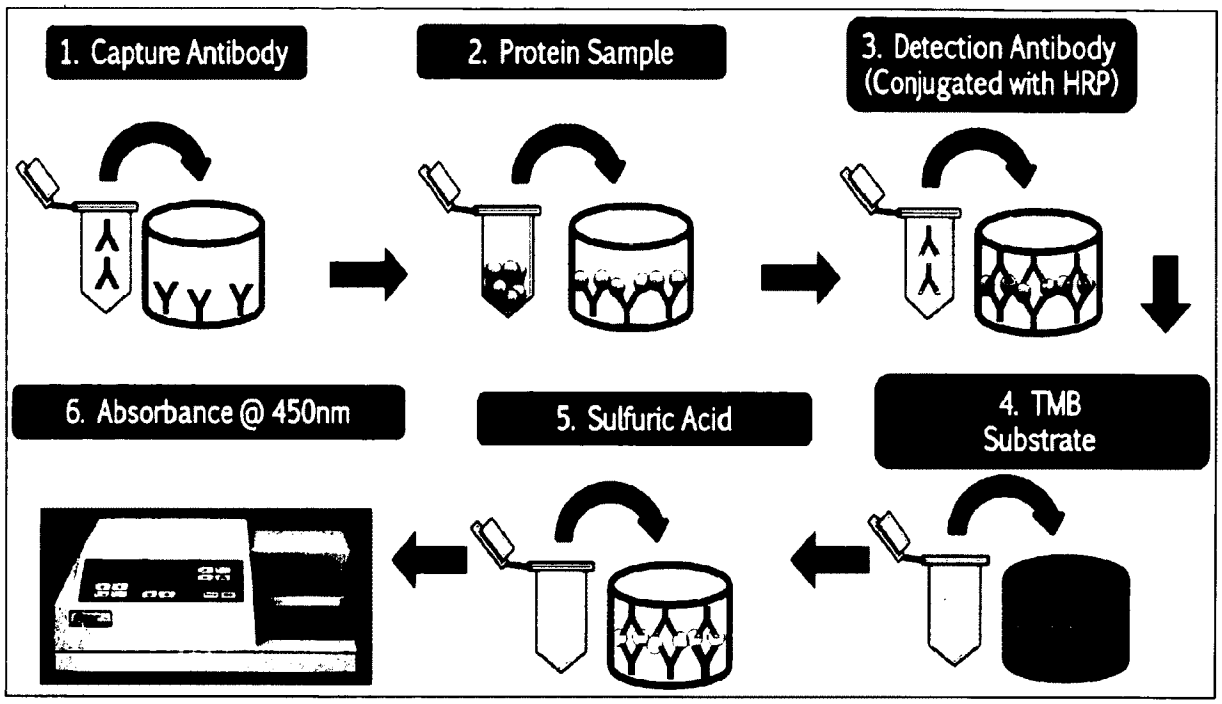

Figure 4. Schematic overview of basic Capture ELISA assay.

1. Capture antibody (purple) is coated to 96-well microplate. After incubation period, excess antibody is washed away with PBST and 2. different concentrations of the analyte, i.e. antigen, sieved house dust, spores (light purple circles) are mixed in 1%BSA-PBST are added to the plate. After an incubation period, unbound analyte is removed by washing with PBST buffer and 3. a detection antibody that recognizes the antigen (anti-analyte, orange) and that has been conjugated with horseradish peroxidase (HRP) is added. After an incubation period the unbound detection antibody is removed by washing with PBST and 4. 3,3',5,5'-Tetramethylbenzidine (TMB) is added to the plate. TMB acts as a substrate to horseradish peroxidase (HRP) and is oxidized to form a blue reaction product. 5. The reaction is stopped using sulfuric acid which produces a yellow reaction product and 6. The absorbance is then read at 450nm using a plate reader. Using a standard curve for protein concentration (e.g. with bovine serum albumin), the quantity of antigen (or allergen) in each well is determined. This protocol has been adapted from (Shi et al., 2011) and other general ELISA protocols.

Figure 5. Schematic representation of a mixture of polyclonal antibodies (green, blue, red, purple) binding 4 different epitopes on an antigen.

Monoclonal antibody-based enzyme-linked immunosorbent assays (mAb-ELISA) have become the reference standard for indoor allergen analysis due to their specificity, accuracy and high throughput (Chapman et al. ,2001). This technology relies on prior isolation of species-specific allergens for the as biomarkers for allergen detection and production of monoclonal antibodies (mAb) (Platts-Mills et al., 1997). A number of kits based on mAb-ELISA technology have been developed to measure dust-mite, cat, dog, cockroach, mouse and rat allergens in indoor spaces (Chapman et al., 2001).

Most of the exposure to indoor particles occurs from settled dust that has been disturbed by room traffic and ventilation system activity and has subsequently become airborne (Foto et al., 2005). Consequently, airborne particle concentrations can vary by several orders of magnitude over a short period of time, and collection and analysis of settled dust offers a more robust estimate of exposure (Tovey & Ferro, 2012).

The identification of fungi and the environmental assessment of fungal allergens has proved to be more difficult, partly because very few fungal allergens have been characterized, and patterns of cross-reactivity among fungal allergens have not been thoroughly documented (Sporik et al., 1993), (National Academy of Sciences, 2000). Earlier fungal immunoassays that have been developed for the identification of *A. alternata* and *A. fumigatus* have proven to be unsuitable for environmental allergen assessment (Sporik et al., 1993), (Platts-Mills et al., 1997). While both *A. alternata* and *A. fumigatus* are known to be allergenic, the selected allergen targets, Alt a 1 and Asp f 1, respectively are not commonly found in the indoor environment (Sporik et al., 1993). These assays could therefore not be used to validate exposure-response relationships (Chapman et al., 2001).

Fungal immunoassays are very promising in their ability to use major species-specific allergens as biomarkers to specifically and selectively detect indoor mould species (Shi et al., 2011). However, a significant limitation to this assessment method is that many biomarker allergens have yet to be identified for most fungal species and therefore the specific antibodies needed for the immunoassays are not available (Keswanie et al. ,2005).

1.8 Development of Suitable Immunoassay for the Detection of Indoor Fungi

1.8.1 Fungal Extract Preparation - Protein Isolation

Potent protein extracts can be prepared from culture media, spores and mycelia (Miller et al., 2010). Extracellular proteins can be extracted from culture by first removing the cells by filtration through cheesecloth, then centrifuging the filtrate to precipitate lipids and dialysing the supernatant to remove low molecular-weight compounds (Horner et al., 1995). Protein extraction from spores and mycelia can prove to be more difficult given the rigidity of fungal cell walls. Methods of extractions include enzymatic, chemical and physical treatments (Nandakumar & Marten, 2002). In chemical methods, non-denaturing detergents such as Triton X, SDS (sodium dodecyl sulfate) and Tween 20 can be used to increase protein solubility (Horner et al., 1995), (Kniemeyer et al., 2006). Other reagents like phosphate buffered saline (PBS) or Tris buffered saline (TBS) can be added to intact spores and shaking can extract a fraction of proteins passively. Often however, passive methods of extraction are not sufficient and more rigorous extraction techniques are required. This is true in the case of many studied fungi, including *W. sebi*. Physical approaches involving cell disruption methods may provide greater protein yields. This can be accomplished by homogenization using

glass bead milling (Horner et al.,1995). A disadvantage of such techniques is that excessive cell disruption can cause contamination of the extract by non-protein components and even the release of proteases that can decrease protein yield (Paris et al.,1990). The addition of protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) can increase allergen extraction yields from spores and mycelia (Paris et al., 1990). This method has proven to successfully yield quality extracts of the fungal protein allergens discussed below. Finally, intracellular proteins can also be released by enzymatic digestion of the cell wall with β-glucanases and chitanases, but often the enzymes used contain protease impurities (Marcilla et al.,1998).

1.8.2 Suitable Fungal Protein Allergens

At present, fungal antigens and antibodies suitable for their detection have been reported for moulds commonly isolated from the built environment such as *Stachybotrys chartarum sensu latto* (Xu et al., 2008), (Shi et al., 2011), *P. chrysogenum* clade 4 (Wilson et al., 2009), (Luo et al., 2010), *A. versicolor* (Shi et al., 2011), and most recently, *C. globosum* (Provost et al., 2012). Two antigenic glycoproteins, SchS21 and SchS34 have been identified in *S. chartarum* (Shi et al., 2011). SchS21 is an alkaline secretory Mg-dependent exodeoxyribonuclease comprised of 144 amino acids, SchS34 is a 221 amino acid protein of unknown function (Shi et al. ,2011). Both proteins have acidic isoelectric points, SchS21 has a pI of 3.9 and SchS34 of 4.2 (Xu et al., 2007). A 52-kDa exoallergen produced by 16 different strains of *P. chrysogenum* was identified by Wilson et al. (Wilson et al., 2009). Additional investigations showed that this modestly glycosylated protein with a pI of 5.3 is seemingly identical to a glycoamylase enzyme produced by a *P. chrysogenum* mutant tolerant to aluminium (Luo et al., 2010).

Shi et al. characterized the major *Aspergillus versicolor* allergen Asp v 13, a 41kDa non-glycosylated protein (corresponding to 403 amino acids) with a pI of 4.5 (Shi & Miller, 2011). Asp v 13 is a secretory subtilisin-like serine protease (Shi & Miller, 2011). Finally, a 47kDa chitonase produced by *C. globosum* was isolated by Provost et al. and found to be non-glycosylated with a pI of 4.5 (Provost et al., 2012).

All of these antigens have been determined as being allergenic in humans and diagnostic for the respective spores, mycelia and cultures of the species from which they were produced (Xu et al.,2008), (Wilson et al.,2009), (Provost et al.,2012). The patterns of cross reactivity to a taxonomically and ecologically diverse group of fungi have been well documented for the antibodies produced against these respective proteins, making them promising candidates for the development of immunoassays to detect antigens in spore and spore/mycelium fragments in dust (Xu et al.,2008), (Wilson et al.,2009), (Provost et al.,2012).

1.8.3 Immunoassays for the Detection of Fungal Allergens in House Dust

More recently, sensitive immunoassays are being successfully developed for the detection of fungal species in house dust (Xu et al.,2008), (Shi et al.,2011). Xu et al. (2008) were able to produce an immunoassay for *S. chartarum* with a limit of detection for the target antigen of 0.2 ng/g dry weight house dust, which is comparable to assessment methods used house dust mite allergens. The general approach followed by the researchers included first screening total *S. chartarum* protein against a large collection of human sera from atopic patients known to have mould allergies to identify which proteins are allergens, then isolating and purifying the major allergenic protein to use as a biomarker for the fungal species (Xu et al.,2008). The purified protein was

then inoculated in rabbits and polyclonal antibodies produced in the rabbit serum were purified and used to perform cross-reactivity tests with protein extracts from various other fungal species (Xu et al.,2008). Subsequent to this course of action, monoclonal antibodies against the target antigen were produced in mice and mAb-immunoassays were optimized for detection of the allergen in house dust (Figure 6, B) (Xu et al.,2008). Using a similar methodology, Shi et al. (2011) have successfully developed a sensitive assay for the quantification of a major *A. versicolor* allergen, Asp v 13, using a double polyclonal antibody capture ELISA assay (Figure 6, B). The immunoassay has a detection limit of 1.0ng and 7.8 µg per gram dry weight based house dust for Asp v 13 and *A. versicolor* spores, respectively. In this assay a fraction of the polyclonal antibody generated against Asp v 13 was biotinylated (bpAb) so that it may directly bind the horseradish peroxidase enzyme (TMB substrate).

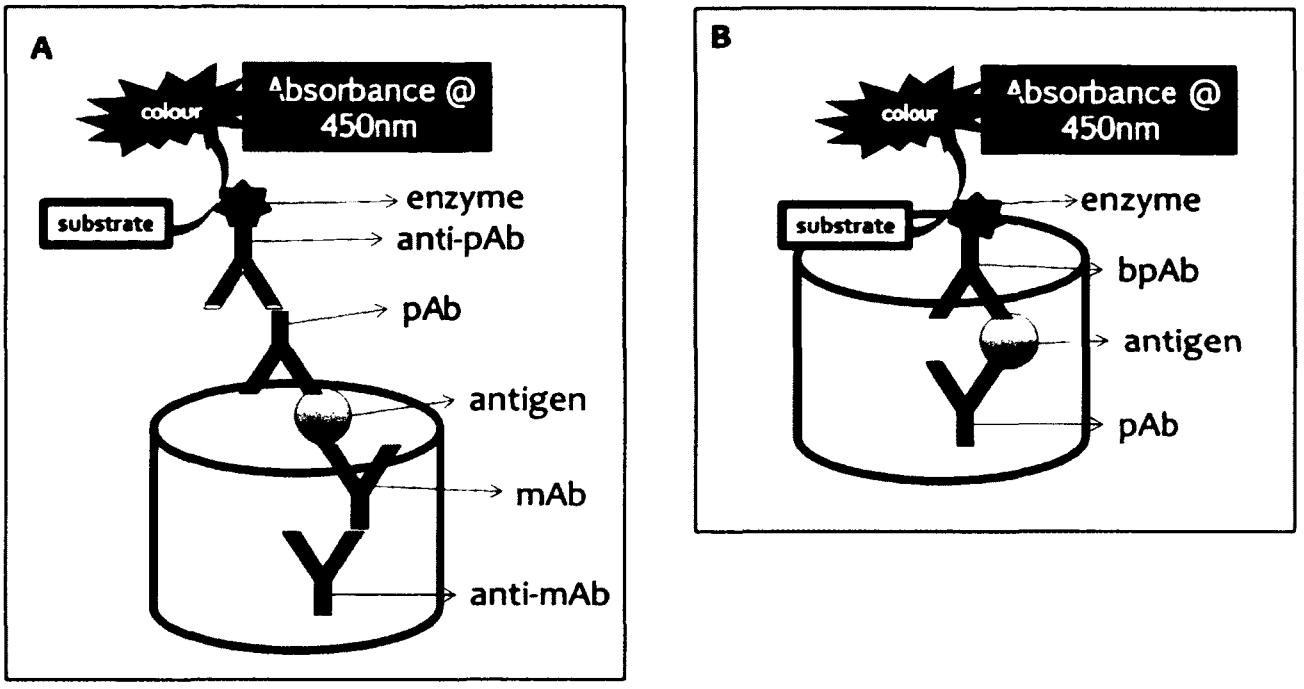

Figure 6. mAb-Capture ELISA (A) and double pAb-Capture ELISA (B) designs. Adapted from immunoassay designs discussed in **(Xu et al.,2008)** and **(Shi et al.,2011)**.

1.9 Objective

The purpose of this project is to isolate and characterize a human allergen from the moderate xerophile *W. sebi*. This obscure fungus turns out to be very common in some homes all over the world. It is known that it is allergenic but until now, the identity of the protein was unknown. The second purpose is to develop antibodies for its detection in house dust.

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.

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^{o} 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 µL 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 µL 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 H_2O) just prior to developing GE Healthcare, Piscataway, NJ).

Ennatin medium containing 1% glycerol- 50 g maltose (Sigma-Aldrch, Oakville, ON); 8 g peptone (Difco, Lawrence, KS); 5 g yeast extract (Sigma-Aldrich, Oakville, ON); 0.75 g KH_2PO_4(Sigma-Aldrich, Oakville, ON); 0.5 g $MgSO_4$-$7H_2O$ (J.T. Baker, Phillipsburg, NJ); 0.067 g $CaCl_2$-$2H_2O$ (Difco, Lawrence, KS) and 20 g glycerol per L of ultrapure H_2O.

Fixation solution- 10 mL ethanol, 2.5 mL glacial acetic acid made up to 25 mL with ddH_2O (GE Healthcare, Piscataway, NJ).

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 H_2O 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 ddH_2O 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.

IEF Standards pI4.45-9.6 – (Bio-Rad, Hercules, CA)

Novex® IEF anode buffer- (Carlsbad, CA). Diluted 50X with ultrapure H_2O.

Novex® IEF cathode buffer- (Carlsbad, CA). Diluted 20X with ultrapure H_2O.

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 Na_2HPO_4-$7H_2O$, 2 g KCl, 2.4 g KH_2PO_4 and 1.0 g Tween 20 made up to 1 L with ultrapure H_2O. pH 7.4.

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

Protease inhibitor cocktail- contains 4-(2aminoethyl) 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 H_2O.
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 H_2O.

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 H_2O 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 H_2O. 10 µL of formaldehyde (37% w/v) added immediately before use. (GE Healthcare, Piscataway, NJ).

Stopping solution- 0.365 g of EDTA-$Na_2{\cdot}2H_2O$ made up to 25 mL ultrapure H_2O. (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 H_2O. pH 7.4.

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

Transfer buffer (immunoblot)- 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 H_2O.

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

Tris buffer (50mM)- 6.057 g/L ddH_2O Trizma base ($NH_2C(CH_2OH)_3$), pH 7.5. (Sigma-Aldrich, Oakville, ON).

2. MATERIALS AND METHODS

2.1 Culture production

The six strains of *W. sebi* studied in this work were isolated from indoor air and dust samples recovered across Canada. Strains obtained from culture collections included DAOM (National Mycological Herbarium at the Department of Agriculture, Ottawa, Ontario) 226641 and 226642, UAMH (University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta) 7897, and CBS (Centraalbureau voor Schimmelcultures, Royal Netherlands Academy of Arts and Sciences, Utrecht, Netherlands) 463.91.

Other strains P8038 and P8522 were isolated by Paracel Laboratories Ltd., and identified by Dr. J.D. Miller. DNA was extracted from the mycelium of each strain using an UltraClean DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Polymerase Chain Reaction (PCR) and subsequent sequencing was carried out by Laboratory Services, University of Guelph (Guelph, ON) using the primers ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3') and ITS1F (5' -CTT GGT CAT TTA GAG GAA GTA -3'). The PCR fragments were sequenced, then aligned using the MAFFT multiple sequence alignment program and compared to each other and the sequence of *W. sebi* employing the BLASTN algorithm against the NCBI nucleotide collection data base. All strain information is summarized in Appendix Table A1.1.

Subcultures were prepared by aseptically transferring mycelium onto sterile yeast extract agar slants or plates supplemented with 20% sucrose. After inoculation, cultures were incubated at 25°C in the dark until sufficient growth was reached (2-3

weeks) and then sealed with parafilm and stored at 4°C until further inoculation. Liquid batch cultures were prepared by inoculating Roux bottles containing 200ml of sterile 2% yeast extract media supplemented with 20% sucrose with a 10ml aliquot (5% v/v) of inoculum produced by macerating a subculture slant in sterile ultrapure water using a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON). Cultures were incubated at 25°C in the dark until sufficient growth was reached (7-14 days, depending on the strain), and then filtered through cheesecloth to separate the mycelium from culture filtrate. The mycelium was rinsed with ultrapure water, then frozen at -20°C prior to freeze drying. The culture filtrate was used within 2 hours to yield extracellular proteins.

2.2 Arthrospore production

W. sebi arthroconidia were produced on agar consisting of potato carrot agar and 3% glycerol (potato carrot agar, PCA). The agar was prepared by separately washing, peeling and boiling 40g of carrots and 40g of potatoes in 1L of ultrapure water for 5 minutes. Each extract was subsequently strained through cheesecloth and 250ml of potato water and 250ml of carrot water were collected and combined, along with 30g of agar and 30g of glycerol. Ultrapure water was added to the solution to bring the final volume to 1L. The PCA medium was sterilized by autoclaving at 121°C for 12 minutes, cooled in a 45°C-water bath for 10 minutes and then poured into sterile petri dishes. The plates were allowed to solidify for 1 hour under sterile conditions. *W. sebi* subcultures (strain UMAH 7897) were used to inoculate PCA plates, which were subsequently incubated in the dark at 25°C for 4 weeks. The plates were placed in a fumehood and air dried for 1 week to allow the agar to dry. Arthrospores were

harvested using a filter-equipped suction vacuum then placed in a glass vial and stored at 4°C (Provost, 2010).

2.3 Extracellular protein extraction

Two hundred (200mM) NaCl, 10 ppt (0.007mM) protease inhibitor cocktail (Sigma-Aldrich) and 0.075ppt (0.43mM) phenylmethylsulfonyl fluoride (PFMS, Sigma-Aldrich) were added to the culture filtrate (filtered supernatant) and the pH of the dissolved solution was adjusted to 9.0. To remove cellular debris and lipids from the culture filtrate, the culture filtrate was centrifuged at 30,000g for 20 minutes at 4°C and filtered through cheesecloth. The culture filtrate (4L) was concentrated 40X using a Cole Parmer Masterflex L/S concentrator (Vernon Hills, IL, USA) equipped with a Sartorius Stedim Biotech (Germany) Vivavflow 200 with Hydrosart 10,000 molecular weight cut off (MWCO) membrane. To remove excess salt from the supernatant, buffer exchanges were performed 3 times: 900ml of 20mM Tris Base (Tris(hydroxymethyl)aminomethane), pH 9 was added to 100ml of concentrated supernatant, and the diluted supernatant was concentrated down again to 100ml (10X buffer exchange), and repeated. Ten (10) parts per thousand (ppt) of protease inhibitor cocktail was added and the buffer-exchanged concentrated filtrate containing the extracellular protein fraction was frozen at -20 °C until further use.

2.4 Protein extraction from spores

Ten (10) mg of spores (including arthrospores) were weighed using a Mettler AE-163 Analytical Balance (±0.0001g), transferred to a plastic vial with a 3/8 methacrylate bead

(ATS Scientific Inc., Burlington, ON) and milled for 2X30 miinutes using a Spex-Certiprep mixer mill (Model 5100, Metuchen, NJ). Spore fragments were dissolved in 1ml of 0.5M Tris-buffer with 0.05% Tween 20 (TBST, pH 7.4) and the mixture was sonicated for 1 hour using a VWR Scientific Products Aquasonic sonicator (Model 50T, Mississauga, ON). After sonification, the resulting spore solution was transferred to a 10kDa cut off Amicon Ultra-15 centrifuge tube (Millipore, Billerica, MA) and 10ml of TBST (pH7.4) were added. The spore solution was centrifuged at 4,500g for 30 minutes at 4 °C (down to a volume of 1ml). The supernatant was subsequently transferred to a 1.5ml plastic microtube and kept on ice until use in Capture ELISA (1-2 hours maximum).

2.5 Protein extraction from cells

Eighty-five (85) mg of lyophilized fungal cells were weighed using a Mettler AE-163 Analytical Balance (±0.0001g) and resuspended in 8.5 ml 20mM Tris buffer (pH7.4) with 5µl Tween. The mixture was vortexed for 5 minutes then macerated in an ice bath using a Polytron rotor-stator homogenizer (Kinematica, Lucerne, Switzerland) at maximum speed for 3X5minutes at 10 minute intervals. The homogenized cell mixture was then transferred to a 10kDa cut off Amicon Ultra-15 centrifuge tube (Millipore) and centrifuged at 4,500g for 10 minutes at 4°C. The supernatant was subsequently transferred to a 1.5ml plastic microtube and stored at -20°C.

2.6 Protein concentration determination

All protein concentrations were determined using the Bradford Protein Assay. Protein samples were serially diluted with ultrapure water and 150µl of each were added to

separate wells in a 96-well Nunc-immuno MaxiSorp plate (Sigma-Aldrich). A volume of 150µl of ultrapure water was added separately to an empty well to serve as a blank. An equal volume of Quick Dye Bradford Reagent (Bio-Rad, Hercules, CA) was added to all protein samples and blank and incubated at room temperature for 10 minutes. Optical density was measured at 595nm using a Molecular Device Spectramax 340PC microplate reader (Sunnyvale, California). Protein concentrations were estimated using the line equation from a BSA standard curve.

2.7 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were separated by SDS-PAGE using the mini VE Vertical Electrophoresis System and Amersham Biosciences electrophoresis tank (GE Healthcare, Baie d'Urfe, QC) powered by a Power Pac 1000 gel electrophoresis power supply (Bio-Rad). Prior to electrophoresis, samples were mixed with 5X SDS PAGE Loading Buffer in a 4:1 ratio (v/v), boiled for 5 minutes then immediately cooled in ice for a minimum of 5 minutes. The protein samples were loaded onto a 10% acrylamide gel containing 10 or 15 lanes and gels were placed into the electrophoresis tank. The SeeBlue® Plus2 Pre-Stained Standard (Invitrogen, Burlington, ON) was loaded in a separate lane for unknown protein molecular weight determination. Adequate resolution was achieved by running samples using the Laemmli running buffer system (25mM Tris 192mM glycine, 0.1 % SDS), at a constant voltage of 100V for 20 minutes, followed by a voltage of 200V for 80 minutes.

2.8 Protein visualisation (acrylamide gel staining)

Following electrophoresis, protein bands were visualized on the polyacrylamide gel by either Commassie staining or Silver staining.

Silver staining was performed to visualize proteins of low concentration (less than 50ng total protein). All incubation steps involved in the Silver stain process were performed on a Thermolyne Aros 160 orbital shaker (Thermo Fisher Scientific) set at 60 RPM. In the first step, the gel was treated with a fixation solution consisting of 50% ethanol, 5% acetic acid in ultrapure water and incubated for 30 minutes, then washed for 10 minutes in 50%ethanol and 2X10minutes in ultrapure water. The gel was then placed in a freshly prepared sensitizer solution consisting of 0.02% sodium thiosulphate in ultrapure water for 30 minutes then washed 2X5 minutes with ultrapure water. A silver stain solution consisting of 0.1% silver nitrate in ultrapure water was added for 30 minutes and the gel was washed with ultrapure water for 2X1 minute. The gel was placed in a developing solution, consisting of 0.04% formalin in 2% sodium carbonate in ultrapure water, and once the desired protein band intensity was reached (30 seconds – 5 minutes), the gel was transferred to 5% acetic acid in ultrapure water to stop the reaction (further reduction of silver). The gel was washed with ultrapure water, then scanned with a GS 800 densitometer (Bio-Rad), and later stored in 1% acetic acid in ultrapure water.

Coomassie staining was performed to visualize proteins having a total concentration greater than 50ng. The gel was placed in Coomassie Brilliant Blue (CBB) stain reagent (Thermo Fisher Scientific, Ottawa, ON), incubated overnight at room temperature on an

orbital shaker set at 60 RPM. The following day, the CBB reagent was discarded and the gel was destained with several changes of with 20%ethanol solution, until the background was transparent, then rinsed with ultrapure water and scanned using a GS 800 densitometer (Bio-Rad). The gel was stored in 20% ethanol.

2.9 Protein molecular weight determination

Protein sample molecular weights were determined by running samples alongside a protein ladder containing a set of molecular weight standards during gel electrophoresis. SeeBlue® Plus2 Pre-Stained Standard (Invitrogen) was used as a ladder and the gel was stained with CBB (Thermo Fisher Scientific) to visualize protein sample bands. In the Tris-glycine system, the SeeBlue® Plus2 ladder consists of the following molecular weight size markers: 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). For each molecular weight marker, the relative migration distance (R_f) was measured in the gel and used to plot the log molecular weight vs R_f value. This graph was used as a standard to determine the molecular weight of all unknown protein sample bands by interpolation from their measured R_f in the gel.

2.10 Indirect Enzyme Linked Immuno-Sorbent Assay (Indirect ELISA)

Indirect ELISA was carried out by coating 100µl of different antigen concentrations in coating buffer (50mM carbonate-bicarbonate, pH 9.6, Sigma-Aldrich) to each well of a 96-well Nunc-immuno MaxiSorp plate (Sigma-Aldrich). Coating buffer devoid of protein

sample was used as a blank in this assay. The plate was incubated overnight at 4°C on a microplate shaker then washed 3X with 150µl TBST (pH7.4). The plate was subsequently blocked with 100µl of TBST solution with 1% BSA (pH7.4) for 1 hour at room temperature on a microplate shaker. After washing 3X with TBST (pH7.4), 100µl of a given concentration of primary antibody in 1%BSA-TBST was added to the plate and incubated 1 hour at room temperature on a microplate shaker. Optimal dilutions for human sera (HpAbs) and rabbit polyclonal antibodies (RpAbs) when used as primary antibodies in indirect ELISA were 1:4000 and 1:32,000 respectively. After washing 3X with TBST (pH7.4), 100µl of the respective horseradish peroxidase (HRP) conjugated secondary antibody (Sigma-Aldrich) diluted in 1%BSA-TBST was added to each well and the plate was incubated for 1 hour at room temperature on a microplate shaker. For human sera, HRP conjugated anti-human IgG was used at a dilution of 1:30,000; for rabbit polyclonal antibodies, HRP conjugated anti-rabbit IgG, was used at a dilution of 1:5,000. After a final wash with 3XTBST (pH7.4), 100µl of TMB substrate (Sigma-Aldrich) was applied per well and the plate was incubated for 10 minutes at room temperature on a microplate shaker, to allow the blue colour to develop. The reaction was stopped by adding 50µL of 0.5M sulfuric acid per well. The optical density was read at 450nm using a Molecular Devices Spectra Max 340PC reader (Sunnyvale, California).

2.11 Immunoblotting

Following protein separation by SDS-PAGE, proteins were transferred from the gel to a Hybond-polyvinylidene difluoride (PVDF) membrane (GE HealthCare, NJ) using a Hoefer miniVE electrotransfer unit (GE Healthcare Life Sciences). The transfer was

carried out at a constant current of 350mA in an ice bath for 60 minutes. Following the transfer, the PVDF membrane was blocked in 25ml of 1%BSA-TBST for 1 hour at room temperature, then the membrane was incubated in 25ml of primary antibody overnight at 4°C. All incubation periods during the immunoblotting steps were carried out on an orbital shaker set at 60 RPM, Optimal dilutions for human sera (HpAbs) and rabbit polyclonal antibodies (RpAbs) when used as primary antibodies in immunoblotting were 1:2000 and 1:50,000, respectively. After washing 3X with TBST (pH7.4), the membrane was placed in 25ml of the respective alkaline phosphatase (AP) conjugated secondary antibody (Sigma-Aldrich), diluted in 1%BSA-TBST and shaken using an orbital shaker set at 60 RPM for 1 hour at room temperature. For human sera, AP conjugated anti-human IgG was used at a dilution of 1:10,000; for rabbit polyclonal antibodies, AP conjugated anti-rabbit IgG, was used at a dilution of 1:30,000. After a final wash with 3XTBST (pH7.4), detection was achieved by incubating the membrane in 5ml of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma-Aldrich) for 5 to 10 minutes. The membrane was rinsed with ultrapure water, dried between blotting pads and scanned with a GS 800 densitometer (Bio-Rad).

2.12 Purification of crude protein by ion exchange chromatography

The purification of the crude extracellular protein was achieved by Fast Protein Liquid Chromatography (FPLC) anion exchange chromatography using a Q-sepharose fast flow (GE Healthcare Life Sciences) packed glass column (1 x 17cm). All buffers used in anion exchange chromatography were previously filtered using a 0.22µm polyethersulfone membrane (Corning Incorporated Life Sciences, Lowell, MA). The column was equilibrated by washing with 200ml of Buffer A (20mM Tris, pH7.5) at a flow

rate of 2ml/min using a peristaltic pump (GE Healthcare Life Sciences). The previously concentrated filtrate containing the extracellular protein fraction (approximately 300mg of total protein) was applied to the Q-column at a flow rate of 0.8ml/min. The column was washed with 3 column volumes of Buffer A. The AKTAprime™ FPLC system (GE Healthcare Life Sciences) Buffer Prep function was used to mix Buffer A and Buffer B (20mM Tris with 1M NaCl, pH7.5) and create an increasing salt gradient (from 0 to 0.5M NaCl, total volume 800ml). This resulted in an increase in the ionic strength of the mobile phase and allowed more negatively charged bound proteins to be eluted the column. Fractions of 12mL were collected in 70 tubes using the ÄKTAprime™ plus fraction collector (GE Healthcare Bio-Sciences Inc., QC). A 50µl aliquot of each fraction was mixed with 10µl 5X SDS PAGE Loading Buffer and an SDS-PAGE was run and subsequently stained with CBB to assess the presence and purity of the target protein. The different fractions containing the target proteins were pooled together and centrifuged at 4,500g and 4°C in a 10kDa cut off Amicon Ultra-15 centrifuge tube (Millipore) until a volume of 0.5ml was obtained. The resulting concentrated and desalted sample was mixed 50:50 (v/v) with glycerol and 20µl protease inhibitor cocktail (Sigma-Aldrich) was added prior to storing at -20°C. Residual proteins were removed by washing with 1M NaCl Tris buffer. The column was stored in 20% ethanol. Column regeneration was achieved with 200ml of 50mM Tris, pH 7.5.

2.13 Antibody production

2.13.1 Human polyclonal antibodies

Human sera samples, containing human polyclonal antibodies (HpAbs), were obtained from atopic patients by ProGene Ltd. (Lenexa, KS). An ImmunoCAP® Specific IgE test was performed on the sera samples by Thermo Fisher Scientific (formely Pharmacia Diagnostics, Kalamazoo, MI) to accurately and specifically identify fungal sensitivity. Following clinical diagnosis, human sera demonstrating a positive response to various fungi were selected and provided to our laboratory. Positive human sera that were used in this experiment are listed in Appendix table A1.2. Unless otherwise stated, human sera samples were diluted 1000X with 1% BSA-TBST when used for Immunoblot testing and 2000X with 1%BSA-TBST when used for Indirect ELISA.

2.13.2 Rabbit polyclonal antibodies

The target protein that was purified by anion exchange chromatography was used to produce polyclonal antibodies in rabbits at Cedarlane Laboratories, Ltd. (Hornby, Ontario; meets the requirements of the Canadian Council on Animal Care). Two rabbits (772 and 1B38) were subjected to a pre-immune bleed then immunized with 0.5 mg of purified target protein (day 0). Both rabbits were then boosted 3 times at 21 day intervals (after 28, 47 and 66 days) with 0.5 mg of purified 47kDa *W. sebi* protein extract to increase the titre of antibodies to the target protein. The final bleed of the immunized rabbit was carried out at day 78.

2.14 Rabbit polyclonal antibody optimization

Rabbit serum 772 was chosen for optimization (purification and decontamination) following the protocol of Shi et al. (Shi et al.,2011) outlined below.

2.14.1 Sample desalting

Sample desalting and Tris → PBS buffer exchanges were carried out using 5 ml G-25 column (GE Healthcare). The column was first equilibrated with 10ml of Buffer A (50mM PBS, pH 7.4) then 500ul of sample (purified antigen, biotinylated antigen, or biotinylated affinity purified RpAb) was applied to the column using a 1ml syringe. Buffer A was added to column and the eluate was collected in 0.5ml fractions; a Bradford assay was conducted on eluted fractions to determine in which fraction the protein had been eluted. Fractions containing protein were concentrated using a 10kDa cut off Amicon Ultra-15 centrifuge tube (Millipore) and centrifuged at 4,500g for 10 minutes at 4°C. Desalted samples were mixed 50% (v/v) with glycerol and stored at -20°C until further use.

2.14.2 Sampled biotinylation

The purified, desalted antigen was biotinylated for use in the antigen-specific pAb purification. The antigen-specific RpAb was biotinylated for use as the detection antibody in all capture ELISA. An excess (2mg) of EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Fisher Scientific) was solubilised in 50µl DMSO and added to 500µl purified, desalted antigen (4mg) or 500µl antigen-specific RpAb (1.5mg) for 2 hours at room temperature on an orbital shaker set at 60 RPM. The biotinylation reaction was

stopped by adding 200µl of 1M Tris, pH 8 and shaking for 10 minutes at room temperature on an orbital shaker set at 60 RPM. The biotinylated sample was then centrifuged for 5 seconds at 20,000g using a desktop centrifuge and loaded onto a 5 ml G-25 column (GE Healthcare Life Sciences) to exchange the buffer and remove unbound biotin molecules.

2.14.3 Rabbit polyclonal antibody affinity purification

A 1ml HiTrap Streptavidin affinity column (GE Healthcare Life Sciences) was equilibrated with 2ml of Buffer A (50mM PBS, pH 7.4). Two (2) mg of biotinylated 47kDa protein was loaded to the column and the later was washed with 20ml of Buffer A. The pH of the rabbit serum was adjusted to 7.4, then filtered through a 0.45µm filter fitted to a 10 ml syringe. Fifteen (15) ml of filtered sera was applied to the column slowly (1ml/min); flowthrough was collected and reloaded onto the column 3X. The column was then washed with 20ml of Buffer A. The antigen-specific PAb was then eluted with Buffer B (400mM PBS, pH 8) and immediately neutralized with Buffer C (10mM acetic acid, pH 2.5) to a pH of 7. The antigen-specific RpAb solution was concentrated from 13ml to 0.5ml using a 30kDa cut off Amicon Ultra-15 centrifuge tube (Millipore) to centrifuge at 4,500g at 4°C. The concentrated solution was mixed 50% (v/v) with glycerol and stored at -20°C until further use. The affinity purification process was repeated for a second aliquot of rabbit sera for biotinylation.

2.14.4 Rabbit polyclonal antibody decontamination

The affinity purified RpAb was further decontaminated to remove self-interaction between the antigen-specific RpAb and the bRpAb. An empty 1ml HiTrap Streptavidin affinity columns (GE Healthcare Life Sciences) column was equilibrated with 10ml of Buffer A (50mM PBS, pH 7.4). A 200µl aliquot of biotinylated affinity purified rabbit polyclonal antibody (bpAb) was loaded onto an empty 1ml HiTrap Streptavidin affinity column (GE Healthcare Life Sciences) slowly (1ml/min). An additional 1ml HiTrap Streptavidin affinity column was connected to the original column containing the biotinylated antibody, to capture any leached bpAb. The affinity purified pAb was slowly applied to column at a flowrate of 1ml/min; flowthrough was collected and reloaded onto the column 5X. The final flowthrough was applied to the column and the column was washed with 20ml of Buffer A. The eluted decontaminated (non-self-interacting) pAb was collected and concentrated from 15ml to less than 650µl using a 30kDa cut off Amicon Ultra-15 centrifuge tube (Millipore) to centrifuge at 4,500g at 4°C. The concentrated solution was mixed 50% (v/v) with glycerol and stored at -20°C until further use. The decontaminated antibody was used as the coating antibody in all capture ELISA.

2.15 Capture Enzyme Linked Immuno-Sorbent Assay (Capture ELISA)

The capture ELISA was performed by coating 10ng of decontaminated affinity purified rabbit pAb in coating buffer (50mM carbonate-bicarbonate, pH 9.6, Sigma-Aldrich, Oakville, ON) to each well of a 96-well Nunc-immuno MaxiSorp plate (Sigma-Aldrich). The plate was incubated overnight at 4°C on a microplate shaker then washed 2X with

200µl TBST (pH7.4). All incubation steps were performed on a microplate shaker. The plate was subsequently blocked with 200µl of TBST solution with 1% BSA-TBST (pH7.4) overnight at 4°C. After washing 2X with 200 µl of TBST (pH7.4), 100µl aliquots of different concentration of purified 47kDa antigen, spores, or spore-spiked dust in 1%BSA-TBST were added to the plate and incubated 1 hour at room temperature. 1% BSA-TBST (pH7.4) devoid of protein sample was used as a blank in this assay. After washing 2X with 200 µl of TBST (pH7.4), 100µl of biotinylated pAb (detection antibody) diluted 10,000X in 1%BSA-TBST were added to each well and incubated 1 hour at room temperature. The plate was washed twice with 200 µl of TBST (pH7.4), and 100µl of streptavidin-HRP conjugate (Jackson), diluted 10,000X in 1%BSA-TBST were added and incubated for 20 minutes. After 2 final washes with 200 µl of TBST (pH7.4), 100µl of TMB substrate (Sigma-Aldrich) was applied per well and the plate was incubated for 10 minutes at room temperature on a microplate shaker, to allow the blue colour to develop. The reaction was stopped by adding 50µL of 0.5M sulfuric acid per well. The optical density was read at 450nm using a Molecular Devices Spectra Max 340PC reader (Sunnyvale, California).

2.16 MS/MS analysis

The target antigen was analyzed by tandem mass spectrometry. All steps were performed with precaution in order to maintain a keratin-free environment. Briefly, all materials were handled while wearing a clean lab coat and vinyl gloves; all work surfaces (i.e. bench tops), equipment and supply containers were wiped down with water, then ethanol; gel caster, buffer tank and glassware were thoroughly cleaned; reagents used were prepared fresh; finally, steps were carried out in a fume hood, when

possible.. SDS-PAGE and CBB staining were performed as described in previous sections. The acrylamide gel was stained overnight, then de-stained using ultrapure. The stained band corresponding to the target 47kDa dimer was carefully excised from the gel using a sterile scalpel, placed in a sterile 1.5ml plastic microtube and covered with 0.1% formic acid. The target antigen was digested using the protease trypsin and the resulting peptides were analyzed at Health Canada by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Linear Trap Quadrupole Fourier Transform (LTQ-FT).

2.17 Determination of isoelectric point (pI)

Two-dimensional gel electrophoresis was performed to determine the isoelectric point of the target protein. Thorough washing of electrophoresis tank and gel caster with ultrapure water took place to avoid SDS contamination during the isoelectric focusing step (1^{st} dimension of two-dimensional gel electrophoresis). A pre-cast IEF gel (Bio-Rad) was placed in the gel caster and the caster was in turn placed into the electrophoresis tank. The Novex® anode running buffer was diluted 50X (490ml ddH_2O to 10ml buffer) and added to the bottom of the electrophoresis tank. The Novex® cathode running buffer was diluted 10X (90ml ddH_2O to 10ml buffer) and added to the upper chamber of the gel caster. The purified protein was mixed 1:4 with the Novex® IEF sample buffer (Bio-Rad) and an IEF standard (Bio-Rad) was 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). Twenty (20) µL of IEF standard (Bio-Rad): loading buffer mixture was loaded into the first well of the IEF gel. Thirty (30)µL of the sample: loading buffer mixture was loaded into the second and fifth wells of the gel. The apparatus was attached to the Power Pac 1000 gel electrophoresis power supply (Bio-Rad) and programmed to run at 100V for 1 hr, followed by 200V for 1 hr, then 500V constant for 30 min. Following electrophoresis, the gel was carefully cut along the 3rd well. The first half of the gel was stained with CBB overnight and de-stained with 20% ethanol in ultrapure water. The forth lane of the IEF gel was carefully excised and placed in 1.0M Tris, pH 6.8 for 15 minutes. A 10% acrylamide solution was prepared and allowed to solidify in a gel caster. Upon solidification, the forth lane of IEF gel (strip) was inserted in the gel caster atop the acrylamide resolving gel and 20mm from the edge of the plate. The bottom of the IEF lane was oriented towards the left of the acrylamide gel, and the exact placement was marked. A single toothed comb was placed in the 20mm gap at the top left of the caster. Stacking solution consisting of 1.4ml ddH_2O, 330µl 30%acrylamide mix, 250 µl 1.0M Tris pH 6.8, 20µl 10% SDS, 20µl 10% ammonium persulfate and 2µl N,N,N',N'-tetramethylethylenediamine was poured on top of the comb and IEF gel strip and allowed to solidify. Once solidified, a second-dimension gel electrophoresis was run as according to the SDS-PAGE protocol (previously described). The resulting gel was stained with CBB overnight and de-stained with 20% ethanol in ultrapure water. The pI of the protein was determined by locating the IEF protein band that corresponds to the 47kDa protein on the SDS-PAGE gel, then comparing its R_f to that of the standard on the IEF gel.

2.18 Glycoprotein staining

Using a Pierce® Glycoprotein Staining Kit (Thermo Fisher Scientific), a glycoprotein assay was performed to determine the glycosylation status of the target 47kDa dimer. The purified protein sample was mixed with loading buffer 4:1 (v/v). The contents of the Positive Control (1mg Horseradish Peroxidase, Thermo Fisher Scientific) vial were reconstituted with 0.5ml of ultrapure water and subsequently diluted in a 4:1 ratio with 5X SDS PAGE Loading Buffer. Similarly, 0.5ml of ultrapure water were added to the Negative Control (1mg Soybean Trypsin Inhibitor, Thermo Fisher Scientific) and the solution was diluted with 5X SDS PAGE Loading Buffer 4:1 (v/v). The protein samples were loaded in duplicates onto two separate 10% acrylamide gels containing 10 or 15 lanes and gels were placed into the electrophoresis tank. Protein samples were separated by SDS-PAGE, as previously described. One gel was immersed in CBB stain following the electrophoresis. The following steps describe the glycoprotein assay performed on the remaining gel. After electrophoresis, the gel was fixed in 100ml of 50% methanol for 30 minutes, then washed in 100ml of 3% acetic acid for 2X10 minutes. All incubation periods were carried out at room temperature on an orbital shaker set at 60 RPM. The gel was transferred to 25ml of Oxidizing Solution, prepared by dissolving the Oxidation Agent (Thermo Fisher Scientific) in 250ml ultrapure water, and agitated for 15 minutes. The gel was washed 3X5 minutes in 3% acetic acid and subsequently transferred to 25ml of Glycoprotein Staining Reagent (Thermo Fisher Scientific) for 15 minutes. After staining, the gel was transferred to 25ml of Reducing Solution, prepared by dissolving the Reducing Agent (Thermo Fisher Scientific) in

250ml ultrapure water, and gently agitated for 5 minutes. The gel was rinsed three times with 3% acetic acid, then with ultrapure water. The protein bands were compared to that of the control samples to determine the glycosylation status of the protein.

2.19 Deglycosylation assay

The target protein was deglycosylated using a trifluoromethanesulphonic acid (TFMS) treatment following a procedure similar to that performed by Luo et al. (Luo et al.,2010). Prior to deglycosylation, the target 47kDa dimer was desalted using 5 ml G-25 column (GE Healthcare Life Sciences), as previously described, and freeze dried. The lyophilized protein (100µg) and the lyophilized positive control (100µg horseradish peroxidase, Thermo Fisher Scientific) were placed in separate glass vials and 45µl of pre-cooled TFMS were added to each at 4°C. Each solution was mixed until dissolved and then kept at -20°C in an ethanol-dry ice bath for 4 hours, with occasional shaking. Afterwards, 150µl of pre-cooled 60% pyridine/water solution were added to each of the vials. Desalting was carried out using a Microcon YM-10 filter (Millipore). The resulting deglycosylated protein samples were run on a SDS-PAGE, as previously described and stained with CBB.

3. RESULTS

3.1 Culture and spore production, protein extraction and concentration

3.1.1 *W. sebi* protein and spore production

Six strains of *W. sebi* were inoculated and grown in two different media: Eniatin, developed to optimize peptide and protein production (Xu et al.,2007) supplemented with 10% glycerol (w/v), and 2% Yeast Extract (YE) supplemented with 20% sucrose (w/v). Glycerol and sucrose were added to reduce water activity of the respective medium. Comparison of the fungal protein profile and yield resulting from growth in each of the media showed great similarity between the different media. The 2% YE with 20% sucrose medium was therefore selected for the remainder of the experiments. *W. sebi* strains under study were recovered from indoor dust samples in Canada and the Netherlands. *W. sebi* sporulation was successfully induced on potato carrot agar (PCA) supplemented with 3% glycerol to reduce water activity; plates yielded an average of 1.83mg spores per plate.

3.1.2 Protein extraction from culture filtrate (extracellular), mycelium (intracellular) and arthrospores

Extracellular proteins were extracted from 4L of culture filtrate and concentrated 40X using a Cole Parmer Masterflex L/S concentrator (Montreal, QC) equipped with a Sartorius Stedim Biotech (Germany) Vivavflow 200 with Hydrosart 10,000 molecular weight cut off (MWCO) membrane. Intracellular proteins were extracted from macerated mycelium using a Polytron rotor-stator homogenizer (Kinematica, Lucerne, Switzerland). Proteins were extracted from spores using a mixer mill and sonicator.

Protein extracts were run on SDS-PAGE and the resulting gel was subsequently stained by CBB to visualize major proteins present (Figure 7 and Figure 8).

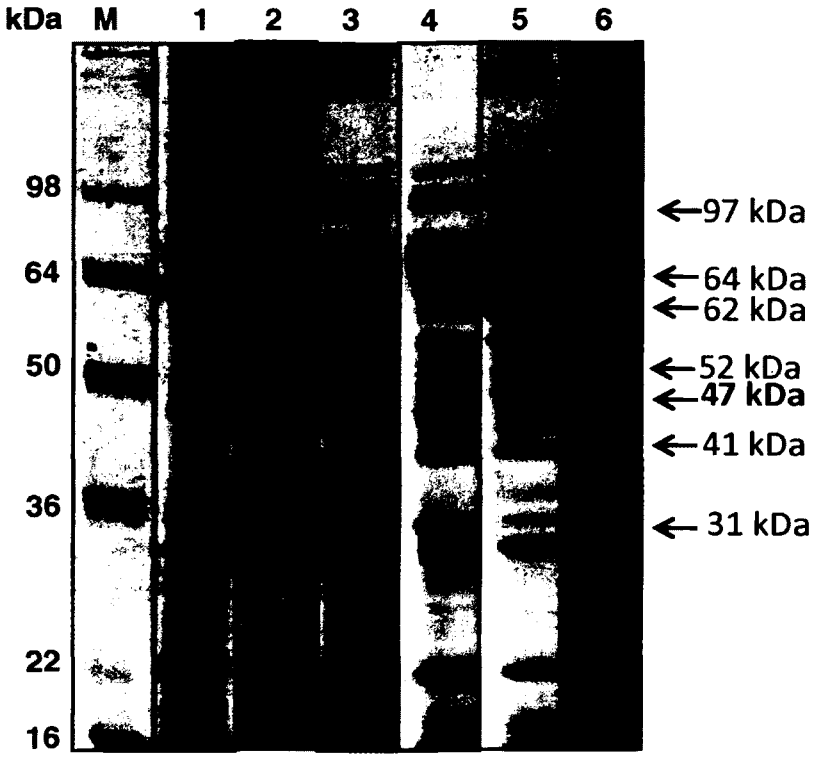

Figure 7. CBB stain illustrating extracellular proteins.

Lane M: SeeBlue® Plus Protein Ladder with molecular weights marked left (8ul)
Lane 1: *W. sebi* crude extracellular protein extract from UAMH 7897 (5ug)
Lane 2: *W. sebi* crude extracellular protein extract from 8522 (5ug)
Lane 3: *W. sebi* crude extracellular protein extract from DAOM 226641(5ug)
Lane 4: *W. sebi* crude extracellular protein extract from DAOM 226642 (5ug)
Lane 5: *W. sebi* crude extracellular protein extract from CBS 463.97 (5ug)
Lane 6: *W. sebi* crude extracellular protein extract from 8038 (5ug)
Major proteins that display an immunogenic response are indicated by arrows

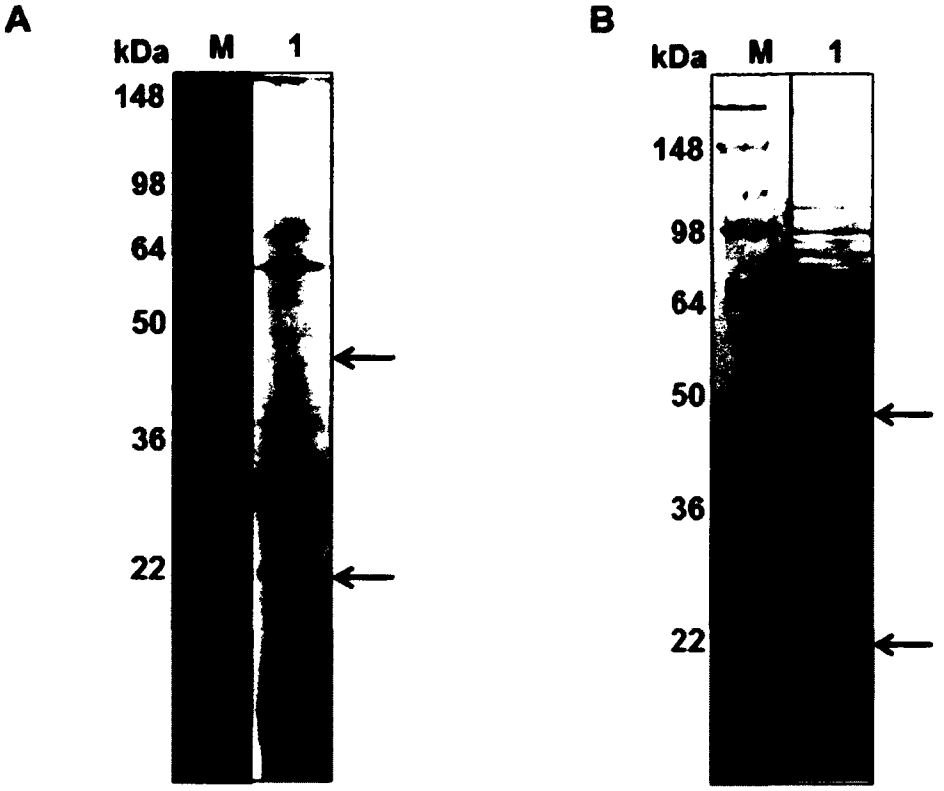

Figure 8. CBB stain illustrating intracellular proteins (A) and proteins found in spores (B).

(A)
Lane M: SeeBlue® Plus Protein Ladder with molecular weights marked left (8ul)
Lane 1: *W. sebi* crude intracellular protein extract from UAMH 7897 (20ug)
(B)
Lane M: SeeBlue® Plus Protein Ladder with molecular weights marked left (8ul)
Lane 1: *W. sebi* crude intracellular protein extract from UAMH 7897 (20ug)

3.1.3 Protein concentration

Protein concentrations were determined using the Bradford Protein Assay. The average extracellular protein yield from the culture filtrate was 369mg of crude protein per batch (4L of media). The average amount of total protein extracted from 1g of mycelium was 5mg. The average amount of total protein extracted from 1mg of spores was 0.11mg.

3.2 *W. sebi* human antigen screening using extracellular protein

3.2.1 Initial antigen screening with ELISA

Indirect ELISA were performed as a preliminary means of assessing the reactivity between crude extracellular extracts and various human polyclonal antibodies (HpAbs) contained in sera samples obtained from atopic patients. The list of human sera used in this study can be found in Appendix Tables A1.2. Optimal conditions for assays were determined as being 80ng crude protein, HpAbs diluted 4000X in 50% glycerol and 1%BSA-TBST, and horseradish peroxidase (HRP) conjugated goat anti-human IgG diluted 10,000X in 1%BSA-TBST.

All strains showed an overall similar average response to HpAbs, as measured by optical density at 450nm (Figure 9). Responses of *W.sebi* strains to individual sera samples are shown in Figures 10-15. Average O.D values elicited by each HpAbs sample with each of the strains are found in Table A1.3. A total of 66 patient sera out of the 84 samples screened gave an OD response of at least 1.0 for all strains tested; 52 of these sera also elicited average O.D. responses ≥1.5.

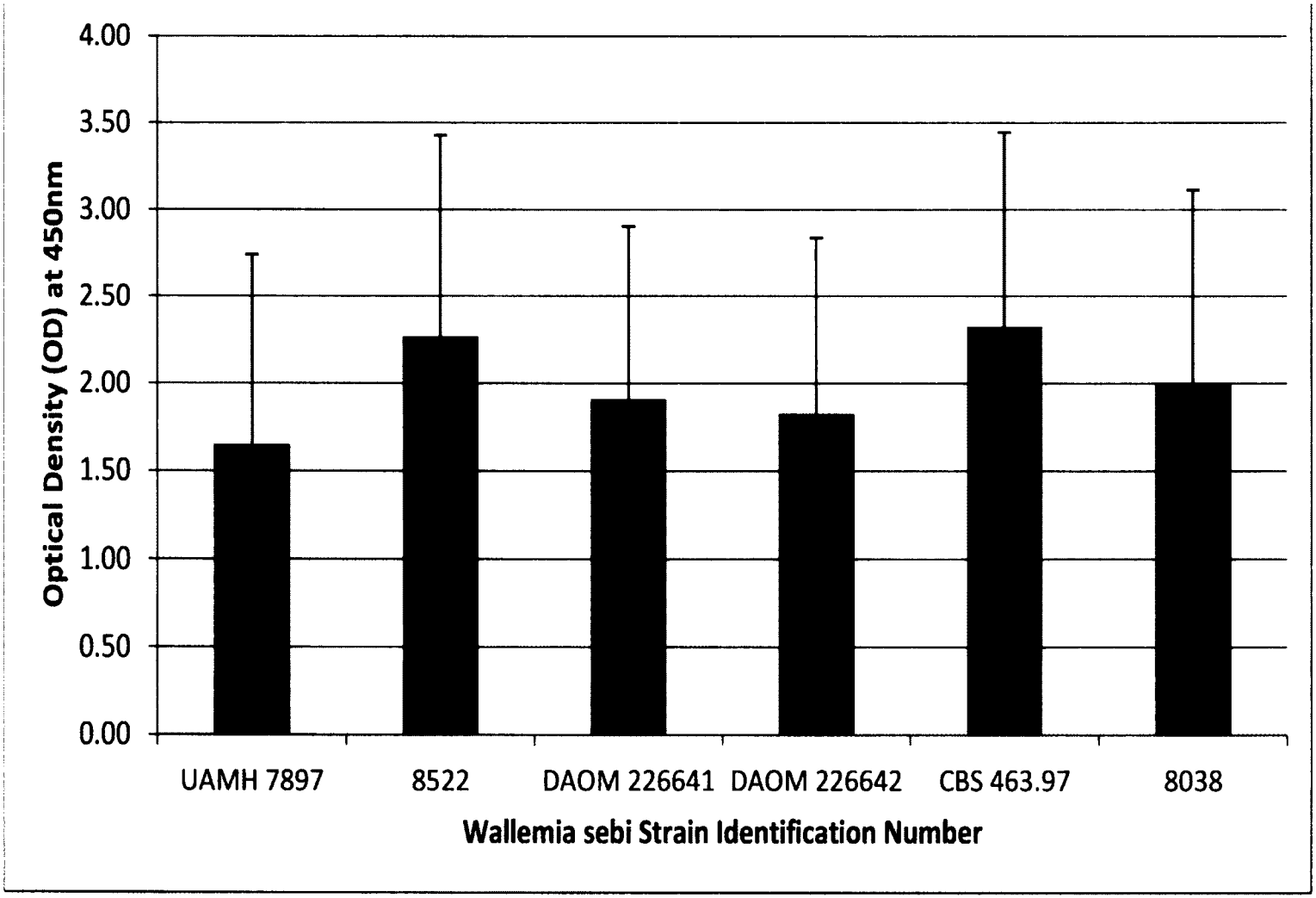

Figure 9. Average ELISA response of all *W. sebi* culture extracts against all HpAbs. Assays were performed in duplicates. Error bars represent standard deviations.

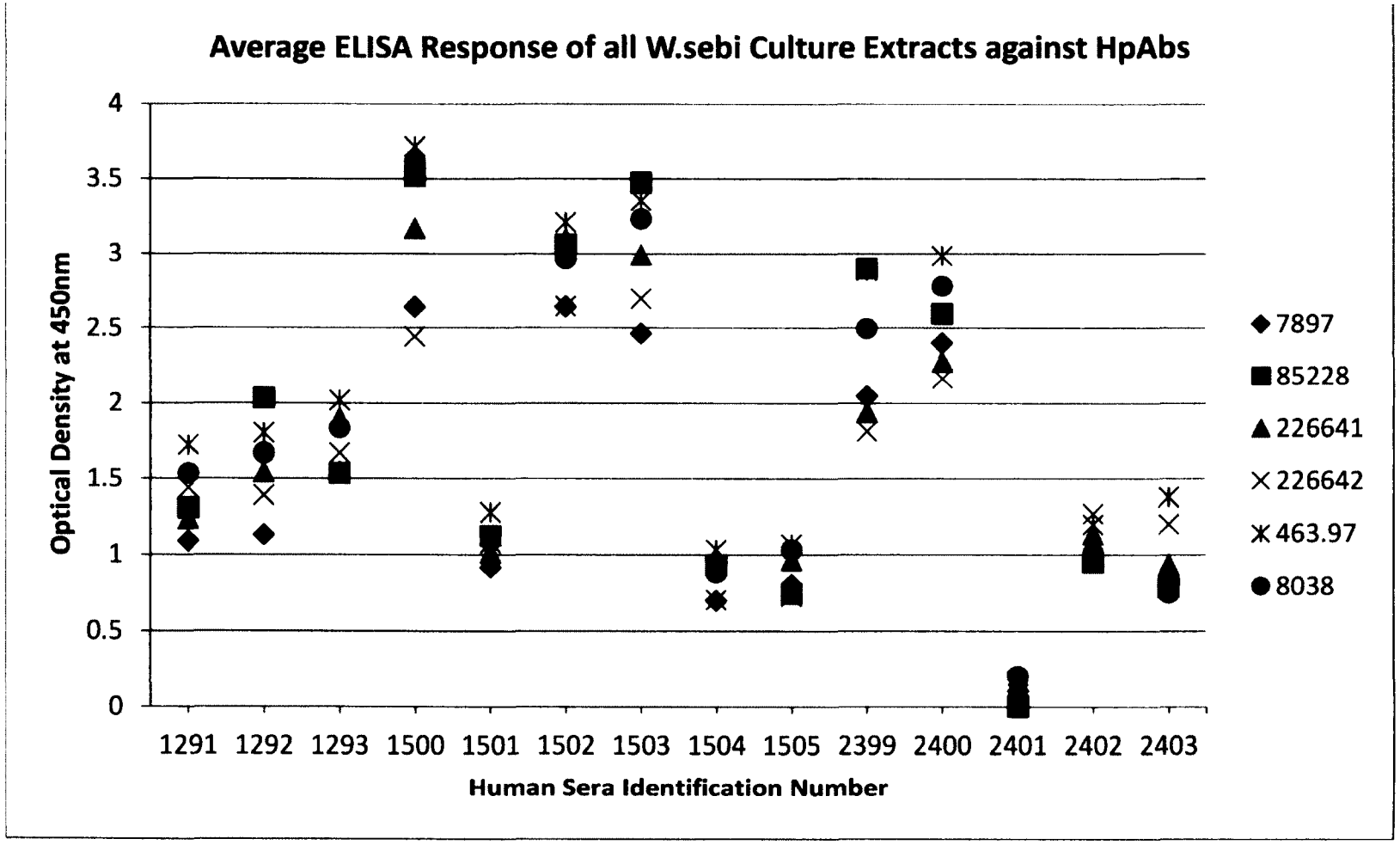

Figure 10. Average ELISA response of all *W.sebi* culture extracts against individual sera samples. Assays were performed in duplicates.

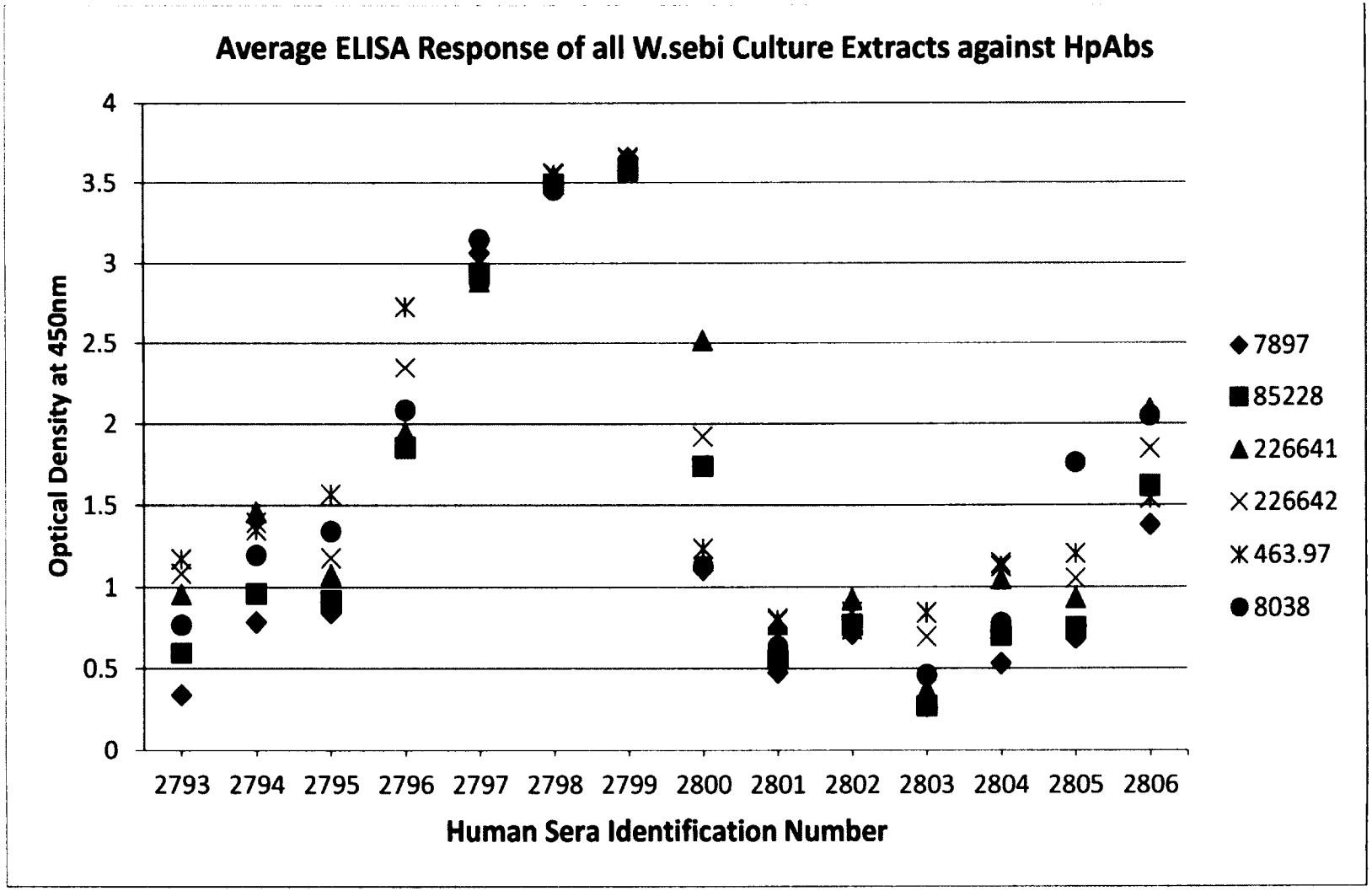

Figure 11. Average ELISA response of all *W.sebi* culture extracts against individual sera samples. Assays were performed in duplicates.

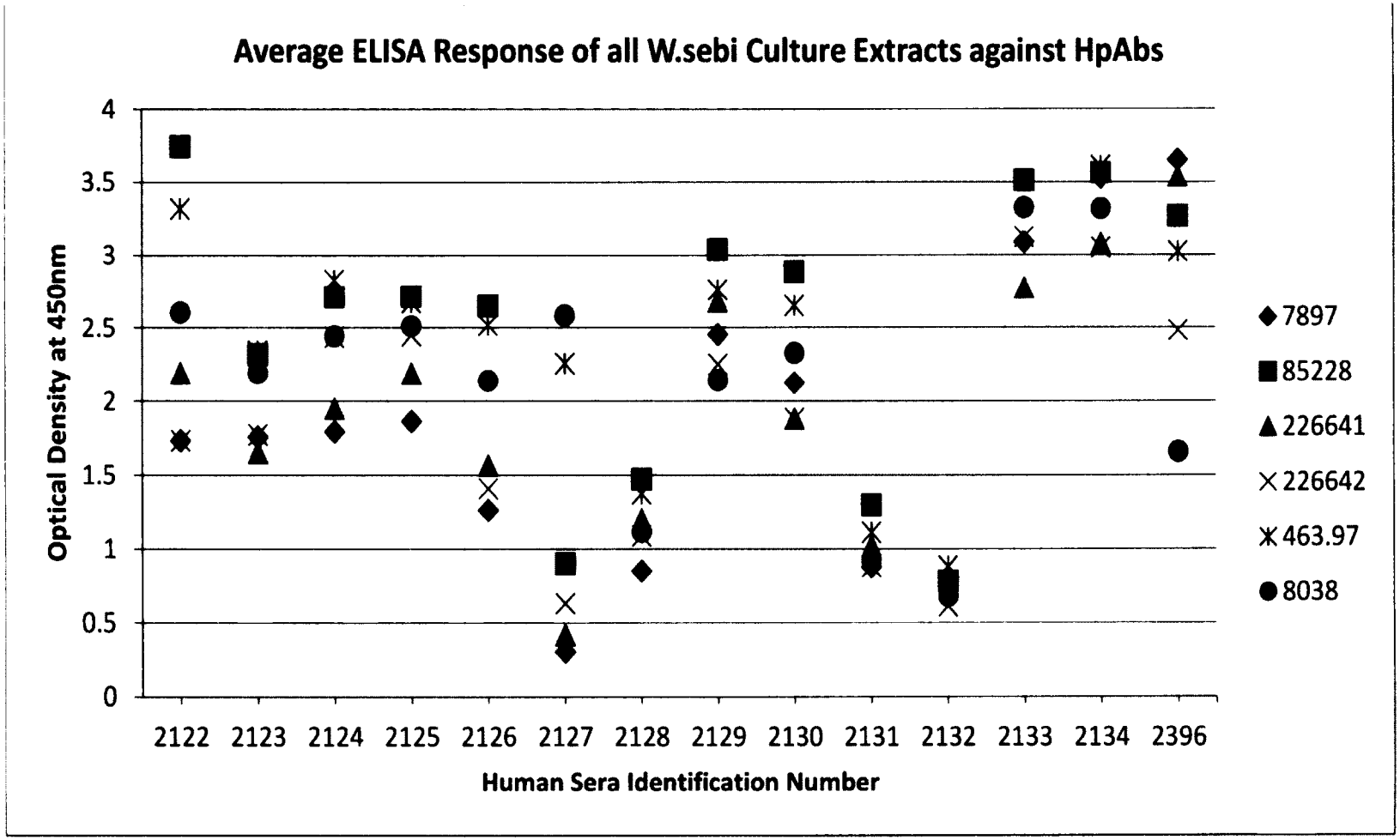

Figure 12. Average ELISA response of all *W.sebi* culture extracts against individual sera samples. Assays were performed in duplicates.

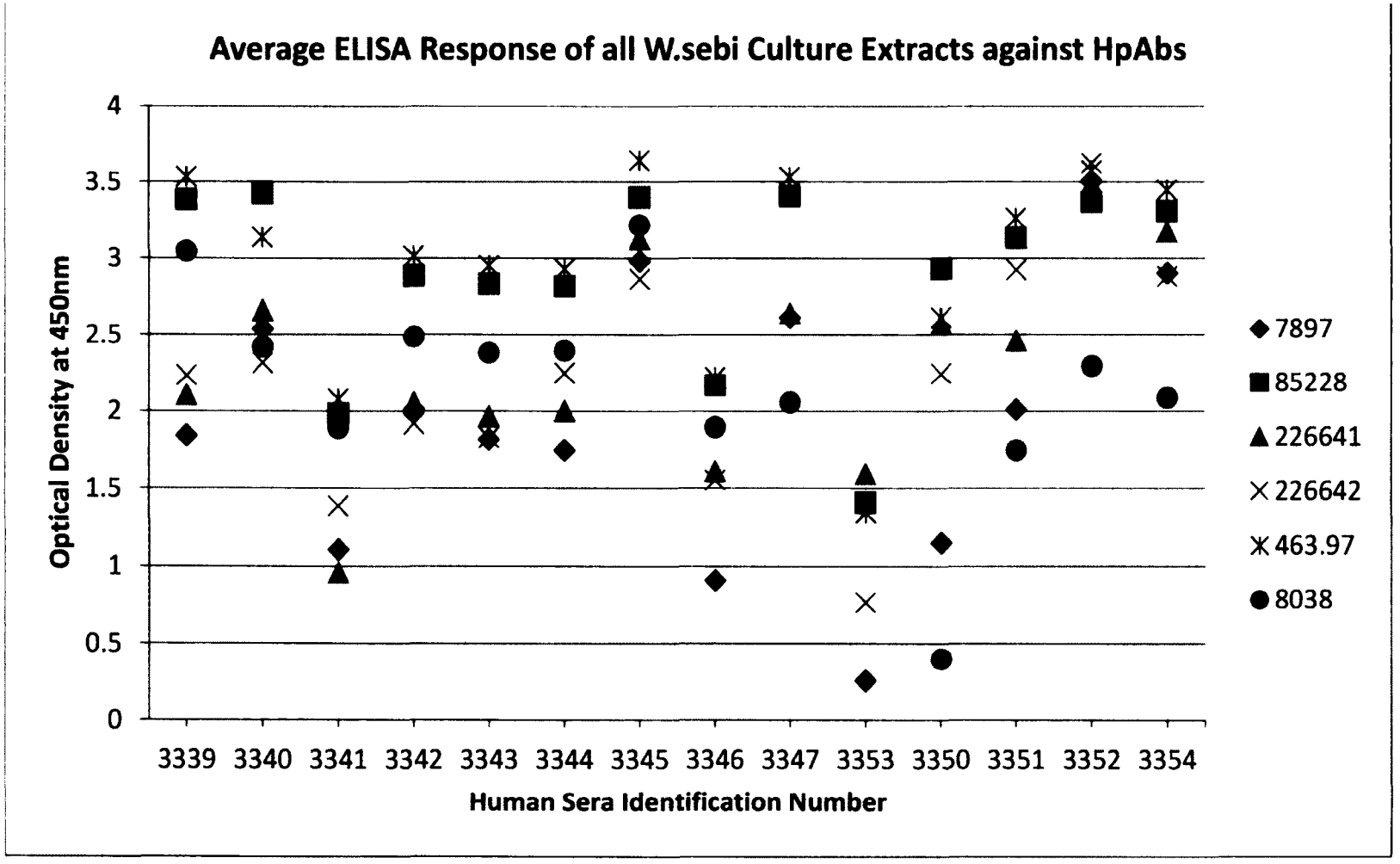

Figure 13. Average ELISA response of all *W.sebi* culture extracts against individual sera samples. Assays were performed in duplicates.

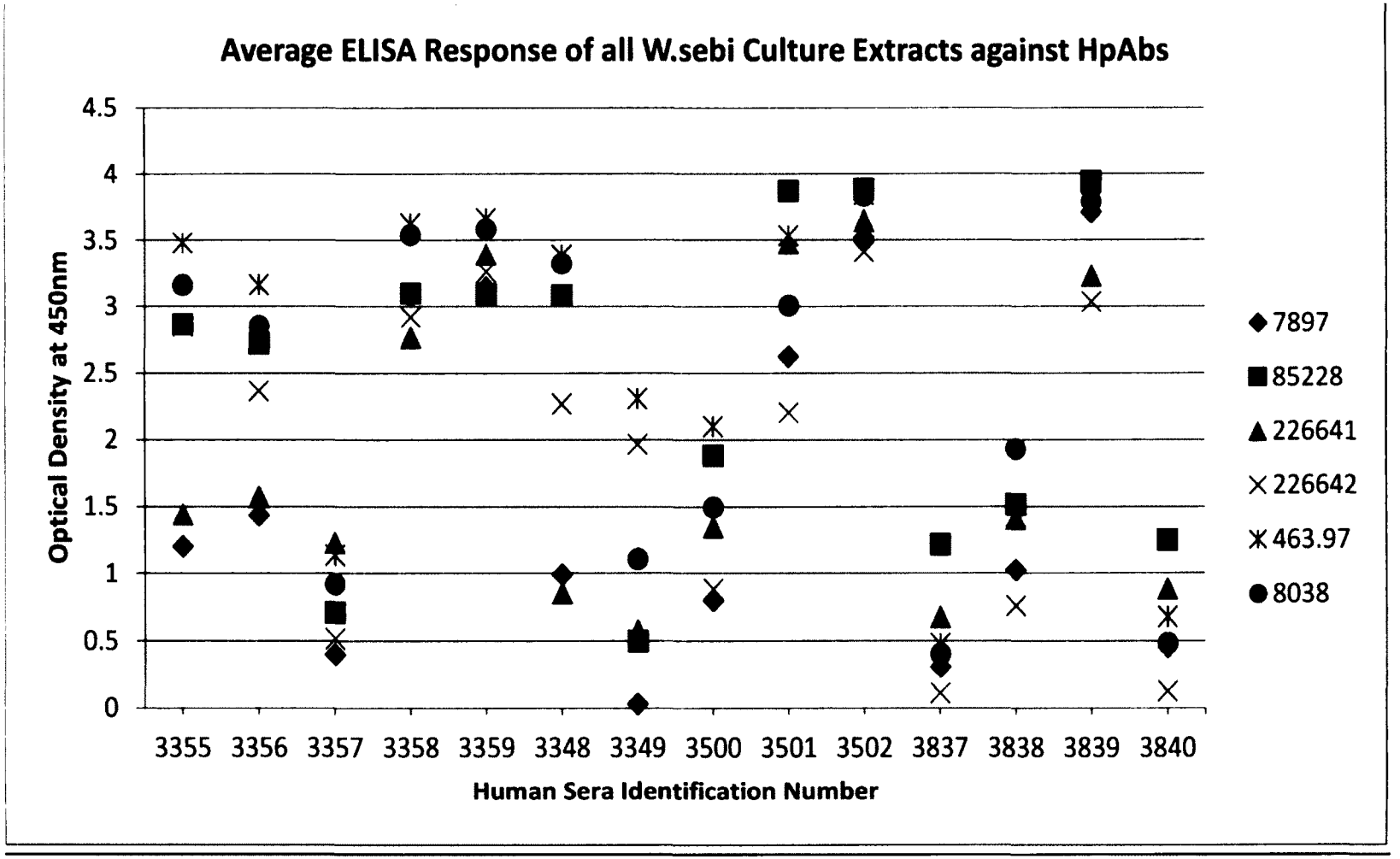

Figure 14. Average ELISA response of all *W.sebi* culture extracts against individual sera samples. Assays were performed in duplicates.

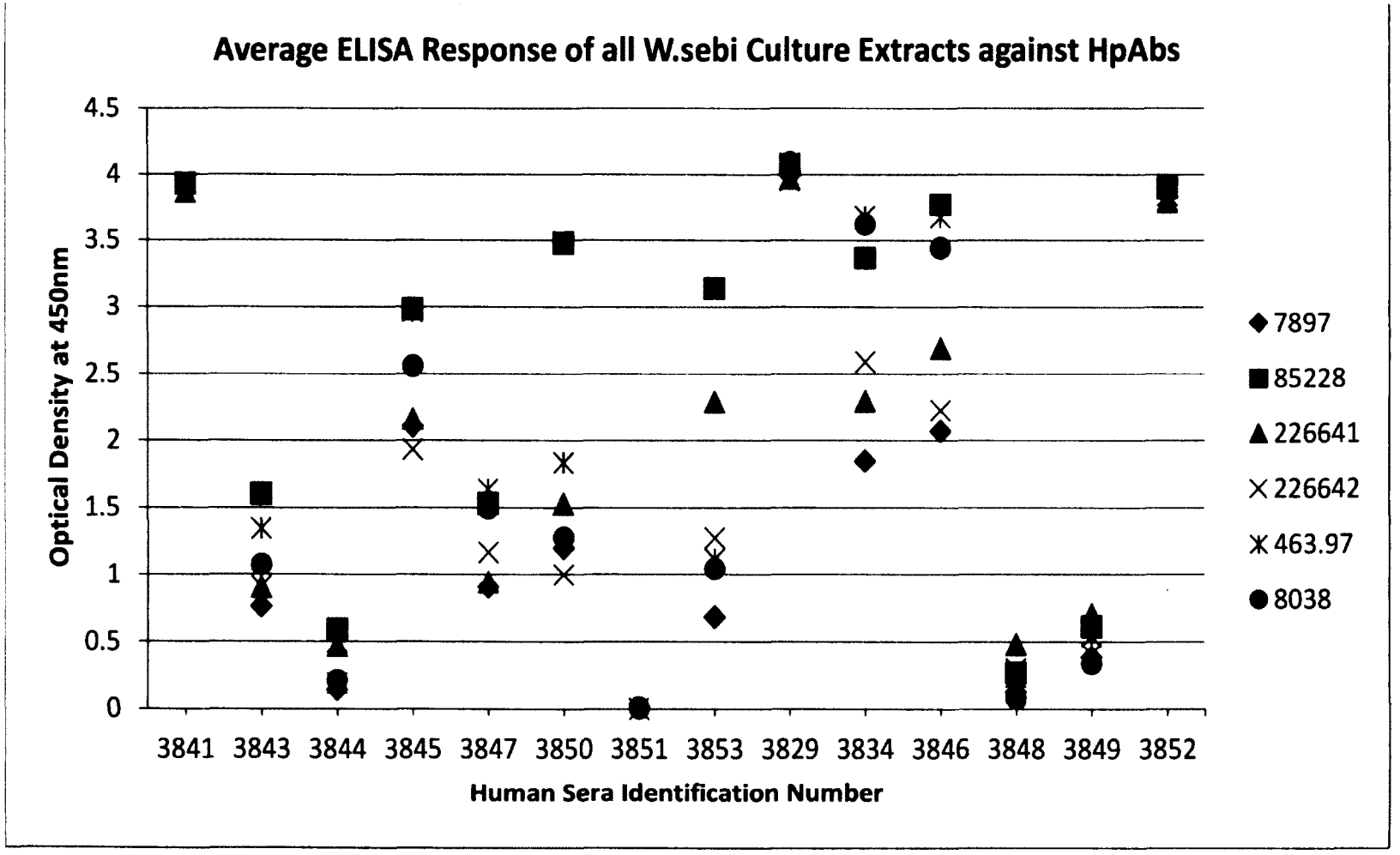

Figure 15. Average ELISA response of all *W.sebi* culture extracts against individual sera samples. Assays were performed in duplicates.

3.2.2 Initial human antigen screening by Western immunoblotting

Immunoblots were performed in addition to ELISAs in order to visualize proteins displaying an antigenic response. Culture extracts from each *W. sebi* strain were screened against a total of 64 different sera. Optimal conditions were identified as being 1µg crude protein, HpAbs diluted 2000X in 50% glycerol and 1%BSA-TBST, and alkaline phosphatase (AP) conjugated mouse anti-human IgG diluted 10,000X in 1% BSA/TBST. Examples of immunoblots are shown in Figures 16-19. Major proteins common to all strains include proteins with approximate molecular weights of: 97kDa, 78kDa, 64kDa, 62kDa, 52kDa, 47kDa, 41kDa, 31kDa) and are indicated by arrows. Most proteins (97kDa, 78kDa, 64kDa, 62kDa, 52kDa, 47kDa, 41kDa, 31kDa) also elicited an immunogenic response with human sera.

Of the 52 different sera that elicited an O.D. of at least 1.5 by Indirect ELISA, 32 also gave a positive immunoblot response to at least one protein from culture filtrate extract.

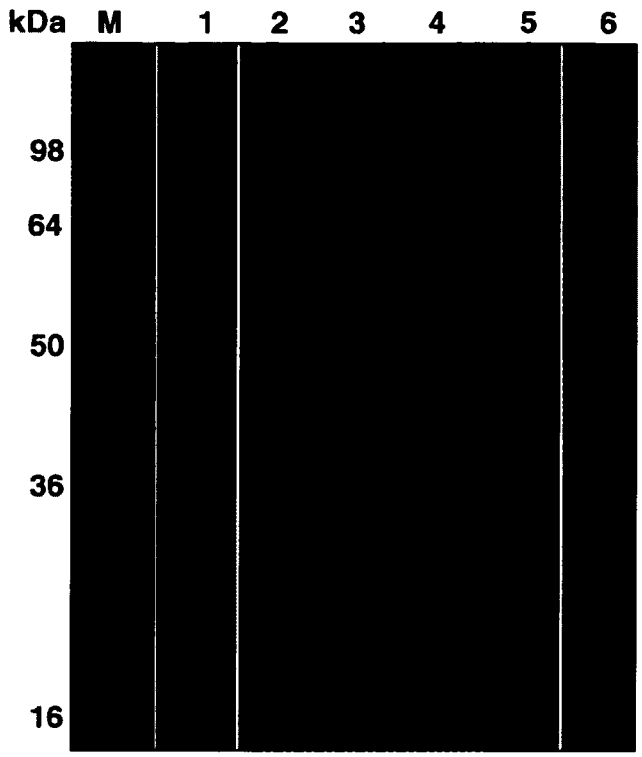

Figure 16. Immunoblot compairing the response of HpAb QC 2124 to different *W.sebi* strains.

Lane M: SeeBlue® Plus Protein Ladder with molecular weights marked left (8ul)
Lane 1: *W. sebi* crude extracellular protein extract from UAMH 7897 (1ug)
Lane 2: *W. sebi* crude extracellular protein extract from P8522 (1ug)
Lane 3: *W. sebi* crude extracellular protein extract from DAOM 226641(1ug)
Lane 4: *W. sebi* crude extracellular protein extract from DAOM 226642 (1ug)
Lane 5: *W. sebi* crude extracellular protein extract from CBS 463.97 (1ug)
Lane 6: *W. sebi* crude extracellular protein extract from P8038 (1ug)
Arrows indicate the 47 kDa protein

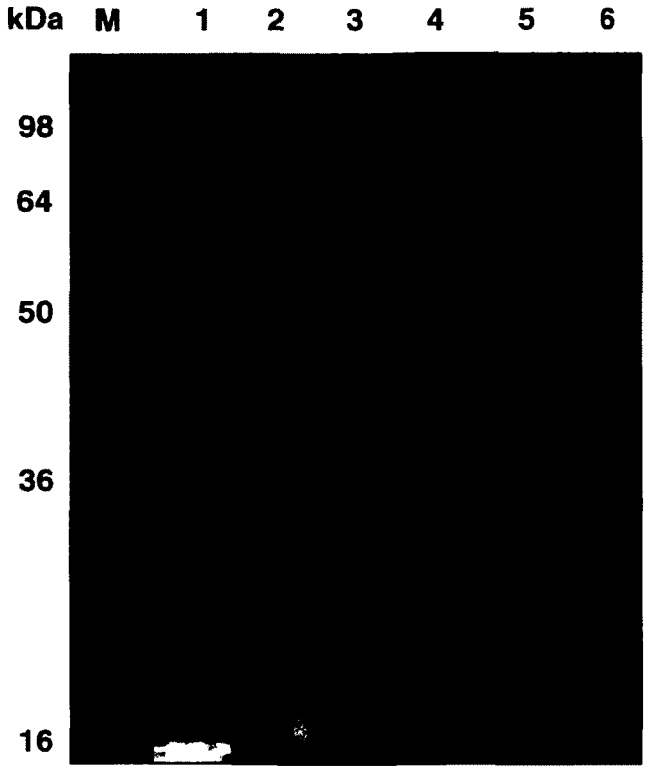

Figure 17. Immunoblot compairing the response of HpAb QC 2125 to different *W.sebi* strains.

Lane M: SeeBlue® Plus Protein Ladder with molecular weights marked left (8ul)
Lane 1: *W. sebi* crude extracellular protein extract from UAMH 7897 (1ug)
Lane 2: *W. sebi* crude extracellular protein extract from P8522 (1ug)
Lane 3: *W. sebi* crude extracellular protein extract from DAOM 226641(1ug)
Lane 4: *W. sebi* crude extracellular protein extract from DAOM 226642 (1ug)
Lane 5: *W. sebi* crude extracellular protein extract from CBS 463.97 (1ug)
Lane 6: *W. sebi* crude extracellular protein extract from P8038 (1ug)
Arrows indicate the 47 kDa protein

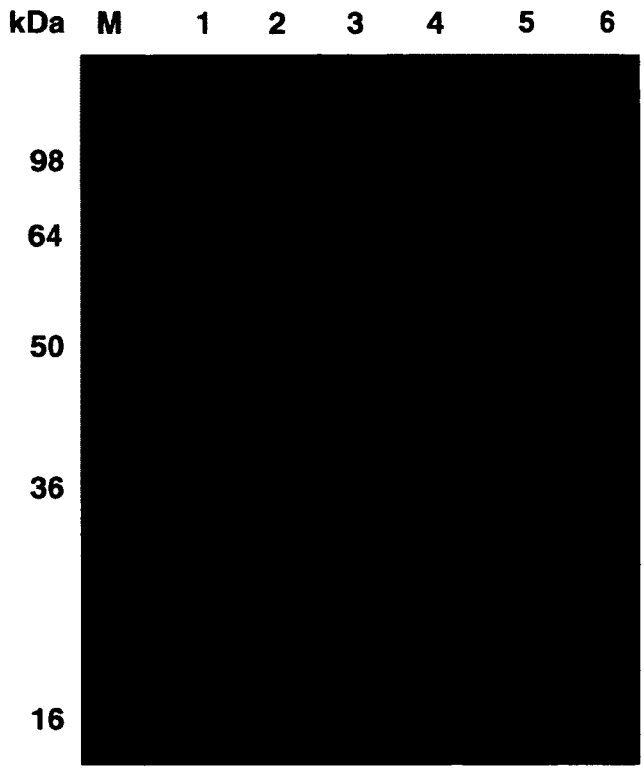

Figure 18. Immunoblot compairing the response of HpAb QC 3343 to different *W.sebi* strains.

Lane M: SeeBlue® Plus Protein Ladder with molecular weights marked left (8ul)
Lane 1: *W. sebi* crude extracellular protein extract from UAMH 7897 (1ug)
Lane 2: *W. sebi* crude extracellular protein extract from P8522 (1ug)
Lane 3: *W. sebi* crude extracellular protein extract from DAOM 226641(1ug)
Lane 4: *W. sebi* crude extracellular protein extract from DAOM 226642 (1ug)
Lane 5: *W. sebi* crude extracellular protein extract from CBS 463.97 (1ug)
Lane 6: *W. sebi* crude extracellular protein extract from P8038 (1ug)
Arrows indicate the 47 kDa protein

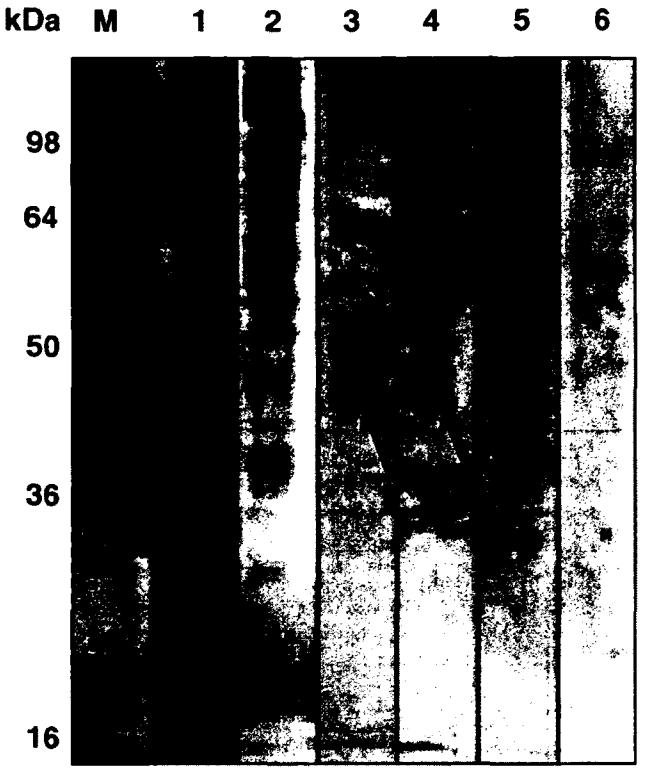

Figure 19. Immunoblot compairing the response of HpAb QC 3347 to different *W.sebi* strains.

Lane M: SeeBlue® Plus Protein Ladder with molecular weights marked left (8ul)
Lane 1: *W. sebi* crude extracellular protein extract from UAMH 7897 (1ug)
Lane 2: *W. sebi* crude extracellular protein extract from P8522 (1ug)
Lane 3: *W. sebi* crude extracellular protein extract from DAOM 226641(1ug)
Lane 4: *W. sebi* crude extracellular protein extract from DAOM 226642 (1ug)
Lane 5: *W. sebi* crude extracellular protein extract from CBS 463.97 (1ug)
Lane 6: *W. sebi* crude extracellular protein extract from P8038 (1ug)
Arrows indicate the 47 kDa protein

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

3.3.1 Selecting *W. sebi* target proteins

Each immunoblot was analyzed and the various reacting proteins were noted (arrows, Figure 7) for each patient serum. The intensity of the protein bands on each immunoblot was graded on a scale of 0 to 3 (zero being no response, 1 being a weak response, 2 being a moderate response and 3 being a strong response), as shown in Table 1. Some human sera samples reacted with several proteins but showed lower responses on ELISA, while other HpAbs responded greatly to both ELISA and immunoblots. The data was carefully analyzed and the most suitable protein for further investigation was identified as a 47kDa protein. This protein was observed in all six *W. sebi* strains studied, but was produced in different yields. Immunoblot responses of the 47kDa protein to the 32 human sera are summarized in Table 2. Strain UAMH 7897 was chosen for further production, isolation and purification of this 47kDa protein as it produced the highest yield of the highest yield of target protein and elicited strong ELISA and immunoblot responses.

Table 1. Summary of immunoblot response of major *W.sebi* proteins against 64 patient sera samples (HpAbs).

Value 1 = weak response; value 2 = moderate response; value 3 = strong response.

QC1293	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa			1	1	1	
78 kDa			1	1	1	
64 kDa						
62 kDa					2	
52 kDa						
47 kDa	1					
41 kDa						
31 kDa						

QC1295	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa				1		
78 kDa				1		
64 kDa		1		1	1	1
62 kDa					3	1
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC1500	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa		1				
62 kDa					2	2
52 kDa					1	1
47 kDa					1	1
41 kDa						
31 kDa						1

QC1502	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa		1	1			
62 kDa		1				
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC1503	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa	2	2	3	3	3	3
62 kDa						
52 kDa				2	1	2
47 kDa	1	1	1	2		2
41 kDa						
31 kDa						

QC2122	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa		1	1		1	1
78 kDa			2			
64 kDa			2			1
62 kDa						
52 kDa						
47 kDa					2	1
41 kDa						1
31 kDa						

QC2123	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa				1	1	
78 kDa				1		
64 kDa		1				
62 kDa					2	1
52 kDa						
47 kDa				2		
41 kDa						
31 kDa	3		1			

QC2124	Strain					
MW	UAMH	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa		1		1	1	2
78 kDa		1		1		
64 kDa						
62 kDa					2	
52 kDa						
47 kDa				2		
41 kDa				2		
31 kDa						

QC2125	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa	1	1	2			
78 kDa	1	1		2		
64 kDa	3	3	3	3	3	
62 kDa			3	3	3	
52 kDa	1	1		2	2	
47 kDa	1	1	2	1	1	
41 kDa						
31 kDa						

QC2126	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa		1	1			
78 kDa						
64 kDa	2	3	1			
62 kDa				2		
52 kDa				1		
47 kDa						
41 kDa				1		
31 kDa						

QC2127	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa			3	1		
78 kDa				3		
64 kDa	2	2	3	3	3	3
62 kDa			3	3	3	2
52 kDa	1	2	3	3	3	3
47 kDa	1	1	3	3	3	3
41 kDa						
31 kDa						

QC2128	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa			2			
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2129	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa			3	1		
62 kDa			1		2	1
52 kDa						
47 kDa						
41 kDa			1			
31 kDa						

QC2130	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa			3			
62 kDa					1	
52 kDa						
47 kDa						
41 kDa						
31 kDa		1				

QC2132	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa				1	1	1
64 kDa			2		2	1
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2133	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa	1	1	2	1		
62 kDa					2	1
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2399	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa	1		2	2	2	
78 kDa		1		1	1	
64 kDa	1	1	1		2	1
62 kDa	1	1	1	2	1	1
52 kDa	1	1	1	1	1	1
47 kDa	1	1	1	1	1	1
41 kDa		1	1	1		
31 kDa						1

QC2400	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa					2	
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2129	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa			3	1		
62 kDa			1		2	1
52 kDa						
47 kDa						
41 kDa			1			
31 kDa						

QC2130	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa			3			
62 kDa					1	
52 kDa						
47 kDa						
41 kDa						
31 kDa		1				

QC2132	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa				1	1	1
64 kDa			2		2	1
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2133	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa	1	1	2	1		
62 kDa					2	1
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2399	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa	1		2	2	2	
78 kDa		1		1	1	
64 kDa	1	1	1		2	1
62 kDa	1	1	1	2	1	1
52 kDa	1	1	1	1	1	1
47 kDa	1	1	1	1	1	1
41 kDa		1	1	1		
31 kDa						1

QC2400	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa					2	
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2797	Strain					
MW	UAMH 7897	8522 (Don)	DAOM 226641	DAOM 226642	CBS 463.97	8038 (Don)
97kDa	1					
78 kDa	1	1	1	1		
64 kDa						
62 kDa					2	1
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2800	Strain					
MW	UAMH 7897	8522 (Don)	DAOM 226641	DAOM 226642	CBS 463.97	8038 (Don)
97kDa	1	1	1	1	1	
78 kDa					2	1
64 kDa	1				1	1
62 kDa						
52 kDa						
47 kDa	1	1				
41 kDa						
31 kDa						

QC2801	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa			2			
64 kDa	1	2	2	1	2	1
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2802	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa					2	
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2803	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa			2			
64 kDa					2	
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC2804	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa	1	2				1
64 kDa	1	2	2	1		
62 kDa					2	1
52 kDa						
47 kDa	1					
41 kDa		1				
31 kDa						1

QC2805	Strain					
MW	UAMH 7897	8522 (Don)	DAOM 226641	DAOM 226642	CBS 463.97	8038 (Don)
97kDa						
78 kDa						
64 kDa		1	1		2	
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC3339	Strain					
MW	UAMH 7897	8522 (Don)	DAOM 226641	DAOM 226642	CBS 463.97	8038 (Don)
97kDa						
78 kDa	1	1	1	1	2	
64 kDa						
62 kDa					3	
52 kDa						
47 kDa	1	1	1	2		
41 kDa				2	2	
31 kDa						

QC3340	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa				1		
62 kDa					3	1
52 kDa						
47 kDa	3				1	
41 kDa			1			
31 kDa						

QC3342	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa		1	1			
78 kDa						
64 kDa	2	2	2	3		
62 kDa			3			
52 kDa						
47 kDa	3	1	1			
41 kDa						
31 kDa		2	2			

QC3343	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa		1	1	1	2	
62 kDa			1	1	1	
52 kDa			1	1	2	
47 kDa	3		3	3	1	
41 kDa						
31 kDa						

QC3345	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa			2	3	1	
78 kDa			1	2	1	
64 kDa				2	1	
62 kDa				3	2	
52 kDa			2	2	2	
47 kDa	3		2	3	2	1
41 kDa						
31 kDa			1	3	3	

QC3347	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa			1	2		1
78 kDa			1	1		1
64 kDa			1	1	1	
62 kDa			1	2	1	1
52 kDa			1	1	1	
47 kDa	2		3	3	1	
41 kDa						
31 kDa						

QC3351	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa	1	2	2			
78 kDa						
64 kDa	1	3	3			
62 kDa		2	2		3	1
52 kDa						
47 kDa	2	2	2	2		
41 kDa			2	2	1	
31 kDa		1	1			

QC3352	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa		2	2			
78 kDa						
64 kDa	1	3	3	2	2	2
62 kDa	1					
52 kDa		2	2			1
47 kDa	3	3	3	1	1	1
41 kDa						
31 kDa						

QC3354	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa			1	1		
78 kDa						
64 kDa		1	1	3	2	
62 kDa		1	1	2	2	
52 kDa						
47 kDa	3	2	2	2	3	
41 kDa	3	2	1	3		
31 kDa		1	1	1		

QC3358	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa	1	1				
78 kDa		1			1	1
64 kDa	1	1				1
62 kDa					2	1
52 kDa				1		1
47 kDa	1	1		1		1
41 kDa	1					
31 kDa						

QC3359	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa			2		1	
78 kDa						
64 kDa	2	3	3	3	2	1
62 kDa		3	1		3	1
52 kDa	2	2	1			
47 kDa	2	2	1	1	1	
41 kDa					2	
31 kDa						

QC3347	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa			1	2		1
78 kDa			1	1		1
64 kDa			1	1	1	
62 kDa			1	2	1	1
52 kDa			1	1	1	
47 kDa	2		3	3	1	
41 kDa						
31 kDa						

QC3351	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa	1	2	2			
78 kDa						
64 kDa	1	3	3			
62 kDa		2	2		3	1
52 kDa						
47 kDa	2	2	2	2		
41 kDa			2	2	1	
31 kDa		1	1			

QC3352	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa		2	2			
78 kDa						
64 kDa	1	3	3	2	2	2
62 kDa	1					
52 kDa		2	2			1
47 kDa	3	3	3	1	1	1
41 kDa						
31 kDa						

QC3354	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa			1	1		
78 kDa						
64 kDa		1	1	3	2	
62 kDa		1	1	2	2	
52 kDa						
47 kDa	3	2	2	2	3	
41 kDa	3	2	1	3		
31 kDa		1	1	1		

QC3358	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa	1	1				
78 kDa		1			1	1
64 kDa	1	1				1
62 kDa					2	1
52 kDa				1		1
47 kDa	1	1		1		1
41 kDa	1					
31 kDa						

QC3359	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa			2		1	
78 kDa						
64 kDa	2	3	3	3	2	1
62 kDa		3	1		3	1
52 kDa	2	2	1			
47 kDa	2	2	1	1	1	
41 kDa					2	
31 kDa						

QC3494	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa						
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC3497	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa						
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC3499	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa			3			
64 kDa	1	1	3	1		1
62 kDa					2	
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC3501	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa	1	1	1			1
62 kDa				1	1	1
52 kDa						
47 kDa				1		1
41 kDa						1
31 kDa						2

QC3502	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa	1	1	1			1
78 kDa						
64 kDa	1	2	2		1	1
62 kDa						
52 kDa						
47 kDa	1	1				
41 kDa						
31 kDa		1				

QC3825	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa				2	2	1
64 kDa	1	1	2	1		2
62 kDa			1	3	3	2
52 kDa				2		
47 kDa				2	1	
41 kDa						
31 kDa						

QC3826	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa	1	3	3	3	3	3
62 kDa	1	2		3	3	1
52 kDa						
47 kDa	1	1	1	2	2	2
41 kDa				2	2	2
31 kDa						

QC3827	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa						
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC3829	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa	1		1	1		1
78 kDa						
64 kDa	2	1	2	1		1
62 kDa					1	1
52 kDa	2	1	1	1		
47 kDa	2	1	1	1		
41 kDa						
31 kDa						

QC3831	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa	1	1	1			
62 kDa						2
52 kDa						
47 kDa	1	1	1			
41 kDa						
31 kDa						

QC3832	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa	1	1	3	2	1	1
62 kDa					3	2
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC3835	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						
78 kDa						
64 kDa						
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC3841	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa			1		1	
78 kDa		1			1	1
64 kDa						
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC3845	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa						1
78 kDa						
64 kDa						
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

QC3846	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa				1	1	
78 kDa						1
64 kDa	1	1	2			1
62 kDa				1	1	
52 kDa						
47 kDa	1	1				
41 kDa						
31 kDa						

QC3852	Strain					
MW	UAMH 7897	8522	DAOM 226641	DAOM 226642	CBS 463.97	8038
97kDa	1	3				3
78 kDa						
64 kDa						
62 kDa						
52 kDa						
47 kDa						
41 kDa						
31 kDa						

Table 2. Immunoblot responses of *W.sebi* target protein (47kDa) against 32 patient sera samples (HpAbs).

Value 1 = weak response; value 2 = moderate response; value 3 = strong response.

Human sera ID	UAMH 7897	P8522	DAOM 226641	DAOM 226642	CBS 463.97	P8038
1293	1	0	0	0	0	0
1500	0	0	0	0	1	1
1502	0	0	0	0	0	0
1503	1	1	1	2	0	2
2122	0	0	0	0	2	1
2123	0	0	0	2	0	0
2124	0	0	0	2	0	0
2125	1	1	2	1	1	0
2129	0	0	0	0	0	0
2130	0	0	0	0	0	0
2133	0	0	0	0	0	0
2399	1	1	1	1	1	0
2400	0	0	0	0	0	1
2796	1	1	1	1	1	0
2797	0	0	0	0	0	0
3339	1	1	1	2	0	0
3342	3	1	1	0	0	0
3343	3	0	3	3	1	0
3345	3	0	2	1	2	0
3347	2	0	3	3	1	1
3351	2	2	2	2	0	0
3352	3	3	3	1	1	1
3354	3	2	2	2	3	0
3358	1	1	0	1	0	1
3359	2	2	1	1	1	0
3501	0	0	0	1	0	1
3502	1	1	0	0	0	0
3829	2	1	1	1	0	0
3841	0	0	0	0	0	0
3845	0	0	0	0	0	0
3846	1	1	0	0	0	0
3852	0	0	0	0	0	0

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

The presence of the target protein in cells and spores was visualized by SDS-PAGE followed by CBB staining (Figure 7). The antigenic response of spores to human sera was verified by immunoblot using sera that showed a positive response in previous testing with *W. sebi* culture filtrate (Figure 20). The arrow in Figure 20 highlights the presence of the target antigenic protein in spores.

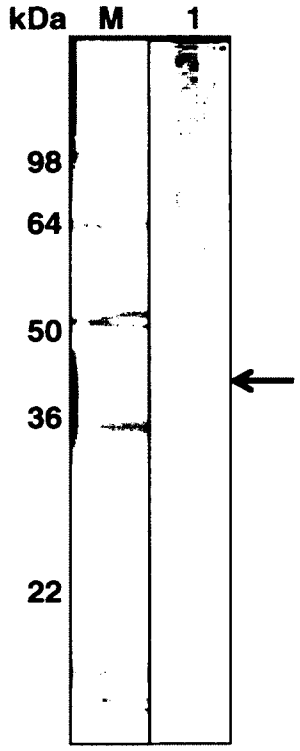

Figure 20. Immunoblot illustrating the response of HpAb QC 2125 to *W.sebi* spore protein extract.

Lane M: SeeBlue® Plus Protein Ladder with molecular weights marked left (6ul)
Lane 1: *W. sebi* spore extract (10ug)
Arrow indicates the 47 kDa protein

3.4 Purification of *W. sebi* target protein

The target 47kDa protein was purified from the crude extracellular protein extract obtained from the concentrated UAMH 7897 culture filtrate using FPLC anion exchange chromatography. Approximately 350mg of crude protein was loaded onto the column. The different proteins in the sample were separated and eluted by increasing the ionic strength in the mobile phase, using an increasing concentration of 1M NaCl in 20mM Tris buffer, pH 7.5. Aliquots of eluted fractions were run on an SDS-PAGE and CBB staining allowed visualization of fractions containing the 47kDa target protein (Figure 21). Fractions 31-34 contained the 47kDa protein; these fractions were combined and pooled together. Once these fractions were pooled, they were run on an SDS-PAGE and stained with CBB (Figure 22). It was noted that several bands were present on the stained gel. To verify the purity of the protein contained in the pooled fraction, the sample was run several different times by SDS-PAGE and subsequently stained by either CBB (Figure 22A) or Silver (Figure 22B). In some of the runs, single bands corresponding to different molecular weights were observed, confirming that the target protein is pure but can adopt different conformations. The lowest molecular weight observed was of 22kDa, suggesting that the previously observed 47kDa was a dimer of this protein. The total amount of purified target protein recovered was approximately 12mg /4L batch. The degree of purity was judged adequate for further study and no additional purification steps were required.

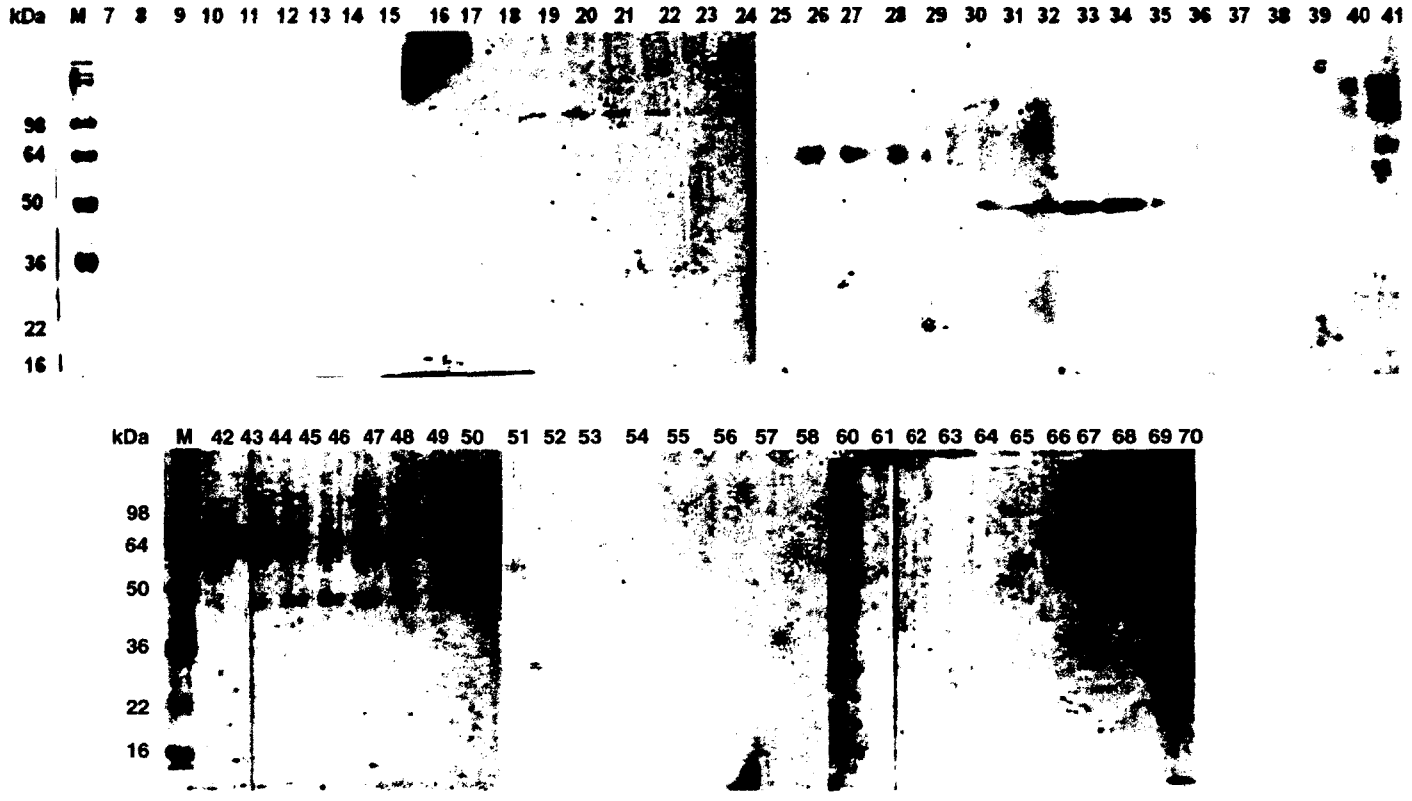

Figure 21. CBB stain illustrating the anion exchange (Q) column fractions obtained.

Lane M: SeeBlue® Plus Protein Ladder with molecular weights marked left (8µl)
Lane 7-70: Q anion exchange column eluted protein fractions (50µl)

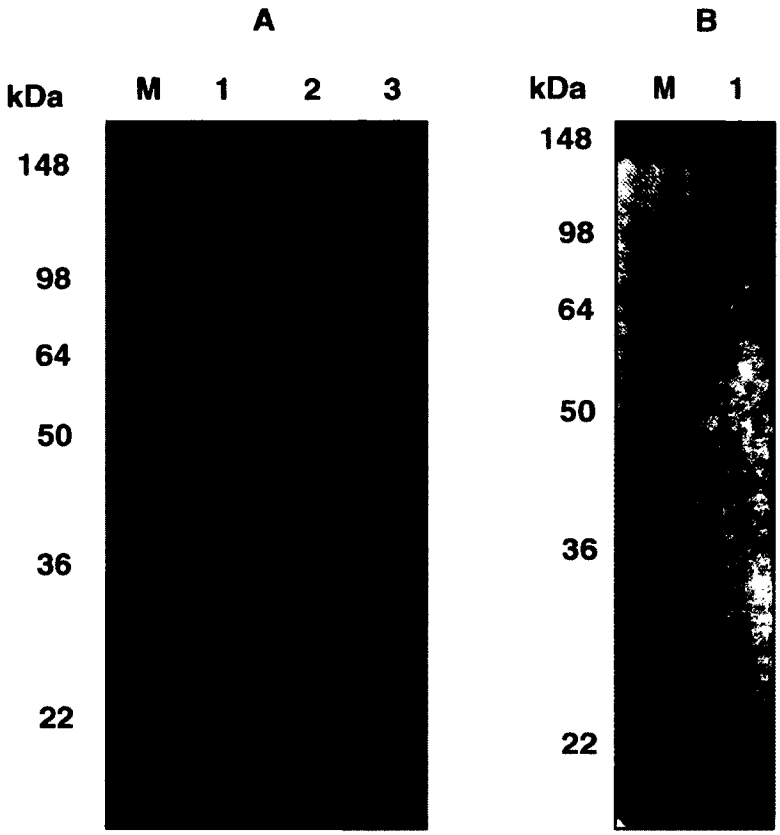

Figure 22. CBB stain (A) and Silver stain (B) of *W.sebi* target protein.

(A)
Lane M: protein standard with molecular weights marked left (8ul)
Lane 1: *W. sebi* purified target protein (6ug)
Lane 2: *W. sebi* purified target protein (6ug)
Lane 3: *W. sebi* purified target protein (6ug)
(B)
Lane M: protein standard with molecular weights marked left (8ul)
Lane 1: *W. sebi* purified target protein (2ug)

3.5 Polyclonal antibody production

3.5.1 Polyclonal antibodies produced in rabbits

Rabbit polyclonal antibodies (RpAbs) were produced by inoculating and boosting rabbits with the target antigen. The RpAbs showed a response with the purified target protein (Figure 23), as well as with the intracellular protein and spore protein extracts (Figure 24) on Western immunoblots, thereby confirming the antigenicity of the target protein in rabbits, in addition to that in humans.

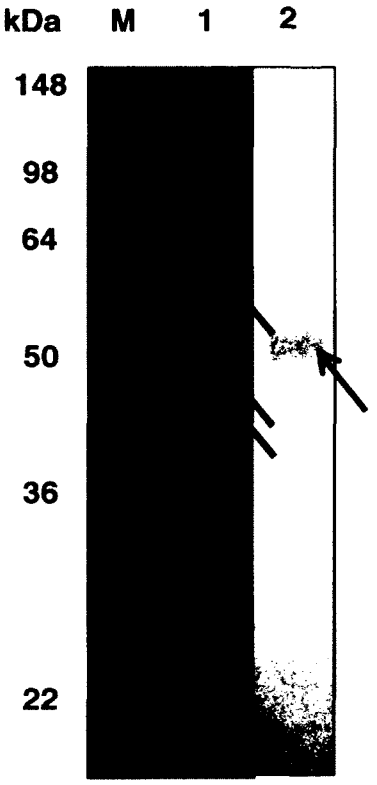

Figure 23. Immunoblot response of *W.sebi* target protein against RpAbs from rabbit sera 772.

Lane M: protein standard with molecular weights marked left (8ul)
Lane 1: *W. sebi* purified protein (0.5ug)
Lane 2: *W. sebi* purified protein (0.5ug)

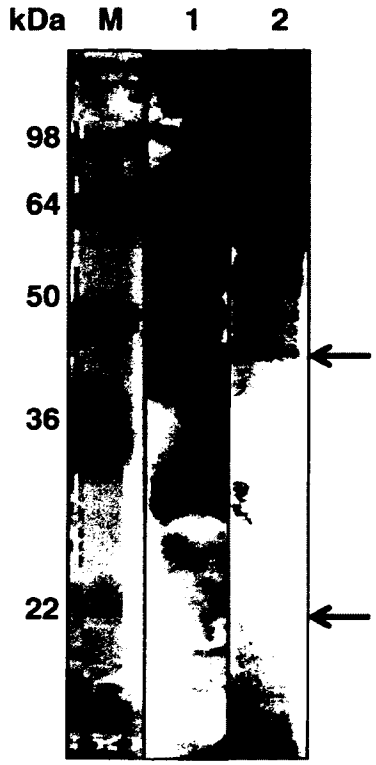

Figure 24. Immunoblot illustrating the response of Rabbit polyclonal antibody 772 to *W.sebi* spore and intracellular protein extracts.

Lane M: SeeBlue® Plus Protein Ladder with molecular weights marked left (6ul)
Lane 1: *W. sebi* spore extract (10ug)
Lane 2: *W. sebi* intracellular protein extract (10ug)

3.5.2 Purification of polyclonal antibodies

Antigen-specific RpAb purification was achieved by loading the RpAbs onto a streptavidin column containing the biotinylated target antigen. A fraction of the purified RpAb was biotinylated. An additional purification step was carried out to remove self-interacting non-biotinylated RpAbs and biotinylated RpAbs, by binding biotinylated antibody to an empty streptavidin column and loading the purified RpAb sample onto the column.

3.6 Development of Capture ELISA assay for *W. sebi* target antigen and arthrospores

In the Capture ELISA assay, the doubly purified pAb was used as the capture antibody, while the biotinylated antibody was used as the detection antibody (anti-analyte).

3.6.1 Capture ELISA assay for *W. sebi* target antigen

The response of the *W. sebi* target protein to RbAbs from rabbit sera 772 was evaluated by Capture ELISA using purified protein extract under different experimental conditions (Figure 25). Optimal assay conditions have been demonstrated as being: coating the microplate with 10ng decontaminated affinity purified pAb; blocking overnight with 1%BSA-TBST; developing with 1:10,000X decontaminated biotinylated affinity purified pAb and detecting the signal with 1:10,000X strep-HRP. O.D values measured at 450nm for each set of conditions are summarized in Appendix Table A1.4.

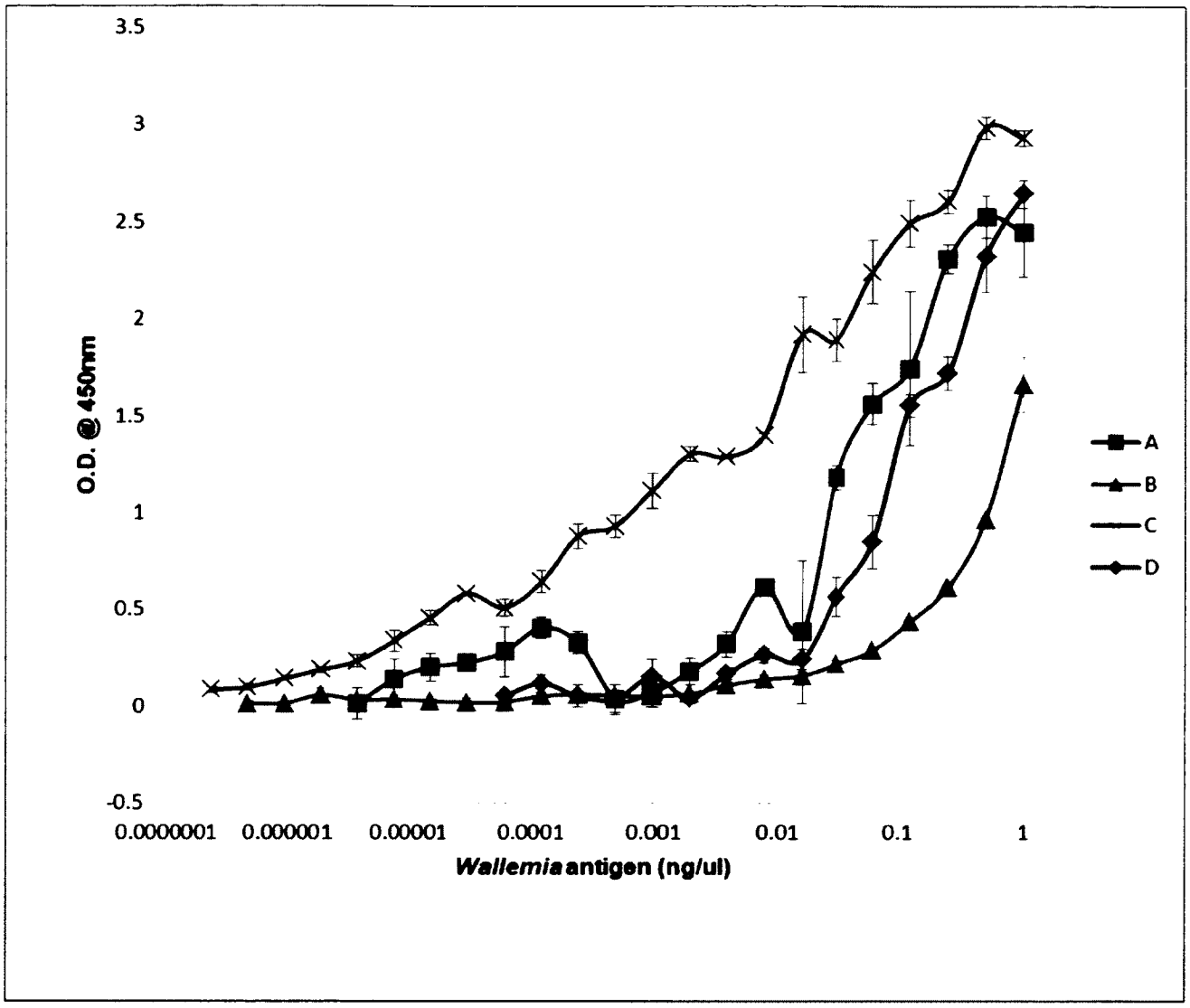

Figure 25. Capture ELISA of W.sebi antigen and RpAbs from rabbit sera 772. (A) coating: 10ng affinity purified pAb; blocking: 4hrs with 1%BSA-TBST; developing antibody: 1:10,000X biotinylated affinity purified pAb; detection: 1:10,000X strep-HRP. (B) coating: 10ng affinity purified pAb; blocking: overnight with 1%BSA-TBST; developing antibody: 1:10,000X biotinylated affinity purified pAb; detection: 1:10,000X strep-HRP. (C) coating: 10ng decontaminated affinity purified pAb; developing antibody: 1:4000X decontaminated biotinylated affinity purified pAb; detection: 1:10,000X strep-HRP. (D) coating: 10ng decontaminated affinity purified pAb; blocking: overnight with 1%BSA-TBST; developing antibody: 1:10,000X decontaminated biotinylated affinity purified pAb; detection: 1:10,000X strep-HRP.

3.6.2 Capture ELISA assay for *W. sebi* arthrospores

The response of the *W. sebi* spores to RbAbs from rabbit sera 772 was evaluated by Capture ELISA using arthrospore protein extract different experimental conditions (Figure 26). Optimal assay conditions have been demonstrated as being the same as those for the purified protein extract: coating the microplate with 10ng decontaminated affinity purified pAb; blocking overnight with 1%BSA-TBST; developing with 1:10,000X decontaminated biotinylated affinity purified pAb and detecting the signal with 1:10,000X strep-HRP. O.D values measured at 450nm for each set of conditions are summarized in Appendix Table A1.5.

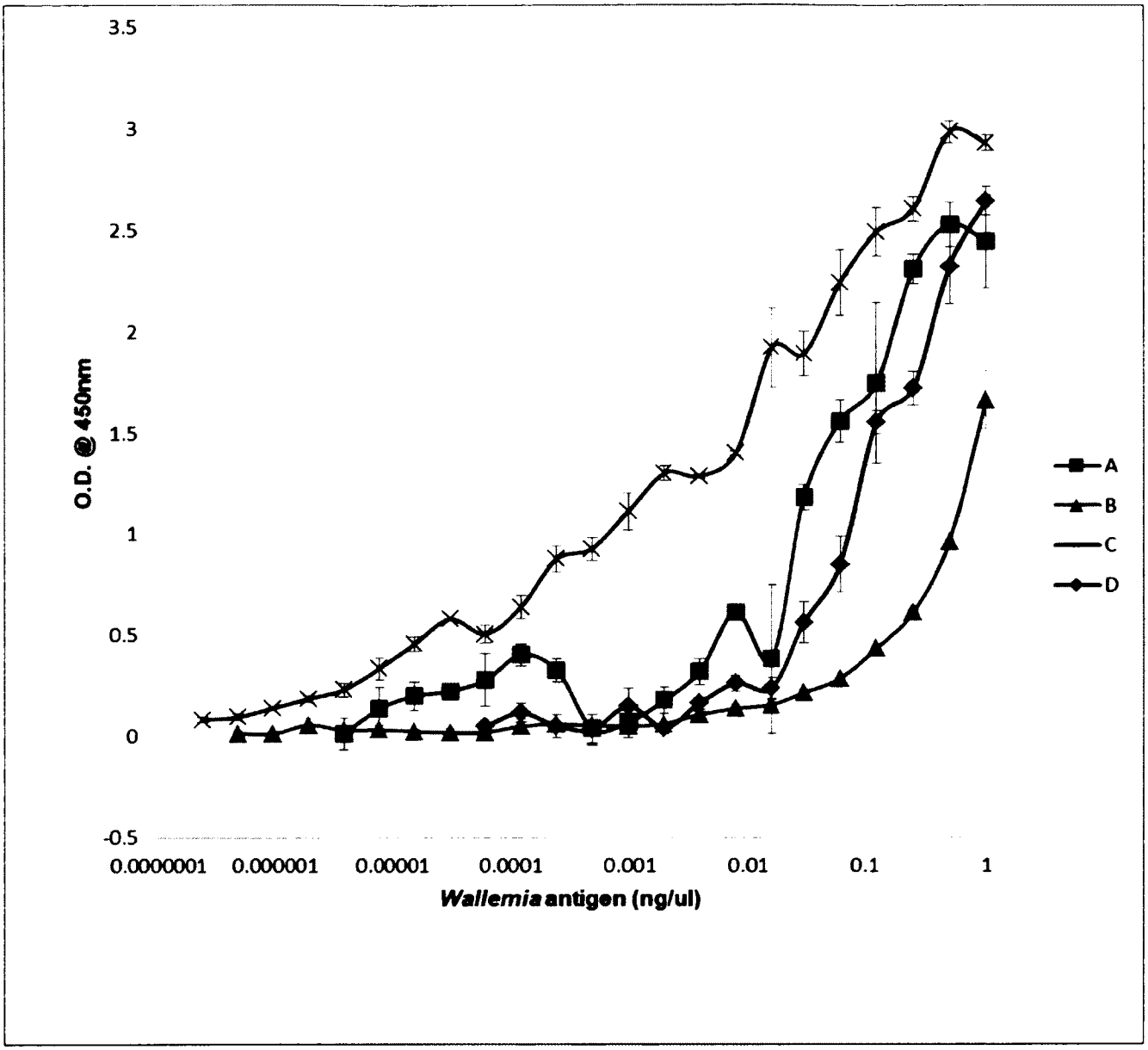

Figure 26. Capture ELISA of W.sebi arthrospores and RpAbs from rabbit sera 772. (A) coating: 40ng decontaminated affinity purified pAb; developing antibody: 1:10,000X decontaminated biotinylated affinity purified pAb; detection: 1:10,000X strep-HRP. (B) coating: 20ng decontaminated affinity purified pAb; blocking: overnight with 1%BSA-TBST; developing antibody: 1:10,000X decontaminated biotinylated affinity purified pAb; detection: 1:10,000X strep-HRP. (C) coating: 10ng decontaminated affinity purified pAb; blocking: overnight with 1%BSA-TBST; developing antibody: 1:10,000X decontaminated biotinylated affinity purified pAb; detection: 1:10,000X strep-HRP.

3.6.3 Capture ELISA assay for *W. sebi* arthrospore-spiked fine dust

The response of *W. sebi* arthrospores in fine dust to RbAbs from rabbit sera 772 was evaluated by Capture ELISA with spore-spiked dust samples under different experimental conditions (Figure 27). Optimal assay conditions have been demonstrated as being the same as those for the purified protein extract and spores alone: coating the microplate with 10ng decontaminated affinity purified pAb; blocking overnight with 1%BSA-TBST; developing with 1:10,000X decontaminated biotinylated affinity purified pAb and detecting the signal with 1:10,000X strep-HRP. O.D values measured at 450nm for each set of conditions are summarized in Appendix Table A1.6.

3.6.4 Cross-reactivity assessment by Capture ELISA using fungal spores

To test the cross reactivity of the RpAb, double pAb-Capture ELISA was performed with using spore protein extracts from various fungal species that are commonly found in the built environment. O.D. responses for the different species were expressed as a fraction of the *W. sebi* response at different spore concentrations (Figure 28). Spore protein from the other fungal species tested elicited low relative responses (weak O.D. signals) to the rabbit sera 772, indicated low cross-reactivity. The average O.D. values measured at 450nm and the calculated relative O.D responses for cross-reactivity are found in Appendices A1.7 and A1.8, respectively.

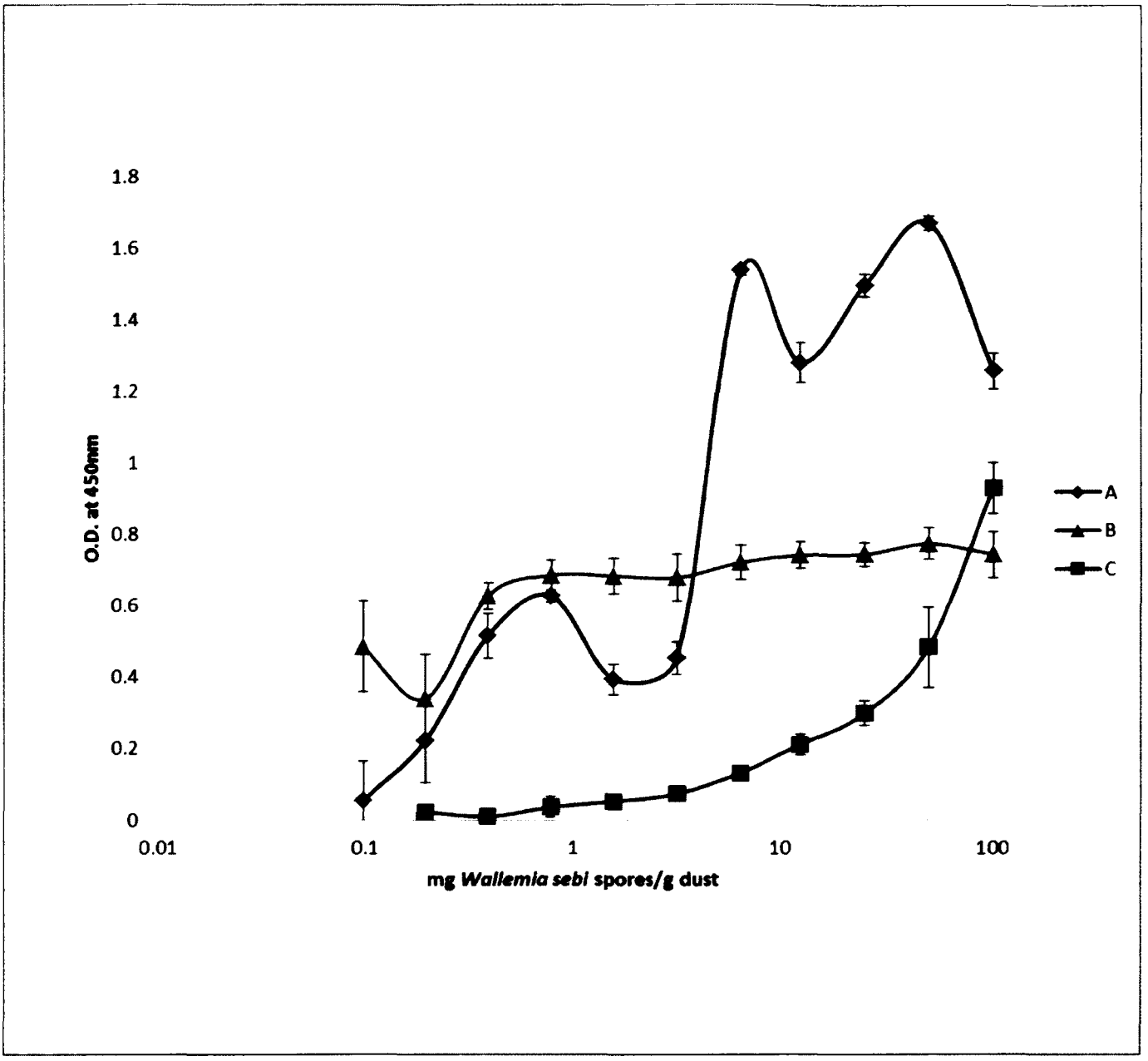

Figure 27. Capture ELISA of W.sebi spore-spiked dust and RpAbs from rabbit sera 772. (A) coating: 20ng decontaminated affinity purified pAb; blocking: overnight with 1%BSA-TBST; developing antibody: 1:10,000X decontaminated biotinylated affinity purified pAb; detection: 1:10,000X strep-HRP. (B) coating: 40ng decontaminated affinity purified pAb; blocking: overnight with 1%BSA-TBST ST; developing antibody: 1:10,000X decontaminated biotinylated affinity purified pAb; detection: 1:10,000X strep-HRP. (C) coating: 10ng decontaminated affinity purified pAb; blocking: overnight with 1%BSA-TBST; developing antibody: 1:10,000X decontaminated biotinylated affinity purified pAb; detection: 1:10,000X strep-HRP.

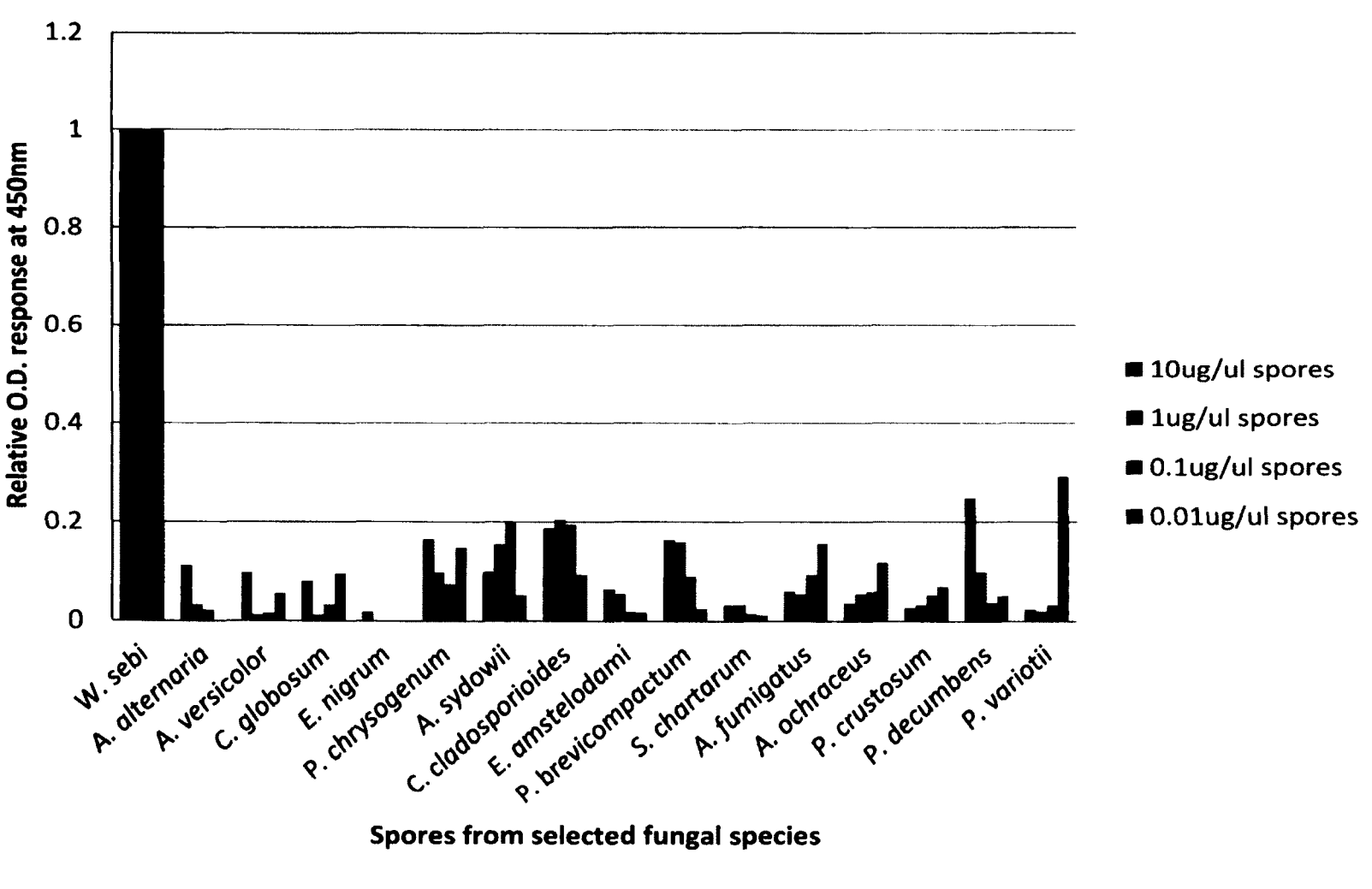

Figure 28. Relative Response of pAb 772 to spores from selected indoor fungal species

3.7 Characterization of the target antigens

3.7.1 Tandem mass spectrometry analysis

Tandem mass spectrometry was performed in an attempt to identify the target antigen. The 47kDa dimer was excised from a CBB-stained acrylamide gel and sent to Health Canada for MS/MS determination. The liquid chromatography tandem mass spectroscopy (LC-MS/MS) was performed by Fourier transform ion cyclotron resonance (FTICR). The total ion chromatograph (TIC) is displayed in Figure 29. Several peptide sequences were manually extracted from the MS/MS (de novo sequencing) and queried using NCBI/ BLAST. The sequences are summarized in Appendix Table A.10. The queried peptides did not give consistent alignments, however one abundant peptide (Appendix Figure A1.1) partially aligned with a glycoside hydrolase.

3.7.2 Determination of isoelectric point (pI)

A two-dimensional gel electrophoresis was carried out to determine the isoelectric point of the target protein (Figure 30). The first dimension was performed to separate the protein by isoelectric point (isoelectric focusing), using a pH gradient across the gel (Figure 30, A). Given that the target protein can adopt several conformations (22kDa, 47kDa, 66kDa), a second separation step was required. The second dimension allowed protein separation according to size (Figure 30, B). The pI of the protein was determined by locating the IEF protein band that corresponds to the 47kDa protein on the SDS-PAGE gel, then comparing its R_f to that of the standard on the IEF gel. The isoelectric point was calculated as being 4.9 for the 47kDa dimer.

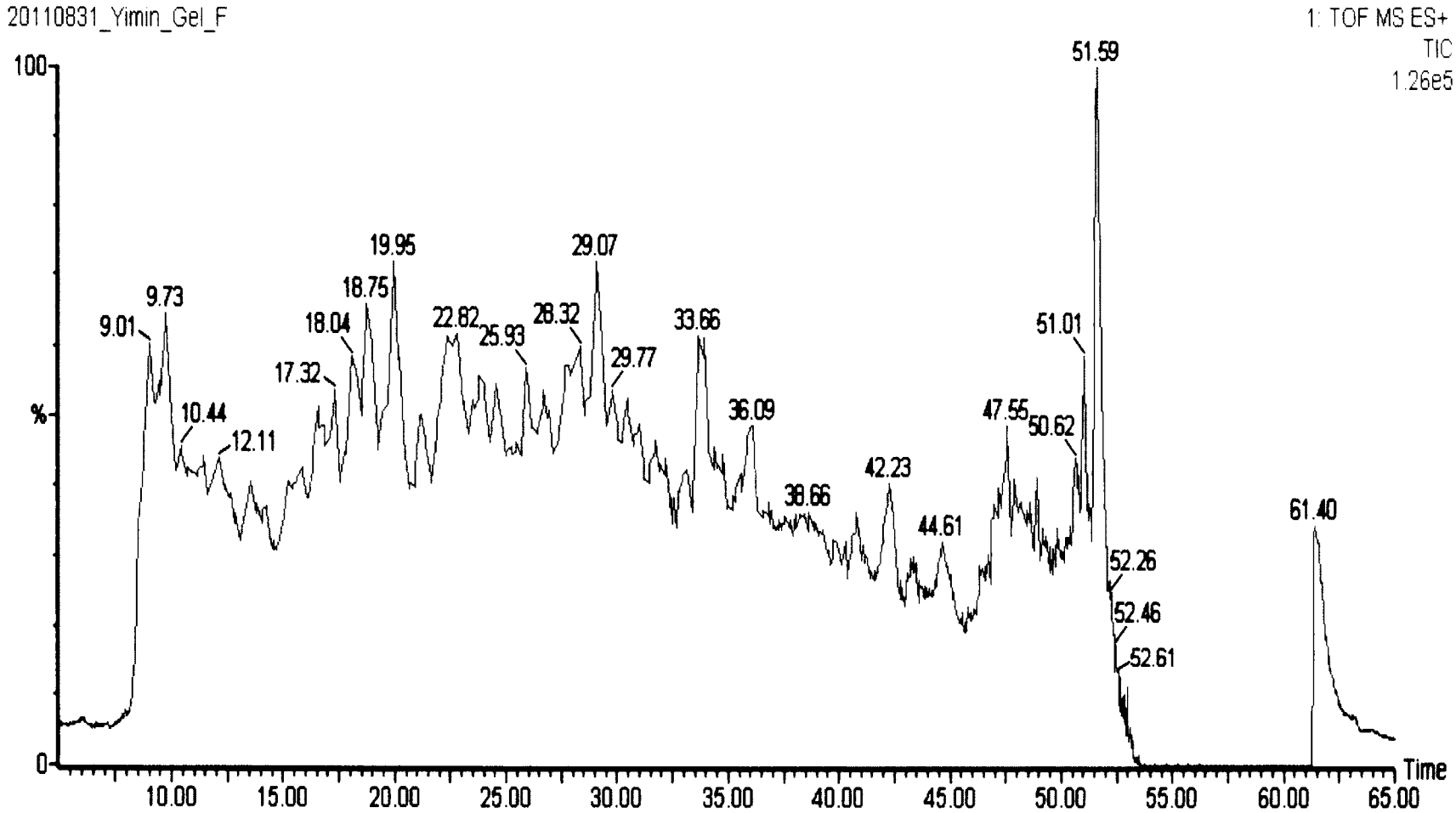

Figure 29. Total Ion Chromatograpm (TIC)

3.7.3 Glycoprotein assay

A glycoprotein stain was used to determine the glycosylation status of the target protein. One gel was stained with CBB (Figure 31, B), a second gel was stained with the glycoprotein staining kit (Figure 31, A) and then compared for evidence of glycosylation. The target protein was shown to be glycosylated.

3.7.4 Deglycosylation assay

The target protein was deglycosylated using a TFMS (trifluoromethanesulphonic acid) treatment (Figure 32). Arrows on Figure 32 illustrate protein bands at positions corresponding to differential molecular weights pre and post TFMS treatment.

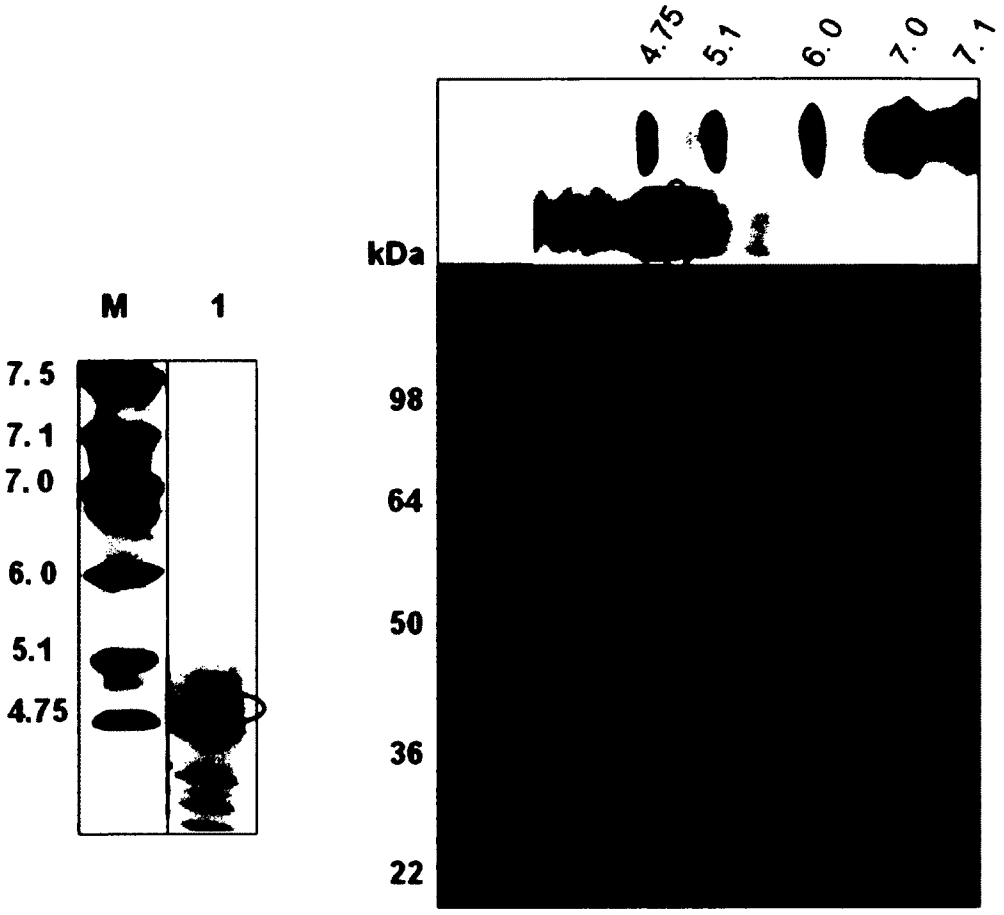

Figure 30. CBB stain illustrating (A) 1st dimension isoelectric focusing and (B) 2nd dimention SDS-PAGE

Lane M: Bio-Rad IEF standard with molecular weights marked left (20ul)
Lane 1: *W. sebi* purified protein (7.5ug)

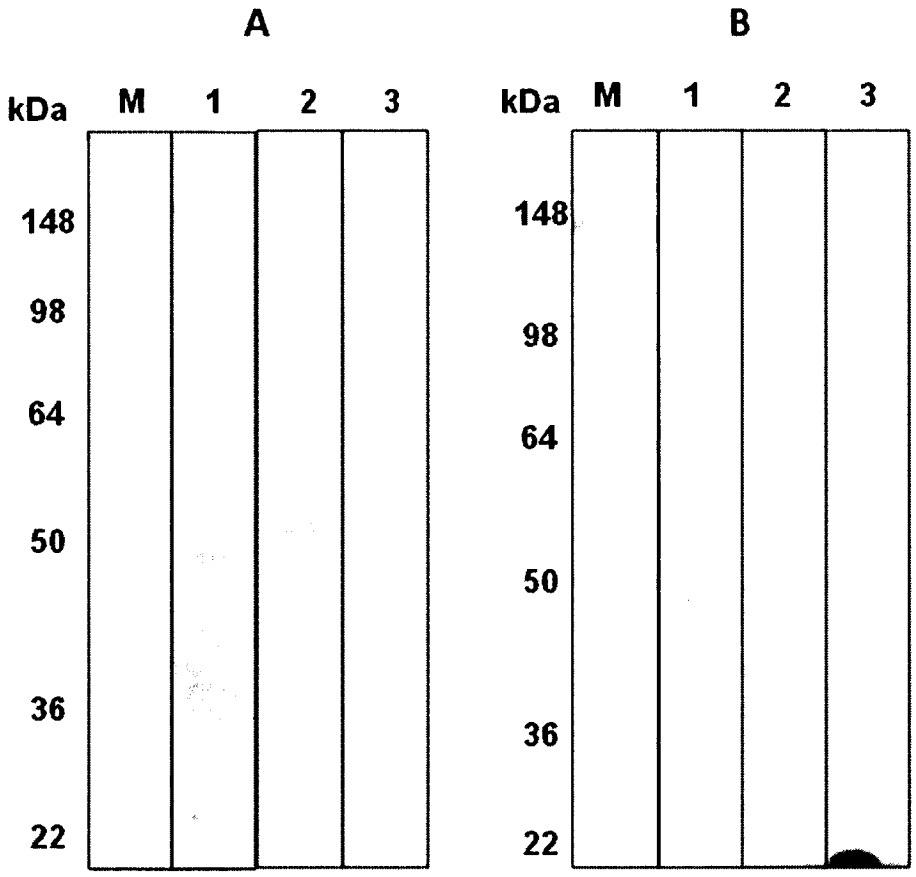

Figure 31. CBB stain illustrating (A) Glycoprotein staining and (B) CBB staining

Lane M: protein standard with molecular weights marked left
Lane 1: *W. sebi* purified protein
Lane 2: Horseradish peroxidase (positive control)
Lane 3: Trypsin inhibitor (negative control)

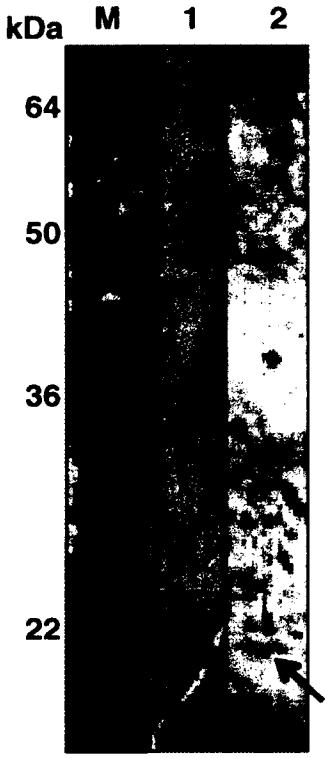

Figure 32. CBB stain illustrating target protein post-TFMS treatment.

Lane M: protein standard with molecular weights marked left
Lane 1: *W. sebi* purified protein, prior to TFMS treatment (5µg)
Lane 2: *W. sebi* purified protein, post TFMS treatment (5µg)

4. DISCUSSION

The increasing incidence of asthma and allergy is a globally relevant public health concern (Ozdoganoglu & Songu, 2012). There are an estimated 300 million people living with asthma; by 2025, this number is projected to reach 400 million as countries become more urbanized (To et al.,2012). In Canada, the prevalence of asthma amongst adults has risen significantly from 2.3% in 1979 to 7.5% in 1998 (Boulet et al.,1999). In 2010, the percentage of the population aged 12 and over having been diagnosed with asthma escalated to 8.5%, or roughly 2.2 million (Statistics Canada, 2010). The socioeconomic impacts of allergic diseases are substantial. In the United States, direct and indirect costs for asthma care are estimated to be as high as $6 billion US dollars (WHO, 2011). Based on current trends of allergy incidence, annual costs are expected to rise in incidence (Krahn et al.,1990), (Dillon et al.,1999).

Environmental allergen exposures and lifestyle factors are critical determinants of asthma and symptom manifestation (Tovey & Ferro, 2012). Many biologically-derived allergens can be found in the indoor environment, including proteins excreted from house dust mites, pet animals and rodents, cockroaches, plants and fungi (Chapman et al.,2001). There is strong evidence provided by epidemiology studies conducted in several countries that exposures from building/house dampness and mould have been associated with elevated risk for respiratory symptoms, asthma, hypersensitivity pneumonitis, rhinosinusitis, bronchitis, and respiratory infections (Mendell et al.,2011), (Quansah et al.,2012). Furthermore, additional studies have demonstrated that asthma and hypersensitivity pneumonitis in non-industrial settings can increase in severity if the

relationship between respiratory health effects fails to be recognized and exposure persists (Park & Cox-Ganser, 2011). Systemic reviews of the existing evidence conclude that exposure to mould and resulting respiratory symptoms, including the new onset of asthma can be reduced by implementing interventions that combine the elimination of moisture intrusion and leaks with the removal of mould-contaminated materials (Krieger et al.,2010); (Mendell et al.,2011); (Quansah et al.,2012). This position has also been adopted internationally by the World Health Organization (WHO, 2009), by many State governments (Health Canada, 2004; 2007) and by NIOSH (NIOSH, 2012).

Exposure assessments to fungal allergens have been complicated due to recurrent identification of many species- and genus-specific, but minor allergens (Soeria-Atmadja et al.,2010); (Crameri, 2011). For this reason, our lab has focused on the identification and characterization of major 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 (Xu et al., 2008), (Wilson et al., 2009), (Provost et al., 2012).

At present, 105 fungal allergens have been registered in the official allergen nomenclature list of allergens administered by Allergen Nomenclature Sub-Committee of the World Health Organization (WHO) and the International Union of Immunological Societies (IUIS) (www.allergen.org; 2012). Of these, 82 allergens belong to the Ascomycota and 23 to the Basidiomycota. The actual number of fungal allergens is significantly higher. As of yet, no allergens produced by *W. sebi* are listed within the database.

Proteins that are accumulated and released in culture are relevant for detection methods. In order to obtain consistent protein yields during the isolation of fungal antigens, growth conditions need to be carefully monitored. Parameters such as temperature, oxygen content, growth time, moisture content and nutrient availability are determining factors in protein accumulation in culture. Because *Wallemia* species are xerophilic, media consisting of 2% yeast extract was supplemented with 20% glucose (w/v) to lower the a_w and to promote a more favourable growth environment. Protein yields obtained with this media were comparable to those obtained with other media types known to produce high concentrations of protein (Xu et al.,2008), (Wilson et al.,2009).

The preliminary step in this research involved screening *W. sebi* culture filtrate crude protein extracts against HpAbs by immunoblotting and ELISA methods. The objective was to assess the reactivity of HpAbs to *W. sebi* extracellular proteins from different strains by ELISA and to visualize those proteins displaying an antigenic response by immunoblot.

Since IgG antibodies represent approximately 75% of circulating immunoglobulins and have a much longer half-life than IgE antibodies (Faquim-Mauro et al.,2003); (Nissen et al.,1998) IgG and corresponding anti-IgG antibodies were chosen for recognition of fungal antigens. The human immune response to the presence of an antigen elicits an almost immediate IgM antibody response, followed shortly by production of IgG antibodies. Without continued exposure, the IgE antibody serum titer declines quickly, whereas IgG antibodies remain in the serum for much longer (Abbas et al.,2007)

Several different antibody and antigen concentrations were tested to optimize the Indirect ELISA detection signal (O.D. response). Coating the microplate with too much antigen can cause passive binding and increase the background noise. Similarly, applying excess primary antibody (sera) can result in non-specific binding. Conversely, a poor signal will be obtained if the plate is coated with too little antigen (low epitope density) or if over-diluted primary antibody is applied, as it will fail to detect the epitopes that are present. Working dilutions of sera and crude protein extract were assessed by checkerboard titrations i.e. by diluting both reagents against each other and examining the responses of resulting combinations. Optimal amounts of coating antigen and primary antibody were identified to maximize the detection signal while maintaining minimal background.

All the *W. sebi* strains studied demonstrated an antigenic response to the HpAbs when assessed by Indirect ELISA, and each of the 6 strains had a comparable average reactivity against all HpAbs screened (Figure 9). A total of 84 sera samples were screened by Indirect ELISA. Variable responses could be observed in the average responses of *W. sebi* strains to individual sera samples (Figures 10-15). A total of 52 sera were very responsive and elicited an average O.D. value above 1.5; an additional 14 sera gave average response above 1.0 (Table 3). Of the 18 sera that elicited lower average signals below (i.e. below 1.0), 5 were hardly responsive and gave responses below 0.5 (Table 3).

Table 3. Summary of Sera Response in ELISA

	O.D ≥ 1.5	1.5 > O.D ≥ 1.0	1.0 > O.D ≥ 0.5	O.D <0.5	Total
# of Responding Patient Sera	52	14	13	5	84

Similar to ELISA, immunoblot analysis (Figures 16-19) of the *W. sebi* crude extracellular protein extracts against a collection of patient HpAbs displayed a variable response. With this technique, however, individual proteins having an antigenic response could be observed. Several different proteins were found to react with HpAbs, as shown by the arrows in Figure 7. Major reacting proteins that were present in all 6 strains included proteins corresponding to 97kDa, 78kDa, 64kDa, 62kDa, 52kDa, 47kDa, 41kDa, and 31kDa molecular weights, as assessed by SDS PAGE. When the protein extracts for each strain were compared, all reacted with at least 50% of the sera tested. Protein from strain P8038 had the lowest response, reacting with 56% of HpAbs tested, while that of strain DAOM 226641 had the highest response, giving a signal with 78% of the screened sera. Protein extracted from UAMH 7897 had a % sera response of 66, P8522 protein of 69, and both DAOM 226642 and CBS 463.97 protein extracts had responses of 70%. The variations between strains can be attributed to each strain producing variable amounts of allergens. When considering responses with strain UAMH 7897, most of the major proteins reacted with a smaller subset of patient sera. Exceptions were the 64kDa and the 47kDa protein (Table 4). The 64kDa protein appeared to be the most responsive, reacting to the greatest number of HpAbs. The 64kDa protein extracted from the UAMH strain reacted with 50% of the patient sera. The 47kDa also was responsive to many HpAbs screened, but to a slightly lesser

extent, responding to 39% of the patient sera. Other reacting proteins, i.e. 78kDa, 62kDa, 52kDa, 41kDa and 31kDa proteins were much less responsive to the screened sera, as shown in Table 4. The strength of the response was measured by grading the intensity of the bands on the immunoblots, on a scale of 0 to 3. As can be seen in Table 1, 0 corresponds to the absence of a band, 1 to a weak signal, 2 to a moderate signal and 3 to a strong signal to the target proteins.

Table 4. Sera immunoblot responses to various *W. sebi* antigens

Protein Molecular Weight (kDa)	# Sera reacting to *W. sebi* UAMH 7897 Proteins (% of sera)
97	10 (16)
78	6 (9)
64	32 (50)
62	3 (5)
52	5 (8)
47	25 (39)
41	2 (3)
31	2 (3)

Variations in reactivity can be explained by the fact that responses are dependent on the exposure characteristic of the individual. Antibody titer in individuals is directly proportional to the duration of exposure to the fungal propagules and inversely proportional to the length of time elapsed since encounter with the allergen. Therefore, individuals having had more recent exposure to *W. sebi,* or longer durations of exposure, can be expected to have higher concentrations of circulating antibody. This would translate into HpAbs from those individuals eliciting higher ELISA responses (e.g., QC1500, QC3829) Conversely, patients with lower ELISA responses were either not recently exposed to *W. sebi* allergens or were exposed for a shorter period of time (e.g., QC2803, QC3844, QC3851).

Additional considerations to choosing a suitable protein for antibody production include its molecular weight and whether it is produced in spores and mycelium. Smaller proteins are often highly conserved and more likely share common epitopes with protein produced by other fungi. Similarly, larger proteins could have a greater number of epitopes, thereby also increasing the probability of cross-reactivity. Furthermore, large proteins may not be as readily excreted due to modifications. Therefore, based its reactivity and size, the 47kDa protein is considered to be the best choice for antibody production; however its presence of this protein must be confirmed in both in mycelium and spores prior to immunization of rabbits for polyclonal production.

A 3-dimentional graph was created to visualize collective ELISA and immunoblot responses (Figure 33). The ELISA and immunoblot responses of each strain tested against the 32 different sera eliciting an OD of 1.5 or higher were combined (Appendix Table A1.9). ELISA OD responses are on the x axis; the immunoblot responses, graded on a scale of 1 (weak) to 3 (strong), are on the y axis; the 32 individual human sera are on the z axis. Generally, all strains seem to react similarly and the observed patient responses appear to fall within three distinct groups. The first cluster of combined responses includes sera from individuals that demonstrated high total protein activity in ELISA and gave intense immunoblot responses to the target 47kDa protein. This implies that these patients are mostly allergic to *Wallemia* species, and more specifically, have a high antibody titer that corresponds to IgG directed against the 47kDa protein. A second group is represented by patient sera displaying a moderate to high total protein activity in ELISA and a weak to moderate immunoblot response to the 47kDa protein. These individuals would have been exposed to the 47kDa protein along

with other *W. sebi* allergens or homologous fungal proteins bearing similar epitopes. Finally, a third cluster of individuals have combined sera responses that display high ELISA activity to total protein but low or absent immunoblot reactivity to the 47kDa. This signifies that these patients have been primarily exposed to protein allergens other than those produced by *W. sebi* and are likely allergic to proteins produced by other fungi that bear homologous epitopes, most likely from outdoor sources of basidiomycetes.

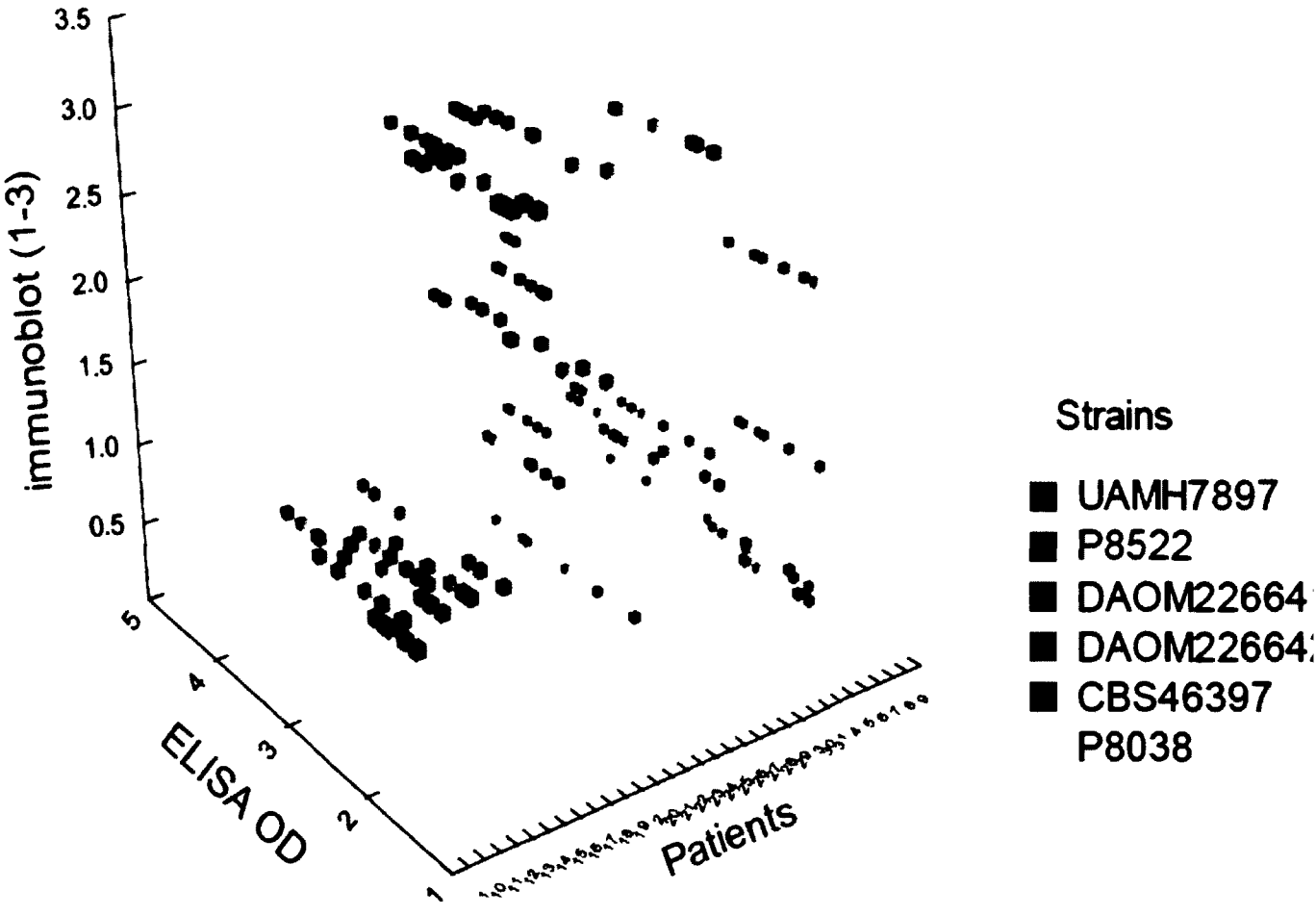

Figure 33 A three-dimensional graph illustrating ELISA (OD values; x axis) and immunoblot (y axis) response of *W.sebi* strains against 32 individual human sera (z axis)

Strain UAMH 7897 was chosen for the purification of the target protein based on its target protein yield, its response to the 47kDa protein on immunoblots and its total protein activity, as determined by ELISA.

The acetone precipitation method is commonly used to extract protein from solution. This technique is based on the altered solubility of peptides in the presence of acetone. Aqueous buffers have large dielectric constants that favour protein stabilitzation and dissolution. The addition of an organic solvent with a small dielectric constant, such as acetone induces the aggregation and precipitation of proteins (Rosenberg, 2005). Although relatively high protein yields can be achieved by this method (Xu et al.,2007), (Wilson et al.,2009), protein extraction using a concentrator and a 10,000 Da cut-off membrane has been shown to be very effective at extracting large amounts of target proteins (i.e proteins larger than 10kDa) (Provost et al.,2012). In this experiment, crude protein was extracted from the culture filtrate and concentrated 40X using a concentrated using a concentrator equipped with a 10,000 MWCO membrane. Approximately 369mg of crude protein was obtained per 4L`batch. The 47kDa protein represented approximately 3% of the total protein yield.

Spores, and spore/mycelium fragments are typically carriers of antigenic proteins and are thought to play a role in adverse health outcomes. For this reason, it was necessary to confirm the presence of the 47kDa antigenic protein in spores and cells. As visualized by SDS PAGE, the 47kDa protein was present in spores and mycelia, although to a lesser extent than when compared to the culture filtrate extract (Figure 8). A plausible explanation for this observation would be that the allergic proteins are principally exported from the mycelia into the culture filtrate. Subsequent purification

steps for target protein isolation were therefore carried out with culture filtrate rather than with mycelium or spores. An immunoblot response with HpAb 2125 was also observed for a 47kDa protein in spores (Figure 20).

Based on its response in both ELISA and immunoblot, as well as its presence in spores and mycelium, the 47kDa appeared to be a promising candidate for the development of immunoassays to detect *W. sebi* antigens in spore and spore/mycelium fragments in dust. This protein was therefore selected as the target antigen for purification, antibody production and characterization.

Purification for the 47kDa protein was performed using anion exchange chromatography. This technique is simple, fast and effective, and allows proteins separation based on net charge. In anion exchange chromatography, the positively charged stationary phase, a beaded, cross-linked agarose with a bound charged group ($-OH-CH_2CHOHCH_2OCH_2CHOHCH_2N^+(CH_3)_3$, interacts with negatively charged amino groups on the proteins in the mobile phase. Applying an NaCl gradient increases the ionic strength of the mobile phase; the anion has strong affinity for the column and thereby competes with the bound proteins. With increased NaCl concentrations, more negatively charged bound proteins can be eluted from the column. Fractions 31-34 contained the 47kDa protein and no further purification steps were required (Figure 22).

After purification, when the pooled fractions containing the target protein were run by SDS-PAGE and silver-stained to confirm the purity of the protein. A single band could be observed in the gel, thereby confirming the purity of the protein, however the band corresponded at 23kDa, rather than at the expected 47kDa mark, implying that the

target protein is a dimerized molecule of a smaller 23kDa monomer. Subsequent electrophoresis runs with the purified protein yielded bands corresponding to 3 different molecular weights - 23kDa, 47kDa, and 66kDa. This suggests that following purification, the protein can adopt 3 different protein confirmations (Figure 23).

Rabbit polyclonal antibodies were produced by immunizing two rabbits (10B48 and 772) with the purified *W. sebi* 47kDa target protein. Both rabbits were boosted with the 47kDa protein to increase the titer of antibodies to the target antigen. Activity of the RpAbs was verified by Indirect ELISA with the purified antigen and high responses were observed with both rabbit test bleeds and final bleeds. Rabbit sera 772 was selected for optimization and subsequent experimentation. Studies by Shi et al. (2011) successfully demonstrated that RpAb performance in Capture ELISA can be increased by prior sera purification and decontamination.

The activity of purified RpAb 772 was verified by Capture ELISA, using the *W. sebi* purified antigen (Figure 26), the spore protein extract (Figure 27) and spore-spiked dust (Figure 29). Double polyclonal antibody based-Capture ELISA have been shown to be suitable for the detection of major fungal allergens in house dust (Shi et al.,2011). In such assays, a purified pAb is coated to the plate and a biotinylated purified pAb is used to detect the analyte. Antigen and antibody concentrations were optimized to increase the ELISA detection signal while maintaining the lowest possible background levels. Following optimization, the RpAb 772 reacted proportionally at every dilution tested. The immunobloting reactivity of the RpAbs was also confirmed as the sera was shown to react to the target protein in culture filtrate (Figure 24), spores and mycelium (Figure

25). This confirms that the 47kDa protein is not only antigenic but also immunogenic, being capable of eliciting an immune response in rabbits.

In addition to confirming the hypothesis of the target protein being an antigen, the RpAb were also produced to verify their potential for incorporation in detection methods. Cross-reactivity was also assessed by Capture ELISA, using spore protein extracts from fungi commonly found indoors. Proteins that share some structural homology can have similar B-cell epitopes; this results in cross-reactivity – the ability of an IgE directed against a given allergen to bind another (Simon-Nobbe et al.,2008). A sequence identity of greater than 50% is required for cross-reactivity to occur (Simon-Nobbe et al.,2008). Capture ELISA showed much higher ELISA O.D. values for *W. sebi* spores relative to other species (Figure 29). In all instances, the response was found to below 30% that of *Wallemia* spore concentrations ranging between 10µg/µl to 0.01µg/µl. These observations are consistent with what is known about *W. sebi*'s taxonomic position. Close evolutionary fungal species typically produce allergens that have a higher degree of similarity and that are likely to induce IgE cross reactivity in exposed sensitized individuals species (Soeria-Atmadja et al.,2010). For this reason, little cross-reactivity is observed between spore protein extracts from the Basidiomycete *Wallemia* and the other common indoor fungi that belong to the Ascomycota. These result demonstrate that the RpAbs could potentially be used as a valid marker for identifying exposure to *W. sebi* in the indoor environment.

Experiments were performed to characterize the 47kDa protein, including glycosylation status determination, isoelectric focusing and mass spectrometry. A glycoprotein assay was also performed and the target protein was found to be glycosylated (Figure

31). Multiple allergens have been identified as glycoproteins and despite the immense diversity between the glycan structures, common patterns have since been identified among glycoprotein allergens produced by invertebrate animals and plants (Altmann, 2007), and are also thought to exist in moulds and yeast allergens (Aalberse et al.,2001). Some glycoproteins have oligosaccharide moieties referred to as carbohydrate cross-reactive determinants (CCD) because of their ability to induce the production of highly cross-reactive IgE. From the difference in the target protein's molecular weight observed in the SDS-PAGE post deglycosylation, it can be concluded that the glycan portion makes but a small contribution to the size of the glycoprotein (Figure 32). It is thus unlikely that the allergenic response to the target protein is caused by these apparently minor carbohydrate chains. This is further supported by previous studies which have found that the IgE binding activity resides in the protein components of fungal glycoproteins (Horner et al.,1995).

The pI of the 47kDa protein was also investigated and determined to be 4.9, which is acidic (Figure 30). This agrees with the previous observation that the 47kDa protein was eluted in later fractions using anion chromatography; meaning that because this protein is acidic and thus negatively charged, it has higher affinity to the positively charged column.

The 47kDa protein was 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). Several peptide sequences were extracted from the MS/MS spectra by de novo sequencing (Appendix Table A1.10). Most peptide sequences were shorter than 10 amino acids. The inferred amino

acid sequences were subjected to NCBI/BLAST alignment in the fungal genome database. Protein identification for the 47kDa protein presented itself to be a challenge, not only due to the small size of the queried peptides, but also because proteins of Basidiomycete-origin have not been extensively studied. Until very recently, the genome had yet to be sequenced and no *Wallemia* proteins were reported in the NCBI protein database. In 2012, Padamsee et al. (2012) sequenced the *W. sebi* genome (CBS 633.66), which turns out to be the second smallest genome reported to date within the Basiodiomycetes, with an estimated size of 9.8 megabase pairs (Mbp). Based on expressed sequence tag (EST) data and homology to other Basidiomycetes, *W. sebi* is predicted to have 5284 genes, which constitutes the smallest gene count in the phylum. The authors found that approximately 30% of the the genome (1689 proteins) had no homolog in any of the fungi they studied (Padamsee et al.,2012). Based on results obtained when queried employing NCBI/BLAST, the target 47kDa protein is unknown. This is not surprising given the very few studies involving *W. sebi.* An abundantly generated peptide gave partial alignment against a glycoside hydrolase of comparable molecular weight. This could suggest that the target protein is a glycoside hydrolase.

Glycoside hydrolases constitute a diverse and complex class of enzymes that catalyze the hydrolysis of glycoside bonds between two or more carbohydrates or between a carbohydrate and non-carbohydrate moiety (CAZy, 2012). These enzymes are classified in 135 different families based on sequence similarity, ather than substrate specificity (CAZy, 2012). Given that they are essential for carbohydrate metabolism, these enzymes are ubiquitous among species. In fungi, glycoside hydrolases are

extracellular enzymes involved the biomass degradation. They are capable of breaking down of plant cell wall components such as cellulose, lignin and hemicellulose. At present, a total of 453 glycoside hydrolases, of 46 different enzymatic activity modes have been characterized from 131 different fungal species. Although most identified fungal glycoside hydrolases are produced by Ascomycota, a number of Basidomycetes have been found to produce enzymes belonging to this family (Murphy et al.,2011); (CAZy, 2012). At least 41 glycoside hydrolases have been characterized by 17 different Basidiomycetes. The white rot fungus, *Phanerochaete chrysosporium,* produces at least 11 glycoside hydrolases involved in the degradation of lignin. Typical molecular size ranges for fungal glycoside hydrolases vary between 20kDa and 145kDa (Murphy et al.,2011).

In *Wallemia sebi,* 30 glycoside hydrolases have been predicted based on conceptual translations of DNA (CAZy, 2012) (www.uniprot.org; 2012). More specifically, glycoside hydrolases are thought to play a role in the morphogenic changes that xerophilic fungi must undergo in order to survive osmotic stress. Although genes implicated in these responses have not been fully elucidated, morphogenetic changes in *Wallemia* species are thought to be regulated by an increase in EXG1 expression, a cellulose/exo-1,3-β-glucanase gene. It has been speculated that this enzyme, belonging to the glucoside hydrolase family, contributes to the observed morphogenic changes by catalyzing the hydrolosis of cell wall glycoside linkages (Krieger et al.,2010).

A substantial number of plant glycoside hydrolases are known to be glycoproteins (Minic, 2008). Furthermore, some fungal glycoside hydrolase have been shown to have similar isoelectric points to that of the 47kDa protein, although being of a very diverse

class, these enzymes can be either acidic or basic (Li et al.,1993). Additionally, all of the previous allegens discovered in the laboratory from damp building fungi have also been excreted proteins (Liang et al.,2011); (Provost et al.,2012); (Wilson et al.,2009); (Xu et al.,2007). It cannot be confirmed at this point whether or not the target protein is a glycoside hydrolase and further characterization would be required. It is, however, clear that the 47 kDa protein is not produced by a diverse range of taxonomically unrelated species found in house dust

Overall, findings in this work would suggest a greater incidence of exposure to *W. sebi* propagules capable of eliciting allergic reactions that was has generally been thought. Albeit few, some studies have demonstrated that *Wallemia* is prevalent in the built environment in Canada and elsewhere. In a study of ca. 400 homes in Wallaceburg, Ontario, a geographical area with homes representative of Canadian housing, *W. sebi* was amongst the top 15 species recovered in culturable dust samples, on a colony forming units per gram settled dust basis and its occurrence was as high as 20% in some homes (Miller & Day, 1997). Similarly, Miller et al. found a 30% occurrence of *W. sebi* in homes in PEI (Miller et al. unpublished). Again, dust sample analysis using next generation sequencing found homes in Regina, SK to have a high prevalence of *W. sebi,* and the occurrence appeared to be higher than in eastern Canada (Amend et al.,2010). Although allergic sensitization to *W. sebi* has been demonstrated in children (Kolossa-Gehring et al.,2007) and adults (Sakamoto et al.,1989) no immunogenic proteins have been reported. The human sera used came mainly from across the USA. The fact that as many as 40% of the sera reacted to the 47 kDa protein indicates

that this fungus is quite common. Previous studies found that allergy to building moulds is a function of their prevalence on mouldy building materials (Miller et al.,2008). For example, it was found that 10% of mould reactive atopic sera had antibodies to *S. chartarum* sensu latto. The most common species of *Penicillium* indoors is the indoor clade of *P. chyrsogenum* (Wilson et al.,2009). Half of mould atopics reacted to its allergen. *A. versicolor* is less common, and the prevalence was around 20% (Liang et al.,2011). The pattern observed with *W. sebi* was also seen in the patient sera response to *C. globosum*. *C. globosum* is often not reported from air samples or building material samples because it takes time to produce reproductive structures. However, it is common on wet building materials worldwide. Provost et al. (2012) found that 50% of the sera were reactive.

Identification of a major allergenic protein in *W.sebi* is not only relevant an indoor air quality perspective. Seeing as *W. sebi* appears to play an important role in occupational disease, this work may also have implications from an occupational health standpoint. *W. sebi* is common in agriculture. Elevated serum IgG levels have been observed among Finnish farmers exposed to *W. sebi,* and studies in Eastern France, where farmer's lung is common, have concluded that the fungus is amongst the likely contributors to disease etiology (Reboux et al.,2001). Given the common occurrence of *W. sebi* in foodstuff, it is not surprising that the fungus is also present in bakery settings. *W. sebi* has been identified in bakery plants as a contaminant during the production process of a coconut type of gingerbread (Vytrasova et al.,2002). Additionally, recent studies have found evidence suggesting that *W. sebi* may be implicated in occupational symptoms experienced in in non-industrial settings Roussel et al. (2012) detected *W.*

sebi in their study of French archives, and found that worker symptoms such as fatigue, eye irritation, throat irritation, coughing and rhinorrhea were found to be significantly linked to contact with mouldy documents

In summary, a 47kDa dimer was isolated from *W. sebi* and was antigenic to humans and rabbits. The protein has not been previously identified and it is hypothesized to be a glycoside hydrolase. The 47kDa protein was found to be glycosylated and had an acidic pI. This protein was present in spores, mycelium and culture filtrate and RpAbs were successfully produced. The anti-47kDa RpAbs were found to react with the target antigen in culture filtrate, mycelium and spores. Capture ELISA with spore protein extracts from various other fungal species revealed very little cross reactivity with produced anti-47kDa RpAbs. The methods involving the production of *W. sebi* antibodies directed against a target antigenic protein are expected to be useful in assessing the presence of *W. sebi* in both indoor environments and agricultural settings. Further research could be accomplished to confirm the identity of the 47kDa protein and the production of anti-47kDa monoclonal antibodies could provide a more sensitive means of *W. sebi* detection.

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6. APPENDIX

Table A1.1. *W. sebi* strain information

Species	Deposited accession number	Paracel Laboratories number	Location	Sampling material
W. sebi	CBS 463.97	N/A	South Holland, the Netherlands	indoor dust
W. sebi	UAMH 7897	N/A	Saskatchewan, Canada	indoor dust
W. sebi	DAOM 226641	N/A	Ottawa, Ontario, Canada	indoor dust
W. sebi	DAOM 226642	N/A	Ottawa, Ontario, Canada	indoor dust
W. sebi	N/A	P8522	Ottawa, Ontario, Canada	indoor dust
W. sebi	N/A	P8038	Ottawa, Ontario, Canada	indoor dust

Table A1.2. List of human sera

QC 1291	QC 2127	QC 2793	QC 3339	QC 3354	QC 3841
QC 1292	QC 2128	QC 2794	QC 3340	QC 3355	QC 3843
QC 1293	QC 2129	QC 2795	QC 3341	QC3356	QC 3844
QC 1500	QC 2130	QC 2796	QC 3342	QC 3357	QC 3845
QC 1501	QC 2131	QC 2797	QC 3343	QC3358	QC 3847
QC 1502	QC 2132	QC 2798	QC 3344	QC 3359	QC 3850
QC 1503	QC 2133	QC 2799	QC 3345	QC 3348	QC 3851
QC 1504	QC 2134	QC 2800	QC 3346	QC 3500	QC 3853
QC 1505	QC 2396	QC 2801	QC 3347	QC 3501	QC 3829
QC 2122	QC 2399	QC 2802	QC 3349	QC 3502	QC 3834
QC 2123	QC 2400	QC 2803	QC 3350	QC 3837	QC 3846
QC 2124	QC 2401	QC 2804	QC 3351	QC 3838	QC 3848
QC 2125	QC 2402	QC 2805	QC 3352	QC 3839	QC 3849
QC 2126	QC 2403	QC 2806	QC 3353	QC 3840	QC 3852

Table A1.3. Indirect ELISA average OD responses at 450nm for *W. sebi* culture filtrate extracts screened against individual HpAbs.

	Average O.D. Response per Strain							Average O.D. Response per Strain					
Human Sera ID	UAMH 7897	P8522	DAOM 226641	DAOM 226642	CBS 463.97	P8038	Human Sera ID	UAMH 7897	P8522	DAOM 226641	DAOM 226642	CBS 463.97	P8038
QC 1291	1.090	1.308	1.235	1.439	1.724	1.537	QC 3339	1.840667	3.383	2.102	2.235	3.535	3.046
QC 1292	1.130	2.035	1.544	1.390	1.803	1.670	QC 3340	2.5385	3.424	2.659	2.315	3.137	2.420
QC 1293	1.546	1.537	1.900	1.671	2.018	1.836	QC 3341	1.10625	1.982	0.957	1.388	2.076	1.891
QC 1500	2.643	3.518	3.168	2.444	3.715	3.616	QC 3342	2.013	2.888	2.053	1.918	3.012	2.489
QC 1501	0.918	1.124	1.012	1.078	1.280	1.097	QC 3343	1.81875	2.834	1.966	1.829	2.954	2.382
QC 1502	2.644	3.062	3.097	2.648	3.210	2.965	QC 3344	1.74725	2.817	2.001	2.250	2.929	2.395
QC 1503	2.465	3.474	2.991	2.698	3.354	3.233	QC 3345	2.981	3.401	3.126	2.863	3.644	3.217
QC 1504	0.698	0.933	0.977	0.702	1.032	0.883	QC 3346	0.907667	2.171	1.612	1.555	2.222	1.898
QC 1505	0.806	0.744	0.966	0.724	1.069	1.034	QC 3347	2.614	3.407	2.641	3.422	3.530	2.056
QC 2122	1.734	3.749	2.194	1.734	3.318	2.606	QC 3348	0.9915	3.088	0.852	2.268	3.388	3.323
QC 2123	1.759	2.318	1.654	1.774	2.341	2.196	QC 3349	0.0315	0.503	0.577	1.965	2.307	1.107
QC 2124	1.793	2.715	1.950	2.438	2.829	2.445	QC 3350	1.152	2.932	2.571	2.245	2.614	0.400
QC 2125	1.863	2.717	2.191	2.447	2.676	2.514	QC 3351	2.009333	3.135	2.462	2.924	3.263	1.748
QC 2126	1.264	2.654	1.568	1.409	2.519	2.142	QC 3352	3.502	3.371	3.488	3.625	3.575	2.296
QC 2127	0.308	0.903	0.423	0.629	2.258	2.586	QC 3353	0.257	1.409	1.595	0.765	1.345	0.000
QC 2128	0.852	1.475	1.201	1.091	1.376	1.120	QC 3354	2.902667	3.307	3.172	2.883	3.447	2.086
QC 2129	2.456	3.039	2.682	2.251	2.763	2.145	QC 3355	1.20025	2.866	1.438	2.851	3.484	3.159
QC 2130	2.129	2.887	1.880	1.883	2.653	2.329	QC3356	1.433	2.723	1.571	2.364	3.163	2.850
QC 2131	0.881	1.300	1.019	0.881	1.112	0.922	QC 3357	0.4035	0.710	1.230	0.519	1.137	0.919
QC 2132	0.752	0.784	0.725	0.609	0.883	0.680	QC3358	3.073	3.102	2.764	2.917	3.622	3.542
QC 2133	3.092	3.512	2.774	3.126	3.510	3.325	QC 3359	3.1435	3.097	3.391	3.260	3.660	3.585
QC 2134	3.527	3.564	3.081	3.052	3.612	3.317	QC 3500	0.79725	1.884	1.344	0.883	2.100	1.493
QC 2396	3.653	3.268	3.538	2.483	3.026	1.660	QC 3501	2.62275	3.874	3.479	2.204	3.537	3.006
QC 2399	2.049	2.900	1.940	1.812	2.887	2.501	QC 3502	3.51975	3.886	3.644	3.413	3.840	3.830
QC 2400	2.404	2.598	2.278	2.165	2.986	2.783	QC 3837	0.309	1.218	0.675	0.112	0.481	0.409
QC 2401	0.107	0.006	0.134	0.086	0.174	0.205	QC 3838	1.025	1.512	1.405	0.757	1.426	1.930
QC 2402	0.977	0.953	1.134	1.272	1.200	1.008	QC 3839	3.7095	3.946	3.229	3.033	3.784	3.787
QC 2403	0.760	0.787	0.935	1.203	1.382	0.749	QC 3840	0.4585	1.249	0.884	0.126	0.676	0.482
QC 2793	0.340	0.598	0.959	1.085	1.174	0.771	QC 3841	3.9135	3.932	3.865	3.939	3.942	3.932
QC 2794	0.790	0.962	1.458	1.396	1.351	1.197	QC 3843	0.764	1.605	0.906	0.911	1.346	1.076
QC 2795	0.846	0.918	1.078	1.180	1.568	1.342	QC 3844	0.14075	0.594	0.472	0.190	0.500	0.212
QC 2796	1.872	1.855	1.947	2.347	2.725	2.085	QC 3845	2.10725	2.983	2.160	1.933	2.964	2.560
QC 2797	3.066	2.931	2.884	2.912	3.000	3.149	QC 3847	0.9075	1.530	0.943	1.167	1.635	1.496
QC 2798	3.496	3.494	3.539	3.561	3.549	3.456	QC 3850	1.19775	3.483	1.529	0.999	1.836	1.278
QC 2799	3.655	3.596	3.579	3.647	3.658	3.615	QC 3851	0.00525	0.000	0.000	0.002	0.000	0.009
QC 2800	1.105	1.737	2.517	1.922	1.236	1.127	QC 3853	0.6865	3.140	2.289	1.275	1.116	1.046
QC 2801	0.475	0.566	0.777	0.811	0.801	0.639	QC 3829	3.9955	4.073	3.972	3.954	4.082	4.091
QC 2802	0.717	0.773	0.925	0.846	0.745	0.755	QC 3834	1.848	3.369	2.292	2.591	3.680	3.621
QC 2803	0.266	0.271	0.375	0.698	0.844	0.460	QC 3846	2.07	3.774	2.690	2.224	3.676	3.444
QC 2804	0.530	0.707	1.048	1.147	1.126	0.783	QC 3848	0.1255	0.270	0.483	0.301	0.240	0.080
QC 2805	0.687	0.753	0.935	1.054	1.207	1.762	QC 3849	0.389	0.614	0.707	0.411	0.544	0.338
QC 2806	1.381	1.618	2.090	1.850	1.542	2.050	QC3852	3.844	3.898	3.787	3.808	3.839	3.874

Table A1.4. Average OD responses at 450nm for Capture ELISA with rabbit sera 772 and *W. sebi* purified target protein (antigen)

	Conditions 'A'		Conditions 'B'		Conditions 'C'		Conditions 'D'	
Purified protein concentration (ng/ul)	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation
1	2.444	0.229	1.662	0.143	2.929	0.041	3.408	0.070
0.5	2.526	0.109	0.964	0.011	2.983	0.057	3.086	0.183
0.25	2.305	0.073	0.612	0.011	2.602	0.059	2.484	0.085
0.125	1.742	0.396	0.434	0.015	2.489	0.120	2.319	0.057
0.0625	1.557	0.106	0.285	0.005	2.239	0.164	1.614	0.138
0.03125	1.181	0.063	0.215	0.018	1.888	0.109	1.329	0.101
0.015625	0.382	0.368	0.157	0.022	1.919	0.195	1.004	0.051
0.0078125	0.615	0.036	0.138	0.022	1.399	0.011	1.028	0.039
0.00390625	0.320	0.066	0.107	0.017	1.286	0.010	0.930	0.028
0.001953125	0.178	0.066	0.062	0.012	1.301	0.036	0.806	0.028
0.000976563	0.071	0.077	0.051	0.003	1.112	0.092	0.914	0.090
0.000488281	0.038	0.072	0.054	0.012	0.928	0.057	0.798	0.077
0.000244141	0.326	0.056	0.060	0.007	0.877	0.064	0.817	0.058
0.00012207	0.403	0.055	0.051	0.034	0.639	0.057	0.882	0.047
6.10352E-05	0.278	0.130	0.020	0.006	0.506	0.043	0.817	0.005
3.05176E-05	0.224	0.039	0.018	0.020	0.580	0.003	0.000	N/A
1.52588E-05	0.199	0.072	0.025	0.011	0.455	0.035	0.000	N/A
7.62939E-06	0.137	0.105	0.033	0.022	0.336	0.054	0.000	N/A
3.8147E-06	0.012	0.078	0.032	0.012	0.231	0.032	0.000	N/A
1.90735E-06	0.000	N/A	0.057	0.035	0.187	0.016	0.000	N/A
9.53674E-07	0.000	N/A	0.015	0.010	0.141	0.002	0.000	N/A
4.76837E-07	0.000	N/A	0.015	0.012	0.097	0.016	0.000	N/A
2.38419E-07	0.000	N/A	0.000	N/A	0.084	0.013	0.000	N/A

Table A1.5. Average OD responses at 450nm for Capture ELISA with rabbit sera 772 and *W. sebi* arthrospore protein extract.

	Conditions 'A'		Conditions 'B'		Conditions 'C'	
Arthrospore protein extract concentration (ng/ul)	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation
10,000	3.887	0.018	1.520	0.053	3.092	0.089
5000	3.949	0.061	2.301	0.019	2.961	0.010
2500	3.935	0.051	2.457	0.118	2.618	0.083
1250	3.981	0.025	2.152	0.039	2.109	0.043
625	3.855	0.018	1.742	0.079	1.851	0.144
312.5	3.871	0.049	1.309	0.027	1.568	0.038
156.25	3.851	0.051	0.955	0.011	1.261	0.072
78.125	3.842	0.062	1.035	0.070	1.011	0.041
39.0625	3.774	0.060	0.816	0.053	0.914	0.030
19.53125	3.663	0.175	0.562	0.060	0.815	0.104
9.765625	3.623	0.049	0.345	0.017	0.651	0.017
4.8828125	N/A	N/A	N/A	N/A	0.635	0.029
2.44140625	N/A	N/A	N/A	N/A	0.650	0.025
1.220703125	N/A	N/A	N/A	N/A	0.614	0.019
0.610351563	N/A	N/A	N/A	N/A	0.608	0.035
0.305175781	N/A	N/A	N/A	N/A	0.563	0.022
0.152587891	N/A	N/A	N/A	N/A	0.540	0.010
0.076293945	N/A	N/A	N/A	N/A	0.539	0.027
0.038146973	N/A	N/A	N/A	N/A	0.506	0.025
0.019073486	N/A	N/A	N/A	N/A	0.518	0.049
0.009536743	N/A	N/A	N/A	N/A	0.529	0.068
0.004768372	N/A	N/A	N/A	N/A	0.482	0.004

Table A1.6. Average OD responses at 450nm for Capture ELISA with rabbit sera 772 and *W. sebi* arthrospore-spiked dust.

	Conditions 'A'		Conditions 'B'		Conditions 'C'	
mg *W. sebi* arthrospores/ g dust	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation
100	1.257	0.050	0.744	0.064	0.929	0.072
50	1.669	0.020	0.773	0.044	0.484	0.113
25	1.496	0.031	0.744	0.032	0.299	0.035
12.5	1.279	0.056	0.741	0.037	0.211	0.030
6.25	1.537	0.012	0.721	0.049	0.132	0.006
3.125	0.453	0.045	0.678	0.066	0.073	0.007
1.5625	0.393	0.044	0.683	0.051	0.052	0.015
0.78125	0.627	0.018	0.685	0.042	0.036	0.028
0.390625	0.517	0.063	0.628	0.037	0.010	0.011
0.1953125	0.222	0.118	0.340	0.124	0.023	0.005
0.09765625	0.053	0.111	0.485	0.127	N/A	N/A

Table A1.7. Average OD responses at 450nm for Capture ELISA with rabbit sera 772 and spore protein extracts from common indoor moulds.

Plate 1	Spores (ug/ul)			
Species	**10**	**1**	**0.1**	**0.01**
W. sebi	3.020	2.966	2.698	1.243
A. alternaria	0.338	0.091	0.055	0.000
A. versicolor	0.291	0.033	0.038	0.066
C. globosum	0.242	0.031	0.083	0.118
E. nigrum	0.048	0.000	0.000	0.000
P. chrysogenum	0.496	0.288	0.195	0.184
Plate 2	**Spores (ug/ul)**			
Species	**10**	**1**	**0.1**	**0.01**
W.sebi	2.275	1.493	0.832	0.369
A. fumigatus	0.132	0.079	0.077	0.057
A. ochraceus	0.077	0.079	0.048	0.044
P. crustosum	0.056	0.044	0.041	0.025
P. decumbens	0.564	0.146	0.030	0.018
P. variotii	0.048	0.027	0.025	0.108
Plate 3	**Spores (ug/ul)**			
Species	**10**	**1**	**0.1**	**0.01**
W. sebi	3.562	2.267	2.554	1.433
A. sydowii	0.350	0.346	0.503	0.071
C. cladosporioides	0.664	0.464	0.496	0.133
E. amstelodami	0.221	0.119	0.042	0.023
P. brevicompactum	0.580	0.359	0.226	0.033
S. chartarum	0.105	0.069	0.033	0.014

Table A1.8. Relative O.D. responses at 450nm Capture ELISA with rabbit sera 772 using spore protein extracts from common indoor moulds expressed as a fraction of W. sebi O.D. responses at 450nm

	Spores (ug/ul)			
Species	**10**	**1**	**0.1**	**0.01**
W. sebi	1.000	1.000	1.000	1.000
A. alternaria	0.112	0.031	0.020	0.000
A. versicolor	0.096	0.011	0.014	0.053
C. globosum	0.080	0.010	0.031	0.095
E. nigrum	0.016	0.000	0.000	0.000
P. chrysogenum	0.164	0.097	0.072	0.148
A. sydowii	0.098	0.152	0.197	0.049
C. cladosporioides	0.186	0.205	0.194	0.093
E. amstelodami	0.062	0.053	0.017	0.016
P. brevicompactum	0.163	0.158	0.088	0.023
S. chartarum	0.029	0.030	0.013	0.010
A. fumigatus	0.058	0.053	0.092	0.155
A. ochraceus	0.034	0.053	0.058	0.118
P. crustosum	0.024	0.030	0.049	0.067
P. decumbens	0.248	0.098	0.036	0.049
P. variotii	0.021	0.018	0.030	0.292

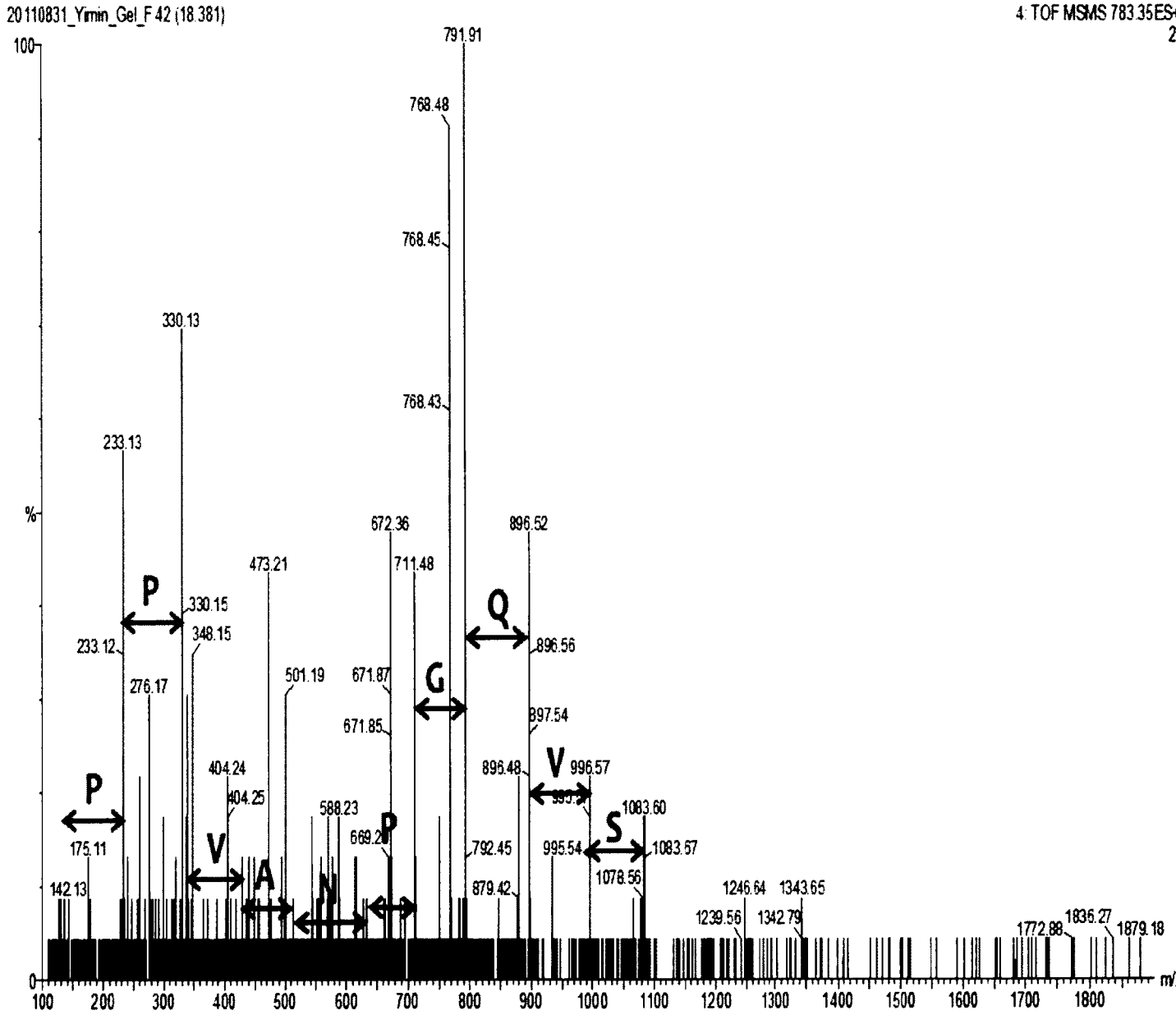
Table A1.9. Raw data for combined ELISA and Immunoblot responses to 32 patient sera

		OD Value per Strain						Blot Response per Strain					
Human Sera ID#	Human Sera ID#	UAMH 7897	P8522	DAOM 226641	DAOM 226642	CBS 463.97	P8038	UAMH 7897	P8522	DAOM 226641	DAOM 226642	CBS 463.97	P8038
1	**1293**	1.546	1.537	1.900	1.671	2.018	1.836	1	0	0	0	0	0
2	**1500**	2.643	3.518	3.168	2.444	3.715	3.616	0	0	0	0	1	1
3	**1502**	2.644	3.062	3.097	2.648	3.210	2.965	0	0	0	0	0	0
4	**1503**	2.465	3.474	2.991	2.698	3.354	3.233	1	1	1	2	0	2
5	**2122**	1.734	3.749	2.194	1.734	3.318	2.606	0	0	0	0	2	1
6	**2123**	1.759	2.318	1.654	1.774	2.341	2.196	0	0	0	2	0	0
7	**2124**	1.793	2.715	1.950	2.438	2.829	2.445	0	0	0	2	0	0
8	**2125**	1.863	2.717	2.191	2.447	2.676	2.514	1	1	2	1	1	0
9	**2129**	2.456	3.039	2.682	2.251	2.763	2.145	0	0	0	0	0	0
10	**2130**	2.129	2.887	1.880	1.883	2.653	2.329	0	0	0	0	0	0
11	**2133**	3.092	3.512	2.774	3.126	3.510	3.325	0	0	0	0	0	0
12	**2399**	2.049	2.900	1.940	1.812	2.887	2.501	1	1	1	1	1	1
13	**2400**	2.404	2.598	2.278	2.165	2.986	2.783	0	0	0	0	0	0
14	**2796**	1.872	1.855	1.947	2.347	2.725	2.085	1	1	1	1	1	0
15	**2797**	3.066	2.931	2.884	2.912	3.000	3.149	0	0	0	0	0	0
16	**3339**	1.841	3.383	2.102	2.235	3.535	3.046	1	1	1	2	0	0
17	**3342**	2.013	2.888	2.053	1.918	3.012	2.489	3	1	1	0	0	0
18	**3343**	1.819	2.834	1.966	1.829	2.954	2.382	3	0	3	3	1	0
19	**3345**	2.981	3.401	3.126	2.863	3.644	3.217	3	0	2	1	2	1
20	**3347**	2.614	3.407	2.641	3.422	3.530	2.056	2	0	3	3	1	0
21	**3351**	2.009	3.135	2.462	2.924	3.263	1.748	2	2	2	2	0	0
22	**3352**	3.502	3.371	3.488	3.625	3.575	2.296	3	3	3	1	1	1
23	**3354**	2.903	3.307	3.172	2.883	3.447	2.086	3	2	2	2	3	0
24	**3358**	3.073	3.102	2.764	2.917	3.622	3.542	1	1	0	1	0	1
25	**3359**	3.144	3.097	3.391	3.260	3.660	3.585	2	2	1	1	1	0
26	**3501**	2.623	3.874	3.479	2.204	3.537	3.006	0	0	0	1	0	1
27	**3502**	3.520	3.886	3.644	3.413	3.840	3.830	1	1	0	0	0	0
28	**3829**	3.996	4.073	3.972	3.954	4.082	4.091	2	1	1	1	0	0
29	**3841**	3.914	3.932	3.865	3.939	3.942	3.932	0	0	0	0	0	0
30	**3845**	2.107	2.983	2.160	1.933	2.964	2.560	0	0	0	0	0	0
31	**3846**	2.070	3.774	2.690	2.224	3.676	3.444	1	1	0	0	0	0
32	**3852**	3.844	3.898	3.787	3.808	3.839	3.874	0	0	0	0	0	0

Table A1.10. De novo sequenced peptides

Peptide m/z (retention time)	Peptide Sequences
436.71 (13.685)	#1: GDVTN(GG)
991.96 (15.257)	#2: GN(GG)DSPPTPI(L)A
791.41 (17.182) 783.35 (18.381)	#3: SVQGPN(GG)AVPP
662.85 (19.815) 664.35 (20.000)	#4: PYSVQGP
792.90 (17.367)	#5: SVQGP
487.56 (18.566)	#6: APGADSVPGGA
855.38 (18.852)	#7: CGI(L)I(L)DDEAGSPG
636.78 (21.434)	#8: SVQSYVTC
644.83 (24.137)	#9: VNGEQLTI(L)
750.89 (27.683)	#10: EVIDNI(L)TSVEA
1233.07 (27.733)	#11: HDSPPTGQNCN(GG)
680.31 (31.986)	#12: I(L)FDVDYI(L)
878.48 (40.657)	#13: PSTDVDTI(L)YDGPVA
680.31 (31.986)	#14: I(L)FDVDYI(L)

Figure A1.1. Spectrum used for 783.35 (18.381) de novo sequencing



6. APPENDIX

Table A1.1. *W. sebi* strain information

Species	Deposited accession number	Paracel Laboratories number	Location	Sampling material
W. sebi	CBS 463.97	N/A	South Holland, the Netherlands	indoor dust
W. sebi	UAMH 7897	N/A	Saskatchewan, Canada	indoor dust
W. sebi	DAOM 226641	N/A	Ottawa, Ontario, Canada	indoor dust
W. sebi	DAOM 226642	N/A	Ottawa, Ontario, Canada	indoor dust
W. sebi	N/A	P8522	Ottawa, Ontario, Canada	indoor dust
W. sebi	N/A	P8038	Ottawa, Ontario, Canada	indoor dust

Table A1.2. List of human sera

QC 1291	QC 2127	QC 2793	QC 3339	QC 3354	QC 3841
QC 1292	QC 2128	QC 2794	QC 3340	QC 3355	QC 3843
QC 1293	QC 2129	QC 2795	QC 3341	QC3356	QC 3844
QC 1500	QC 2130	QC 2796	QC 3342	QC 3357	QC 3845
QC 1501	QC 2131	QC 2797	QC 3343	QC3358	QC 3847
QC 1502	QC 2132	QC 2798	QC 3344	QC 3359	QC 3850
QC 1503	QC 2133	QC 2799	QC 3345	QC 3348	QC 3851
QC 1504	QC 2134	QC 2800	QC 3346	QC 3500	QC 3853
QC 1505	QC 2396	QC 2801	QC 3347	QC 3501	QC 3829
QC 2122	QC 2399	QC 2802	QC 3349	QC 3502	QC 3834
QC 2123	QC 2400	QC 2803	QC 3350	QC 3837	QC 3846
QC 2124	QC 2401	QC 2804	QC 3351	QC 3838	QC 3848
QC 2125	QC 2402	QC 2805	QC 3352	QC 3839	QC 3849
QC 2126	QC 2403	QC 2806	QC 3353	QC 3840	QC 3852

Table A1.3. Indirect ELISA average OD responses at 450nm for *W. sebi* culture filtrate extracts screened against individual HpAbs.

	Average O.D. Response per Strain							Average O.D. Response per Strain					
Human Sera ID	UAMH 7897	P8522	DAOM 226641	DAOM 226642	CBS 463.97	P8038	Human Sera ID	UAMH 7897	P8522	DAOM 226641	DAOM 226642	CBS 463.97	P8038
QC 1291	1.090	1.308	1.235	1.439	1.724	1.537	QC 3339	1.840667	3.383	2.102	2.235	3.535	3.046
QC 1292	1.130	2.035	1.544	1.390	1.803	1.670	QC 3340	2.5385	3.424	2.659	2.315	3.137	2.420
QC 1293	1.546	1.537	1.900	1.671	2.018	1.836	QC 3341	1.10625	1.982	0.957	1.388	2.076	1.891
QC 1500	2.643	3.518	3.168	2.444	3.715	3.616	QC 3342	2.013	2.888	2.053	1.918	3.012	2.489
QC 1501	0.918	1.124	1.012	1.078	1.280	1.097	QC 3343	1.81875	2.834	1.966	1.829	2.954	2.382
QC 1502	2.644	3.062	3.097	2.648	3.210	2.965	QC 3344	1.74725	2.817	2.001	2.250	2.929	2.395
QC 1503	2.465	3.474	2.991	2.698	3.354	3.233	QC 3345	2.981	3.401	3.126	2.863	3.644	3.217
QC 1504	0.698	0.933	0.977	0.702	1.032	0.883	QC 3346	0.907667	2.171	1.612	1.555	2.222	1.898
QC 1505	0.806	0.744	0.966	0.724	1.069	1.034	QC 3347	2.614	3.407	2.641	3.422	3.530	2.056
QC 2122	1.734	3.749	2.194	1.734	3.318	2.606	QC 3348	0.9915	3.088	0.852	2.268	3.388	3.323
QC 2123	1.759	2.318	1.654	1.774	2.341	2.196	QC 3349	0.0315	0.503	0.577	1.965	2.307	1.107
QC 2124	1.793	2.715	1.950	2.438	2.829	2.445	QC 3350	1.152	2.932	2.571	2.245	2.614	0.400
QC 2125	1.863	2.717	2.191	2.447	2.676	2.514	QC 3351	2.009333	3.135	2.462	2.924	3.263	1.748
QC 2126	1.264	2.654	1.568	1.409	2.519	2.142	QC 3352	3.502	3.371	3.488	3.625	3.575	2.296
QC 2127	0.308	0.903	0.423	0.629	2.258	2.586	QC 3353	0.257	1.409	1.595	0.765	1.345	0.000
QC 2128	0.852	1.475	1.201	1.091	1.376	1.120	QC 3354	2.902667	3.307	3.172	2.883	3.447	2.086
QC 2129	2.456	3.039	2.682	2.251	2.763	2.145	QC 3355	1.20025	2.866	1.438	2.851	3.484	3.159
QC 2130	2.129	2.887	1.880	1.883	2.653	2.329	QC3356	1.433	2.723	1.571	2.364	3.163	2.850
QC 2131	0.881	1.300	1.019	0.881	1.112	0.922	QC 3357	0.4035	0.710	1.230	0.519	1.137	0.919
QC 2132	0.752	0.784	0.725	0.609	0.883	0.680	QC3358	3.073	3.102	2.764	2.917	3.622	3.542
QC 2133	3.092	3.512	2.774	3.126	3.510	3.325	QC 3359	3.1435	3.097	3.391	3.260	3.660	3.585
QC 2134	3.527	3.564	3.081	3.052	3.612	3.317	QC 3500	0.79725	1.884	1.344	0.883	2.100	1.493
QC 2396	3.653	3.268	3.538	2.483	3.026	1.660	QC 3501	2.62275	3.874	3.479	2.204	3.537	3.006
QC 2399	2.049	2.900	1.940	1.812	2.887	2.501	QC 3502	3.51975	3.886	3.644	3.413	3.840	3.830
QC 2400	2.404	2.598	2.278	2.165	2.986	2.783	QC 3837	0.309	1.218	0.675	0.112	0.481	0.409
QC 2401	0.107	0.006	0.134	0.086	0.174	0.205	QC 3838	1.025	1.512	1.405	0.757	1.426	1.930
QC 2402	0.977	0.953	1.134	1.272	1.200	1.008	QC 3839	3.7095	3.946	3.229	3.033	3.784	3.787
QC 2403	0.760	0.787	0.935	1.203	1.382	0.749	QC 3840	0.4585	1.249	0.884	0.126	0.676	0.482
QC 2793	0.340	0.598	0.959	1.085	1.174	0.771	QC 3841	3.9135	3.932	3.865	3.939	3.942	3.932
QC 2794	0.790	0.962	1.458	1.396	1.351	1.197	QC 3843	0.764	1.605	0.906	0.911	1.346	1.076
QC 2795	0.846	0.918	1.078	1.180	1.568	1.342	QC 3844	0.14075	0.594	0.472	0.190	0.500	0.212
QC 2796	1.872	1.855	1.947	2.347	2.725	2.085	QC 3845	2.10725	2.983	2.160	1.933	2.964	2.560
QC 2797	3.066	2.931	2.884	2.912	3.000	3.149	QC 3847	0.9075	1.530	0.943	1.167	1.635	1.496
QC 2798	3.496	3.494	3.539	3.561	3.549	3.456	QC 3850	1.19775	3.483	1.529	0.999	1.836	1.278
QC 2799	3.655	3.596	3.579	3.647	3.658	3.615	QC 3851	0.00525	0.000	0.000	0.002	0.000	0.009
QC 2800	1.105	1.737	2.517	1.922	1.236	1.127	QC 3853	0.6865	3.140	2.289	1.275	1.116	1.046
QC 2801	0.475	0.566	0.777	0.811	0.801	0.639	QC 3829	3.9955	4.073	3.972	3.954	4.082	4.091
QC 2802	0.717	0.773	0.925	0.846	0.745	0.755	QC 3834	1.848	3.369	2.292	2.591	3.680	3.621
QC 2803	0.266	0.271	0.375	0.698	0.844	0.460	QC 3846	2.07	3.774	2.690	2.224	3.676	3.444
QC 2804	0.530	0.707	1.048	1.147	1.126	0.783	QC 3848	0.1255	0.270	0.483	0.301	0.240	0.080
QC 2805	0.687	0.753	0.935	1.054	1.207	1.762	QC 3849	0.389	0.614	0.707	0.411	0.544	0.338
QC 2806	1.381	1.618	2.090	1.850	1.542	2.050	QC3852	3.844	3.898	3.787	3.808	3.839	3.874

Table A1.4. Average OD responses at 450nm for Capture ELISA with rabbit sera 772 and *W. sebi* purified target protein (antigen)

	Conditions 'A'		Conditions 'B'		Conditions 'C'		Conditions 'D'	
Purified protein concentration (ng/ul)	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation
1	2.444	0.229	1.662	0.143	2.929	0.041	3.408	0.070
0.5	2.526	0.109	0.964	0.011	2.983	0.057	3.086	0.183
0.25	2.305	0.073	0.612	0.011	2.602	0.059	2.484	0.085
0.125	1.742	0.396	0.434	0.015	2.489	0.120	2.319	0.057
0.0625	1.557	0.106	0.285	0.005	2.239	0.164	1.614	0.138
0.03125	1.181	0.063	0.215	0.018	1.888	0.109	1.329	0.101
0.015625	0.382	0.368	0.157	0.022	1.919	0.195	1.004	0.051
0.0078125	0.615	0.036	0.138	0.022	1.399	0.011	1.028	0.039
0.00390625	0.320	0.066	0.107	0.017	1.286	0.010	0.930	0.028
0.001953125	0.178	0.066	0.062	0.012	1.301	0.036	0.806	0.028
0.000976563	0.071	0.077	0.051	0.003	1.112	0.092	0.914	0.090
0.000488281	0.038	0.072	0.054	0.012	0.928	0.057	0.798	0.077
0.000244141	0.326	0.056	0.060	0.007	0.877	0.064	0.817	0.058
0.00012207	0.403	0.055	0.051	0.034	0.639	0.057	0.882	0.047
6.10352E-05	0.278	0.130	0.020	0.006	0.506	0.043	0.817	0.005
3.05176E-05	0.224	0.039	0.018	0.020	0.580	0.003	0.000	N/A
1.52588E-05	0.199	0.072	0.025	0.011	0.455	0.035	0.000	N/A
7.62939E-06	0.137	0.105	0.033	0.022	0.336	0.054	0.000	N/A
3.8147E-06	0.012	0.078	0.032	0.012	0.231	0.032	0.000	N/A
1.90735E-06	0.000	N/A	0.057	0.035	0.187	0.016	0.000	N/A
9.53674E-07	0.000	N/A	0.015	0.010	0.141	0.002	0.000	N/A
4.76837E-07	0.000	N/A	0.015	0.012	0.097	0.016	0.000	N/A
2.38419E-07	0.000	N/A	0.000	N/A	0.084	0.013	0.000	N/A

Table A1.5. Average OD responses at 450nm for Capture ELISA with rabbit sera 772 and *W. sebi* arthrospore protein extract.

	Conditions 'A'		Conditions 'B'		Conditions 'C'	
Arthrospore protein extract concentration (ng/ul)	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation
10,000	3.887	0.018	1.520	0.053	3.092	0.089
5000	3.949	0.061	2.301	0.019	2.961	0.010
2500	3.935	0.051	2.457	0.118	2.618	0.083
1250	3.981	0.025	2.152	0.039	2.109	0.043
625	3.855	0.018	1.742	0.079	1.851	0.144
312.5	3.871	0.049	1.309	0.027	1.568	0.038
156.25	3.851	0.051	0.955	0.011	1.261	0.072
78.125	3.842	0.062	1.035	0.070	1.011	0.041
39.0625	3.774	0.060	0.816	0.053	0.914	0.030
19.53125	3.663	0.175	0.562	0.060	0.815	0.104
9.765625	3.623	0.049	0.345	0.017	0.651	0.017
4.8828125	N/A	N/A	N/A	N/A	0.635	0.029
2.44140625	N/A	N/A	N/A	N/A	0.650	0.025
1.220703125	N/A	N/A	N/A	N/A	0.614	0.019
0.610351563	N/A	N/A	N/A	N/A	0.608	0.035
0.305175781	N/A	N/A	N/A	N/A	0.563	0.022
0.152587891	N/A	N/A	N/A	N/A	0.540	0.010
0.076293945	N/A	N/A	N/A	N/A	0.539	0.027
0.038146973	N/A	N/A	N/A	N/A	0.506	0.025
0.019073486	N/A	N/A	N/A	N/A	0.518	0.049
0.009536743	N/A	N/A	N/A	N/A	0.529	0.068
0.004768372	N/A	N/A	N/A	N/A	0.482	0.004

Table A1.6. Average OD responses at 450nm for Capture ELISA with rabbit sera 772 and *W. sebi* arthrospore-spiked dust.

	Conditions 'A'		Conditions 'B'		Conditions 'C'	
mg *W. sebi* arthrospores/ g dust	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation	Average O.D. value at 450nm	Standard deviation
100	1.257	0.050	0.744	0.064	0.929	0.072
50	1.669	0.020	0.773	0.044	0.484	0.113
25	1.496	0.031	0.744	0.032	0.299	0.035
12.5	1.279	0.056	0.741	0.037	0.211	0.030
6.25	1.537	0.012	0.721	0.049	0.132	0.006
3.125	0.453	0.045	0.678	0.066	0.073	0.007
1.5625	0.393	0.044	0.683	0.051	0.052	0.015
0.78125	0.627	0.018	0.685	0.042	0.036	0.028
0.390625	0.517	0.063	0.628	0.037	0.010	0.011
0.1953125	0.222	0.118	0.340	0.124	0.023	0.005
0.09765625	0.053	0.111	0.485	0.127	N/A	N/A

Table A1.7. Average OD responses at 450nm for Capture ELISA with rabbit sera 772 and spore protein extracts from common indoor moulds.

Plate 1	Spores (ug/ul)			
Species	**10**	**1**	**0.1**	**0.01**
W. sebi	3.020	2.966	2.698	1.243
A. alternaria	0.338	0.091	0.055	0.000
A. versicolor	0.291	0.033	0.038	0.066
C. globosum	0.242	0.031	0.083	0.118
E. nigrum	0.048	0.000	0.000	0.000
P. chrysogenum	0.496	0.288	0.195	0.184
Plate 2	**Spores (ug/ul)**			
Species	**10**	**1**	**0.1**	**0.01**
W.sebi	2.275	1.493	0.832	0.369
A. fumigatus	0.132	0.079	0.077	0.057
A. ochraceus	0.077	0.079	0.048	0.044
P. crustosum	0.056	0.044	0.041	0.025
P. decumbens	0.564	0.146	0.030	0.018
P. variotii	0.048	0.027	0.025	0.108
Plate 3	**Spores (ug/ul)**			
Species	**10**	**1**	**0.1**	**0.01**
W. sebi	3.562	2.267	2.554	1.433
A. sydowii	0.350	0.346	0.503	0.071
C. cladosporioides	0.664	0.464	0.496	0.133
E. amstelodami	0.221	0.119	0.042	0.023
P. brevicompactum	0.580	0.359	0.226	0.033
S. chartarum	0.105	0.069	0.033	0.014

Table A1.8. Relative O.D. responses at 450nm Capture ELISA with rabbit sera 772 using spore protein extracts from common indoor moulds expressed as a fraction of W. sebi O.D. responses at 450nm

	Spores (ug/ul)			
Species	**10**	**1**	**0.1**	**0.01**
W. sebi	1.000	1.000	1.000	1.000
A. alternaria	0.112	0.031	0.020	0.000
A. versicolor	0.096	0.011	0.014	0.053
C. globosum	0.080	0.010	0.031	0.095
E. nigrum	0.016	0.000	0.000	0.000
P. chrysogenum	0.164	0.097	0.072	0.148
A. sydowii	0.098	0.152	0.197	0.049
C. cladosporioides	0.186	0.205	0.194	0.093
E. amstelodami	0.062	0.053	0.017	0.016
P. brevicompactum	0.163	0.158	0.088	0.023
S. chartarum	0.029	0.030	0.013	0.010
A. fumigatus	0.058	0.053	0.092	0.155
A. ochraceus	0.034	0.053	0.058	0.118
P. crustosum	0.024	0.030	0.049	0.067
P. decumbens	0.248	0.098	0.036	0.049
P. variotii	0.021	0.018	0.030	0.292

Table A1.9. Raw data for combined ELISA and Immunoblot responses to 32 patient sera

		OD Value per Strain						Blot Response per Strain					
Human Sera ID#	Human Sera ID#	UAMH 7897	P8522	DAOM 226641	DAOM 226642	CBS 463.97	P8038	UAMH 7897	P8522	DAOM 226641	DAOM 226642	CBS 463.97	P8038
1	**1293**	1.546	1.537	1.900	1.671	2.018	1.836	1	0	0	0	0	0
2	**1500**	2.643	3.518	3.168	2.444	3.715	3.616	0	0	0	0	1	1
3	**1502**	2.644	3.062	3.097	2.648	3.210	2.965	0	0	0	0	0	0
4	**1503**	2.465	3.474	2.991	2.698	3.354	3.233	1	1	1	2	0	2
5	**2122**	1.734	3.749	2.194	1.734	3.318	2.606	0	0	0	0	2	1
6	**2123**	1.759	2.318	1.654	1.774	2.341	2.196	0	0	0	2	0	0
7	**2124**	1.793	2.715	1.950	2.438	2.829	2.445	0	0	0	2	0	0
8	**2125**	1.863	2.717	2.191	2.447	2.676	2.514	1	1	2	1	1	0
9	**2129**	2.456	3.039	2.682	2.251	2.763	2.145	0	0	0	0	0	0
10	**2130**	2.129	2.887	1.880	1.883	2.653	2.329	0	0	0	0	0	0
11	**2133**	3.092	3.512	2.774	3.126	3.510	3.325	0	0	0	0	0	0
12	**2399**	2.049	2.900	1.940	1.812	2.887	2.501	1	1	1	1	1	1
13	**2400**	2.404	2.598	2.278	2.165	2.986	2.783	0	0	0	0	0	0
14	**2796**	1.872	1.855	1.947	2.347	2.725	2.085	1	1	1	1	1	0
15	**2797**	3.066	2.931	2.884	2.912	3.000	3.149	0	0	0	0	0	0
16	**3339**	1.841	3.383	2.102	2.235	3.535	3.046	1	1	1	2	0	0
17	**3342**	2.013	2.888	2.053	1.918	3.012	2.489	3	1	1	0	0	0
18	**3343**	1.819	2.834	1.966	1.829	2.954	2.382	3	0	3	3	1	0
19	**3345**	2.981	3.401	3.126	2.863	3.644	3.217	3	0	2	1	2	1
20	**3347**	2.614	3.407	2.641	3.422	3.530	2.056	2	0	3	3	1	0
21	**3351**	2.009	3.135	2.462	2.924	3.263	1.748	2	2	2	2	0	0
22	**3352**	3.502	3.371	3.488	3.625	3.575	2.296	3	3	3	1	1	1
23	**3354**	2.903	3.307	3.172	2.883	3.447	2.086	3	2	2	2	3	0
24	**3358**	3.073	3.102	2.764	2.917	3.622	3.542	1	1	0	1	0	1
25	**3359**	3.144	3.097	3.391	3.260	3.660	3.585	2	2	1	1	1	0
26	**3501**	2.623	3.874	3.479	2.204	3.537	3.006	0	0	0	1	0	1
27	**3502**	3.520	3.886	3.644	3.413	3.840	3.830	1	1	0	0	0	0
28	**3829**	3.996	4.073	3.972	3.954	4.082	4.091	2	1	1	1	0	0
29	**3841**	3.914	3.932	3.865	3.939	3.942	3.932	0	0	0	0	0	0
30	**3845**	2.107	2.983	2.160	1.933	2.964	2.560	0	0	0	0	0	0
31	**3846**	2.070	3.774	2.690	2.224	3.676	3.444	1	1	0	0	0	0
32	**3852**	3.844	3.898	3.787	3.808	3.839	3.874	0	0	0	0	0	0

Table A1.10. De novo sequenced peptides

Peptide m/z (retention time)	Peptide Sequences
436.71 (13.685)	#1: GDVTN(GG)
991.96 (15.257)	#2: GN(GG)DSPPTPI(L)A
791.41 (17.182) 783.35 (18.381)	#3: SVQGPN(GG)AVPP
662.85 (19.815) 664.35 (20.000)	#4: PYSVQGP
792.90 (17.367)	#5: SVQGP
487.56 (18.566)	#6: APGADSVPGGA
855.38 (18.852)	#7: CGI(L)I(L)DDEAGSPG
636.78 (21.434)	#8: SVQSYVTC
644.83 (24.137)	#9: VNGEQLTI(L)
750.89 (27.683)	#10: EVIDNI(L)TSVEA
1233.07 (27.733)	#11: HDSPPTGQNCN(GG)
680.31 (31.986)	#12: I(L)FDVDYI(L)
878.48 (40.657)	#13: PSTDVDTI(L)YDGPVA
680.31 (31.986)	#14: I(L)FDVDYI(L)

Figure A1.1. Spectrum used for 783.35 (18.381) de novo sequencing