Studies on human antigenic protein AVS 41kDa from *Aspergillus versicolor*

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1 Abstract

A. versicolor is a common fungus found on damp building materials in indoor environments. An antigenic protein at 41kDa from *A. versicolor* (AVS 41kDa) was successfully purified and characterized in this study. From the partial N-terminal sequence obtained from Edman sequencing, the protein was identified as an unprocessed alkaline serine proteinase. The protein was not glycosylated and had an acidic pI of 4.5.

There were a total of 5 different mAbs produced and they were all of the IgM isotype. Three different purification methods were tested to purify these antibodies such that they could be used in various applications. Gel filtration was found to be the best way to purify these IgM antibodies from mouse ascites compared to two other affinity purification methods: HiTrap IgM purification HP and an IgM purification kit. The responses of these mAbs against extracts of spores from different strains of *A. versicolor*, as well as culture supernatants from different fungi were examined for cross reactivity on Western blots. AVS 41kDa was found to be present in the different strains of AV spores tested. The ELISA responses of one of the mAbs 6E2 were tested against different *A. versicolor* spore strains as well as the spores from different fungal species. The results showed very good ELISA response against AV spores and specificity was reasonably good.

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+ve control for glycoprotein stain – horseradish peroxidise
°C - degree Celsius
3x – 3x acetone pellet
ABPA – allergic bronchopulmonary aspergillosis
AP – alkaline phosphatase
AS – ammonium sulphate
AV – *Aspergillus versicolor*
AVS 41kDa – *Aspergillus versicolor* 41kDa protein from culture supernatant
a_w – water activity
BCIP – 5-Bromo-4-chloro-3-indolyl phosphate liquid substrate system
BLAST – Basic Local Alignment Search Tool
BSA – bovine serum albumin
CBB – commassie brilliant blue stain
CBS – The Centraalbureau voor Schimmelcultures (CBS), an institute of the Royal Netherlands Academy of Arts and Sciences in Utrecht
CF – culture filtrate
DAOM – Department of Agriculture, Ottawa, Mycology, Ottawa ON
DTT – 1,4-dithiothreitol
EDTA – ethylenediaminetetraacetic acid
ELISA – enzyme linked immuno sorbent assay
FT – flow through
H_2O – water
HP – hypersensitivity pneumonitis
IEX – ion exchange
IgE – immunoglobulin E
IgG – immunoglobulin G
IgM – immunoglobulin M
K_2SO_4 – potassium sulphate
kDa – kilodaltons
L – Litre
LMW – low molecular weight marker
M – Molarity
mAb – monoclonal antibody
MEA – Malt extract agar
mg – milligram
min – minutes
mL – millilitre
mM – milliMolarity
MW – molecular weight, expressed in kDa
NaCl – sodium chloride
NCBI – National Center for Biotechnology Information
ng – nanogram
OD – optical density

PBST – PBST – phosphate buffered saline, pH 7.4, 0.05% Tween 20
PCR – polymerase chain reaction
pI – isoelectric point
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST – Tris-Buffered Saline, pH 7.4, 0.05% Tween 20
-ve control for glycoprotein stain – soybean trypsin inhibitor
μg – microgram
μL – microlitre

7 Introduction

7.1 Fungi

There are approximately 120,000 known species of fungi representing less than 10% of the estimated diversity (Hawksworth, 2001). Fungi are eukaryotic, heterotrophic organisms that contain nuclei. They degrade various sources of organic carbon to grow. In the environment, fungi are the degraders that break down 50% of dead plant and animals each year in tropical rain forests. Fungi come in different forms and shapes. Mushrooms, yeasts and molds are the most well known fungi. Yeasts in particular have been used for making bread and perform alcoholic fermentations for a long time. Yeasts are now used to produce biologically active proteins that can be used as therapeutics. They are one of the microorganisms that can be used for to produce vaccines, vitamins, immune therapy proteins, etc. (Buckley, 1997).

Some fungi are very important to plants. For example, mycorrhizal fungi live in and around the surface of the plant and help the plant to solubilize and access inorganic nutrients. Some orchid species depend entirely on mycorrhizal fungi to grow and to reproduce (Buckley, 1997).

7.2 Introduction to Ascomycotina

A. versicolor is an anamorphic, filamentous fungus that commonly occurs in damp building material indoors (Miller *et al.*, 2008). It belongs to kingdom Eumycota, phylum Dikaryomycota and subphylum Ascomycotina (Kendrick, 1992). Ascomycetes have two productive stages, asexual reproduction (anamorph) and sexual reproduction (teleomorph). Their anamorph productive stages are sometimes called molds. Many molds can produce a large amount of spores in a very short time. The meiosporangia of ascomycetes are called asci which can release the spores into the air after maturation (Kendrick, 1992). Since the spores are quite small in size and they are released in large numbers into the air, they are present everywhere. If the spores land in damp places, they germinate to produce hyphae that grow to form a mycelium, and then to eventually make more spores for reproduction (Kendrick, 1992). From approximately 200 reports around the world, *Cladosporium* was shown to be present at an average of 33% of spore counts in air samples. *Aspergillus* and *Penicillium* species were present at only 3.5% of samples (Kendrick, 1992; Smith, 1990).

7.3 Fungal diseases

Fungi in outdoor air are an important cause of allergy and hay fever. Studies have shown that fungal exposures could cause skin and nail mycoses as well as some lung infections such as aspergillosis, histoplasmosis and coccidiosis (Kendrick, 1992; Miller, 1992). For instance, approximately 200,000 Americans develop coccidiosis each year, 200 of those

people are hospitalized and 200 of them die each year (Buckley, 1997). Inhalation of mold spores causes respiratory allergic symptoms (Salvaggio and Aukrust, 1981).

Dales *et al.* (2004) have studied 60,066 asthma admissions to 108 hospitals in 10 different cities with Canada. The results showed that increases in asthma admissions was directly associated with increases in mean value of each allergen in the air especially from fungal spores. Several skin tests were done on 11 and 99 patients who were admitted to hospital with and without respiratory arrests from asthma. The results showed that 10 of 11 patients were positive against *Alternaria alternata* for those who had respiratory arrest and 31% of 99 patients showed positive to *Alternaria alternata* without respiratory arrests (Dales *et al.,* 2004).

Severe asthma is defined as when forced expiratory volume in one second is measured to be less than 70% of expected volume. Studies from O'Driscoll *et al.* (2005) and Zureik *et al.* (2002) showed that the sensitivity to mold was correlated with severity of asthma. Patients with high sensitivity to *Alternaria* species can be associated with severe asthma (Zureik *et al.*, 2002). O'Driscoll *et al.* (2005) studied 181 asthma patients who were admitted to Hope Hospital multiple times in the U.K. The results have shown that 76% of the patients studied with multiple admissions to the hospital had at least one positive skin test to mold. Some 37% of the patients who had multiple admissions to the hospital were allergic to *Aspergillus* species.

The German environmental survey for children in 2003-2006 included a total of 1790 children aged 3-14. Their exposure to environmental pollutants including different fungal species was tested. The mould specific IgE was analyzed in serum samples against commonly occurring fungal species such as *Penicillium chrysogenum*, *Aspergillus versicolor*, *Wallemia sebi*, *Eurotium* species and *Alternaria alternata*. The results showed that 9.5% of the children were sensitive to at least one of the moulds and approximately 2.2% of the children were allergic to *A. versicolor* (Kolossa-Gehring *et al.,* 2007).

7.4 Fungal allergies

Penicillium and *Aspergillus* are the most common indoor fungal species (Beaumont *et al.,* 1985; Flannigan & Miller; 2001; Miller *et al.,* 2008; Salvaggio and Aukrust, 1981). *Aspergillus* species have evolved over the past 200 million years (Galagan *et al.,* 2005). However, of the ~185 species of Aspergillus, there are only a few of them that cause invasive diseases (Galagan *et al.,* 2005). Some *Aspergillus* species produce toxic metabolites and others could cause allergies or mycoses (Geiser *et al.,* 2007).

There have been many studies associated with respiratory problems caused by mold and damp house conditions in Europe and North America since 1982. Studies done as part of the Harvard six-cities program (for outdoor air and health) showed that the presence of mold and dampness in their house were associated with increased asthma and increased upper respiratory disease (Brunekreef *et al.*, 1989). Similar studies were done associated with the Canadian Long Range Transport of Air Pollutant Program of Health Canada.

These involved a total of 30 communities, 15,000 children and 18,000 adults. The results were similar to those from the US study except that the larger Canadian study showed a dose response in the association with respiratory symptoms and mold and dampness (Dales *et al.*, 1991a; 199b).

Another study for testing biological contaminants in building materials was done in Wallaceburg, Ontario. It was chosen because it had a good population size with 400 homes which were representative of Canadian housing. A total of 270 fungal species were isolated from the homes in Wallaceburg. The most frequent species found in indoor contaminants were *Aureobasidium pullulans*, mushroom spores, *Alternaria alternata, Penicillium chrysogenum* and *Aspergillus versicolor.*

In studies of 3713 Norwegian children in 1999-2000 (Rydjord *et al.*, 2008), blood samples of 190 children were taken and mold-specific antibody IgE was tested against *A. versicolor*, *P. chrysogenum* and *C. herbarum.* These were felt to be common mold species in Scandinavia. However, only 1 child was tested to be positive to *A. versicolor.*

An allergic reaction is defined as hypersensitivity of the immune response triggered by a specific antigen and it generally occurs within half an hour of exposure. Based on the mechanism of hypersensitivity, there are four different categories. Human allergies to mold belongs to type I hypersensitivity which generally involves the interaction of an antigen with immunoglobulin E (IgE). When associated with an expression of disease,

the antigen is called an allergen. Common symptoms are allergic rhinitis and asthma (Horner *et al.*, 1995).

Type II hypersensitivity is called cytotoxic hypersensitivity. When antigens bound to the cell wall and being recognized by antigen-specific IgG or IgM, the cell lysis occurs (Horner, *et al.*, 1995). Type III hypersensitivity is usually immune complex induced. It is triggered by recognition of antigen-antibody complex when it attaches vessel walls. An example of type III hypersensitivity is serum sickness (Horner, *et al.*, 1995). Type IV hypersensitivity is a delayed response from the cells and usually peaks at 24-72 hours after exposure to an antigen. It is a cell mediated reaction and it is triggered by recognition of lymphokines released by antigen-specific T cells when an antigen enters the cells (Horner, *et al.*, 1995).

7.4.1 Rhinitis and asthma

Rhinitis is a disease caused by an inflammation of nasal mucous membrane resulting in nasal discharge, sneezing and congestion (Day and Ellis, 2001). Mold spores are an important cause of rhinitis, because of their small particle size. Persistent cold-like symptoms in children are clinically similar to rhinitis, but the symptoms are much worse. Children who suffer from rhinitis usually have symptoms similar to cold and the symptoms usually persist for months during the winter (Day and Ellis, 2001).

Asthma is a disease caused by inflammation and hyperirritability of the bronchial mucosa resulting in changes of expiratory flow rate. Patients who suffer from asthma usually have shortness of breath as well as wheeze or cough (Day and Ellis, 2001). Dales *et al.* (2003) reported that 10% of the children who were admitted to Childrens' Hospital of Eastern Ontario suffered from asthma due to inhalation of fungal spores after heavy rain.

7.4.2 Hypersensitivity pneumonitis (HP) and allergic bronchopulmonary aspergillosis (ABPA)

Exposure to high concentration of fungal spores in dust could cause hypersensitivity pneumonitis (HP) and allergic bronchopulmonary aspergillosis (ABPA) (Day and Ellis, 2001; Horner, *et al.*, 1995). HP is an immunologic lung disorder which is caused by a combination of type III and type IV hypersensitivities. It occurs at 4-8 hours after exposure. National Institute for Occupational Safety and Health (NIOSH) suggested that HP is one of the primary diseases of farmers. In the U.K, pigeon breeding is the second most frequent cause of the disease. In USA, buildings and ventilation systems with moisture and elevated temperature are also recognized as a source of HP. The first occupational HP was from the farmers handling with moldy hay (farmer's lung), described by Campbell (1932). It is one of the best known occupational HP and was caused by thermophilic Ascomycetes. However, many workplaces, such as those involved in malting and enzyme detergent manufacture and some residential heating systems have similar problems; because they all have an environment that favors the growth of Ascomycetes and causes HP (Hodgson and Flannigan, 2001).

ABPA is a serious inflammatory lung disease when persons have been long term exposure to a high concentration of *A. fumigatus* (Dillon *et al.*, 2007). When *A. fumigatus* spores are inhaled for long periods of time, some people become allergic to the fungus. This results in inflammation and in some cases, the spores trapped in the lower respiratory tract in patients' lungs can grow, colonize and germinate to form a mycelium. This causes permanent damage of the bronchial wall and surrounding pulmonary tissue. The antigens and toxins released from *A. fumigatus* spores can continue to cause a series of immunological responses (Day and Ellis, 2001). The proposed mechanism of ABPA is associated with IgE- and IgG-mediated immune response involving both type I and type III hypersensitivities (Horner, *et al.*, 1995).

Allergy tests usually involve injecting a commercially prepared protein extract or allergens from fungal culture extracts into the skin. People who are allergic to these proteins will have a reaction because of immunoglobulin E (IgE) antibodies developed against these allergens in the body. However, not all the allergens are commercially available and the allergens from high concentration of mold found in contaminated buildings are not yet fully understood. The skin tests are not reliable because of some cross reactivities against different fungal allergens. It is very difficult to diagnose the allergies due to chronic exposure of a particular mold unless the skin test for the allergen is commercially available (Tarlo *et al.*, 1988).

Fungal allergies are very difficult to diagnose because there are different allergens from different species within the same genus. Due to lack of standard extraction methods,

allergenic extracts can vary from strain-to-strain and batch-to-batch (Horner, *et al.*, 1995). There are 7 allergen product manufacturers in US that offer a total of 236 fungal allergenic extract products. However, the cross-reactivity among different fungal genera within fungal species made it very difficult for specific diagnostic tests. Extraction buffer, pH, ratio, time and temperature are all the factors that can affect the allergenic extracts (Esch, 2004). Esch (2004) measured the contents of allergenic extracts from silver stain and ELISA inhibition potency of *Alternaria alternata* from 7 different manufacturers. The protein contents of the extracts were quite different among different companies and there was no significant correlation between allergen concentration and their relative potency values. Aas *et al.* (1980) compared different commercial available allergenic extracts from *Alternaria*, *Cladosporium*, *Mucor* and *Penicillium* by skin test. The results showed that the extracts from the same species from different companies had different potency. In a study of skin tests for various mold species from several different companies, Day *et al.* (cited in Day and Miller 1996) found that extracts from the same species would produce conflicting results (positive or negative) depending on the source.

7.5 Taxonomy

The traditional methods to distinguish different fungal species were entirely based on the colony color and morphology (Gams *et al.*, 1985). The *Asperigillus* species have condiophores that have a stipe with either a bent or T-shaped base connected with an aspergillum and an enlarged vesicle and conidia-containing (Klich, 1993). *Aspergillus* species traditionally represents a monophyletic group that has an aspergillum. *Aspergillus*

species are now divided into different genera based on their teleomorphs. The *Aspergillus* species has 18 sections within 6 subgenera since there is more DNA or RNA sequence information available to analysis the taxonomy for *Aspergillus* species. *A. versicolor* is in subgenus *Nidulantes*, section *Versicolores* (Peterson, 2008). The Section *Versicolores* members have biseriate aspergilla which have a thin layer of cells between the vesicle and conidia-containing cells. The color of the conidia is green to blue-green (Klich, 1993). Section *Versicolores* comprises of *A. versicolor* (Figure 1) and *A. sydowii* (Peterson, 2008). *A. versicolor* can be separated from *A. sydowii* by the shape of the vesicles. The shape of the vesicle for *A. versicolor* was pyriform or clavate whereas *A. sydowii* had a shape of globose or spathulate based on the cultures by Klich (1993).

Figure 1 – picture of *A. versicolor* conidiophores (used with permission of CBS, the Netherlands)

7.6 Measurements of exposure

The United States National Academy of Sciences has considered the relationship of exposure to fungi growing in damp buildings to allergic respiratory disease in two reports. These reports identified two phenomena associated with mold growth in housing and health. First, when the extent of mold in a building is above some threshold (Haverinen *et al.,* 2001; Hyndman, 1990; Miller *et al.,* 1999), it is associated with exacerbation of asthma and increased upper respiratory disease in mold-sensitized individuals (Health Canada, 2004; NAS, 2000; NAS, 2004). Second, there is epidemiological evidence for an increased rate of asthma under such conditions (Dales *et al.,* 1991a; 1991b; Jaakkola *et al.,* 2005). The critical limitation of these studies is the lack of reliable measurements of exposure (Dillon *et al.,* 1999; Douwes *et al.,* 2003; Horner, 2005).

Strategies to quantitate fungi in indoor environments have involved either measuring the amount of total mold or specific building-associated molds. The former studies involve fungal culture or spore counts using light microscopy; or the detection of total fungal biomass by the fungal ergosterol in air samples by GC-MS and the fungal inflammatory agent β-1,3-D-glucan. When long duration air samples (5-7 days) were taken in houses, both measures were highly correlated with the area of mold growth in the buildings. From the latter study, exposures to fungal spores represented a small component of the net exposure, with recognizable spore and mycelial fragments and much smaller sized fragments representing the majority (Foto *et al.,* 2005).

The most commonly used method is dilution plates. However, culturing plates do not provide accurate estimation on biomass among different species. The results usually lack comparability and reproducibility. The general procedure for this method is described as follows. The samples are collected from building materials and dust. The sample is placed into an appropriate buffer and different dilution series are made. Each dilution is cultured evenly on agar media in triplicates. The plates with different dilutions are incubated for days under appropriate temperature. Then the colonies were counted. Each colony is then transferred into a 2% malt extract agar (MEA) or Czapek-yeast extract agar for identification. The purpose of the procedure is to dilute until no or very few colonies develope on the plates. However, the number of fungal propagules determined by this method is only approximately 1-50% of the total number of spores. There are a few problems: 1) different fungi have different growth requirements, so the growth condition may not be suitable for the some species; 2) certain spores such as spores from *Stachybotrys chartarum* have a half life of a few months, so the viable spores are largely decreased through time; 3) some fungi such as *Trichoderma* species can produce anti-fungal agents which prohibits the growth of other fungi on the culture plates (Miller, 2001).

Ergosterol can provide a measurement of fungal biomass because it is the primary membrane sterol of ascomycetes. Areas of visible mold damage can be directly measured by ergosterol concentration in indoor environment (Dillion *et al.*, 2007). However, this method does not provide any qualitative information (Miller, 2001).

More recent studies have shown that the design of DNA primers and probes and production of monoclonal antibodies (mAbs) are the possible ways to measure the exposure. Pietarinen *et al.* (2008) reported a study using quantitative PCR (qPCR) method to analyze fungi and bacteria in building materials. A total of 184 different types of building materials were collected and both culture plates and qPCR were used to measure the presence of different fungal species. The results have shown that the prevalence of fungi measured by qPCR was much higher than culture-based prevalence for almost all fungal species, except *Acremonium* species and *A. versicolor*. There are several advantages to qPCR: 1) it is a more suitable assay for slow growing fungi such as *Stachybotrys*; 2) it can detect uncultivable and non-viable fungi that have lost their activity because of their short half life; 3) it could be a good method to detect fungi that are difficult to grow (Pietarinen *et al.*, 2008).

In our lab, we have taken the approach of looking for fungal components that are antigenic in humans from building-associated fungi as a tool to assess exposure. Experience with other building-associated allergens suggests that a reliable way to assess disease associated with fungi in the environment would be to develop "standardized methods for assessing exposure to fungal allergens, preferably based on measurements of allergens" (NAS, 2000).

An example where the measurement of an allergen for exposure assessment of fungi (as suggested by the NAS report quoted above) can be seen in a comparison of different methods for exposure assessment to *A. fumigatus* in relation to ABPA (Dillion *et al.*,

2007). Air samples were collected from a wood chip recycling plant. There were four major techniques being used in this experiment: viable spore counts, qPCR, ergosterol and Asp f1. Asp f1 is a major allergen of *A. fumigatus*. The results indicated that viable spore counts did not provide quantitative results. QPCR did not provide reliable information because it did not relate to any of the traditional measurements used. Ergosterol and Asp f1 were two quantitative methods that showed promise. This special situation, the majority of ergosterol (96%) was from *A. fumigatus*. Asp f1 showed a high correlation with viable *A. fumigatus* propagule counts and in this case, the disease-causing agent.

Different fungi could grow on different building materials. Miller *et al.* (2008) studied the occurrence of fungi that grow on different damp building materials in North America. Gypsum wallboard contains different concentration of calcium and other salts depending on the gypsum ore, so all the fungi that can grow on wallboard are salt tolerant. Water activity, nutritional differences and concentration of calcium salts are the three factors that determine the species of fungi that grow on wallboard. There are also various fungi that can grow on different wood product and ceiling tiles. The growth factor is cellulose based, so different species of fungi may be found depending on the cellulose concentration (Miller *et al.*, 2008).

In the collection of data from four large environmental labs (two in Canada and two in the USA), *Aspergillus versicolor* was "common" on insulation, textiles, ceiling tiles, paper-faced gypsum wallboard, and manufactured wood. This is the only fungus that was

listed as common in all but one of the substrates studied (missing only from analyses of wood; Miller *et al.,* 2008). *A. versicolor* was also very common on moldy building material samples in Denmark and the U.K. (Flanningan & Miller, 2001; Gravesen *et al.*, 1999).

7.7 Water activity

The growth of a microorganism depends on the availability of nutrient and the water level in a material (Flannigan and Miller, 2001). Water activity (a_w) is used by biologists to express the availability of the free water which is loosely held by molecular interaction as well as the absorbed water weakly held in spaces. The definition of a_w is "the ratio of the vapour pressure exerted by water in the (hygroscopic or porous) material to the vapour pressure of pure water at the same temperature and pressure" (Flannigan and Miller, 2001). Each organism has its unique minimum a_w and optimum a_w for growth. The filamentous fungi which are generally associated with indoor air problems could be classified into four groups according to different a_w at 25°C: hydrophilic ("water-loving", $a_w > 0.90$), mildly xerophilic (mildly favors dry condition, minimum a_w: 0.80-0.09), moderately xerophilic (moderately favors dry condition, minimum a_w: 0.75-0.79) and extremely xerophilic ("dry-loving", $a_w<0.75$) (Flannigan and Miller, 2001). The representative species for hydrophilic species are *Stachybotrys chartarum* and *Chaetomium globosum*; for slightly xerophilic species are *Alternaria alternata* and *A. fumigatus*; for moderately xerophilic species is *A. versicolor* and for xerophiles is

Wallemia sebi (Flannigan and Miller, 2001). The optimal a_w of *A. versicolor* is 0.79 (Grant *et al.*, 1989).

7.8 Temperature

Similar to water activity, temperature is also very important for the growth of the microorganisms. Psychrophiles, such as some species of *Cladosporium* and *Penicillium*, are defined as those organisms that have an optimal growth temperature between 0-15°C and a maximum temperature of 20°C. Thermophiles are the microorganisms that could grow at a minimum temperature of 50-55°C. Most fungi found in building materials are classified as mesophiles which means that they are neither psychrophilic nor thermophilic. There are two different groups for mesophiles. The first group is called psychrotolerant mesophiles, which could grow at 0°C, but the optimal growth conditions are above 20°C. The second group such as *A. fumigatus* has a minimum growth temperature of 20°C, but is still capable of growing at 50-55°C.

Studies from Ayerst (1969) and Lacey (1984) have shown that xerophilic fungi have the lowest a_w when the temperature was closest to their optimal growth temperature. For example, the optimal growth temperature for *A. versicolor* was around 21-30°C (Smith, 1993). The minimum a_w for *A. versicolor* was around 0.79 at 18°C, but the minimum a_w had increased to 0.87 when the temperature was at 12°C (Flannigan and Miller, 2001).

A. versicolor can grow on wallpaper and gypsum board under condensing conditions. The water in building materials generally comes from condensation in the walls, in the basement as well as the roof space (Miller, 1992). Condensation forms when the warm air from the room comes in contact with the cold air outside. Hunter (1989) reported that different species of mold were collected from the kitchen wall surface during winter in the U.K. The major fungi from the wallpaper were *A. versicolor*, *Cladosporium*, *Ulocladium* and *Stachybotrys*. Air conditioning systems could cause similar problems.

7.9 Mycotoxins

It has been long known that molds are capable of producing mycotoxins that are toxic to human and animals. All known mycotoxins are the secondary metabolites produced by fungi. Secondary metabolites are the compounds produced by fungi that are not directly involved in normal growth and proliferation.

Nielsen *et al.* (2003) reported that the major mycotoxins produced by *A. versicolor* were sterigmatocystin and 5-methoxy-sterigmatocystin. International Agency for Research on Cancer (IARC) has classified sterigmatocystin as a class 2B carcinogen (Tuomi *et al.*, 2000) which means that it is probably carcinogenic to humans. Sterigmatocystin becomes carcinogenic when it has been activated by cytochrome P450 mono-oxidase in the liver. Sterigmatocystin also inhibits the movement of tracheal cilia (Nielsen *et al.*, 2003).

Sterigmatocystin is produced at a high concentration on Czapek yeast autolysate (CYA) and 2% amlt agar. On yeast extract sucrose (YES) agar, *A. versicolor* could produce versicolins as well as a small quantity of sterigmatocystin. Larsen and Frisvad (1994) reported that 50% of the isolate from wallpaper paste contained sterigmatocystin. At high water levels when aw is >0.9, *A. versicolor* produces both 5-methoxy-sterigmatocystin and sterigmatocystin with a concentration of 7 and 20μg/cm^2 respectively. In the report of Engelhart *et al.* (2002), 20% of household dust samples contained sterigmatocystin with a concentration up to 4ng/g. In the study of Gravesen *et al.* (1999), 72 mold-infected building materials were collected from 23 different water damaged buildings in Denmark. Twenty-three samples contained *Aspergillus*, 19 contained sterigmatocystin.

7.10 Antibody production

Monoclonal antibody technology was first developed by Kohler and Milstein when they successfully fused B lymphocytes with immortal myeloma cells in 1975 (Kohler and Milstein, 1975). The resulting hybridoma cells were capable of producing specific antibodies with infinite growth. Due to their generally high specificity and long life, mAbs from mouse ascites are produced in this study for human antigenic protein from *A. versicolor*. The general procedure for mAb production is discussed in the following section.

Mice are repeatedly immunized with target antigen through the tail veins every 2-3 weeks until the desired antibody titer is reached in the serum (National Research Council, 1999). Then the mouse is euthanized and the spleen is removed. The cells from the spleen are used to fuse with myeloma cells for *in vitro* hybridoma cell production. Myeloma cells are immortal tumor cells of lymphocytes. Once the antibody producing spleen cells are fused with myeloma cells, the resulting hybridoma cells can have unlimited growth. The fusion needs to be completed at the presence of polyethylene glycol which can fuse the cell membranes of freshly harvested spleen cells and myeloma cells by co-centrifuging. The fused hybridoma cells are grown in hypoxanthine-aminopterin-thymidine (HAT) which is a selective medium that only allows hybridoma cells to grow due to the presence of aminopterin, an inhibitor for nucleotides synthetic pathway required for normal cell to grow (National Research Council, 1999). The fused cells are transferred into a 96 well plate that contains feeder cells from abdomal cavity of the mouse. The feeder cells can provide the growth factors required for hybridoma cells to grow (Quinlan and Kennedy, 1994). Then hybridoma cells are screened by direct ELISA against the target protein for the positive antigen binding hybridoma cells. The mAb-producing cell lines from each antigen binding hybridoma cell are isolated, cloned and then screened again. The second screening identifies the isotype of the clones (i.e. IgG or IgM), and ensures that the selected mAb producing cell lines are still active and capable of producing antibody, since not all cell lines can survive and keep producing the specific antibody (Campbell, 1991; National Research Council, 1999). The selected clones are then stored at -80°C for future use. The cell supernatants of this step are shipped back for testing. Once the

cell supernatants were tested, the selected supernatants were used to produce mAb either through mouse ascites (*in vivo*) or *in vitro* production.

7.10.1 *In vivo* production

High titer mAbs can be produced by mouse ascites fluid (*in vivo*). The desired antibody producing hybridoma cells are injected intraperitoneally to the mouse, and then the ascites fluids are collected three times after 1-2 weeks (Yokoyama, 2008). Since the beginning of mAb production, *in vivo* production of mAb was preferred because it is easy to produce; it can result in a high antibody concentration; it has a high success rate and it has a low cost per mg of mAbs production. However, there are some disadvantages. For example, mAbs from *in vivo* production contain various mouse proteins that sometimes require further purification. There are some ethical issues and concerns from animal welfare perspective due to the significant pain or distress in mice during mAb production. The *in vivo* method is also generally not suitable for large scale production of mAbs (National Research Council, 1999).

7.10.2 *In vitro* production

There are two general methods for *in vitro* production: batch tissue-culture methods and semipermeable-membrane-based system. Most hybridoma cells are grown in tissue-cultures media containing fetal bovine serum with approximately 50μg/mL of bovine immunoglobulin for batch tissue-culture method (National Research Council,

1999). Since bovine immunoglobulins might contaminate the mAbs and because of the concerns raised from animal welfare, there are some < 1% fetal calf serum (FCS) and serum free media available to support the growth of hybridoma cell lines. However, the majority of hybridoma cells take days to adapt to the low FCS media condition, and 3-5% of the hybridoma cells could never adapt to the low FCS media. The adapted cells are grown under standard tissue culture condition for 10 days and the mAbs are harvest from the medium. The antibody yield is usually very low (less than 20μg/mL). Batch tissue-culture methods are relative easy to perform and it has a low cost, but large amounts of tissue media need to be processed and purified to obtain a high concentration of mAb (Heidel, 1997; Peterson and Peavey, 1998; Vachula *et al.*, 1995).

The second method is called semipermeable-membrane-based system. It uses a barrier (either a hollow fiber or semipermeable membrane depending on the devices) to separate the cells and mAb from nutrients and waste products, so mAb could remain in a small volume at a high concentration in a different chamber, and the media could be removed without affecting the antibody. However, the dead cells could accumulate rapidly, and an expensive purification may be required (National Research Council, 1999).

The *in vitro* tissue-culture method is now widely used for mAb production as compared to the *in vivo* method because it reduces the use of mice and they are suitable for large scale production of antibody. However, there are a lot of problems associated with *in vitro* production of mAb. 1) Modern *in vitro* methods have increased the success rate to

over 90%, but there are still approximately 3-4% failures (Hendriksen *et al.*, 1996; Varmus, 1997). The mAbs could have abnormal growth and secretion or mutated antibody sequence as compare to *in vivo* production due to the absence of feeder cells from the mouse. 2) *In vitro* production may not be suitable for rapid and small scale screening for hybridomas since *in vitro* production is usually more expensive and time-consuming. Their bioreactivity *in vivo* cannot be tested due to the absence of mice. 3) In certain research, high concentration of the antibody is required. The antibody produced *in vitro* needs to be concentrated prior to use causing denaturation of antibody and loss of antigen binding activity since some antibodies such as IgM and IgG3 are both very susceptible to purification procedures (Underwood and Bean, 1985). 4) Certain antibody isotypes such as IgM and IgA which are very important for development of new vaccines against human diseases such as glomerulonephritis and Henoch-Schonlein vasculitis and glomerulitis often fail to grow under *in vitro* conditions (Poncet *et al.*, 1988; Varmus, 1997). 5) Immunoglobulin G could be glycosylated differently *in vitro* as compared to mouse ascites, which may alter the antigen-binding site and lose their biologic functions (Leibiger *et al.*, 1995). 6) *In vitro* production of mAb requires a high purity of hybridoma cells. However, if they are contaminated with yeast or fungi, mAb can still be produced *in vivo* to remove the contaminants (Stein, 1998).

7.11 Antibody isotypes

There are five types of immunoglobulins in human: IgG, IgM, IgA, IgD and IgE and they are glycosylated. The sugar moieties of the immunoglobulins can facilitate secretion from

the plasma cells, can increase the water solubility of the immunoglobulins and can protect the molecules from degradation (Steward, 1984). The major antibody isotypes for producing mAbs are IgG and IgM. Each class has different metabolisms. IgG is a major class of immunoglobulins that comprises 70% of the total immunoglobulins in human serum. It is found both in intra- and extra-vascular compartments (Steward, 1984). The molecular weight of IgG is approximately 150kDa. It is a major antibody for infections in infants because it is the only class of immunoglobulins that can cross the human placenta. There are four different subclasses of IgG: IgG1, IgG2, IgG3 and IgG4 and each subclass has slightly different numbers and positions of disulphide bonds. The structure of IgG was first determined by Porter (1959). He observed three major fragments of IgG from rabbit antibodies when they were incubated with proteolytic enzyme papain. He separated those fragments by ion exchange chromatography, one of the fragments could be crystallized (Fc, fragment crystallizable) and other two fragments were identical and had the ability to bind with the antigen (Fab, fragment antigen binding). The structure of IgG was later determined to have two light chains and two heavy chains which are connected by covalent disulphide bonds and hydrogen bonds (Steward, 1984). The structure is shown in Figure 2.

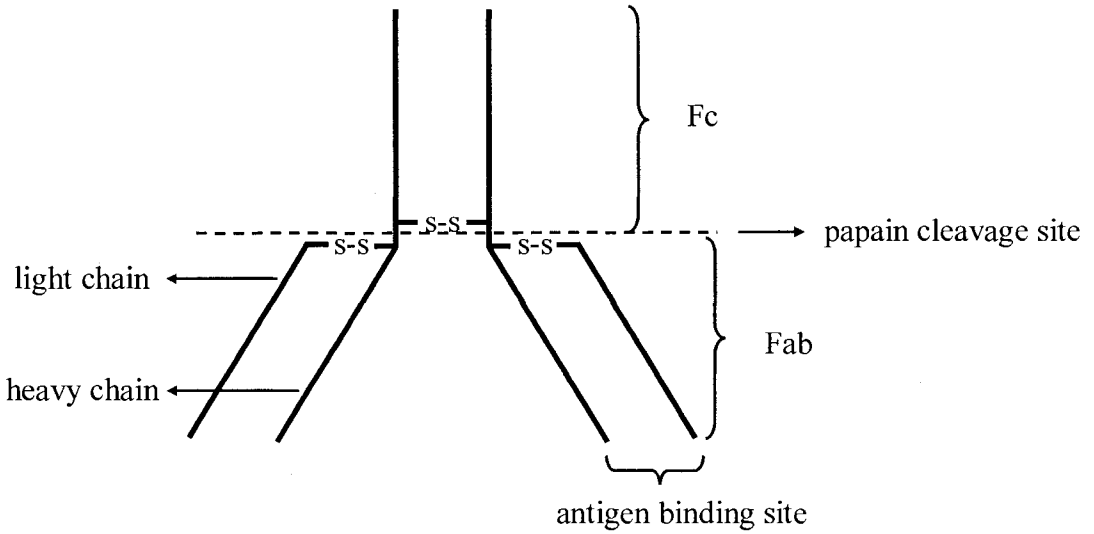

Figure 2 – A general structure for IgG (adapted from Steward, 1984)

IgM is distributed mostly in the intravascular compartment. IgM has a large molecular weight (approximately 900kDa) since it contains five IgG subunits, each is connected through disulphide bonds and has 2 μ chains (Figure 3). The dashed lines represent the disulphide bonds between different subunits. The shape of IgM was identified to be a circular pentameric molecule under the electron microscope. The whole molecule is stabilized by disulphide bonds and the presence of J chains. IgM have 10 potential antigen binding sites due to its unique structure. However, sometimes not all the antigen binding sites are available because of their small flexibility from their large molecular weight (Steward, 1984).

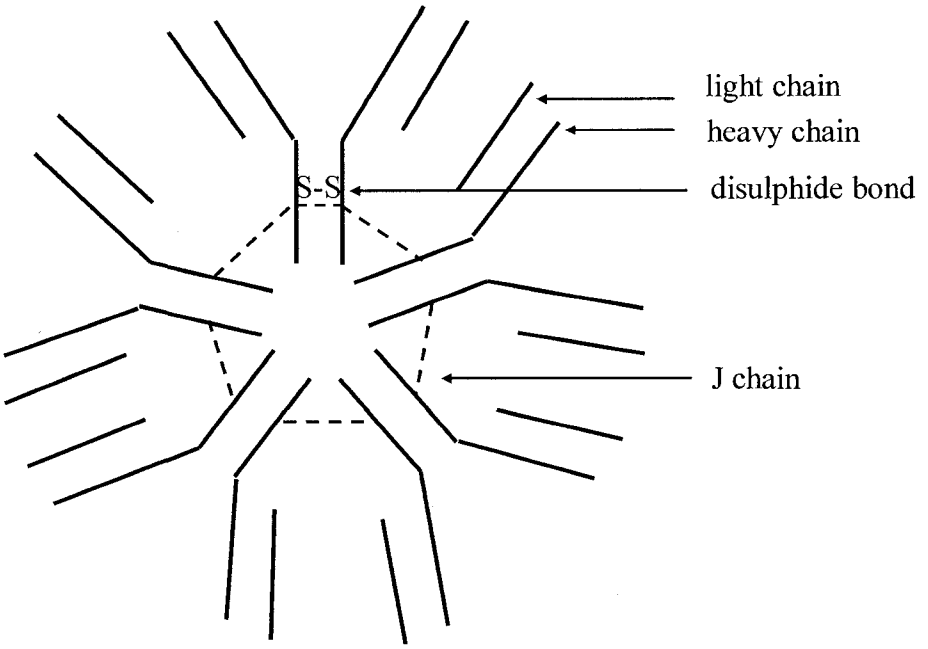

Figure 3 – A general structure for IgM (adapted from Steward, 1984)

7.12 Objective

The purpose of this study is to characterize the human antigenic protein found in *A. versicolor* (Zhao, 2006) and to develop species-specific mAbs against the *A. versicolor* antigen.

8 Material and Methods

8.1 Reagent

Product	Supplier	Catalogue #
3,3',5,5' – Tetramethyl-benzidine (TMB) liquid substrate system for ELISA	Sigma	T0440-1L
Acetic Acid	Anachemia	00598-468
Acetone	Fisher	A18P-4
Acrylamide/Bis solution	Bio-Rad	L16100156
Ammonium persulphate	USB	76322
Beads	Spex Certiprep	3112
BCIP/NBT-purple liquid substrate system for membrane	Sigma	B3679-100ML
Bio-Lyte 3/10 Ampholyte, 40%	Bio-Rad	163-1112
Bovine serum albumin	Sigma	A7030
Bradford reagent	Bio-Rad	500-0205
Bromophenol Blue	USB	12370
Calcium chloride dehydrate	Fisher	C-79
Carbonate-Bicarbonate buffer	Sigma	C3041-100CAP
CHAPS	Sigma	C-5070
Dithiothreitol	Bio-Rad	161-0610
D-(+)-Maltose monohydrate	Sigma	M5885
GelCode Blue stain Reagent	Pierce	24592
GelCode Glycoprotein Staining Kit	Pierce	24562
Gel filtration molecular weight markers	Sigma	MW-GF-200

12,400-200,000

Glycine	MP Biomedicals	808831
GTTP membrane filters, 0.2μm 26mm	Millipore	GTTP04700
HiPrep 16/60 Sephacryl S-300 HR gel filtration column	GE Healthcare	17-1167-01
Iodoacetamide	Sigma	I1149-5G
LMW-SDS Marker Kit	GE Healthcare	17-0446-01
Magnesium sulphate 7-hydrate	J.T.Baker	2504-01
Methanol	Fisher	A421-1
Microtitre plate	Nunc	439454
Peptone	Difco	211677
Peroxidase-conjugated affinipure F(ab')2 goat anti-mouse IgM, μ chain specific	Jackson Laboratories	115-036-075
Potassium chloride	Sigma	P9333-500G
Potassium phosphate monobasic	Sigma	P-5379
Protein G sepharose fast flow	GE Healthcare	17-0618-01
Protein IEF cell	Bio-Rad	165-4001
ReadyStrip IPG strips, pH 3-10	Bio-Rad	163-2000
ReadyStrip IPG strips, pH 3-6	Bio-Rad	163-2003
Rice, converted	Uncle Ben's	N/A
Sephacryl S-200-HR gel filtration resin	Sigma	S200HR-250ML
Skim milk fat free	Nestle Carnation	N/A
Sodium Chloride	Sigma	S9888-10KG
Sodium dodecyl sulphate	J.T.Baker	4095-02
Sodium phosphate	Fisher	S-374

Silver staining kit, protein	GE Healthcare	17-1150-01
Trizma base	Sigma	T1503-1KG
Trizma hydrochloride	Sigma	T3253-1KG
Tween	Sigma	P7949-500ML
Yeast extract	Difco	212750

8.2 Protein production, purification and detection

8.2.1 Culture production

The studied strain of *Aspergillus versicolor* used in this work was DAOM 235361. The copy of this strain was made by single spore isolation and was marked as *A. versicolor* 2-2b (AV 2-2b). This strain was identified by Dr. J. David Miller and confirmed by DAOM.

All the fungal strains were prepared in the same way as follows. Each strain was transferred onto 2% sterile malt extract agar (Difco, Lawrence, KS) slants or 2% malt extract agar plates. The slants and the plates were incubated at 25°C in dark for one week and then stored at 5°C for further inoculation.

Enniatin medium has a high yield of proteins and peptides (Traber *et al.*, 1989) and it was used to produce extracellular protein (e.g. Xu *et al.*, 2008). One liter of Enniatin medium contained 0.75g KH_2PO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.067g $CaCl_2 \cdot 2H_2O$, 8g peptone, 5g yeast extract and 50g maltose. This medium was added to 2.8L Fernbach flasks and 2.0L Erlenmeyer flasks using 560 mL medium and 400 mL medium respectively and autoclaved at 121°C for 12 minutes. A slant was macerated in sterile ultrapure water under aseptic condition and 5% (v/v) of this aliquot was used to inoculate each 2.0L and 2.8L flask at 2.5% v/v. The cultures were then placed on a rotary shaker at 220rpm and incubated for 48 hours in the dark at 25°C. The cultures in the 2L flasks and 2.8L flasks were harvested separately. The mycelium was filtered through cheese cloth. An aliquot of

the culture filtrate (1mL) was concentrated to 200μL and was saved at -20°C for silver stain and Western blot for protein purification analysis. The rest of culture filtrate was used to extract extracellular proteins. The mycelium was washed twice with ultrapure water and wrapped in aluminum foil and stored at -20°C. When the cells were frozen, they were freeze-dried.

Fungal spores were produced on rice cultures using a method similar to that of Murad *et al.* (1993). Uncle Ben's^{TM} converted long grain rice (30g) was placed into a 500 mL wide-mouth Erlenmeyer flask with 30 mL of ultrapure water. The rice was autoclaved for 30 minutes. The rice cultures were inoculated the same way as liquid culture medium, and then they were incubated in the dark for 3 weeks at 25°C. The foam plug of the flask was replaced to cheesecloth and the rice culture was air dried under the fumehood. The spores were then removed from the rice and stored in a glass vial at 4°C.

8.2.2 Extracellular protein extraction

After the culture supernatant was harvested, equal volume of cold acetone (-20°C) was slowly added into the culture supernatant to precipitate out some non-protein materials, resulting a 50% (v/v) acetone solution. This 50% acetone/culture filtration was left at -20°C over night to ensure complete precipitation. The non-target protein materials were removed the next day by centrifugation at 17,400 g for 10 min at -4°C. The pellet was discarded and the supernatant was further dissolved in an equal volume of cold acetone (-20°C), resulting a 75% (v/v) acetone/culture filtration. This acetone/culture filtration

solution was left at -20°C over night. The proteins were precipitated out and were removed from the supernatant the next day by centrifugation at 17,400 g at -4°C for 7 min. The protein pellet was left on ice in the fume hood for 1 hour to allow residual acetone to evaporate. The pellet was then dissolved in 50mM of Tris-HCl, pH 7.5 buffer. Salt precipitation was used as the next step with increasing ammonium sulphate (AS) saturation to further separate the target protein. Some undesired proteins can be removed from 55% AS saturation with constant stirring for 4-5 hours. Non-target proteins were removed from the pellet after centrifugation at 17,400g at 4°C for 15 minutes. More AS was slowly added into the supernatant to make up to 55-60%, 60-65% and 65-70% AS saturation respectively similarly as 0-55% AS precipitation. The pellets were dissolved into Tris buffer respectively and stored at -20°C. After 70% AS saturation was reached, the supernatant was removed and discarded. 55-60%, 60-65%, 65-70% AS precipitation were then dialyzed separately by dialysis tubing (molecular weight cut off 6-8kDa) and concentrated by 10 kDa cut off Amicon Ultra-15 centrifuge tubes (Millipore Corp., Billerica, MA) at 3,000g for further purification.

8.2.3 The determination of protein concentration

The Bradford assay was used to determine protein concentration. Different concentrations of the samples were diluted with ultrapure water and added to a 96 well Nunc-immuno MaxiSorp plate (Sigma, Oakville, ON). The optical density (OD) was measured at 595nm in a microplate spectrophotometer (Max340PC, Molecular Devices, Mississauga, ON). The desired concentration of the unknown protein samples gives an

OD between 0.2-0.3. Diluted protein sample (150μL) and 150μL ultrapure water (blank) were added into different wells on the plate. An equal volume of Bradford reagent (150μL) at room temperature was added into the protein samples and the blank, respectively. After 10 minutes, the OD was read at 595nm. The protein concentration was then determined from the standard curve of different concentration of BSA.

8.2.4 Ion exchange chromatography

The first step of protein purification was anion exchange chromatography. The glass column (1 x 17cm) was packed with 1.5 mL of Q-sepharose fast flow (GE Healthcare, Chicago, IL). All the buffers for ion exchange and gel filtration chromatography were filtered through 0.22μm syringe filter (Millipore corp.). The column was conditioned by washing with 200 mL of 50mM Tris buffer, pH 7.5 until the optical density (OD) was less than 0.05. Approximately 20-26mg of the 55-60% or 60-65% AS samples was applied on the column. The column was washed with more Tris buffer at flow rate of 1.5 mL/min until the OD was less than 0.05. An increasing NaCl concentration in Tris buffer was used to elute the proteins according to their different charges. Different molar concentrations of NaCl Tris buffer were used to elute the protein (0.14M, 0.16M, 0.18M, 0.2M and 0.25M for 2-2b). Each fraction was collected at 5 mL/tube for 30 tubes using ÄKTAprime™ plus fraction collector (GE Healthcare). The proteins from each fraction were then concentrated and desalted immediately using 10kDa cut off Amicon Ultra-15 centrifuge tubes as previously described. The purity of the target protein was examined

on silver stain and the Western response of the target protein was also examined after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

After washing with 1M NaCl Tris buffer, if residual proteins remained on the column, it was washed by 2mL of 1.0M NaOH, followed by 300mL of ultrapure water. Then 10mL of 70% ethanol, followed by additional 200mL of ultrapure water was used to further wash the column. The column was stored at 20% ethanol until the next use. The column could be regenerated by washing with 200mL of 50mM Tris buffer, pH 7.5.

8.2.5 Gel filtration chromatography

After anion exchange chromatography, there were only 3-5 major proteins left in the protein sample as shown on a silver stained gel. If the molecular weight of these proteins was further apart, the *A. versicolor* target protein could be further separated from gel filtration chromatography. The 96 x 1.6cm column was packed with Sephacryl S-200-HR (Sigma-Aldrich) resin. The column was conditioned by washing with 200mL 0.15M NaCl Tris buffer at a flow rate 0.1mL/min. The void volume was measured by Blue Dextran 2000 (~2000 kDa). The calibration curve for the gel filtration was established by using molecular weights 12kDa to 200kDa calibration kit (Sigma) with five different protein standard, cytochrome c (12.4kDa), carbonic anhydrase (29kDa), albumin (66kDa), alcohol dehydrogenase (150kDa) and β-amylase (200kDa).

The ion exchange materials were changed into 0.15M NaCl Tris buffer, using an Amicon Ultra-15 centrifuge tube. The concentrated ion exchange samples (0.5mL, approximately 1.5mg) were applied onto the gel filtration column. The proteins were then eluted by 0.15M NaCl Tris buffer at 0.1mL/min. The fractions were collected in 10mL test tubes at 1mL/tube by ÄKTAprime™ plus fraction collector. Different peaks were concentrated separately and visualized by silver staining and the protein response against the mAb was tested by Western blot after SDS-PAGE. The purified *A. versicolor* 41kDa protein was marked as (AVS 41kDa).

8.2.6 Preparation of spore samples

8.2.6.1 The number of spores

All the spore samples were counted with a microscope and a haemocytometer. The spores (1.00±0.02 mg) were weighed by a Mettler 163 analytical balance in a 500 μL plastic tube. The spores were suspended in a 100 μL of PBST. The spore suspensions were diluted 100x or 500x and applied onto the haemocytomer and number of spores was counted under Wild microscope (Switzerland) at 400 x.

8.2.6.2 Protein extraction from spores

The proteins from different spore samples were extracted as above. Approximately 1mg of the spores was weighted with an analytical balance in a vial with polystyrene bead. A Spex-Certiprep mixer mill (model 5100, Metuchen, NJ) was used to mill the spores for 30 minutes. The crushed spores (0.50±0.02 mg) were then weighted again with an

analytical balance in a 500 µL plastic tube. Tris buffer, pH 7.5 or TBST (50uL) was then used to dissolve the crushed spores. The freshly prepared spore extracts were used for Western blot and ELISA.

8.2.7 SDS-PAGE and silver stain/Commassie blue stain (CBB)

8.2.7.1 SDS-PAGE

The purity of the protein samples were tested by SDS-PAGE followed by silver stain/ CBB stain. SDS-PAGE was done by 10% acrylamide concentration 8.5 x 8cm vertical slab gels with 10-15 wells in Laemmli running buffer system. Different protein samples were first mixed with 5 x loading buffer with 4:1 (v/v) ratio. The samples were then boiled for 5 minutes and were immediately cooled down on ice for 5 minutes. The gels were placed into the electrophoresis tank (GE Healthcare) and the protein samples were carefully loaded into different wells. A low molecular weight marker from LMW-SDS Marker Kit (GE Healthcare) was run in a separate lane for future protein molecular weight determination. A Mini VE Vertical Electrophoresis System (GE Healthcare) with a Power Pac 1000 gel electrophoresis power supply (Bio-Rad, Hercules, CA) was used for SDS-PAGE. The proteins could be successfully separated under electrical current at 100V for 20 minutes and 200V for 1 hour or until the bromophenol blue dye reached 1cm from the bottom of the gel. When the gel was finished running, silver stain (for proteins ~0.5µg range) or CBB stain (for proteins ~5µg range) was used to detect the purity of the sample depending on the amount of the sample.

8.2.7.2 Silver stain

Fixing solution contained 100mL ethanol, 25mL glacial acetic acid. The solution was then made to 250mL with ultrapure water.

Sensitizing solution was made from 75mL ethanol, 10mL sodium thiosulphate and 17g sodium acetate. The solution was diluted to 250mL with ultrapure water.

Silver solution was prepared by diluting 25mL silver nitrate to 250mL with ultrapure water.

Developing solution consisted of 6.25g sodium carbonate in 250mL ultrapure water.

Stop solution was made by dissolving 3.65g EDTA-Na_2•$2H_2O$ into 250mL ultrapure water.

Silver stain was generally used throughout the experiment according to manufacturer's instructions; silver stain was a more sensitive staining assay compare to CBB stain. The different samples were loaded into wells on the gel and run under electrical current same as described previously. After the gel finished running, the gel was immediately placed into fixation solution for 30 minutes. The gel was switched to sensitizing solution with fresh 25% glutardialdehyde each time. After sensitizing, the gel was washed with ultrapure water for 3 times, each with 5 minutes. Silver solution was then added onto the gel with freshly added 37% formaldehyde each time for 20 minutes. The gel was washed twice for 1 minute each with ultrapure water. Developing solution with freshly made 37% formaldehyde was used to develop the color on the gel for approximately 3-5 minutes or until the proteins bands have reached the desired intensity. Stopping solution was added

at last to stop the reaction. After 10 minutes, the gel was then washed with ultrapure water and scanned with a GS800 densitometer imaging system (Bio-Rad).

The molecular weight of the desired protein was obtained by the molecular weight calibration curve from LMW-SDS Marker Kit (GE Healthcare). Five different native protein standards were used for the calibration: phosphorylase B (97kDa), albumin (66kDa), ovalbumin (45kDa), carbonic anhydrase (30kDa), trypsin inhibitor (20.1kDa) and lactoalbumin (14.4kDa).

8.2.7.3 Membrane CBB

Membrane CBB was used to obtain an Edman degradation amino acid sequence. The target *A. versicolor* protein after gel filtration was loaded on the 10% acrylamide gel. The gel after SDS-PAGE was transferred to a Hybond-PVDF membrane (GE Healthcare) with an electro transfer unit (Hoefer miniVE, Amersham Biosciences, U.K). Before the transfer, the membrane was first soaked in methanol for 1 minute and then in Towbin buffer (4:1 v/v ratio of 1x SDS running buffer and methanol) for 30 minutes. The blotting pads and the sponges used for transfer were also placed into Towbin buffer prior to the transfer. The gel and the membrane were both sandwiched between 4 sponges and 6 pieces of blotting pad. The proteins could be transferred onto the membrane at a constant current of 400mA for 30 minutes in an ice bath. When the protein transfer was complete, the membrane was removed from the transfer kit and placed in Gelcode Blue staining overnight. Then 50% methanol/water (v/v) was used the following day to destain the membrane. The destained membrane was kept in ultrapure water. Then the membrane

was scanned with a GS800 densitometer imaging system. The picture and the membrane were both sent to Sick Kids Hospital in Toronto, Ontario for Edman sequencing.

8.2.7.4 Immunoblot (Western blot)

After transfer, the low molecular weight protein marker was cut off and placed into GelCode Blue stain solution, and the rest of the membrane was placed into blocking solution (1% BSA/TBST, TBS buffer with 0.1% Tween-20) for 1 hour at room temperature. After blocking, different primary antibodies were added onto the membrane and incubated overnight on the shaker at 4°C. The membrane was then washed the next day with 3 x 10 minutes of TBST buffer at room temperature. Alkaline phosphatase (AP) conjugated anti-human IgG or AP conjugated anti-mouse IgM with a dilution of 2500x in 1%BSA/TBST was used as the secondary antibody. The membrane was washed again with 3 x 5 minutes of TBST buffer. BCIP (Sigma) was used to stain the membrane for a few minutes. After the color was developed, the membrane was rinsed with water and dried with blotting pads. The membrane was scanned with a GS800 densitometer imaging system.

The primary antibody for testing human antigen was human sera polyclonal antibodies. The crude mouse ascites were used for testing the isotypes of the different mAb. The antibodies were diluted 2000x with 1% BSA/TBST buffer. The Western response of five different cell supernatants was examined by purified *A. versicolor* protein. The supernatant of 2H12, 6E2 and 8A10 for mAbs was used as primary antibody for cross reactivity test against different fungal spore extract and different fungal culture

supernatant. The cell supernatant 8A10 was used to test the activities after each step of protein purification. These cell supernatants were diluted 500x and 1000x depending on the antibody concentration. During the antibody purification, each fraction of the mAb was diluted 1000x and was tested against AV proteins from 60-65% AS precipitation.

8.2.8 Characterization of the protein

8.2.8.1 Glycoprotein stain

Glycoproteins contain a sugar moiety and a polypeptide moiety which are connected through covalent bonds. Previous experiments have shown some proteins have lost their antigenicity after blocking the sugar moiety of the protein (Woodward *et al.,* 1985), so it is important to know whether the antibody binding is affected by the sugar component of the protein. Glycoprotein stain is a powerful technique to detect the sugar moieties on polyacrylamide gels. Glyprotein stain was done by glycoprotein staining kit (Pierce, Nepean, ON) according to manufacturer's instructions. Horseradish peroxidase (4μg) was used as a positive control, since it contains approximately 16% sugar residue. Soybean Trypsin Inhibitor (4μg) was used as a negative control. *A. versicolor* pure protein (4μg) was applied to test for the presence of sugar residue.

After gel electrophoresis, the gel was immersed into 25mL 50% methanol/water (v/v) solution for 30 minutes. After removal of the 50% methanol solution, the gel was washed with 3% acetic acid/water (v/v) 2 x 10 minutes. Then 25mL of the oxidizing solution was directly added onto the gel. After oxidizing step, washing with 3% acetic acid was carried

out 3 times, each for 5 minutes. The GelCode® Glycoprotein Staining Reagent (25mL) was added for 20 minutes. The staining solution was then removed and 25mL of reducing solution was added onto the gel to develop color for 5 minutes. The gel was then washed with 3% acetic acid for additional 5 minutes resulting magenta bands with a light pink background. After scanning, the gel was placed into GelCode Blue stain overnight, and the ultrapure water was used the next day to destain the gel. The gel was scanned with a GS800 densitometer imaging system.

8.2.8.2 Determination of the isoelectric point (pI)

Rehydration buffer (10mL) consisted of 9.41mL 8.5M urea, 0.2g Chaps, 76.7mg DTT, 50μL Ampholytes (Bio-Rad) pH 3-10 and 5μL 1% bromophenol blue.

Equilibration buffer I (20mL) contained 0.91g Trizma Base, 14.12mL 8.5M urea, 0.4g SDS, 4mL glycerol and 0.4g DTT.

Equilibration buffer II (20mL) was similar to equilibration buffer I, except 0.4g DTT was replaced with 0.5g Iodoacetamide.

All the pipette tips were autoclaved prior to use. The ReadyStrip IPG strip (Bio-Rad) with a pH range of 3-10 was first used to determine the range of pI for *A. versicolor* 41kDa protein. Then a narrower pH range of IPG strip (pH 3-6) was used to determine the pI point of *A. versicolor* protein. The IPG strip was left at room temperature for 15 minutes before it was placed into the rehydration buffer. The rehydration buffer (150μL) containing 7.5μg of *A. versicolor* pure protein was placed evenly onto the disposable rehydration/equilibration tray, and the IPG strip was immersed into the rehydration

buffer. After 60 minutes, the rehydration buffer was covered with 800μL of mineral oil and the tray was covered with lid and left at room temperature overnight. The electrode wicks were used to cover each side of the electrode on the focusing tray the next day. Each electrode wick was then covered with 10μL of ultrapure water. The mineral oil was carefully removed from the IPG strip from the rehydration tray. The anode of the IPG strip was placed at the anode of the focusing tray and 1mL of fresh mineral oil was placed on top of the IPG strip. The focusing tray was covered with the lid and was placed in the 90–240 VAC basic unit protein IEF cell (Bio-Rad). Three stages were used to focus the IPG strip at 20°C. The strip was first running at 250V for 15 minutes, and then it was increased to 4000V with rapid ramp for 2 hours for the second stage. After stage 2, the strip was running at 4000V until the voltage hour had reached 20,000V-hr. After the IPG strip finished running, the mineral oil was carefully removed and the strip was placed onto a new rehydration tray. The rehydration tray was covered with lid and wrap with plastic wrap and stored at -20°C overnight. The IPG strip was thawed the next day for 15 minutes. Then the strip was rinsed with ultrapure water, followed by 1mL of equilibration buffer I and II, respectively, each with 15 minutes. Bromophenol blue (1%, 100μL) was added into 1x SDS running buffer and was used to stain the IPG strip for 2 minutes. After staining, the strip was applied onto a 10% acrylamide gel. Low molecular weight marker was applied on a paper wick and placed beside the IPG strip. The gel electrophoresis was performed the same as previously described. The gel was then stained with silver stain.

8.3 Antibody production, purification and detection

8.3.1 Human polyclonal antibody

Human sera were used to test the Western response against *A. versicolor* partially purified protein from 60-65% AS precipitation. The human sera samples were obtained from ProGene Ltd., a large research clinical laboratory (Lenexa, KS). These sera were first screened by Pharmacia ImmunoCAP reagents for clinical diagnostic purposes for their response to various fungi. Once the test was complete, the positive sera were labeled and chosen to ship to Dr. Miller's Laboratory for research purposes. The positive human sera that were used to test *A. versicolor* allergen were QC 1294, QC1297, QC2397 and QC2398.

8.3.2 Mouse monoclonal antibody

The monoclonal antibody (mAb) was produced by Immuno-Precise Antibodies Ltd (Victoria, BC). This was done by injecting 10mg of the 42kDa purified *A. versicolor* protein from gel filtration into the mice. The cell supernatants were screened by dotted blot for their Western response against *A. versicolor* protein at Immuno-Precise and five of the best reacted supernatants were sent back to the Miller laboratory for further screening on Western blotting against the purified protein after gel filtration. The supernatants were made into different dilutions depending on their Western responses, from 100x to 1000x. After testing, ascites were made from the desired clones to obtain mAb, then were sent back to the Miller laboratory to test their sensitivity and specificity.

All the cell supernatant and mouse ascites were filtered through 0.45μm filters prior to use.

8.3.2.1 Affinity purification of IgM from HiTrap IgM purification HP

Both cell supernatant and the mouse ascites were used to perform antibody purification by HiTrap IgM purification HP (GE Healthcare) according to the manufacturer's instructions. Three different binding conditions were used for the antibody purification, 0.8M $(NH_4)_2SO_4$ with 20mM sodium phosphate, 1M $(NH_4)_2SO_4$ with 20mM sodium phosphate, and 0.5M K_2SO_4 with 20mM sodium phosphate, pH 7.5 (binding buffer). The mAbs solutions were prepared by changing the buffer to the binding buffer using 30kDa cut off Amicon Ultra-15 centrifuge tubes. The HiTrap column was prepared by washing 5 column volumes of binding buffer, 20 mM sodium phosphate, pH 7.5 (elution buffer) and 20 mM sodium phosphate, pH 7.5 with 30 % isopropanol (regeneration buffer). The column was equilibrated by washing with 10 column volumes of binding buffer. The mAb solution was then added into the column with a syringe. The column was then washed with 15 column volumes of binding buffer or until the OD was less than 0.05 at 280 nm. The purified IgM was eluted by 12 column volumes of the elution buffer. The column could be regenerated by 7 column volumes of the regeneration buffer. The column was re-equilibrated with 5 column volumes of binding buffer. Each elution fraction was collected in a test tube and concentrated by 30kDa cut off Amicon Ultra-15 centrifuge tube. The Western response was tested for each fraction. If the column was not used, they were stored in 20% ethanol/water (v/v).

8.3.2.2 Affinity purification of IgM from IgM purification kit

IgM purification kit (Pierce) consists of a 5mL immobilized Mannan Binding Protein (MBP) column and an optimized buffer system. The mouse IgM antibody was purified according to manufacturer's instructions. All the buffers were provided by the purification kit. The ascites were changed buffer to 20mM Tris, 1.25M sodium chloride; pH 7.4 using 50kDa cut off Amicon Ultra-15 centrifuge tubes. The ascites were concentrated to 0.5mL and were combined with 0.5mL of IgM binding buffer, pH 7.4. The MBP column was washed with 5mL of MBP column preparation buffer, pH 7.4 at room temperature. Then 20mL of IgM binding buffer was used to wash the column at 4°C. The cold ascites (4°C, 1mL) was added into the column, followed by additional 0.5mL of IgM binding buffer. The column was then sealed and incubated at 4°C for 30 minutes. The unbound proteins were removed by binding buffer at 4°C until the OD was less than 0.05 at 280nm. The elution step was performed at room temperature. Elution buffer (3mL), pH 7.4 was added into the column, and the column was sealed and incubated at room temperature for 2 hours. The eluate was collected at 1mL/tube. The elution fractions (OD >0.02 at 280nm) were pooled together and concentrated by 50kDa cut off Amicon Ultra centrifuge tubes. The concentrated antibody was then stored in 50% (v/v) glycerol/Tris buffer, pH 7.5 at -20°C. The Western response of the purified antibody was tested against AV crude proteins from 60-65% AS precipitation. MBP column was washed with 10mL of ultrapure water and followed by 10mL of IgM binding buffer after use. The column was then stored upright at 4°C.

8.3.2.3 Gel filtration chromatography

Since IgM antibody has a much higher molecular weight than majority of the proteins, HiPrep 16/60 Sephacryl S-300 HR gel filtration column was used as the purification procedure. The column was conditioned by 200mL of 0.15M NaCl Tris buffer at a flow rate 0.5mL/min. Culture ascites (2mL) of the mAb 2H12, 5G5, 5C9, 6E2 and 8A10 were filtered through 0.45μm filters (Millipore Corp.). The ascites were concentrated and changed buffer to 0.15M NaCl Tris buffer by 50kDa cut off Amicon Ultra-15 centrifuge tubes. The final volume was made to 500μL and the mAb was applied to the gel filtration column. The void volume was calculated by Blue Dextran 2000 (~2,000kDa). The fractions were collected at 1mL/tube. The elution peaks were concentrated separately and the Western response against AV crude protein from 60-65% AS precipitation (5μg/lane) was examined. The purified antibody was concentrated to 6.5mg/mL. The molecular weight was determined by the calibration curve made from gel filtration high molecular weight calibration kit (GE Healthcare). The high molecular weight calibration kit contained 5 different protein standards: thyroglobulin (669kDa), ferritin (440kDa), aldolase (158kDa), conalbumin (75kDa) and ovalbumin (43kDa).

8.3.3 Indirect enzyme linked immuno sorbent assay (Indirect ELISA)

All the ELISA was done on the 96 well microtitre plates. Each well contained 100μL of the diluted pure *A. versicolor* protein or the blank. All the experiments were repeated 3 times with duplicate data for each run. The protein standards were directly prepared in coating buffer 50mM pH 9.6 carbonate-bicarbonate (Sigma) in polystyrene microtitre

plate. The blank contained only 100μL of the coating buffer. The standard curve for most concentrated purified protein was 100ng/well, and they were half diluted across the plate. The standard curve for crushed AV spores was prepared in a similar way, except that the first well of each column contained 40μg of the spores. The cross reactivity tests contained 16 different fungal spore samples from 15 different species. They were coated onto the plate in a similar way except that only 3 different dilutions were made. The dilutions of the spores were normalized by the number of spores for each well for each species. After coating, the plate was sealed and placed on a plate shaker for 4 hours at room temperature. After 4 hours, the coating solution was removed and 200 μL of Blotto solution (1% skim milk/TBS) was added as blocking reagent. The plate was covered and blocked for 16 hours at 4°C. The blocking solution was then removed the next day and the plate was washed by TTBS (TBS solution with 0.05% Tween-20) by ELISA plate washer for 3 times each with 250μL of TTBS. The purified pAb from protein G column and the purified mAbs from gel filtration were diluted by Blotto solution and added into the plate. The pAb was diluted 1000x and the mAbs were diluted 600x. After 1 hour incubation on the shaker, the pAb and mAbs were discarded and the plate was washed again with ELISA washing machine by TTBS. Secondary antibody anti-mouse IgM HRP was diluted with Blotto solution 20,000x and was added into each well. After another hour of incubation, the secondary antibody was removed and the plate was washed with TTBS. Substrate TMB (100μL) was added into each well to develop the color. After 10 minutes, the reaction was then stopped by adding 50μL of 0.5M of H_2SO_4. The OD was read by ELISA plate reader at 450nm.

9 Results

9.1 Protein production, purification and detection

9.1.1 Purification of target protein from *A. versicolor* culture filtrate

A. versicolor liquid culture was harvested separately and marked as 2L and 2.8L, respectively. The culture supernatant was concentrated through 3x acetone precipitation. The 3x acetone pellet contained approximately 250 mg of crude proteins/batch. Different concentrations of AS saturation were then used to concentrate the proteins. An aliquot of the protein sample for each extraction step was saved and was tested on silver stain and Western blot for protein contents and the Western response. Approximately 4μg of crude proteins from each fraction was applied for the silver stain and the Western blot. The results are shown in Figure 4 and Figure 5.

From the silver stain, all the fractions except 75% AS from 2.8L flasks showed the presence of the target protein. AS fractions of 55-60%, 60-65% and 65-70% from 2L flasks contained fewer proteins and showed good response on Western blot. The fractions of 55-60% and 60-65% showed good response on Western blot for 2.8L flasks (Figure 5). These were pooled (80mg/batch) for further protein purification.

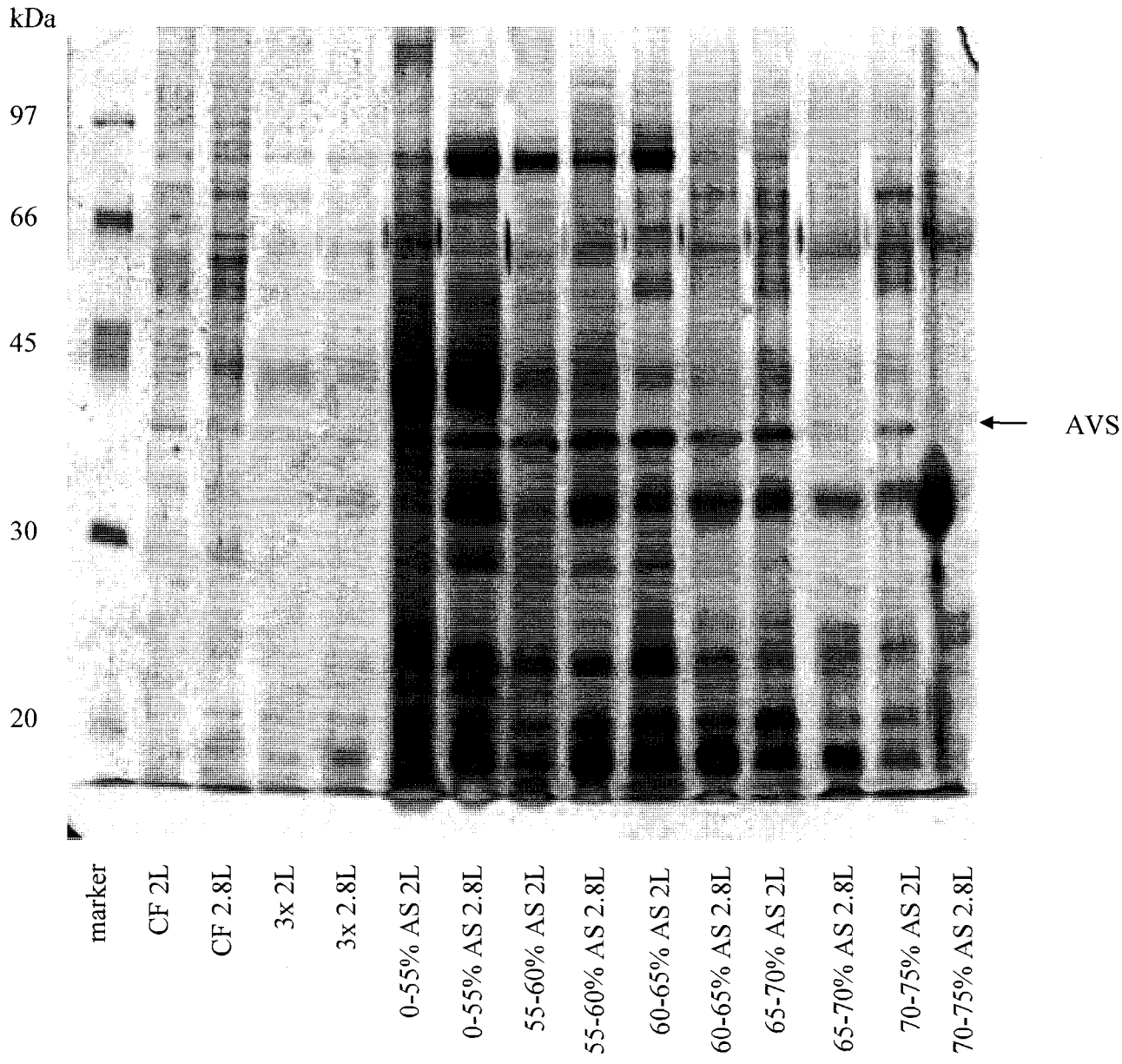

Figure 4 – Silver stain comparing the protein contents for different AS fractions, 4µg/lane of crude proteins, marker: LMW-SDS marker from GE healthcare, AVS: a protein from *A. versicolor* culture supernatant

CF: culture filtrate
3x: 3x acetone pellet
AS: ammonium sulphate

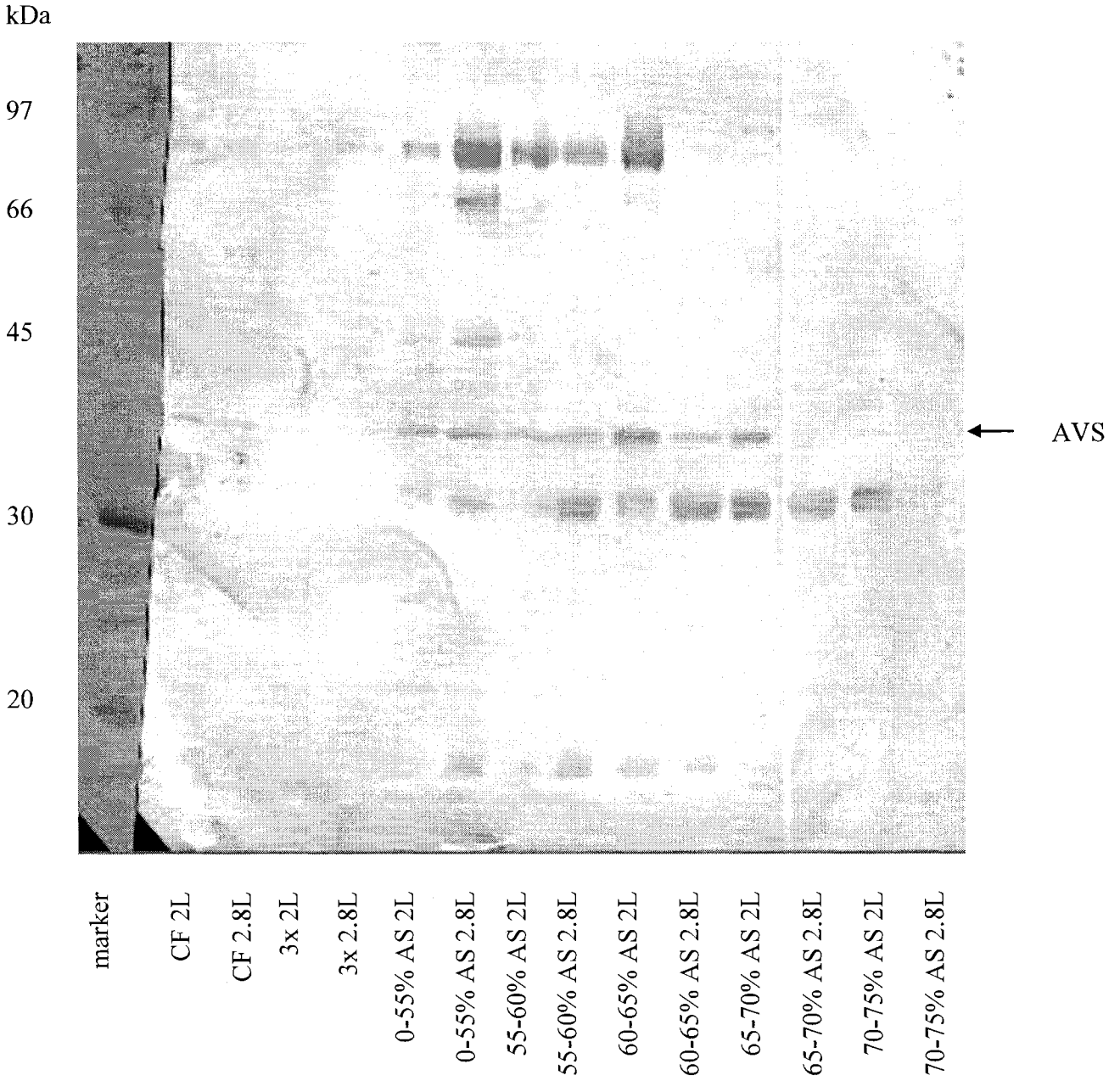

Figure 5 – Immunoblot comparing the response of AV protein for different AS fractions using cell supernatant 8A10 (1000x) as the primary antibody, 4µg/lane of crude proteins, marker: LMW-SDS marker from GE healthcare, AVS: a protein from *A. versicolor* culture supernatant

9.1.2 Ion exchange chromatography

AVS 41kDa was purified by anion exchange chromatography after AS precipitation. 55-65% AS was the sample before ion exchange. Approximately 20mg of the crude proteins was applied to the column for each run. All the fractions from the ion exchange column were collected and concentrated for the silver stain and Western blot. Four different NaCl fractions, 0.16M NaCl, 0.18M NaCl, 0.2M NaCl and 0.22M NaCl Tris buffer showed positive for AVS 41kDa protein shown in Figure 6 as indicated by the arrows. However, the majority of the target protein was eluted from the column at 0.18M NaCl and 0.2M NaCl Tris buffer as shown by the arrows in the Western blot (Figure 7). Since only three major bands were in these two ion exchange fractions and the molecular weight of these three proteins were approximately 30kDa apart, the semi-purified protein from 0.18M NaCl and 0.2M NaCl Tris buffer was suitable for gel filtration chromatography. These two fractions were concentrated and were stored at -20°C until the next step. Total amount of semi-purified protein was determined to be approximately 1.2mg/batch.

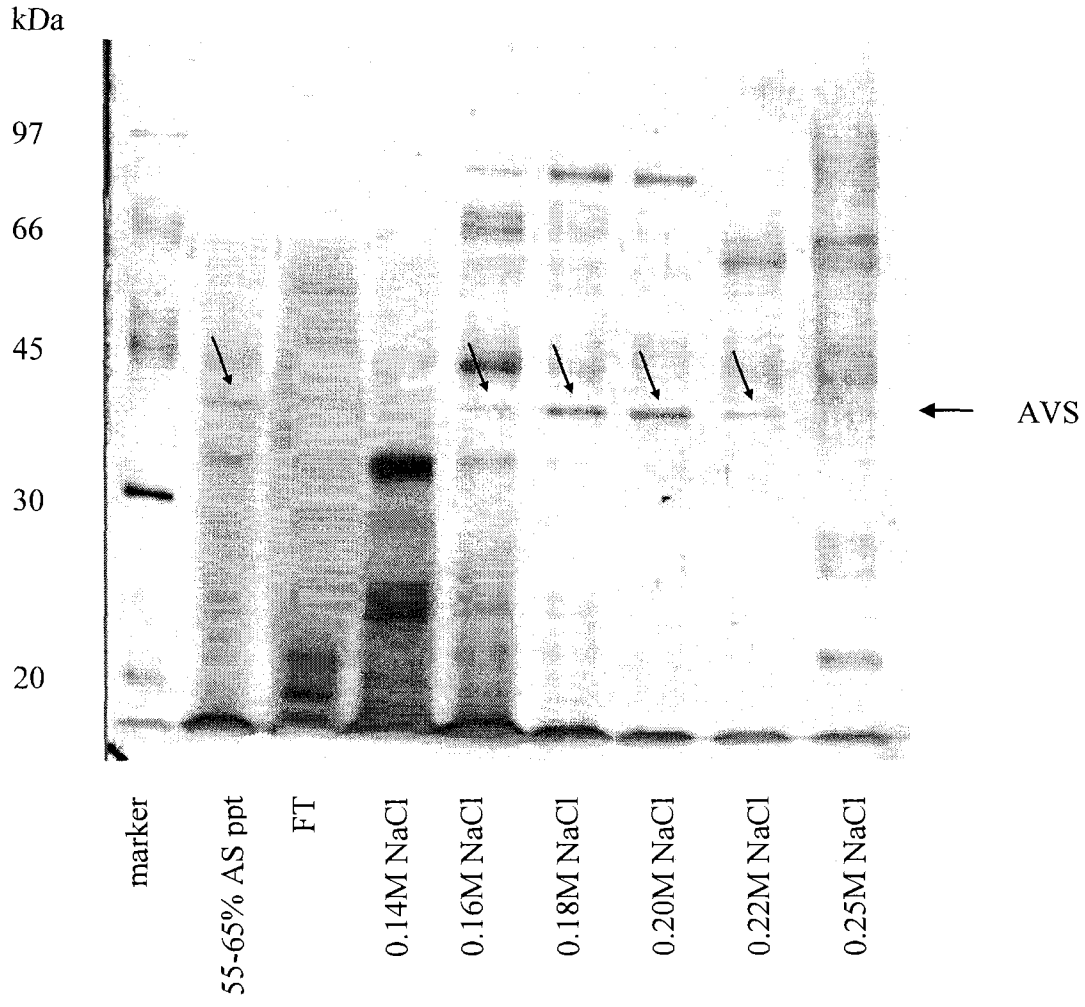

Figure 6 – Silver stain comparing the protein contents for different NaCl concentrations eluted from an anion exchange column, marker: LMW-SDS marker from GE healthcare, AVS: a protein from *A. versicolor* culture supernatant

FT: flow through

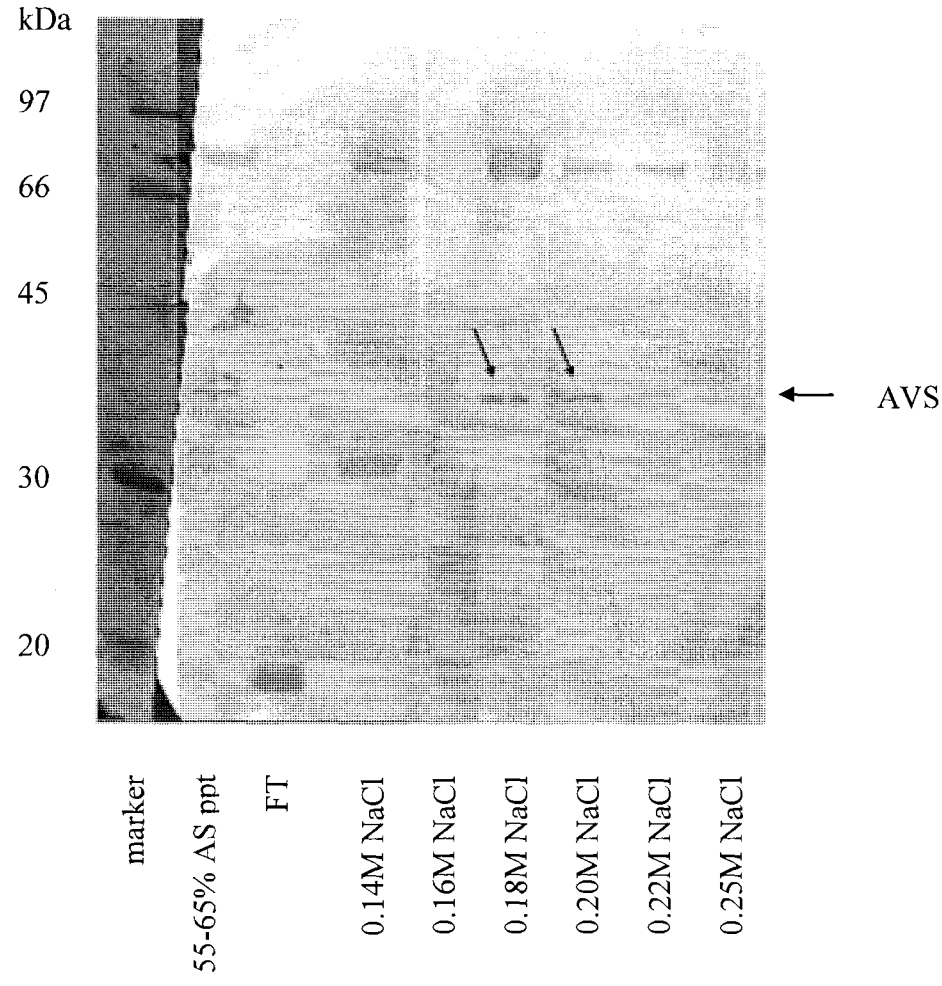

Figure 7 – Immunoblot comparing the response for different NaCl concentrations eluted from an anion exchange column against cell supernatant 8A10 (1000x), marker: LMW-SDS marker from GE healthcare, AVS: the target protein from *A. versicolor* culture supernatant

9.1.3 Gel filtration chromatography

All the fractions from 0.18M and 0.2M NaCl Tris buffer were pooled together and concentrated for gel filtration chromatography. The calibration curve for Sephacryl S-200R gel filtration column was done by using 6 different standards: blue dextran 2000, β-amylase, alcohol dehydrogenase, albumin, carbonic anhydrase and cytochrome c. Gel filtration column separates the proteins by their size, therefore the target AVS protein should be eluted between albumin (66kDa) and carbonic anhydrase (29kDa), i.e. between 73.44mL and 85.60mL. Table 1 showed the names, the molecular weights and their fraction volumes from gel filtration column of 6 standards. The elution fractions and the absorbance of each protein standard were shown in Figure 8. The number for each peak corresponds to the 6 protein standards from highest molecular weight to the lowest one as shown in Table 1. The linear calibration curve for logarithm of the molecular weight versus the fraction column is shown on Figure 9. The R^2 for the calibration curve was 0.9826.

Table 1 – The molecular weight of the standards for the calibration curve for Sephacryl S-200-HR gel filtration column

name of the protein	molecular weight (kDa)	log MW	fraction volume (mL)
1. blue dextran 2000	2000	3.301	37.84
2. β-amylase	200	5.301	62.24
3. alcohol dehydrogenase	150	5.176	67.19
4. albumin	66	4.820	73.44
5. carbonic anhydrase	29	4.462	85.60
6. cytochrome c	12.4	4.093	92.92

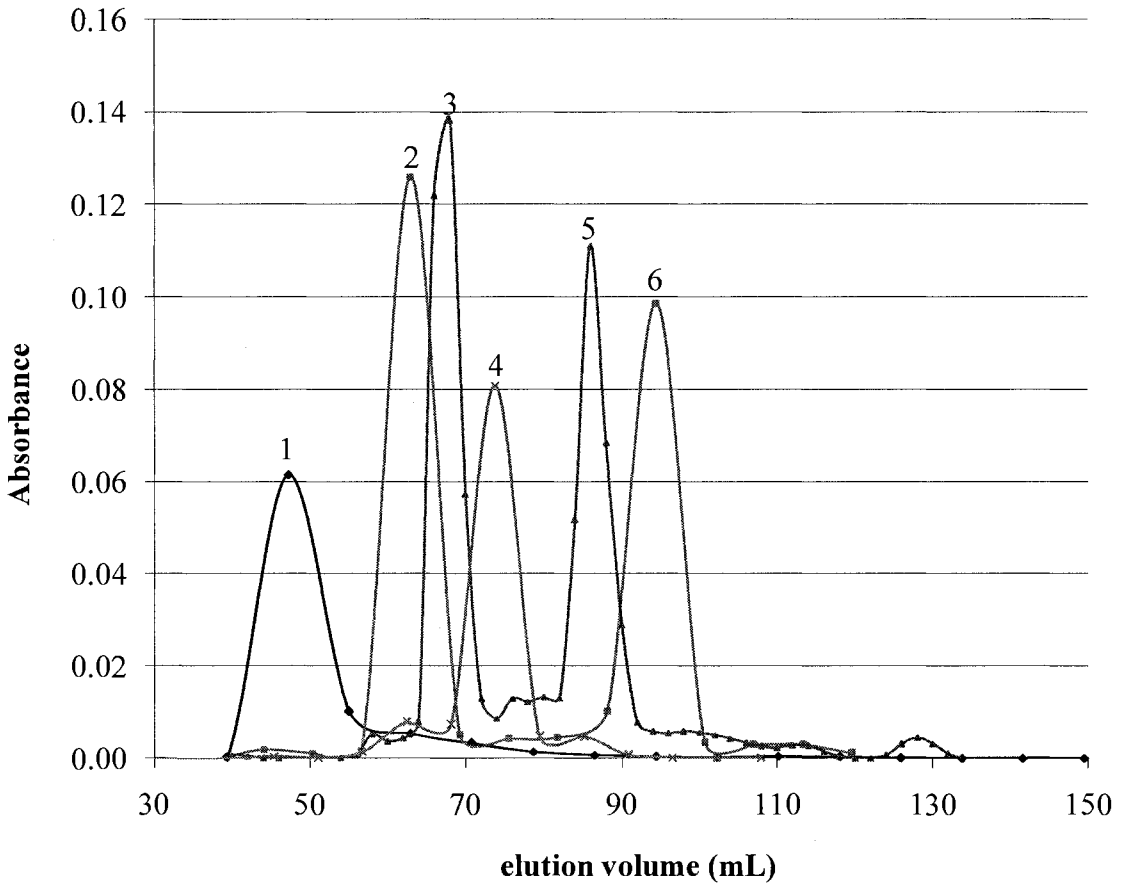

Figure 8 – The elution fractions for the protein standards for Sephacryl S-200-HR gel filtration column; peak 1 - 6 represents blue dextran 2000, β-amylase, alcohol dehydrogenase, albumin, carbonic anhydrase and cytochrome c, respectively

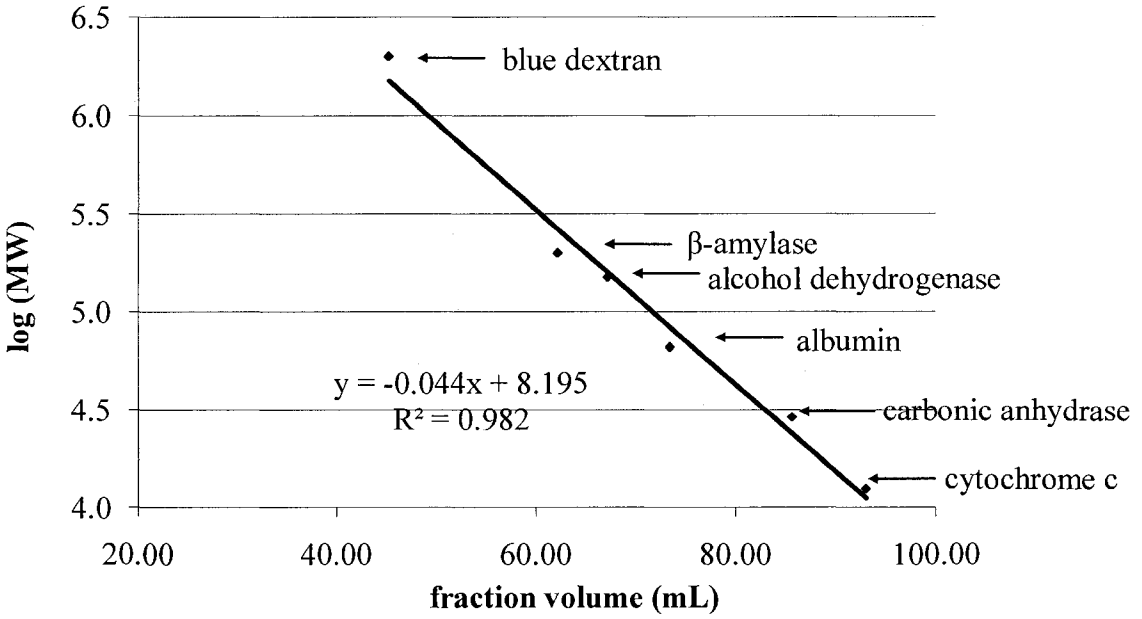

Figure 9 – Standard curve for Sephacryl S-200-HR gel filtration column from 6 standards

The gel filtration curve is shown in Figure 10. Five different fractions from gel filtration column were collected and concentrated separately by Amicon ultra 10kDa cut off centrifuge tubes. The elution volume for these fractions were 19-33mL (fraction 1), 34-44mL (fraction 2), 45-49mL (fraction 3), 50-55mL (fraction 4) and 56-68mL (fraction 5) respectively. The protein concentration was determined by Bradford assay. Silver stain (Figure 11) and Western blot (Figure 12) were then used to determine the protein contents and the Western response against cell supernatant 8A10 before and after gel filtration (2.5μg/lane of proteins). However, fraction 5 was not included because it did not have sufficient protein.

Fraction 3 and fraction 4 (Figure 11) both showed the presence of target protein (AVS 41kDa) on silver stain and they both had immunoblot response on Western as indicated by the arrows. However, fraction 3 contained above 80% of the target protein, and fraction 2 only contained less than 50%. Therefore, fraction 3 was selected as AVS 41kDa pure protein for Edman degradation sequencing, glycoprotein staining, 2D gel electrophoresis and the standard curve for ELISA. A total of approximately 30μg of AVS 41kDa pure protein was stained by membrane CBB (Figure 13). The membrane was then sent to Sick Kids Hospital for N-terminal sequencing. The partial sequence results were compared with the known homologous protein sequence from National Center for Biotechnology Information (ncbi) Swissprot protein sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, 2008) by Basic Local Alignment Search Tool (BLAST) algorithm (Table 2).

AVS 41kDa shared 69%, 62% and 62% identity with homologous proteins from three different Aspergillus species, *A. fumigatus* (34kDa), *A. flavus* (34kDa) and *A. oryzae* (34kDa). All three homologous proteins were predicted to be alkaline serine protease. Based on their complete protein sequence, the N-terminal sequence for all the mature protein starts at 122 amino acid (Yu *et al.*, 1999, Ramesh *et al.*, 1994, http://www.allergen.org/Allergen.aspx, 2008)

The protein recovery for each purification step for AVS 41kDa is shown in Table 3. The protein recovery for AVS 41kDa protein was 0.08% after gel filtration.

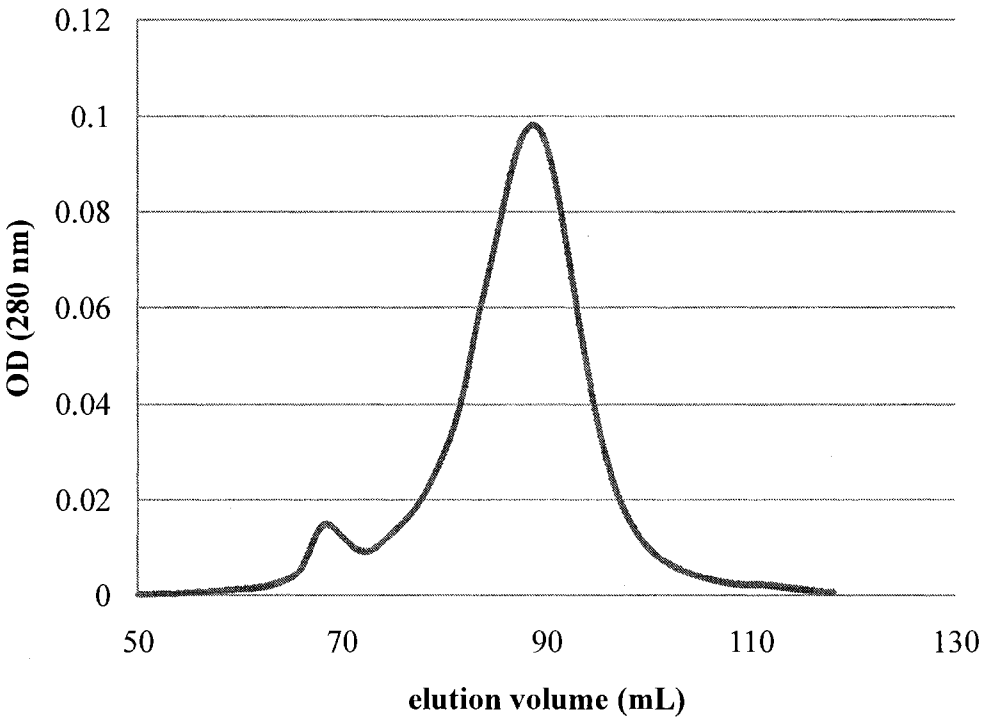

Figure 10 – The gel filtration curve for AVS 41kDa protein purification

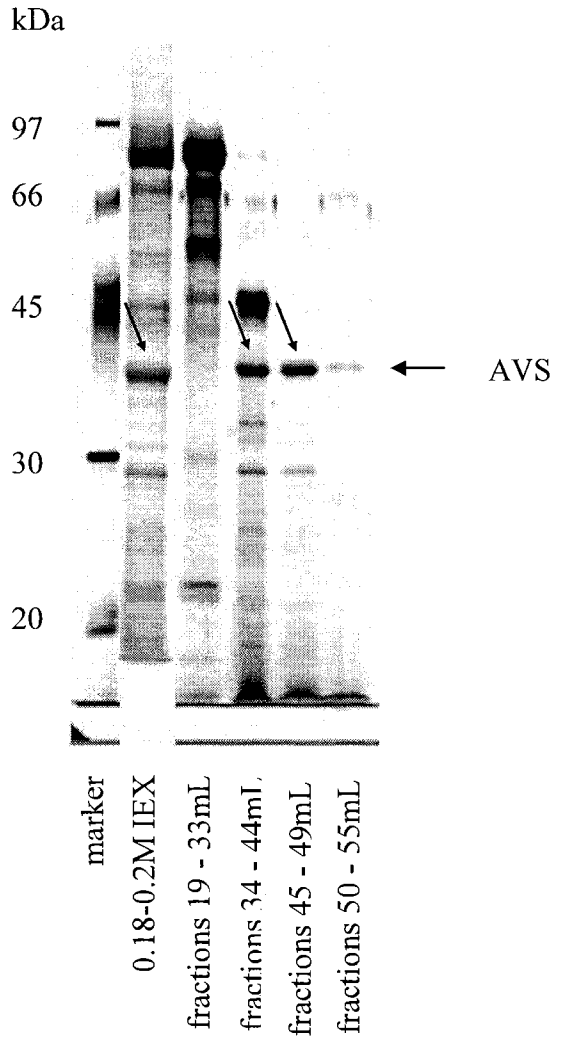

Figure 11 – Silver stain comparing the protein contents before and after gel filtration, 2.5µg/lane of AV proteins, marker: LMW-SDS marker from GE healthcare, AVS: the target protein from *A. versicolor* culture supernatant

IEX: ion exchange

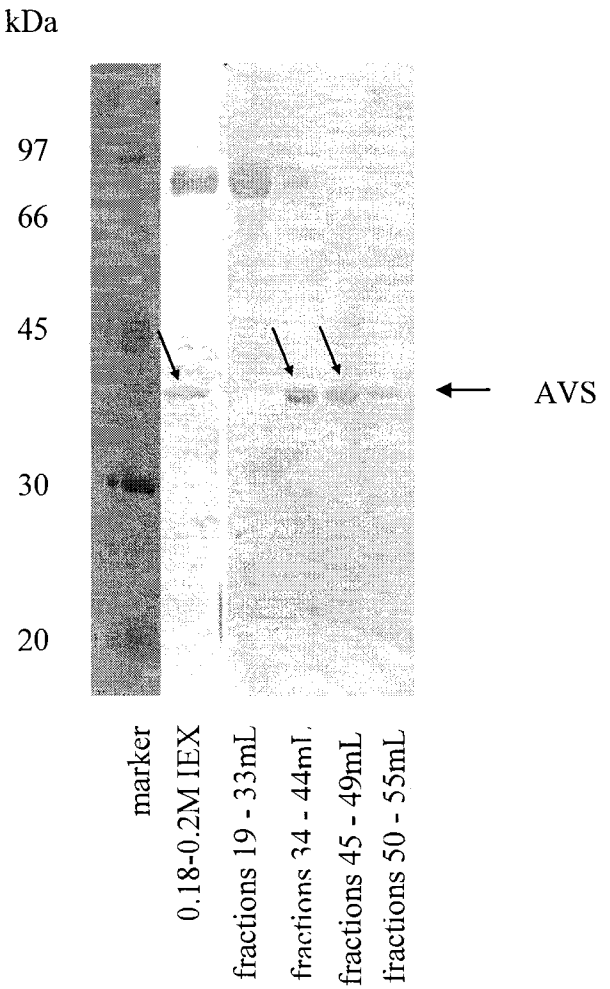

Figure 12 – Immunoblot comparing Western response for target protein before and after gel filtration, 2.5µg/lane of AV proteins, marker: LMW-SDS marker from GE healthcare, AVS: the target protein from *A. versicolor* culture supernatant

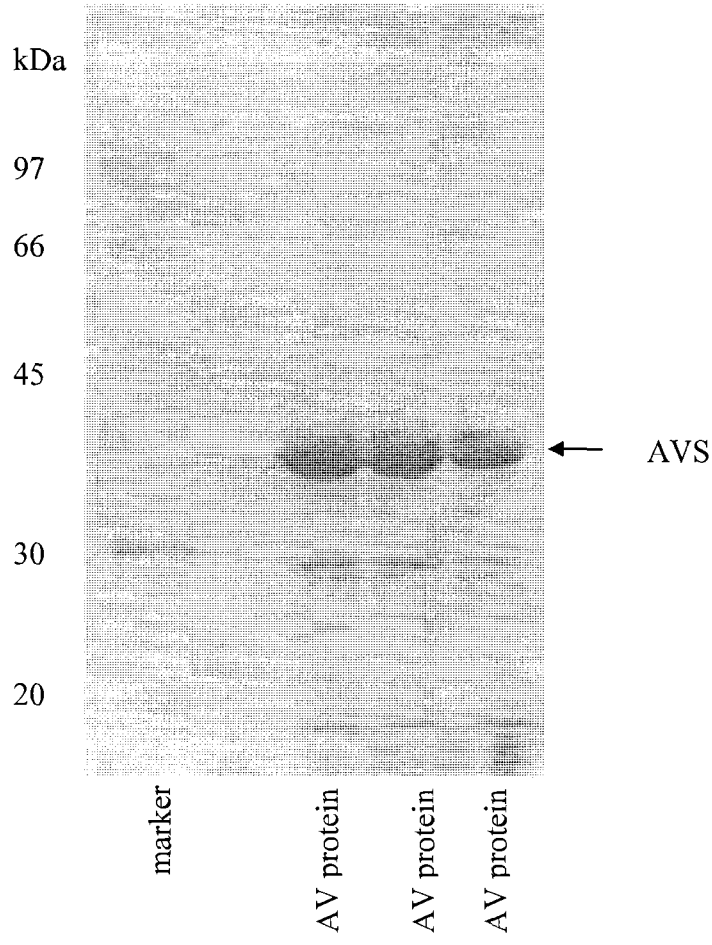

Figure 13 – Membrane CBB for Edman degradation, 20μg/lane of purified AV protein, approximately 10μg/lane of target protein, marker: LMW-SDS marker from GE healthcare, AVS: the target protein from *A. versicolor* culture supernatant

Table 2 – The N-terminal amino acid sequence result and blast search comparison with other *Aspergillus* species

protein	N-terminal amino acid sequence	identity with AVS 41kDa
A. versicolor 41kDa	ALTTQSDAPPGLGAISHQGDAS(/A)SSYI	100%
A. fumigatus 34kDa	ALTTQKGAPWGLGSISHKGQAS TDYI	69%
A. flavus 34kDa	GLTTQKSAPWGLGSISHKGQQS TDYI	62%
A. oryzae 34kDa	GLTTQKSAPWGLGSISHKGQQS TDYI	62%

Table 3 – The protein recovery for each purification step for AVS 41kDa

purification step	% recovery	amount of protein (mg)
3x acetone	100	250
AS precipitation	32	80
ion exchange chromatography	0.48	1.2
gel filtration chromatography	0.08	0.2

9.1.4 Glycoprotein stain for AVS 41kDa

Glycoprotein stain was used to detect the existence of sugar moiety on AVS 41kDa protein. The gel was stained with glycoprotein stain first (Figure 14B) and followed by gel CBB (Figure 14A). The positive control was horseradish peroxidase and the negative control was the soybean trypsin inhibitor. The positive bands (indicated by the arrows) for glycoprotein stain were horseradish peroxidase and an approximately 14kDa protein existed in the AV protein sample. Each lane contained 4μg of protein sample. AVS 41kDa and the soybean trypsin inhibitor were invisible on glycoprotein stained gel, which demonstrated that AVS 41kDa was not glycosylated.

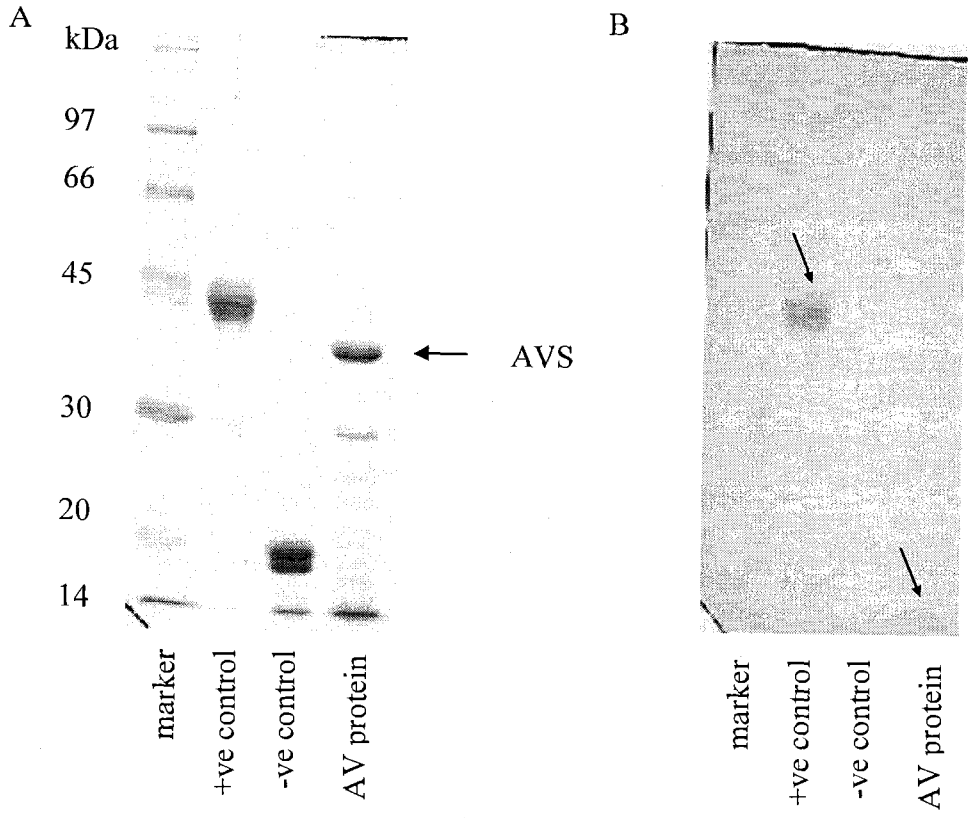

Figure 14 – CBB stain (A) and glycoprotein stain (B) for AV pure protein, 4 µg/lane of each sample, marker: LMW-SDS marker from GE healthcare, AVS: the target protein from *A. versicolor* culture supernatant

9.1.5 Determination of the isoelectric point (pI) for AVS 41kDa

A two dimension gel was used to determine the pH of AVS 41. The first dimension was isoelectric focusing which allows the proteins to separate by their pH horizontally. Then the proteins were separated according to their molecular weight vertically on SDS-PAGE. AVS 41 was indicated by the arrow on the gel. The pH range for 6μg of purified AVS 41kDa was determined by using a pH 3-10 IPG strip during isoelectric focusing step. The calculated pH for AVS 41kDa was around 4.4 (Figure 15). Then a narrower range IPG strip was used to determine the pH of AVS 41kDa. The pH for AVS 41kDa was 4.5, which indicated that AVS 41kDa was an acidic protein (Figure 16). This image was produced by using 7.5μg of purified AVS 41kDa. The molecular weight marker was shown on the side of the gel and pH standards were shown at the bottom of the gel.

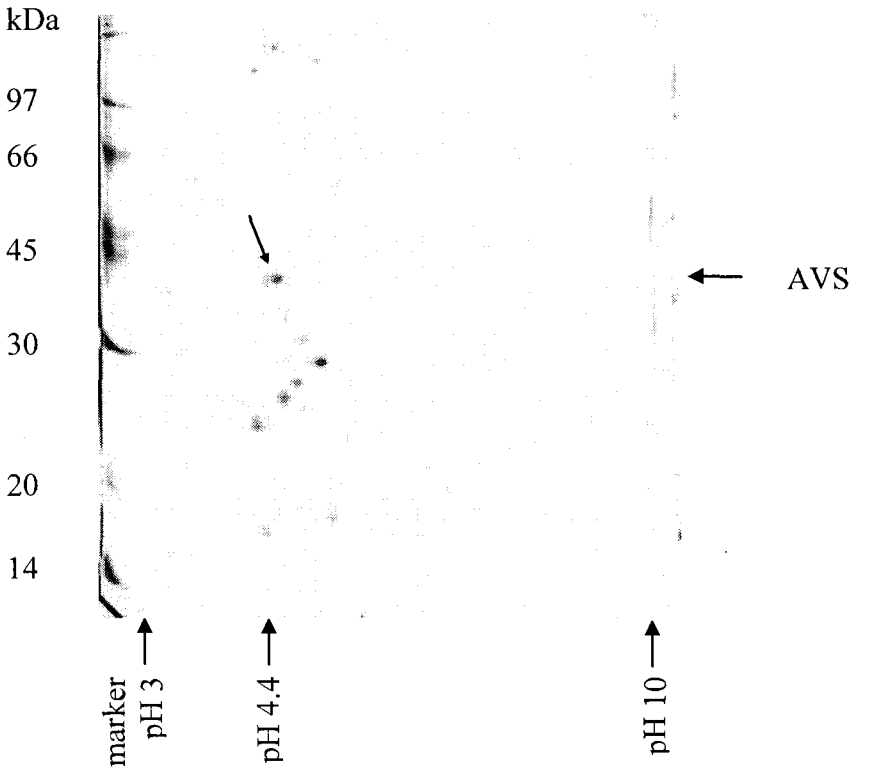

Figure 15 – 2D gel electrophoresis analysis of purified AV protein (6µg). The IPG strip of pH 3-10 was used and the gel was silver stained, marker: LMW-SDS marker from GE healthcare, AVS: the target protein from *A. versicolor* culture supernatant

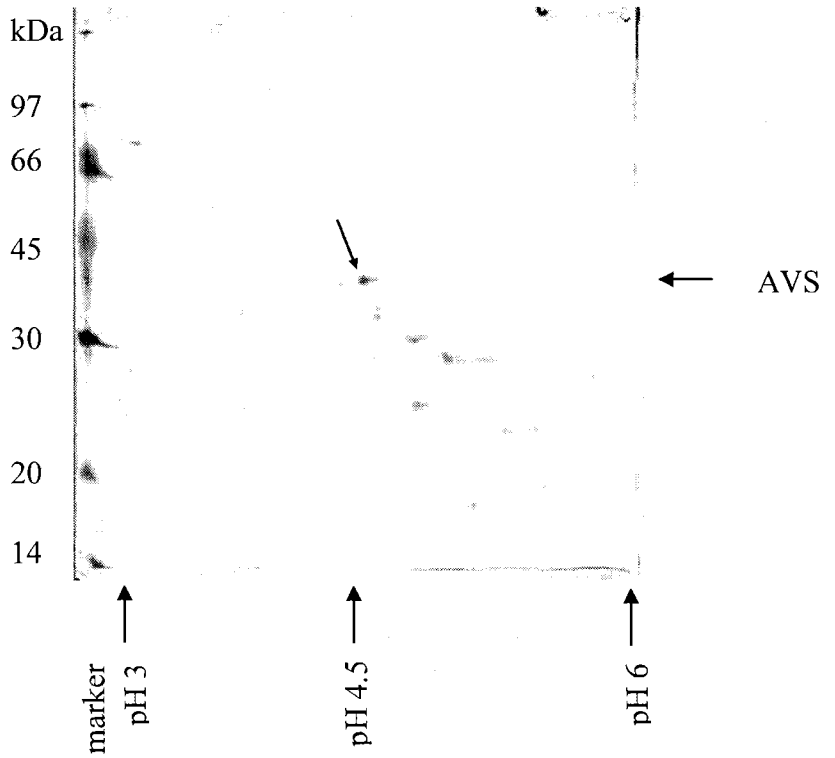

Figure 16 – 2D gel electrophoresis analysis of purified AV protein (7.5µg). The IPG strip of pH 3-6 was used and the gel was silver stained, marker: LMW-SDS marker from GE healthcare, AVS: the target protein from *A. versicolor* culture supernatant

9.2 Antibody production, purification and detection

Previous studies had shown that both 43kDa and 41kDa (AVS 41kDa) protein purified from *A. versicolor* liquid culture supernatant were antigenic to humans (Zhao, 2006). Ten mg of purified AVS 43kDa protein from gel filtration column was sent to Immuno-Precise Antibodies Ltd for mAb production in mouse. The mouse sera (Figure 17) and the mAbs (Figure 18) from the mice from Immuno-Precise were tested against AV crude protein from 60-65% AS precipitation and the cell supernatants (Figure 19) were tested against AVS 41kDa pure protein on Western blot. The mouse sera (last lane in Figure 17) showed the positive response to both AVS 41kDa and AVS 43kDa protein. The human sera, cell supernatants and the mAbs all showed positive responses to AVS 41kDa protein on Western blot. There was another protein at 33kDa (AVS 33kDa) that showed very similar Western response against human sera (Figure 17), cell supernatant (no. 9 in Figure 23, 24, 25) and the mAbs (Figure 18).

9.2.1 Test for human allergenicity of AVS 41kDa against human sera

In testing the human allergenicity of AV crude protein against human sera, mouse serum was used as the primary antibody for the positive control. Four selected human sera, QC1294, QC1297, QC2397, QC2398, were tested against AVS 41kDa crude protein for its allergenicity (Figure 17). All four human sera have evidently shown to be positive for AVS 41kDa protein as indicated by the arrows.

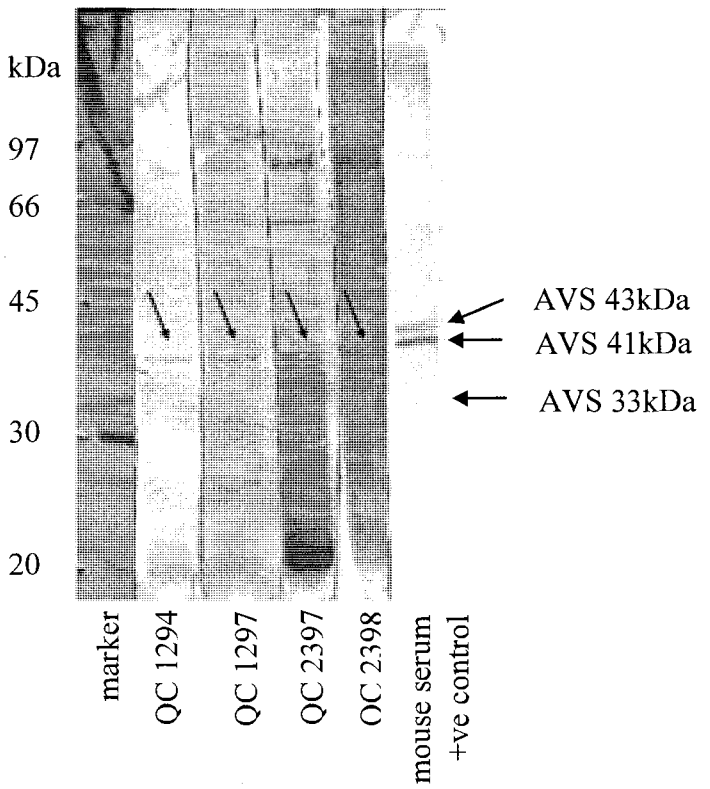

Figure 17 – Immunoblot for 4 selected human sera against AV crude protein from 60-65% AS precipitation, 5µg/lane of crude protein, marker: LMW-SDS marker from GE healthcare, AVS: the protein from *A. versicolor* culture supernatant

9.2.2 The determination of antibody isotype

Some of the 5 mAbs appeared to be IgM and some showed both IgM and IgG activity on ELISA (data from Immuno-Precise Antibodies Ltd). Therefore the antibody isotype was examined in this experiment by Western blot. Approximately 5μg/lane of crude AV protein containing AVS 41kDa protein from 60-65% AS precipitation was applied for each well. After the protein was separated on SDS-PAGE and transferred to a membrane, all 10 lanes were cut individually and were incubated separately with different primary or secondary antibodies (see Figure 18). The 1^{st} and 6^{th} lane was placed into mAb 2H12. Lane 2 and lane 7 were incubated in mAb 5C9. Similarly, the 3^{rd} and 8^{th} lane was placed into mAb 5G5, 4^{th} and 9^{th} lane was placed into mAb 6E2 and 5^{th} and 10^{th} lane was placed into mAb 8A10. Then the first 5 lanes were placed into anti mouse IgM μ chain specific secondary antibody and the last 5 lanes were placed into anti mouse IgG Fc_γ specific secondary antibody respectively. All 5 mAbs showed positive Western response against anti mouse IgM secondary antibody for AVS 41kDa protein. Two of the antibodies, 2H12 and 5C9 showed slightly positive Western response against anti mouse IgG secondary antibody. All the positive lanes are indicated by the arrows in Figure 18. Since all the mAbs were shown to be IgM isotype, anti mouse IgM μ chain specific secondary antibody was used for all other experiments.

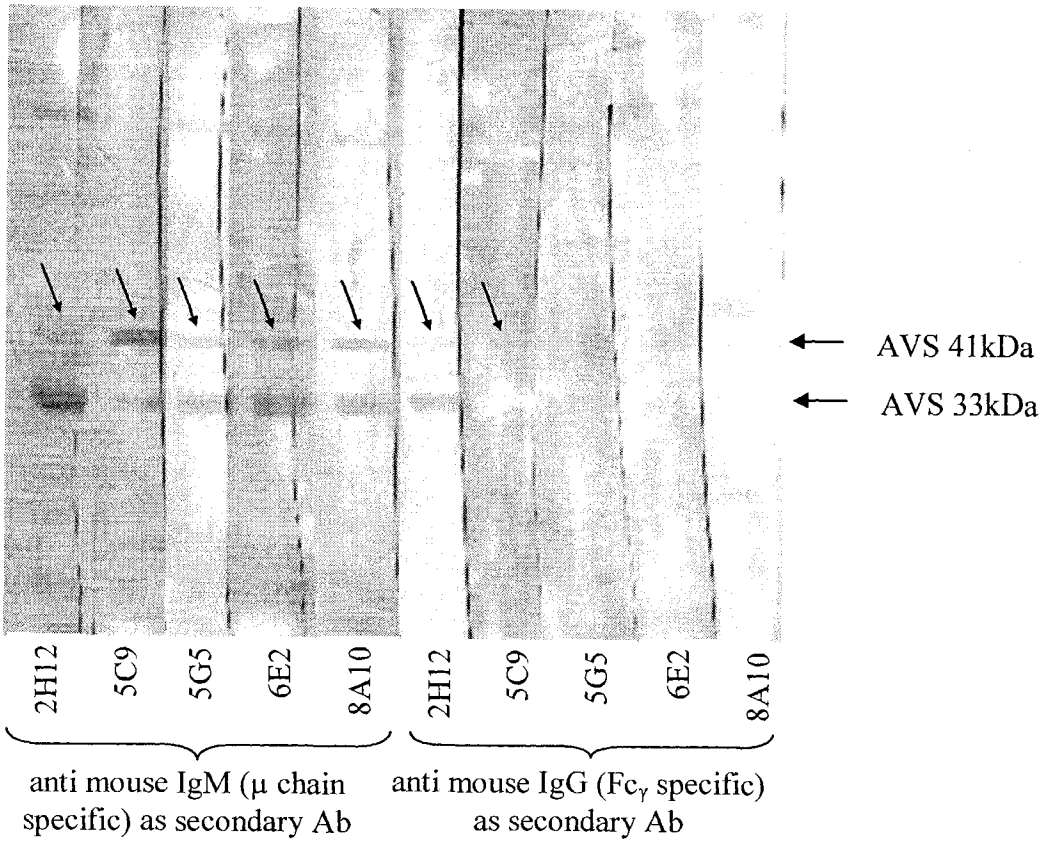

Figure 18 – Immunoblot of antibody isotype determination for mAb 2H12, 5C9, 5G5, 6E2 and 8A10, AVS: the protein from *A. versicolor* culture supernatant

9.2.3 Test for Western response of AVS 41kDa on 5 different cell supernatant against AV pure protein

Five different cell supernatants were tested against AV pure protein on Western blot at 500x (the first five lanes) and 1000x dilution (the last five lanes) for their immunoblot response. Three of these (i.e. 2H12, 6E2 and 8A10) showed positive to AV protein at 500x dilution and only 2H12 and 8A10 showed positive at 1000x dilution (Figure 19). Since only three of the antibodies showed good response on Western blot, these were chosen to test the Western response against different AV spores as well as cross reactivity for different fungal culture supernatants.

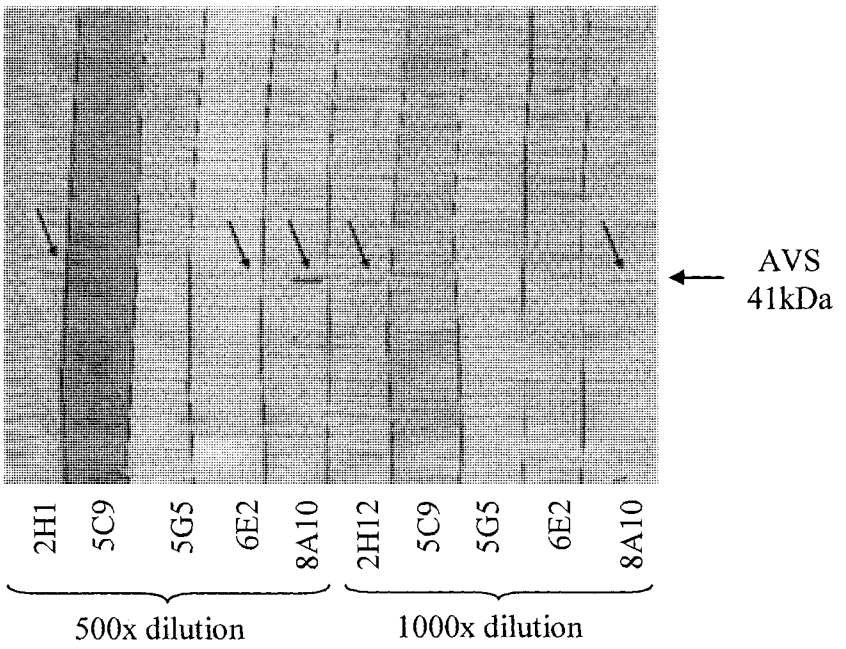

Figure 19 – Immunoblot for testing 5 different cell supernatants with 500x and 1000x dilution against AV pure protein (0.8µg/lane), AVS: the protein from *A. versicolor* culture supernatant

9.2.4 Test for AVS 41kDa Western response on 3 different cell supernatant against AV spores

Different AV spore fragments were dissolved in PBST and were used for examining the Western response against 3 cell supernatants. All tested AV spores (5μg/strain), except strain 1-1a showed to be positive against 2H12 (Figure 20), 6E2 (Figure 21) and 8A10 (Figure 22). AV crude protein from 60-65% AS precipitation was included at the last lane as a positive control. The positive bands for AVS 41kDa from different AV spores were indicated by the arrows. The positive response of AVS 33kDa only showed in the last lane containing AV crude protein, which indicated that AVS 33kDa protein was not common among different AV spores.

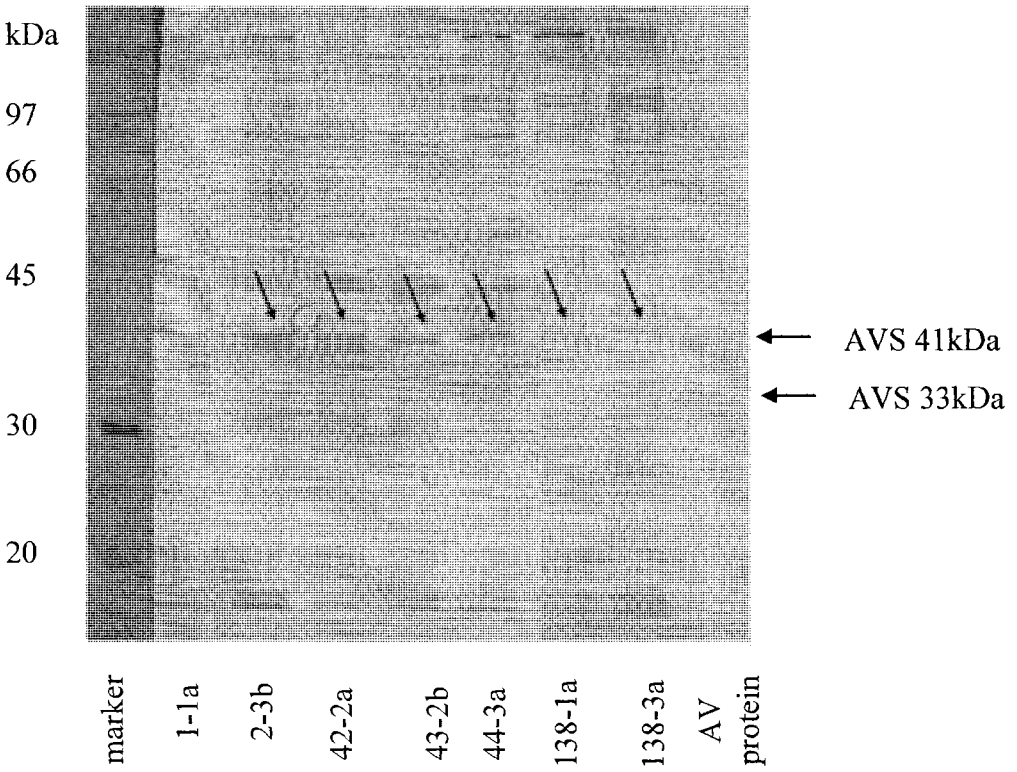

Figure 20 – Immunoblot of different AV strains from spores against cell supernatant 2H12, 50µg/lane of crushed spores, 5µg/lane of AV crude protein, marker: LMW-SDS marker from GE healthcare, AVS: the protein from *A. versicolor* culture supernatant

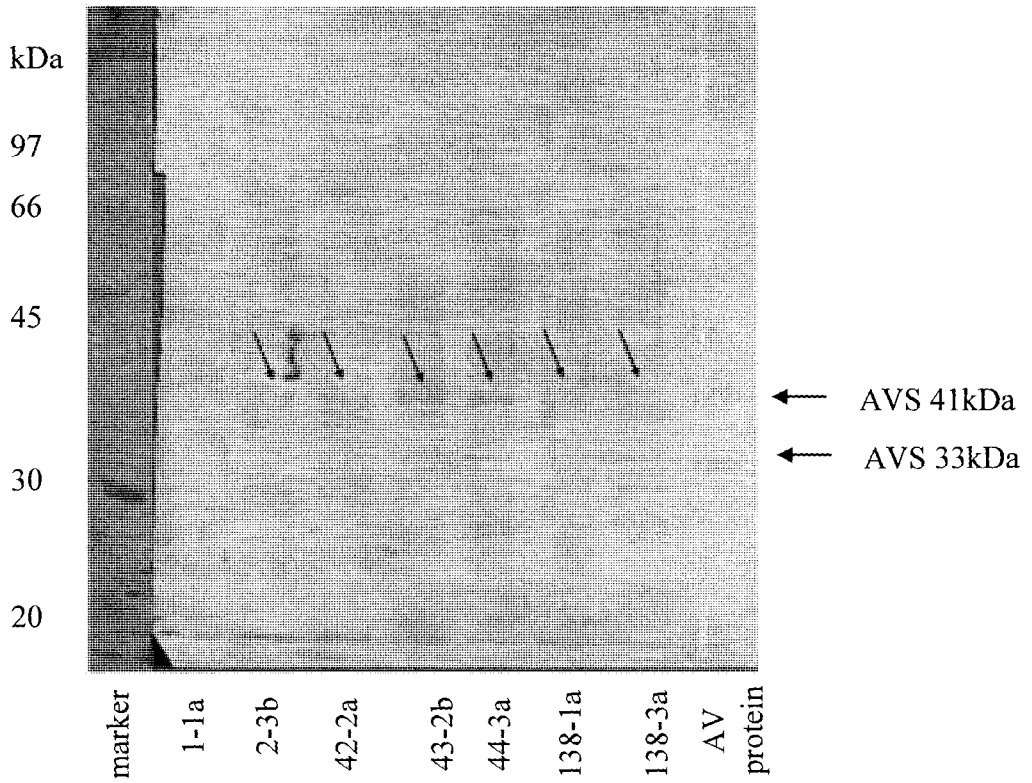

Figure 21 – Immunoblot of different AV strains from spores against cell supernatant 6E2, 50µg/lane of crushed spores, 5µg/lane of AV crude protein, marker: LMW-SDS marker from GE healthcare, AVS: the protein from *A. versicolor* culture supernatant

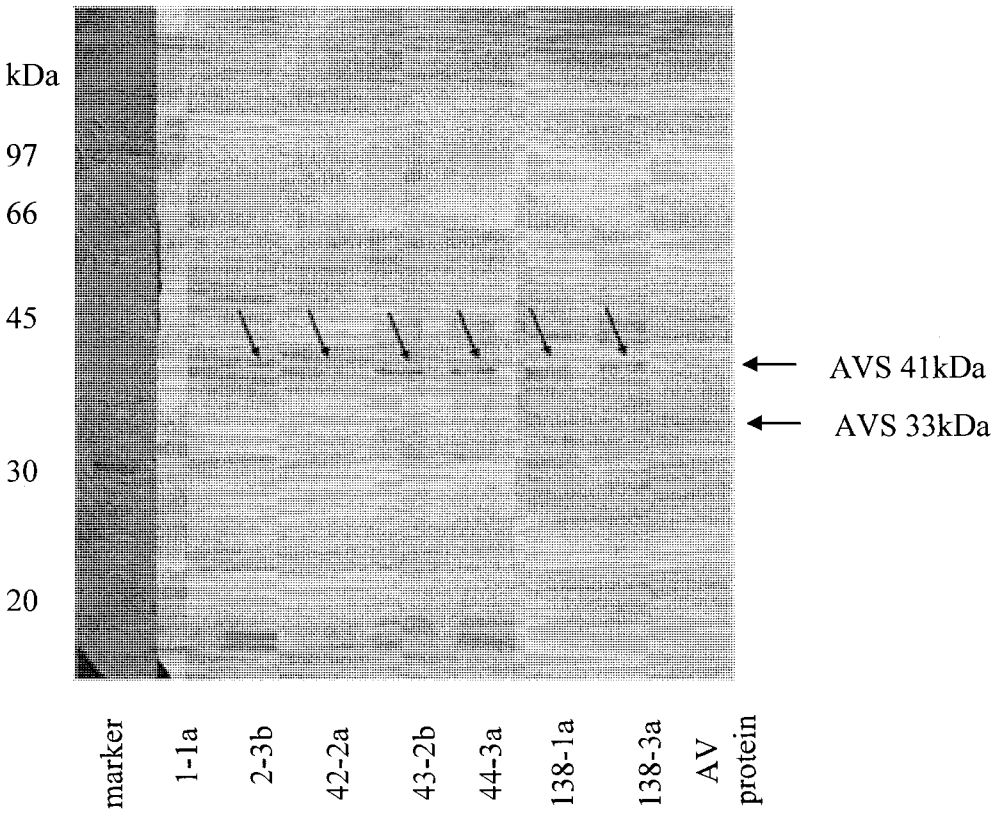

Figure 22 – Immunoblot of different AV strains from spores against cell supernatant 8A10, 50µg/lane of crushed spores, 5µg/lane of AV crude protein, marker: LMW-SDS marker from GE healthcare, AVS: the protein from *A. versicolor* culture supernatant

9.2.5 Cross reactivity test for different fungal culture supernatant

Different fungal cultures were grown as described for *A. versicolor* cultures. When sufficient cells were produced, the cultures were harvested and the supernatants were concentrated by 3x acetone. The 3x acetone pellets were dissolved in Tris buffer and were used for the cross reactivity test. The concentration for each culture supernatant was tested by Bradford Assay. Same amount of crude proteins (2.6μg) for each fungal species was applied into each lane to test the cross reactivity against 3 different cell supernatant from the mouse, 2H12 (Figure 23), 6E2 (Figure 24) and 8A10 (Figure 25). *A. versicolor* crude proteins were used as positive control (No. 9).

A list of fungal culture supernatants tested by immunoblot is from Table 4.

A. unguis, *Eurotium rubrum J9862-3m*, *Paecilomyces variotti 0424-8m*, *Scopulariopsis brevicaulis* showed possible cross reactivity for antibody cell supernatant 2H12.

A. sydowii, *P. crustosum 245045* and *S. brevicaulis* showed possible cross reactivity against cell supernatant 6E2.

The last cell supernatant tested was 8A10. *P. crustosum 245045* and *S. brevicaulis* showed possible bands near 41kDa against this antibody.

Table 4 – List of different fungal culture supernatant for cross reactivity test

no.	Species
1	*Acremonium strictum*
2	*Aspergillus ochraceus*
3	*A. sydowii*
4	*A. unguis*
5	*Chaetomium globosum 5765.2*
6	*Cladosporium cladosporioides*
7	*Eppicoccum nigrum*
8	*Eurotium herbariorum 55.3*
9	*A. versicolor* DAOM *235361*
10	*Eurotium rubrum J9862-3m*
11	*Paecilomyces variotti 8424-8m*
12	*Penicillium brevicompactum* DAOM *234049*
13	*P. chrysogenum CBS 277.067*
14	*P. crustosum* DAOM *245045*
15	*P. decumbens 5566.8*
16	*P. funiculosum L6321-3*
17	*P. thomii L6322-4*
18	*Scopulariopsis brevicaulis 5854*

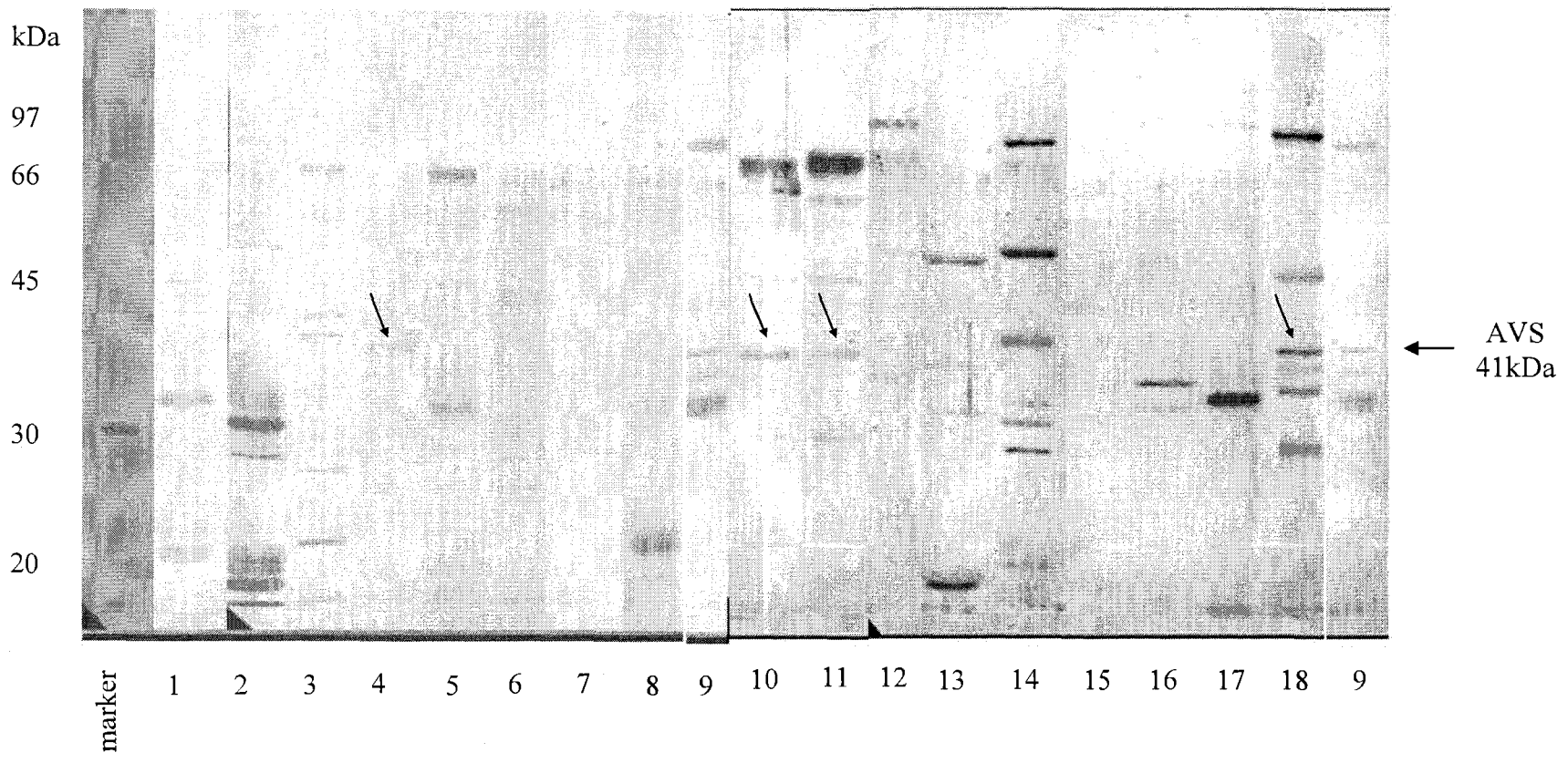

Figure 23 – Immunoblot examining the cross reactivity test for different fungal culture filtrate against cell supernatant 2H12, 2.6 µg/lane of crude protein, marker: LMW-SDS marker from GE healthcare, AVS: the protein from *A. versicolor* culture supernatant, the species for number 1-18 were shown in Table 4

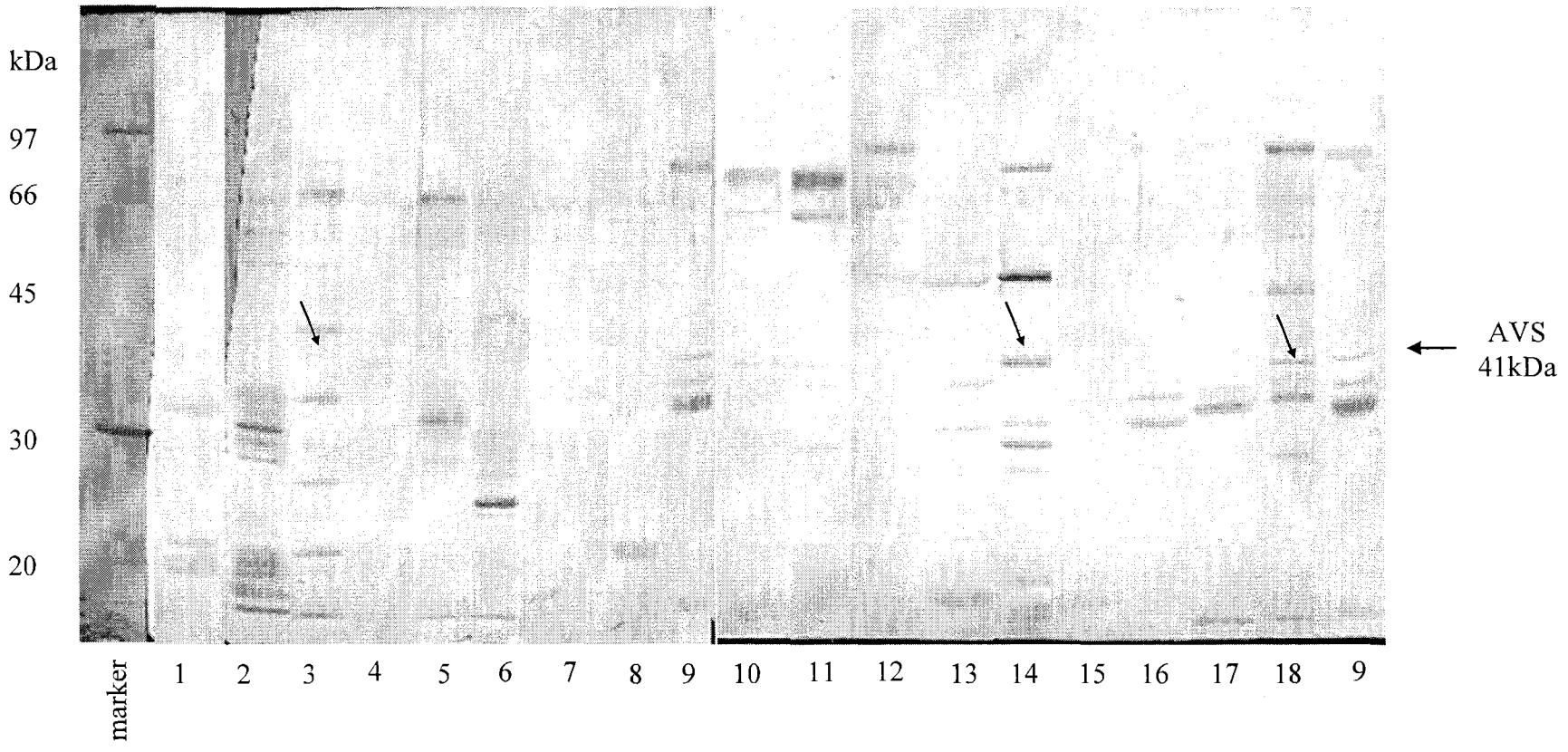

Figure 24 – Immunoblot examining the cross reactivity test for different fungal culture filtrate against cell supernatant 6E2, 2.6 µg/lane of crude protein, marker: LMW-SDS marker from GE healthcare, AVS: the protein from *A. versicolor* culture supernatant, the species for number 1-18 were shown in Table 4

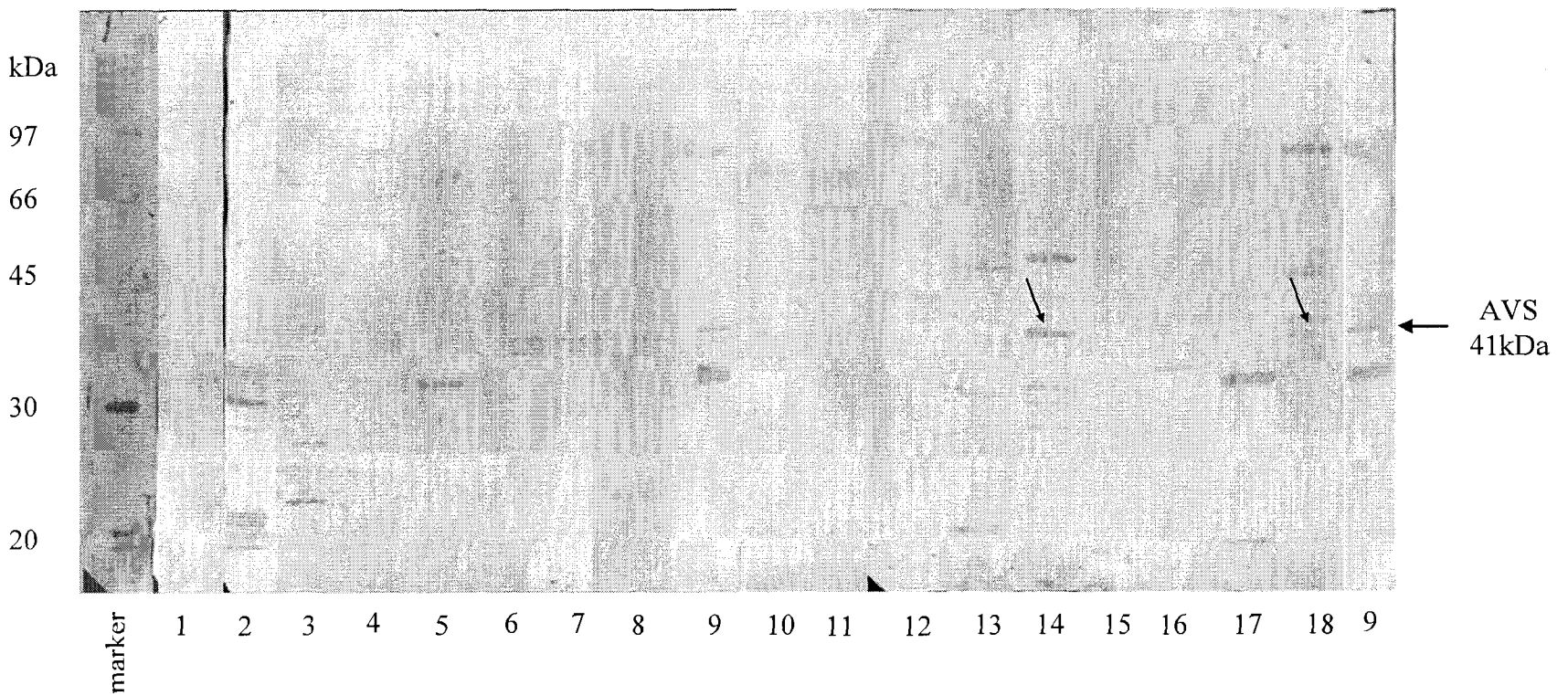

Figure 25 – Immunoblot examining the cross reactivity test for different fungal culture filtrate against cell supernatant 8A10, 2.6 µg/lane of crude protein, marker: LMW-SDS marker from GE healthcare, AVS: the protein from *A. versicolor* culture supernatant, the species for number 1-18 were shown in Table 4

9.2.6 Monoclonal antibody purification

9.2.6.1 Affinity purification of IgM from HiTrap IgM purification HP

Since 2H12 and 8A10 showed the highest Western response from previous experiments, they were both chosen to be purified by HiTrap IgM purification column. Figure 26 showed a comparison of Western response from the unpurified and purified mAbs 2H12 and 8A10. AV crude proteins from 60-65% AS precipitation was used as the protein sample for Western blot. The mAb 8A10 showed very similar Western response before and after purification. However, the purified mAb 2H12 showed a much weaker Western response.

To analyze the problem and the loss of the antibody for 2H12, each fraction from each purification step was collected and concentrated to test the Western response (Figure 27). The ascite 2H12 was first made to 50% AS pellet by dissolved into an equal volume of 100% saturated AS solution. The pellet and the supernatant were both collected for Western response. The 50% AS pellet was dissolved and changed buffer to 1M AS in 20mM sodium phosphate solution. The antibody was then passed through HiTrap IgM purification column. The unbound antibodies (flow through) were collected and concentrated for immunoblot. The purified 2H12 was eluted from the column by 0M AS in 20mM sodium phosphate. The elution fractions were concentrated and marked as for Western blot. Unpurified 2H12 showed the highest Western response, followed by 50% AS pellet. The purified 2H12 showed a very weak Western response.

Since IgM was very easy to degrade under high salt concentration, the binding condition was changed to 0.5M K_2SO_4 in 20mM sodium phosphate. Only mAb 8A10 was tested for this method. The step to make 50% AS pellet was omitted in this purification. The unpurified 8A10 was included as the positive control and it showed the highest Western response. Both the flow through and the purified 8A10 showed very weak Western response (Figure 28). The purification was not successful because the antibody did not bind the column sufficiently, resulting a loss of antibody during purification.

9.2.6.2 Affinity purification of IgM from IgM purification kit

The affinity purification from IgM purification kit generally provides a good purification of IgM antibody from mouse ascites. Monoclonal antibody 8A10 was used for this purification method (Figure 29). However, this purification was not successfully because the purified 8A10 showed almost no Western response as compare to the unpurified antibody. The response was also much weaker compare to the purified 8A10 from HiTrap IgM purification column from Figure 26 and Figure 27.

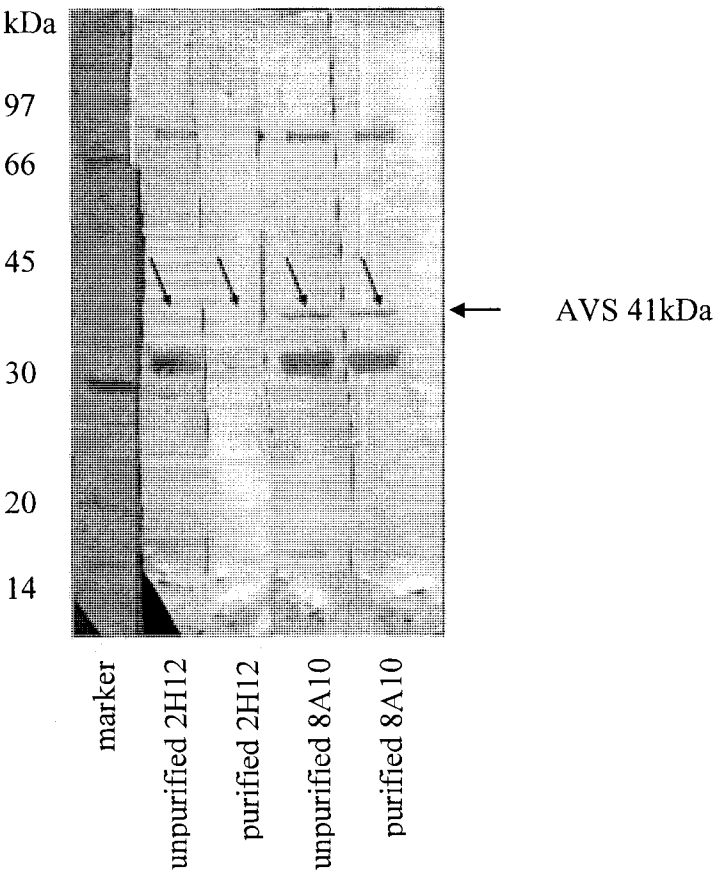

Figure 26 – Immunoblot examining the Western response for mAbs 2H12 and 8A10 before and after purification from HiTrap IgM purification column using 1M AS as binding condition, marker: LMW-SDS marker from GE healthcare, AVS: the protein from *A. versicolor* culture supernatant

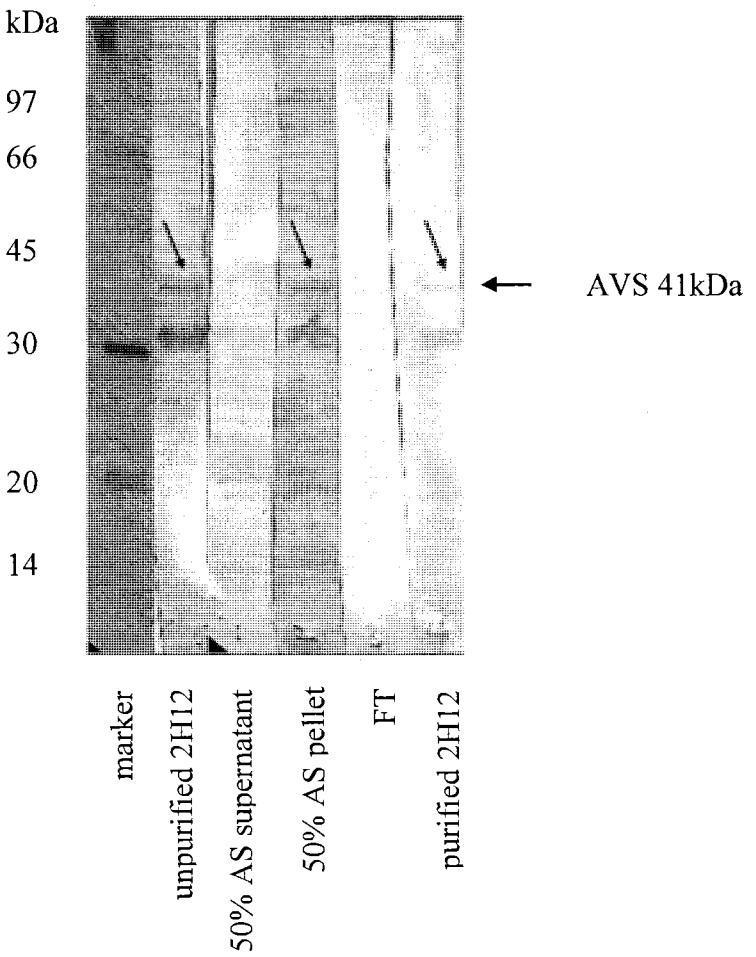

Figure 27 – Immunoblot examining the Western response of mAb 2H12 for each purification step from HiTrap IgM purification column using 1M AS as binding condition, marker: LMW-SDS marker from GE healthcare, AVS: the protein from *A. versicolor* culture supernatant

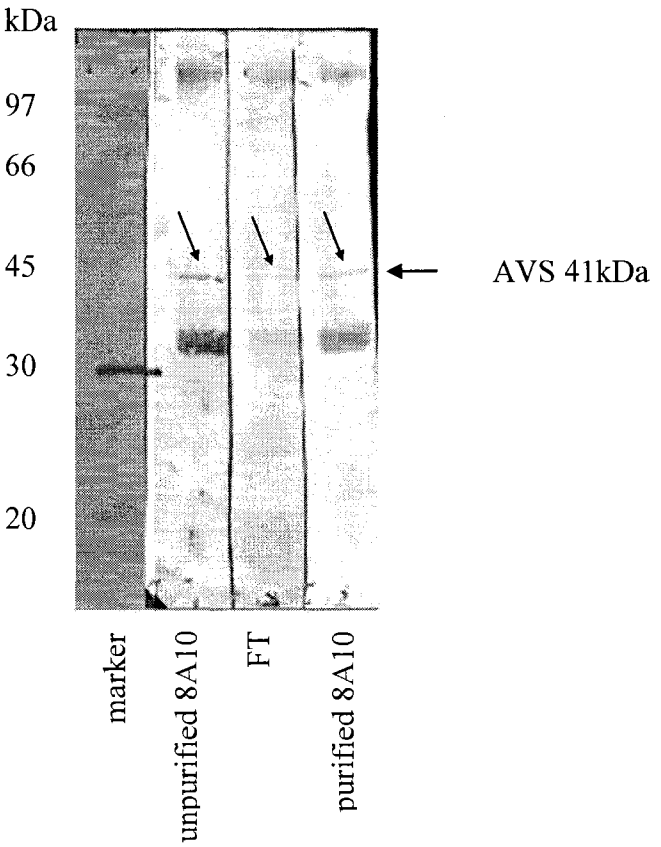

Figure 28 – Immunoblot examining the Western response for mAb 8A10 before and after purification from HiTrap IgM purification column using 0.5M K_2SO_4 as binding condition, marker: LMW-SDS marker from GE healthcare, AVS: the protein from *A. versicolor* culture supernatant

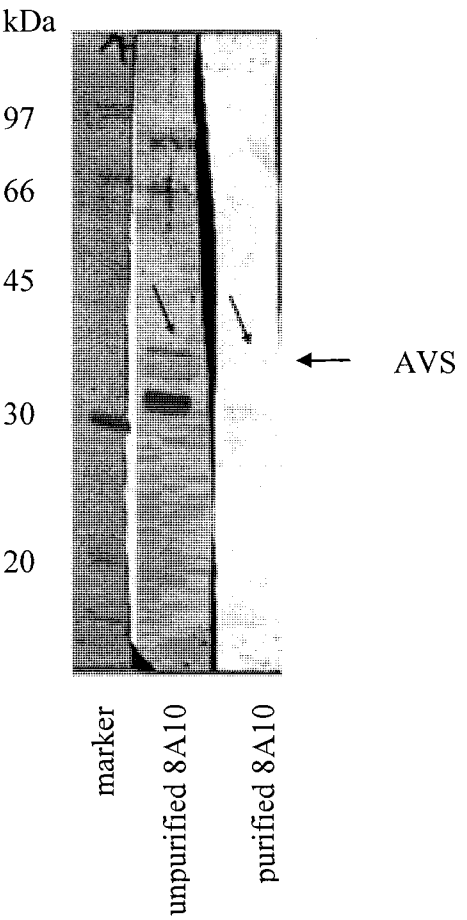

Figure 29 – Immunoblot examining the Western response for mAb 8A10 before and after purification from IgM purification kit, marker: LMW-SDS marker from GE healthcare, AVS: the target protein from *A. versicolor* culture supernatant

9.2.6.3 Partial antibody purification by 50% AS precipitation

Since the strongest Western response for 2H12 during HiTrap purification column was 50% AS precipitation (Figure 27), all 5 mAbs were made to 50% AS precipitation and the small molecular weight proteins were removed by 100kDa cut off membrane. The 50% AS supernatant and the pellet for all the mAbs were both tested on Western blot for the success of partial purification. As indicated by the arrows in Figure 30, the majority of the antibody was all presented in the 50% AS pellet for all 5 mAbs, except that mAbs 2H12 and 5C9 showed slightly positive Western response for 50% AS supernatant.

The success of partial antibody purification was examined by indirect ELISA (Figure 31). All 5 mAbs were tested at 1000x dilution on the same ELISA plate with different pure AVS 41kDa protein concentration (from 100ng/well to 0ng/well). 5C9 showed the highest ELISA response (OD 1.7 at 100ng/well) with a slightly high blank (0.7). The second highest ELISA response was from 6E2 (OD 0.82 at 100ng/well). The OD for mAb 5G5, 2H12 and 8A10 at 100ng/well was 0.58, 0.5 and 0.39 respectively. The ELISA with highest signal (mAb 5C9 and 6E2) was repeated three times. The signal was confirmed and the standard curve of 100ng/well to 0ng/well of AVS 41kDa pure protein with standard deviation was shown in Figure 32 (mAb 5C9) and Figure 33 (mAb 6E2).

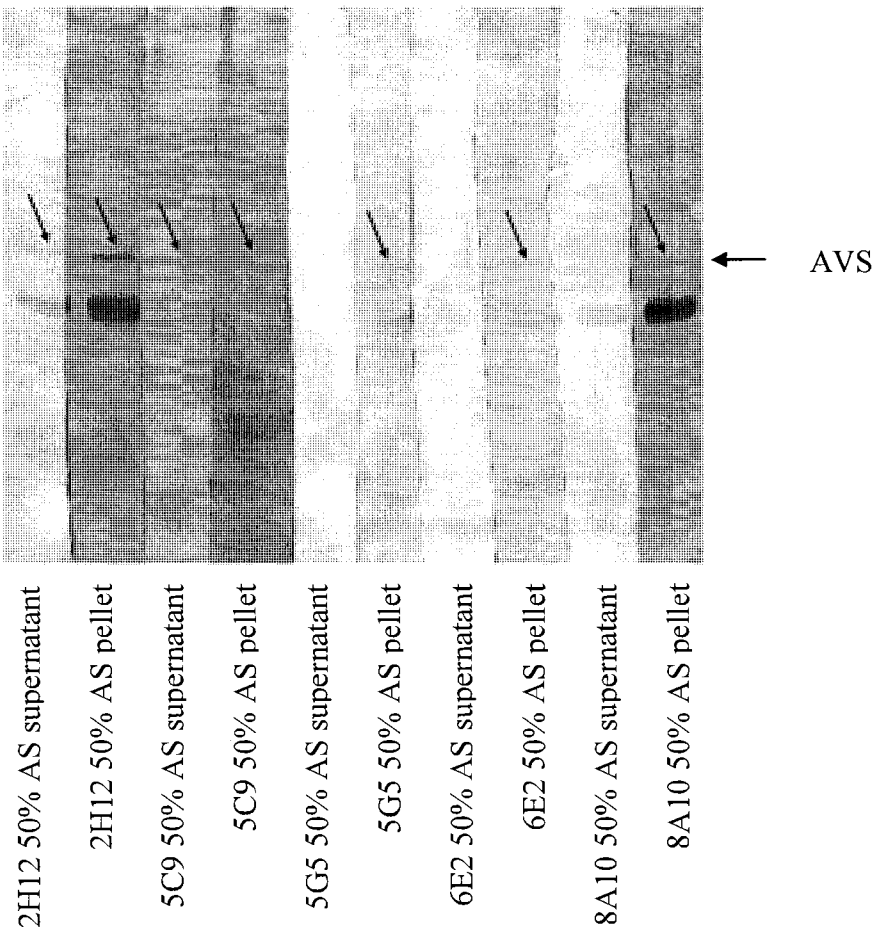

Figure 30 – Immunoblot examining the Western response for 5 mAbs 2H12, 5C9, 5G5, 6E2 and 8A10 before and after purification from 50% AS, AVS: the target protein from *A. versicolor* culture supernatant

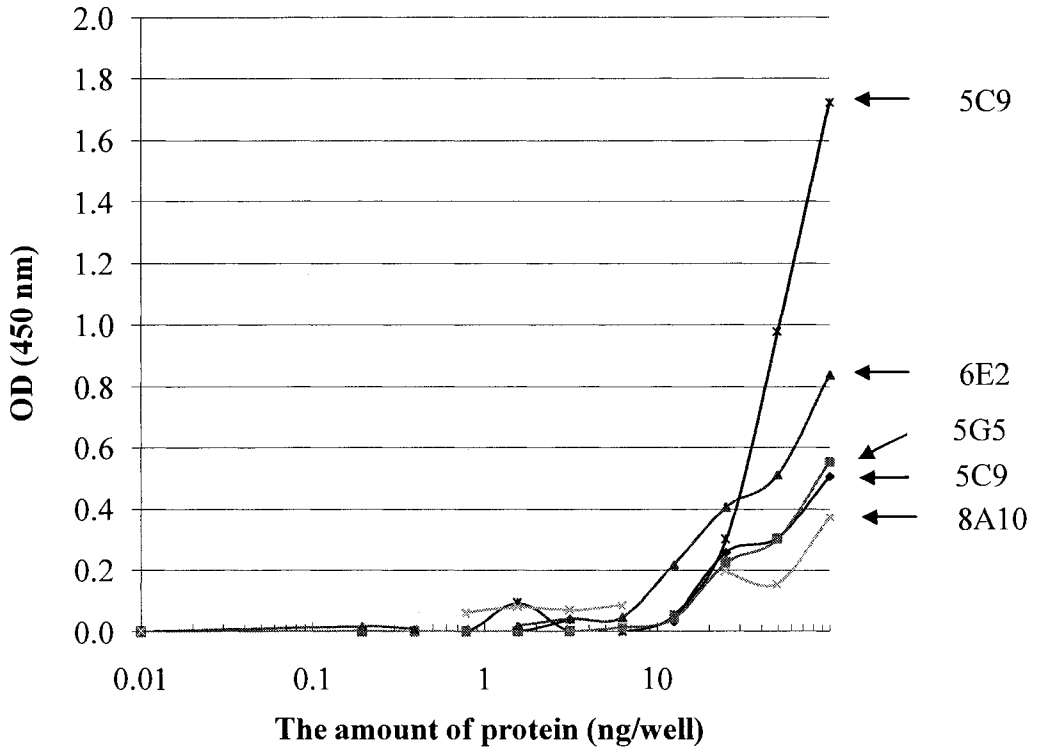

Figure 31 – ELISA response for 5 mAbs 2H12, 5C9, 5G5, 6E2 and 8A10 after purification from 50% AS against AVS 41kDa pure protein

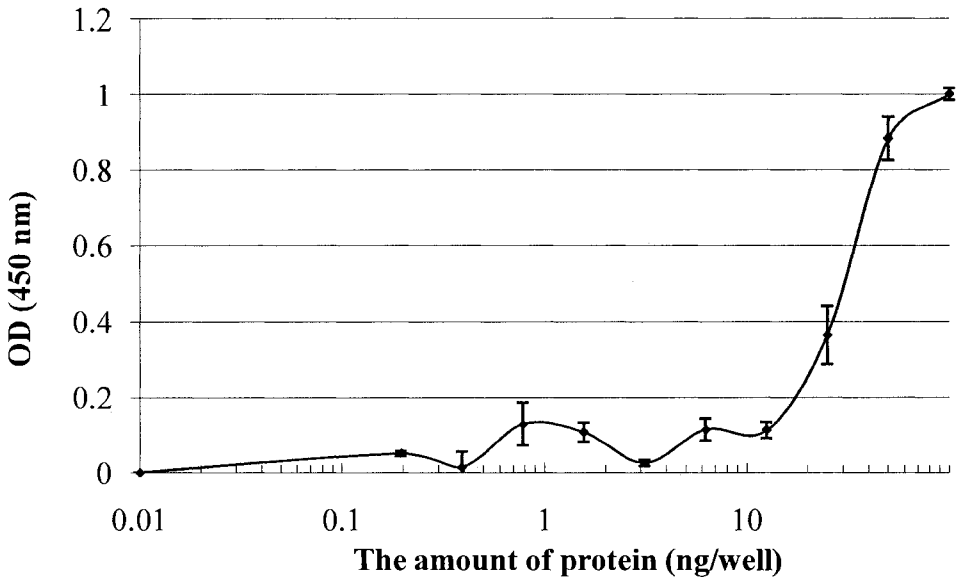

Figure 32 – Average ELISA response for mAb 5C9 after purification from 50% AS against AV pure protein

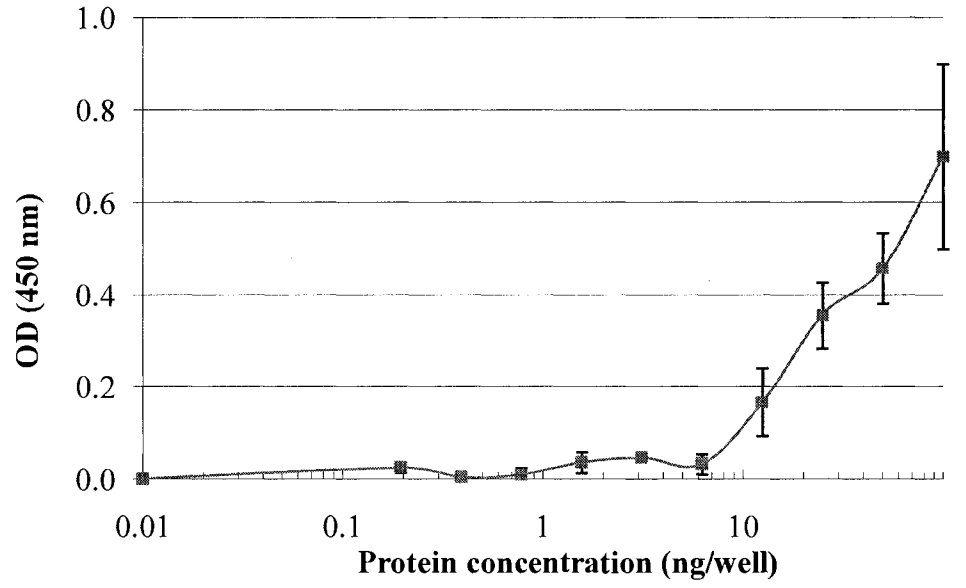

Figure 33 – Average ELISA response for mAb 6E2 after purification from 50% AS against AV pure protein

9.2.6.4 Antibody purification by gel filtration chromatography

The previous experiments showed very good indirect ELISA response for mAb 5C9 and 6E2 except the blank was high (0.7). Highly purified IgM antibody could reduce the background by minimizing the cross reactivity between the impurities from mAb (primary antibody) and secondary antibody. The mouse ascites containing IgM antibody had a molecular weight of approximately 900kDa, which was very far apart from other plasma proteins in the ascites such as such as transferrin and albumin (Feldhoff *et al.*, 1985), so gel filtration column could be used to achieve a high degree of purification.

The calibration curve for HiPrep 16/60 Sephacryl S-300 HR gel filtration column was done by using 6 different standards: blue dextran 2000, thyroglobulin, ferritin, aldolase, conalbumin and ovalbumin. The name of the standards, the molecular weight and the fraction volume were shown in Table 5. Since the blue dextran and thyroglobulin had a molecular weight of 2000kDa and 669kDa, the purified IgM mAb should be eluted within this range, i.e. between fraction volume of 37.835 mL and 45.973 mL. The absorbance for all the standards with the change of elution volume was shown in Figure 34. The peaks for 6 protein standards were numbers according to Table 5. The logarithm of molecular weight and the fraction volume was used to plot the linear calibration curve for this antibody gel filtration column. R^2 for the calibration curve was 0.9911.

The gel filtration curve for mAb 5C9 is shown in Figure 36. Two elution fractions (from 35.0-41.0 mL and from 41.0-46.5 mL) were pooled together separately to test the Western response (Figure 37). The unpurified 5C9 was included as a positive control.

Fraction 35.0-41.0 mL showed almost the same Western response as the unpurified antibody. The molecular weight between fraction 35.0-41.0 mL were in the range between 2000kDa (37.835 mL) and 669kDa (45.973 mL) from the standard calibration curve, which indicated that IgM antibody should be eluted at this fraction. The Western response has also confirmed the immunoblot response against AV crude protein, so this fraction was concentrated and was used later as purified 5C9. Fractions from 41.0 mL to 46.5 mL also showed a weak Western response against AV crude protein.

The gel filtration curve for mAb 6E2 was shown in Figure 38. Four different fractions with elution volume of 35.0-41.5 mL, 41.5-45.0 mL, 45.0-49.0 mL and 49.0-59.0 mL from 6E2 were collected and pooled together to test the Western response against AV crude protein. However, only the unpurified 6E2 and the first fraction with elution volume of 35.0-41.5 mL showed positive Western response as indicated by the arrows in Figure 39, so this fraction was concentrated and marked as purified 6E2.

Purified mAbs 5C9 and 6E2 were tested by indirect ELISA for their activity against AVS 41kDa pure protein. The signal for 5C9 was reduced from 1.7 to 0.8 at 100ng/well of AVS 41kDa pure protein compare to the ELISA signal from partially purified 5C9 from 50% AS pellet (Figure 40), but the background was reduced from 0.7 to 0.4. However, the signal for 6E2 (Figure 41) was increased from 0.82 to 1.1 and the blank was relatively low (0.25).

The antibody purifications by gel filtration column appeared to be successful by their Western response and the blank was reduced for the indirect ELISA for both 5C9 and 6E2, so gel filtration column was used to purify three remaining mAbs: 2H12, 5G5 and 8A10. Their immunoblot response after purification was tested on Western blot and the activity of antibody was tested on ELISA. The gel filtration curve for mAb 2H12, 5G5 and 8A10 was shown in Figure 42, Figure 44 and Figure 46. All gel filtration curves from all 5 mAbs showed very similar trend. The elution fractions for 2H12, 5G5 and 8A10 were collected similarly as mAb 6E2 and the Western response against AV crude protein were shown in Figure 43, Figure 45 and Figure 47 for 2H12, 5G5 and 8A10. All the fractions from 2H12 (Figure 43) showed positive Western response. However, as indicated by the arrows, only the unpurified mAbs and the first peak (35.0 mL – 41.0 mL for 5G5 in Figure 45 and 35.0 mL – 41.5 mL for 8A10 in Figure 47) showed the positive response against AV protein. The fractions from the first elution peak of each mAb were concentrated and were used as purified mAbs for indirect ELISA.

The results of indirect ELISA from 3 purified mAbs 2H12, 5G5 and 8A10 were shown in Figure 48, Figure 49 and Figure 50, respectively. All of the 3 purified mAbs showed improved ELISA response against AVS 41kDa pure protein at 100ng/well concentration compare to the ELISA response from partial purified antibody from 50% AS pellet. The OD signal for 2H12, 5G5 and 8A10 was 0.62 (vs. 0.5 from 50% AS pellet), 0.82 (vs. 0.58 from 50% AS pellet) and 0.73 (vs. 0.39 from 50% AS pellet), respectively.

The antibody concentration was calculated by Bradford assay and all the purified antibodies were concentrated to approximately 6.5mg/mL. The yield of antibodies was shown in Table 6. Antibody 6E2 showed the highest yield (2.1mg/2mL ascites) and 5C9 showed the lowest yield (1.0mg/2mL ascites). The yield for 2H12, 5G5 and 8A10 was 1.2mg/2mL ascites, 1.3mg/2mL ascites and 1.6mg/2mL ascites, respectively.

Table 5 – The molecular weight of the standards for the calibration curve for HiPrep 16/60 Sephacryl S-300 HR gel filtration column

protein	molecular weight (kDa)	log MW	fraction volume (mL)
1. blue dextran 2000	2000	3.301	37.835
2. thyroglobulin	669	2.825	45.973
3. ferritin	440	2.643	53.173
4. aldolase	158	2.199	62.520
5. conalbumin	75	1.875	69.240
6. ovalbumin	43	1.633	72.680

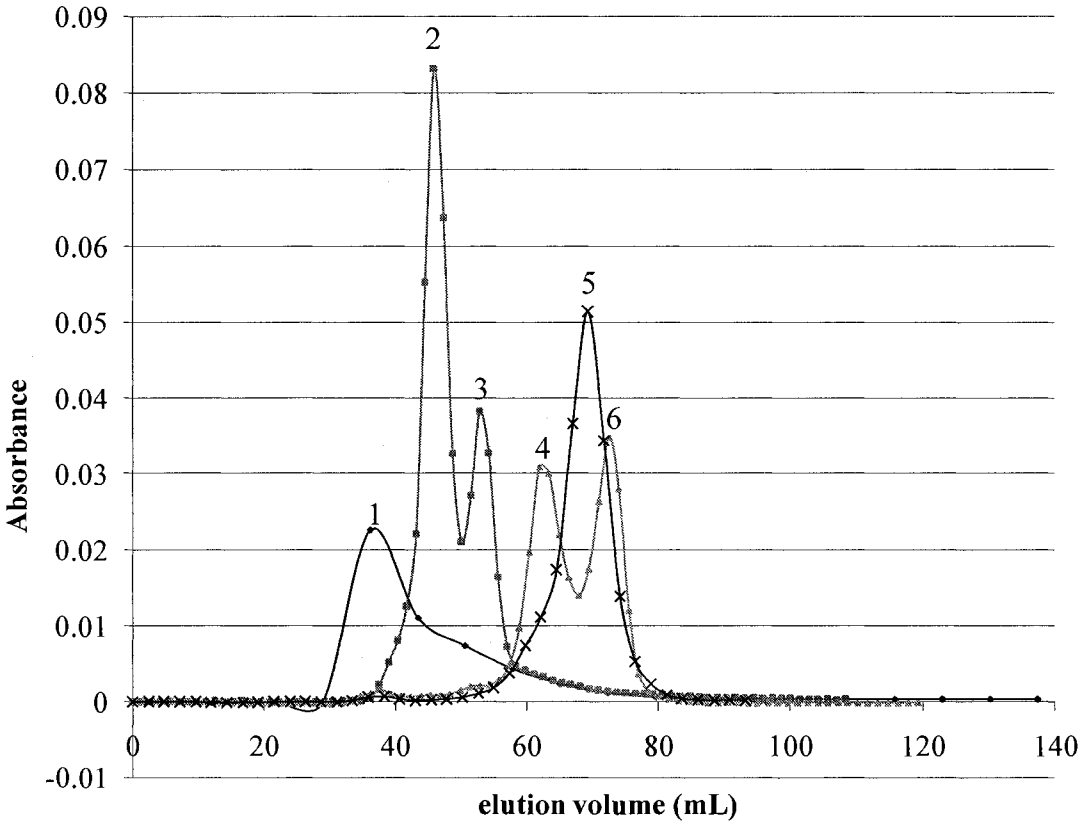

Figure 34 – The elution fractions for the protein standards for HiPrep 16/60 Sephacryl S-300 HR gel filtration column, peak 1-6 represents blue dextran 2000, thyroglobulin, ferritin, aldolase, conalbumin and ovalbumin, respectively

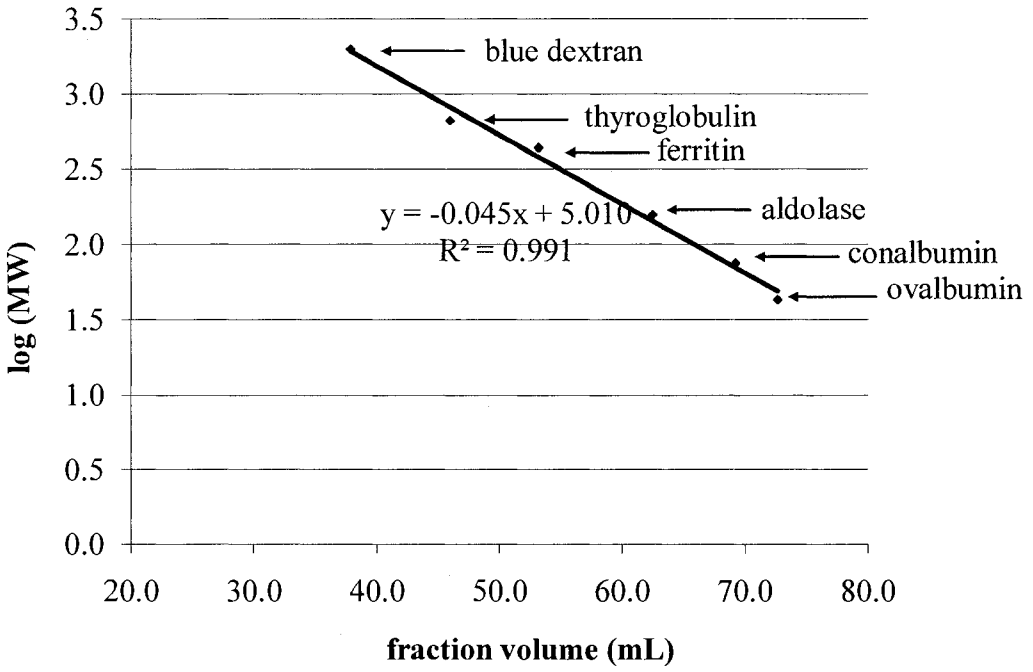

Figure 35 – Standard curve for HiPrep 16/60 Sephacryl S-300 HR gel filtration column from 5 protein standards using high molecular weight calibration kit

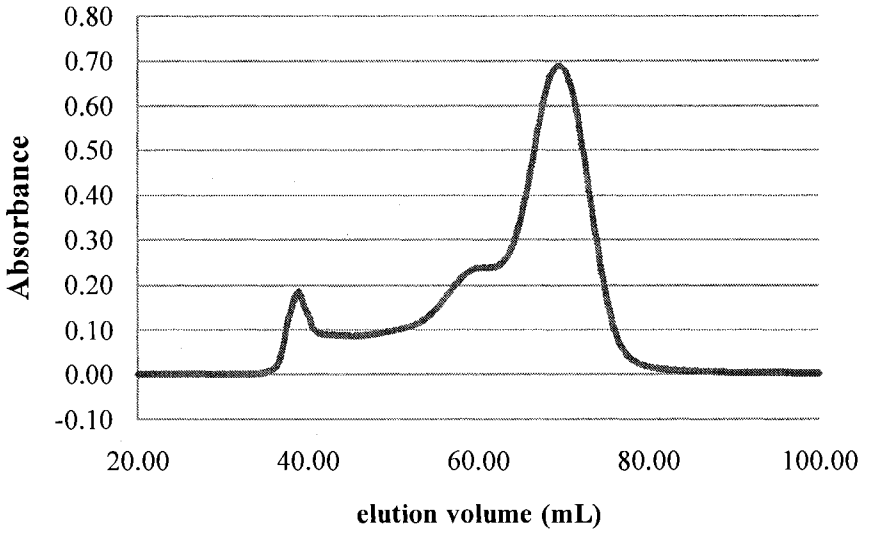

Figure 36 – The gel filtration curve for mAb 5C9

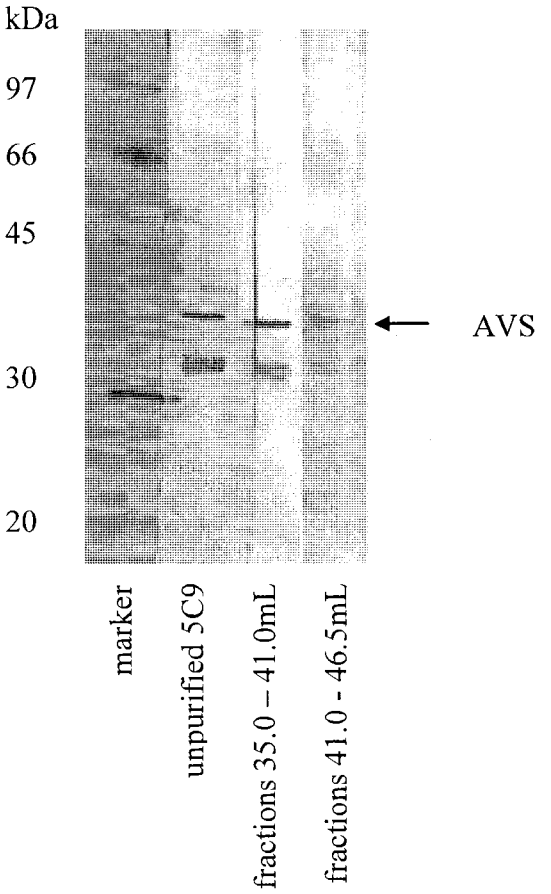

Figure 37 – Immunoblot examining the Western response of mAb 5C9 for different elution fractions after gel filtration column against AV crude protein, AVS: the target protein from *A. versicolor* culture supernatant

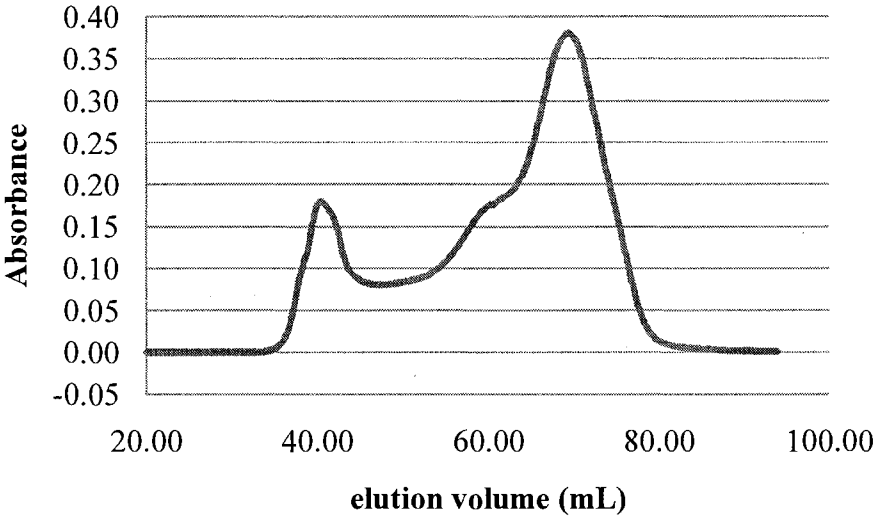

Figure 38 – The gel filtration curve for mAb 6E2

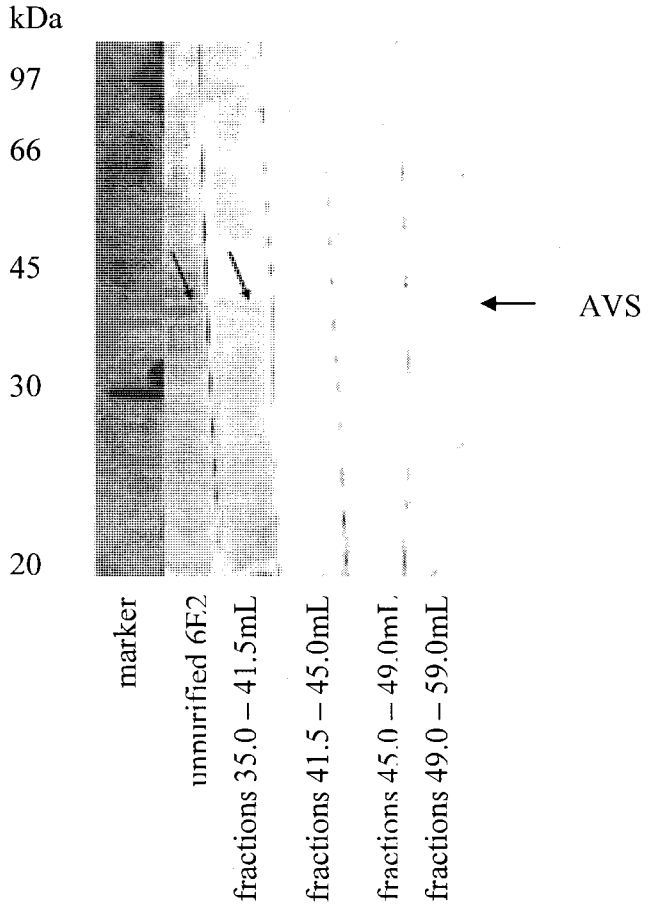

Figure 39 – Immunoblot examining the Western response of mAb 6E2 for different elution fractions after gel filtration column against AV crude protein, AVS: the target protein from *A. versicolor* culture supernatant

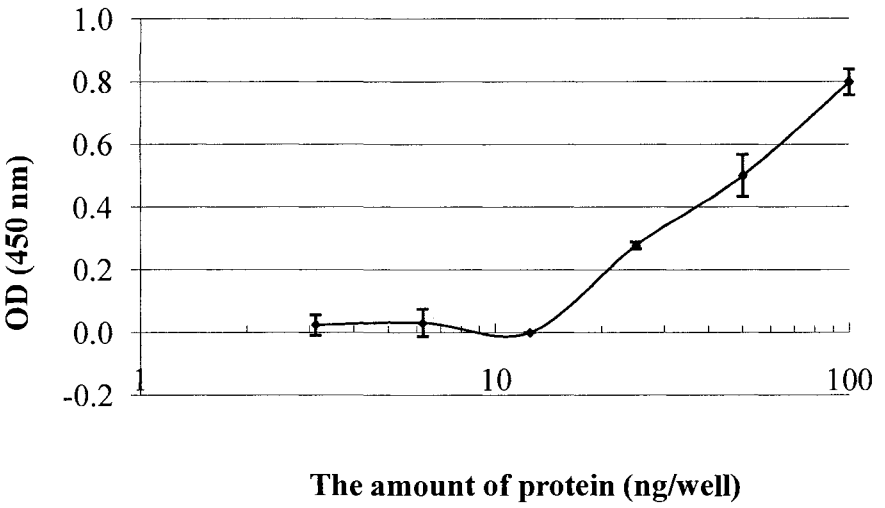

Figure 40 – Average ELISA response for mAb 5C9 after purification from gel filtration column against AV pure protein

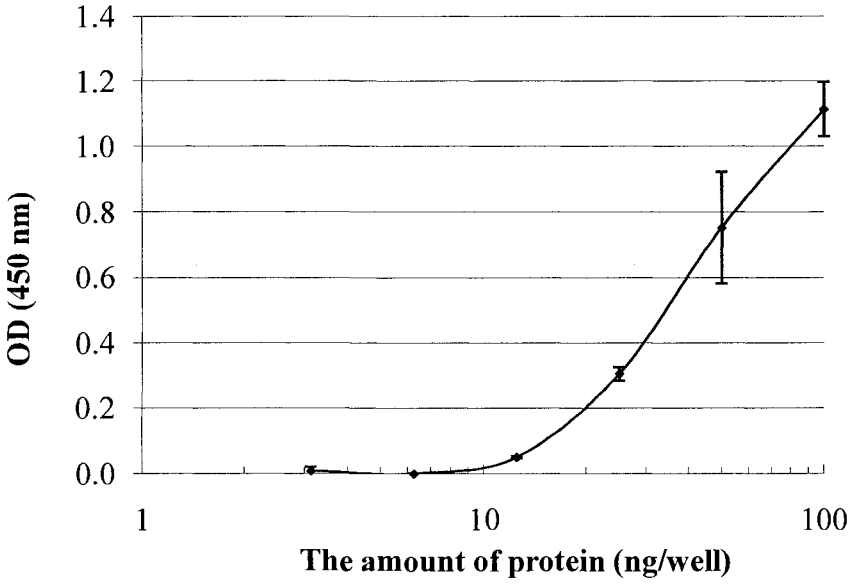

Figure 41 – Average ELISA response for mAb 6E2 after purification from gel filtration column against AV pure protein

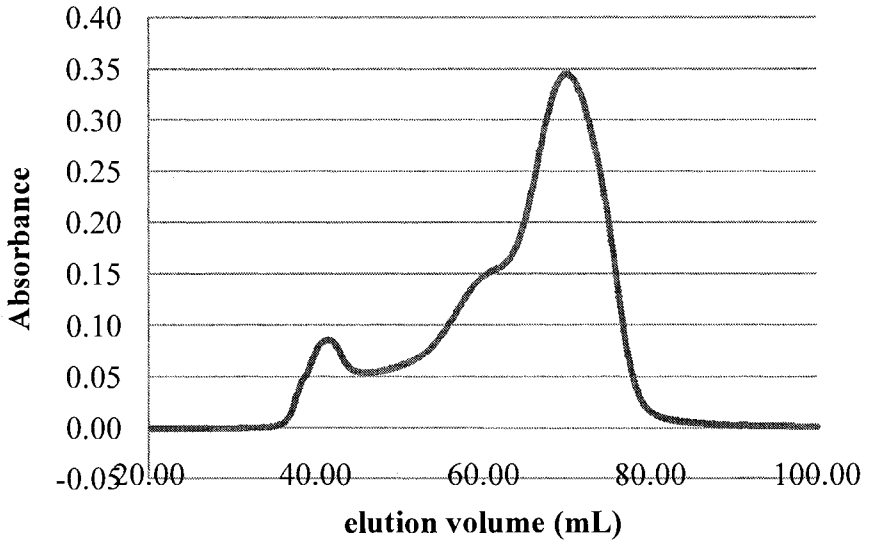

Figure 42 – The gel filtration curve for mAb 2H12

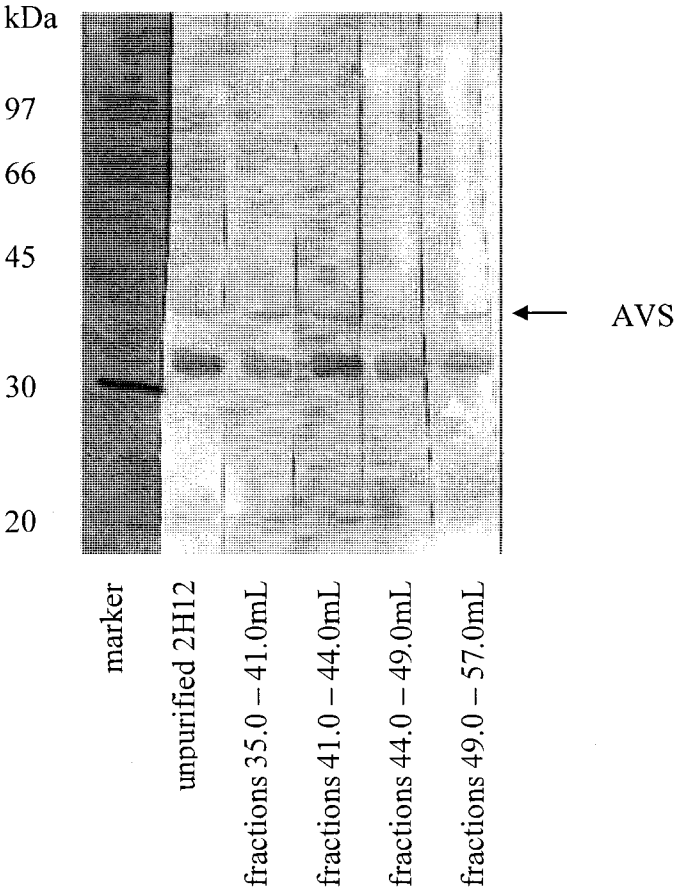

Figure 43 – Immunoblot examining the Western response of mAb 2H12 for different elution fractions after gel filtration column against AV crude protein, AVS: the target protein from *A. versicolor* culture supernatant

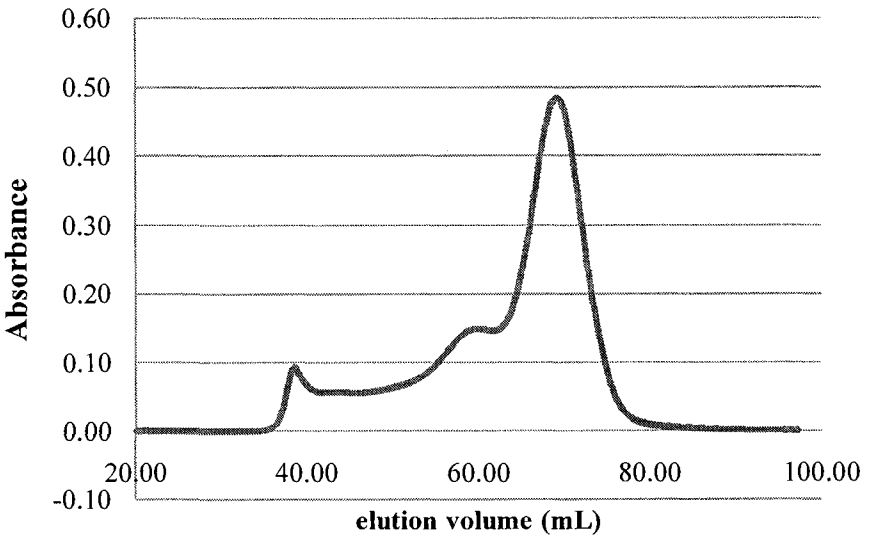

Figure 44 – The gel filtration curve for mAb 5G5

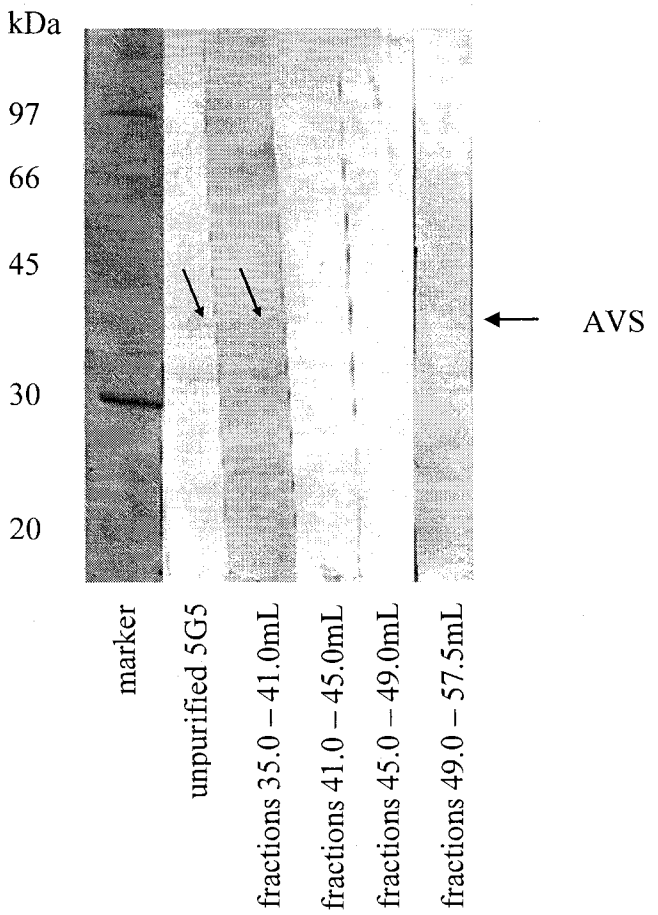

Figure 45 – Immunoblot examining the Western response of mAb 5G5 for different elution fractions after gel filtration column against AV crude protein, AVS: the target protein from *A. versicolor* culture supernatant

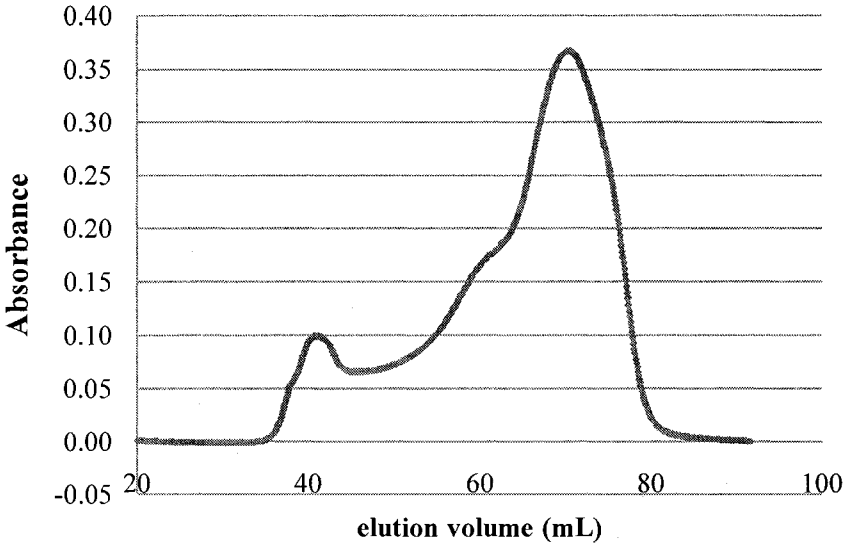

Figure 46 – The gel filtration curve for mAb 8A10

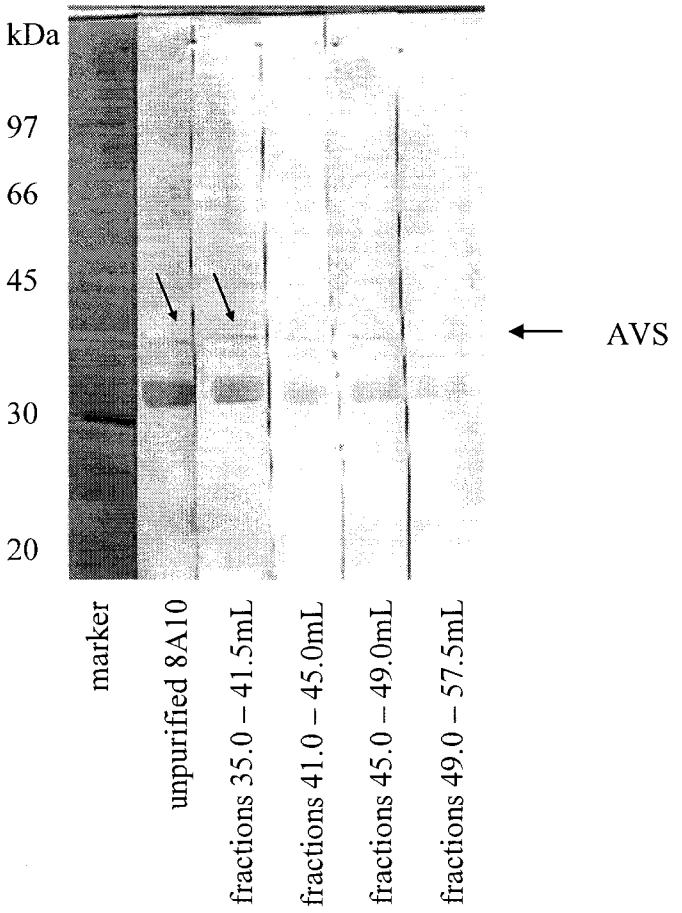

Figure 47 – Immunoblot examining the Western response of mAb 8A10 for different elution fractions after gel filtration column against AV crude protein, AVS: the target protein from *A. versicolor* culture supernatant

Table 6 – The yield for different mAbs after HiPrep 16/60 Sephacryl S-300 HR gel filtration column

antibody	yield of the mAbs
2H12	1.2mg/2mL ascites
5C9	1.0mg/2mL ascites
5G5	1.3mg/2mL ascites
6E2	2.1mg/2mL ascites
8A10	1.6mg/2mL ascites

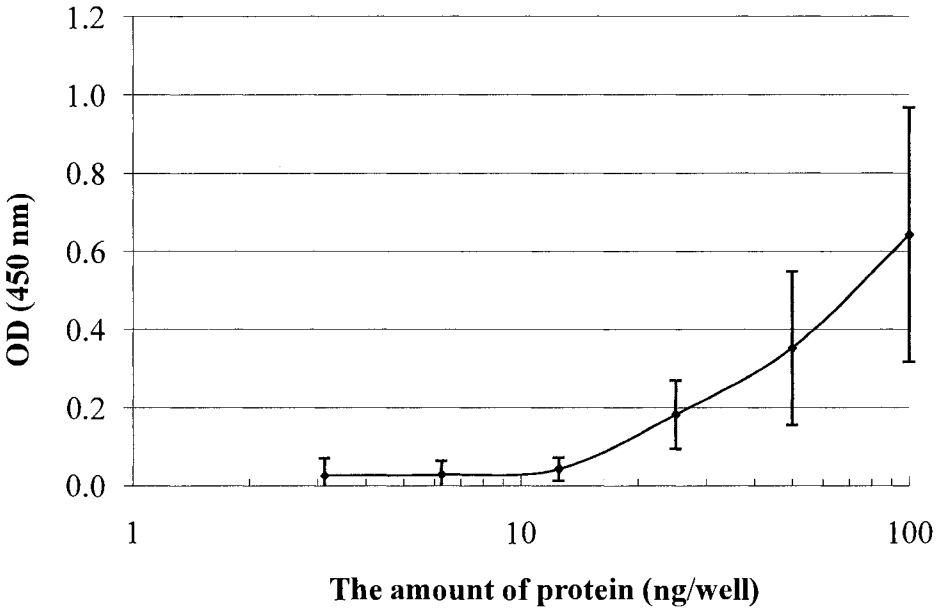

Figure 48 – Average ELISA response for mAb 2H12 after purification from gel filtration column against AV pure protein

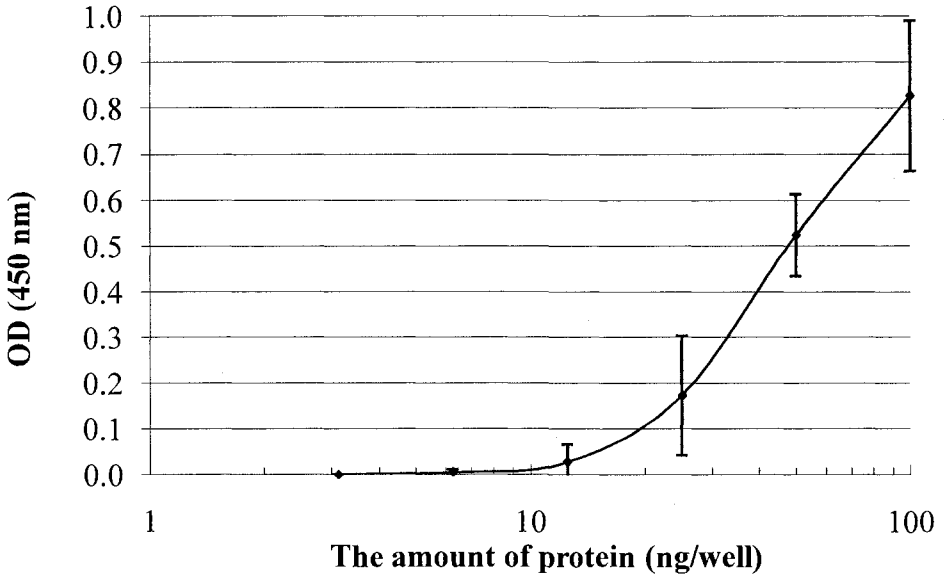

Figure 49 – Average ELISA response for mAb 5G5 after purification from gel filtration column against AV pure protein

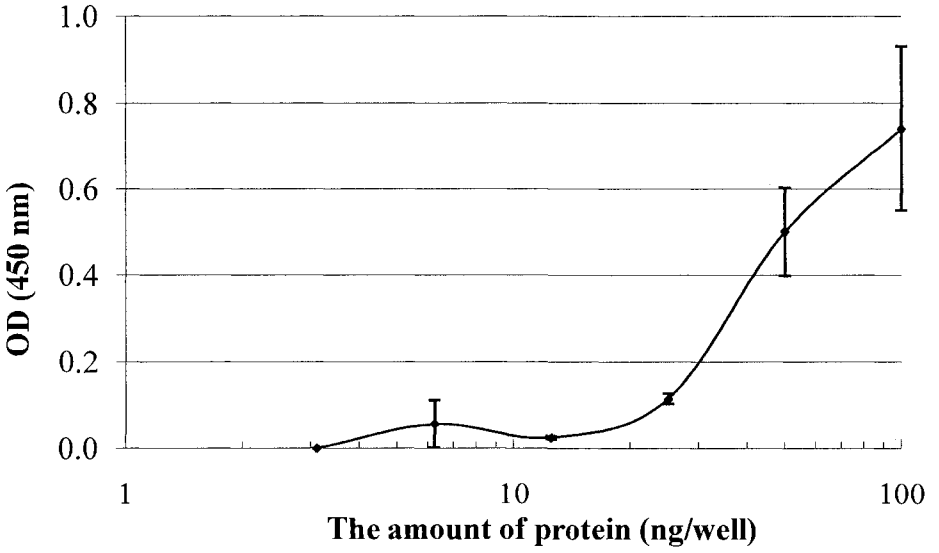

Figure 50 – Average ELISA response for mAb 8A10 after purification from gel filtration column against AV pure protein

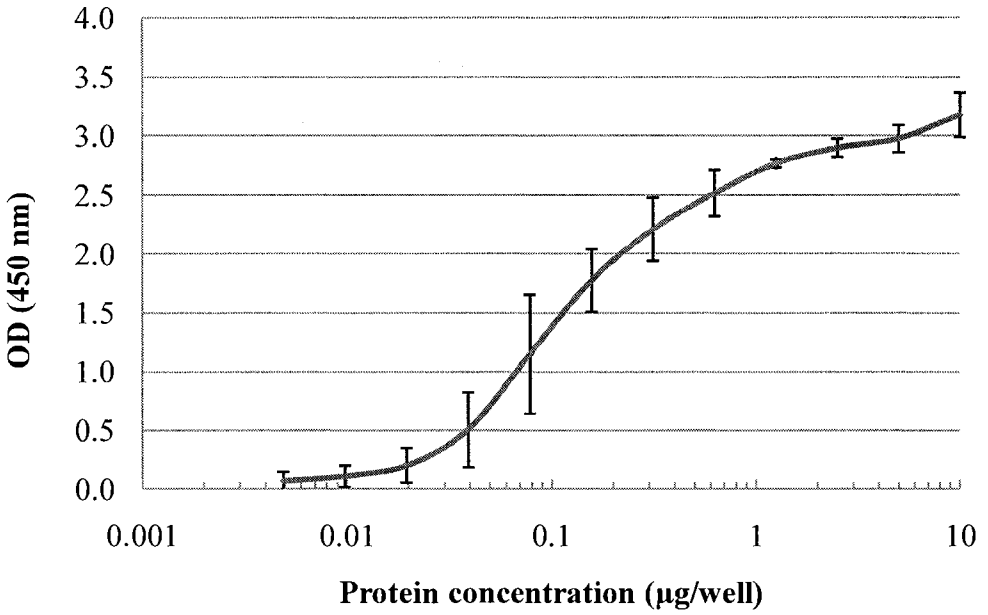

Figure 51 – The standard ELISA response for mAb 6E2 against AVS 41kDa

9.2.7 Cross reactivity test different AV spores on indirect ELISA against purified mAb 6E2

The ELISA response for different strains of AV spores was tested by using purified 6E2 from gel filtration column, since mAb 6E2 showed the best ELISA response against AVS 41kDa pure protein. Six different strains of AV spore fragments (2-2 in Figure 52, 2-2a in Figure 53, 2-3b in Figure 54, 43-2b in Figure 55, 44-3a in Figure 56 and 138-1a in Figure 57) were tested against purified mAb 6E2. The location of origin of each strain was summarized in Table 7. The highest response for AV spore fragments was all shown to be approximately 10μg/well. High spore concentrations above 10μg/well (40μg/well and 20μg/well of spores) showed the inhibition of the ELISA response. The highest ELISA signal was from strain 2-2 (OD 2), followed by 2-3b (OD 1.3), 138-1a (OD 1.25) and 2-2a, 43-2b and 44-3b (OD 1.1). However, the ELISA reactivity of AV spores from strain 2-2a, 2-3b, 43-2b and 44-3a was not very consistent compare to strain 2-2 and 138-1a since the standard deviation was much higher.

The cross reactivity against different fungal spores using purified 6E2 mAb was tested on ELISA plate. The OD values for different fungal species were measured at 450nm and the bar graph is shown in Figure 58. Pure AVS protein (OD 2) and AV spores (OD 1.835) were included as positive standard. *P. spinulosum* showed a relative high cross reactivity (>1).

Table 7 – List of different strains for *A. versicolor* spores

number	location
2-2	Winnipeg
2-2a	Winnipeg
2-3b	Winnipeg
43-2b	Ottawa
44-3a	Vancouver
138-1a	Hawaii

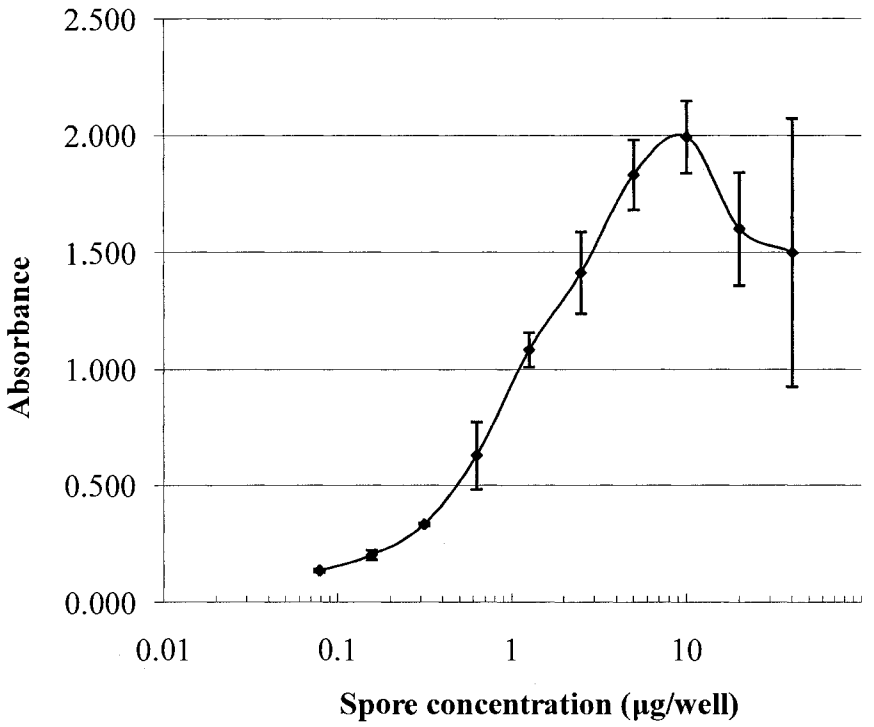

Figure 52 – Average ELISA response for purified mAb 6E2 against 2-2 AV spores

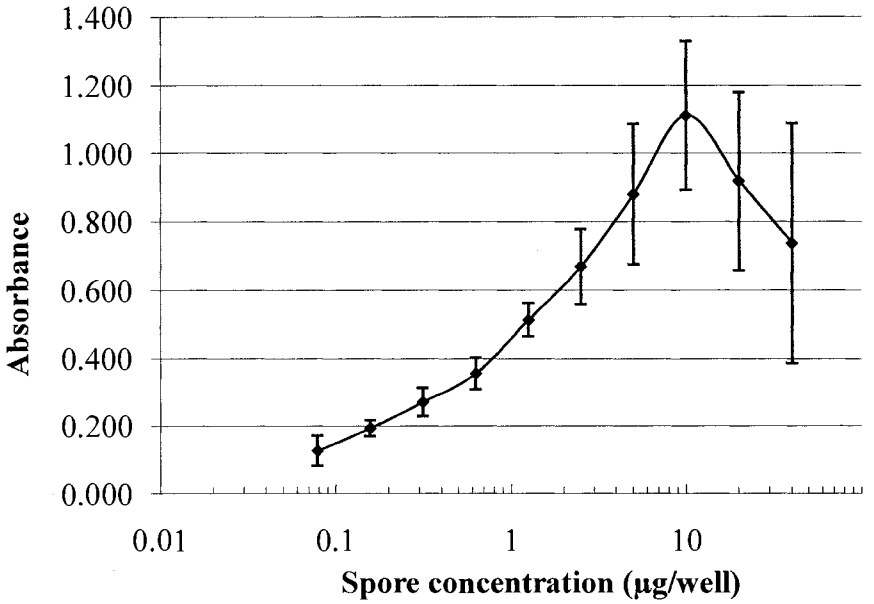

Figure 53 – Average ELISA response for purified mAb 6E2 against 2-2a AV spores

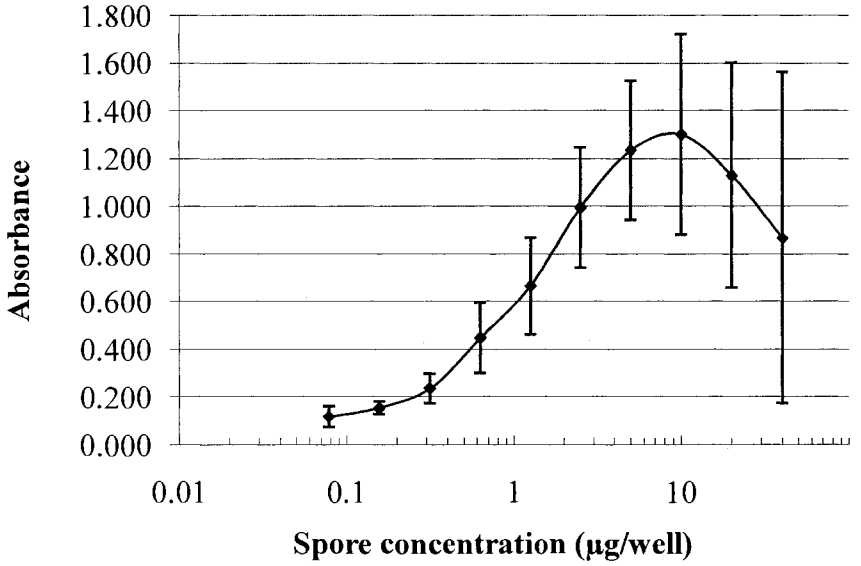

Figure 54 – Average ELISA response for purified mAb 6E2 against 2-3b AV spores

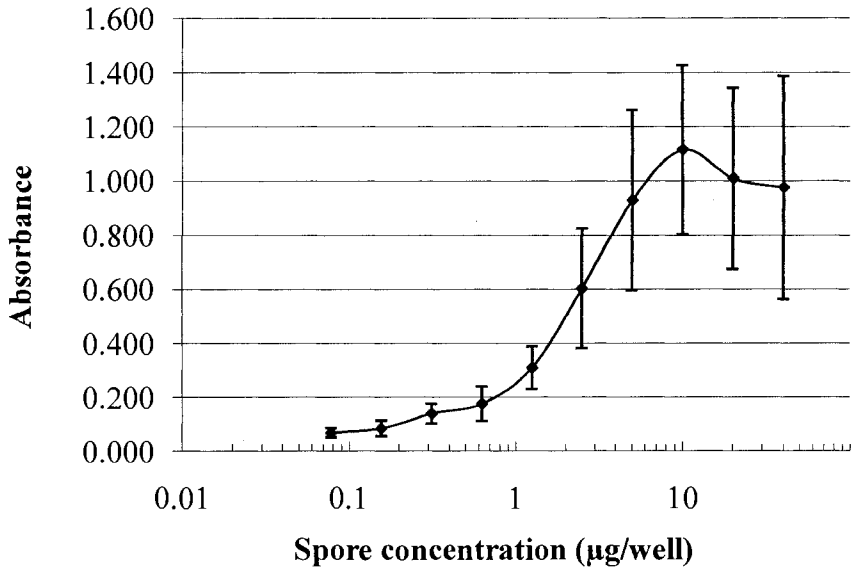

Figure 55 – Average ELISA response for purified mAb 6E2 against 43-2b AV spores

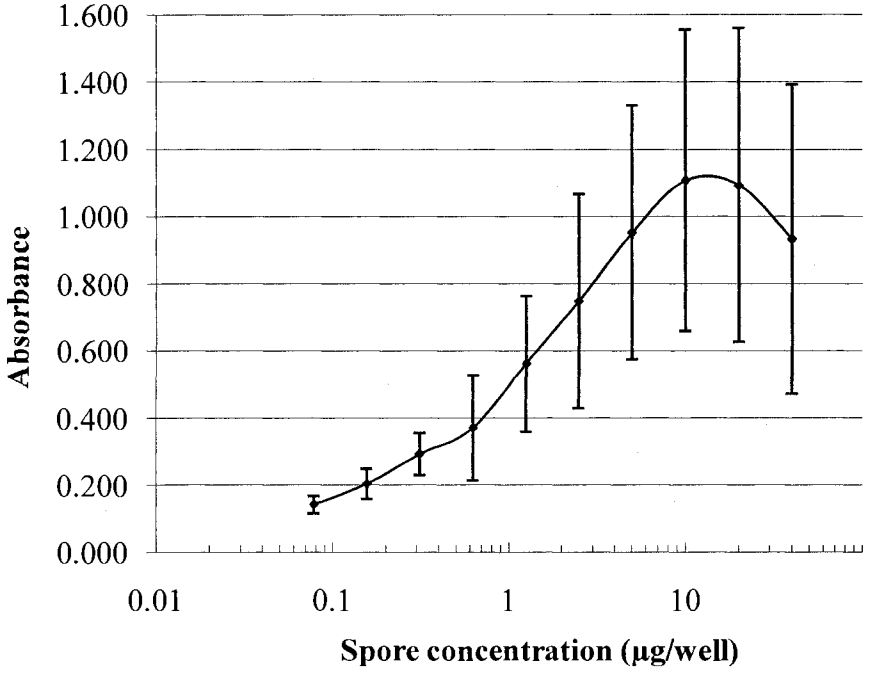

Figure 56 – Average ELISA response for purified mAb 6E2 against 44-3a AV spores

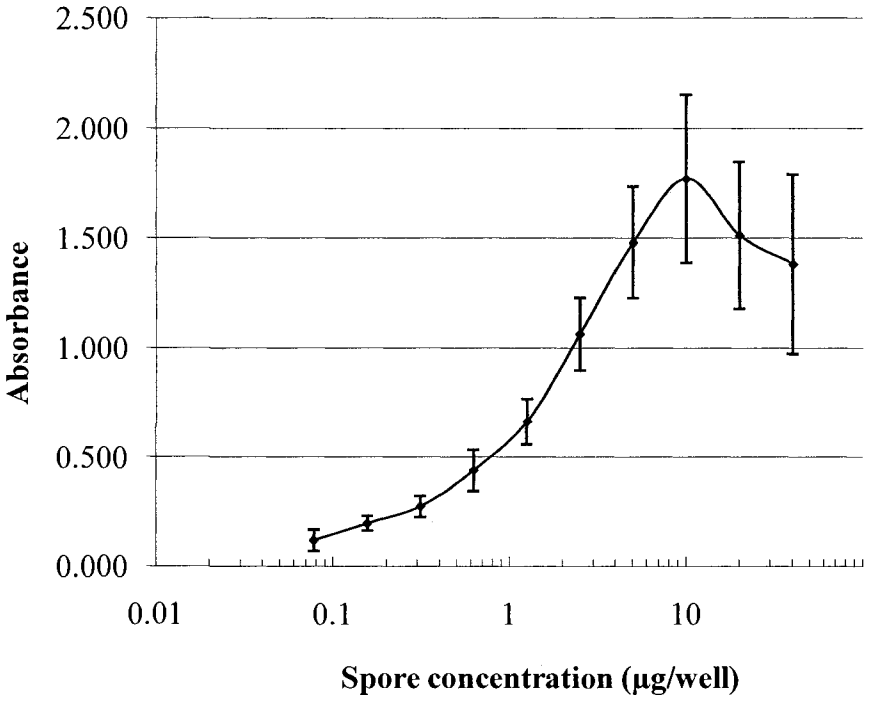

Figure 57 – Average ELISA response for purified mAb 6E2 against 138-1a AV spores

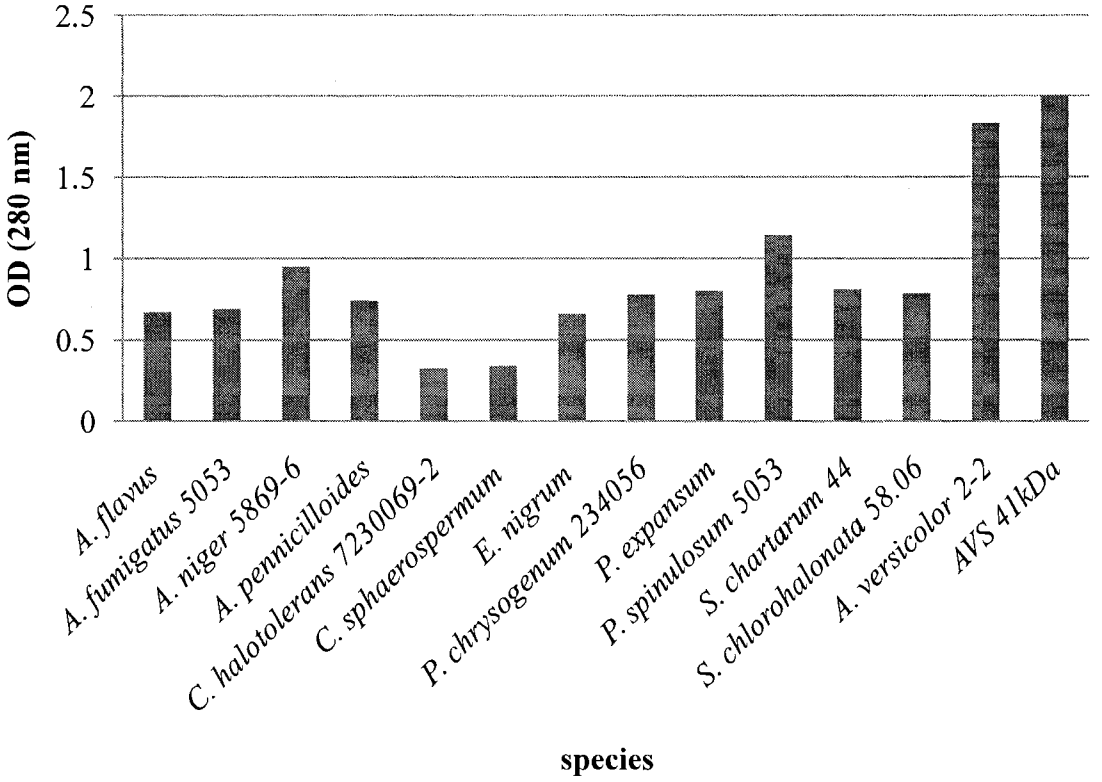

Figure 58 – The cross reactivity of different fungal spores against purified mAb 6E2

10 Discussion

10.1 Identification of the protein

Studies from Germany, Norway, Canada and United States have all shown that fungal exposure is directly related to many respiratory diseases. The fungus I studied, *A. versicolor* is one of the most common indoor fungi found in various damp building materials (Miller *et al.*, 2008). For many reasons, methods to assess fungal exposure still remain a problem. The commonly used methods are culture methods, PCR-probe and the production of monoclonal antibodies.

Previously, a 43 kDa protein from *Aspergillus versicolor* was identified as a human antigen and was purified from the culture supernatant from *A. versicolor* DAOM 235361 in our laboratory (Zhao, 2006). Approximately 10 mg of the protein was used to produce mAbs in four mice (protocol similar to that described in Xu *et al.,* 2008). When the sera were screened on Western against semi-purified protein extract, all had good response, producing the expected antibodies against AVS at 43kDa but also with a response to a 41kDa protein in one mouse (Xu, unpublished data). The two mice with the highest titer were chosen to produce monoclonal antibodies. When this process was completed, ca. 1000 clones were screened on ELISA and using a dot matrix protocol, on Western (performed by Immunoprecise Limited, Victoria, BC). Cell supernatants from these were further screened on Western and five were chosen for ascites production in the Miller laboratory. Immunoprecise test data indicated that these antibodies were almost exclusively IgM isotype, which was confirmed in these studies.

To date, only Schmechel, *et al.* (2005) have reported monoclonals to this species. They were not successful in producing mAbs of adequate specificity to *A. versicolor*. Using the particulate fraction of a spore homogenate as the immunogen, they generated 46 mAbs. None of the antibodies they generated were species-specific for *A. versicolor*. Several antibodies strongly cross-reacted with most *Aspergillus*, *Penicillium* and *Eurotium* species, among others. Schmechel *et al.* (2005) used spore fragments as immunigens and found that that murine antibodies to *A. versicolor* were also dominated by, in their case, highly cross-reactive antibodies of the IgM isotype. This is the isotype of the antibody that we produced from the mice ascites. This is less common and suggests some characteristic of the spore surface proteins results in IgM at the expense of IgG antibodies. The property of the antigenic proteins such as protein folding and how proteins function found in *A. versicolor* species may play an important role in generating mAbs resulting in production of IgMs.

Once the mouse ascites were produced, more *A. versicolor* antigenic proteins were required to test specificity of mAbs. The same strain of *A. versicolor* culture was used to inoculate the same liquid medium and the cultures were then harvested at 48 hours after inoculation as previously described (Zhao, 2006). However, after ion exchange chromatography, the dominant protein at 0.2M NaCl fraction inverted such that the 41kDa protein was accumulated at the expense of the 43 kDa protein. Different experiments were then done to test the properties of the newly discovered 41kDa protein.

These studies have shown that the two proteins AVS 43kDa and 41kDa are very closely related proteins. Western blots confirmed that the 41kDa protein was also a human antigen, since all 4 different randomly selected human sera QC1294, QC1297, QC2397 and QC2398 showed strongly positive IgG Western response against this 41kDa protein in crude fungal extract (Figure 17). This information allowed the further conclusion that both proteins had a similar Western response against human sera compared with the 43kDa protein. Spores from 6 strains from different geographic locations, (DAOM 235361, 2-2a, 43-2b, 44-3a, 138-1a and 138-3a) were all shown to contain 41kDa protein. All had the Western response against the mAbs (Figures 20-22). In addition, both proteins were eluted from 0.2M NaCl on ion exchange column during protein purification, indicating that both proteins have the same ionic net charge on the surface of the protein, since ion exchange chromatography is a technique that separates the proteins based on their ionic strength on the surface of the protein. The similar properties of these two proteins suggested that these two proteins are possibly the same protein but have undergone a slightly different modification during culture production. Zhao (2006) showed that the spores of *A. versicolor* had very high concentrations of the 41kDa protein. Based on ELISA data discussed in more detail below, (Figure 58), there was approximately 1ng of pure AVS 41kDa protein per 10^4 AV spores.

10.2 Comparison of N-terminal sequences among *Aspergillus* species

The N-terminal sequence has been successfully generated by Edman degradation. This 41kDa protein showed a 69% identity with a 34kDa protein from *A. fumigatus*, a 62% identity with 34kDa protein from *A. flavus* and *A. oryzae* on NCBI database. These three 34kDa proteins from *A. fumigatus*, *A. flavus* and *A. oryzae* shared similar characteristics. All were alkaline serine proteinases (Yu *et al.*, 1999, Ramesh *et al.*, 1994, http://www.allergen.org/Allergen.aspx accessed August, 2008).

Alkaline serine proteinase from *A. flavus* shares an 83% identity to *A. fumigatus* (Jaton-Ogay *et al.*, 1992) and a 82% identity to *A. oryzae* (Tatsumi *et al.*, 1989). This among, some other physical data suggests that these three alkaline serine proteinases are very closely related. The cDNA libraries have been made for all three proteinases. These studies have shown that they contain a signal sequence from amino acid 1-21, a pro-sequence from amino acid 22-121 and a mature protein from amino acid 122-403.

All the cDNAs have an open-reading frame that contains 1212 nucleotides. All the three alkaline serine proteinases encode a total of 403 amino acids which have a predicted molecular mass of 42kDa. All the serine proteinases in all three species are processed only at the N terminus (Tatsumi *et al.*, 1989). The first 21 amino acids have the properties of signal sequence of secreted fungal proteins (Periman and Halvorson, 1983; Yu *et al.*, 1999). The function of signal sequence is probably direct transport polypeptide chain

across the ER membrane into the ER lumen (Yu *et al.*, 1999). The proteinases contain three introns, and the amino-terminal sequence for the mature protein was determined to be very similar as the protein sequence deduced from the DNA sequence. The N-terminus of the mature protein all started at amino acid 122. The first 121 amino acids possess the properties of the prepro sequence of the proteinases (Jaton-Ogay *et al.*, 1992; Tatsumi *et al.*, 1989). The mature proteins for all alkaline serine proteinases contains 282 amino acids and have a predicted molecular weight of 34kDa, which was consistent with the molecular weight obtained from SDS-PAGE for all three fungi. This indicated that the alkaline serine proteinases must be synthesized as a pre-proprotein with a signal sequence and then being processed when the enzyme is active (Ramesh *et al.*, 1994). A majority of the proteases from serine protease family have a long propeptide that functions as a precursor structure. This structure can keep the protease from autolysis until it is processed when the protein is active (Yu *et al.*, 1999). The propeptide from *A. fumigatus* has an identical 100 amino acid segment with propeptide of the *A. oryzae* serine proteinase, suggesting that this propeptides are quite conservative within the same species. Since the N-terminal sequence for *A. versicolor* is similar to these three species, it is possible that the 41kDa protein is an alkaline proteinase that has a signal sequence, a prosequence and a mature protein. It is possible that the 43kDa protein is the unprocessed protein with a signal sequence of approximately 21 amino acids and the 41kDa are the same protein, but without the signal sequence. It is interesting to note that there is another 33kDa protein from *A. versicolor* crude culture supernatant extract that has the Western response against different mAbs (Figure 18). It is possible that the mature protein for *A. versicolor* alkaline serine proteinase is the 33kDa protein. The glycoprotein staining test

has confirmed that AVS 41kDa protein is not glycosylated, which was the same as determined by Ramesh *et al.* (1994) for *A. flavus,* Jaton-Ogay *et al.* (1992) for *A. fumigatus* and Tatsumi *et al.* (1989) for *A. oryzae*. However, since the human antibodies respond to the possibly unprocessed protein, this is what accumulates in nature.

There are some differences among these alkaline serine proteinases. 2D gel electrophoresis was used to determine the pI of the protein. The pI for the AVS 41kDa protein was determined to be 4.5 which was acidic. The pHs for all other proteinases were summarized in Error! Not a valid bookmark self-reference.. The pI for the *A. fumigatus* serine proteinase was 7.9 for (Reichard, 1990), 7.0 for *A. oryzae* (Shen *et al.*, 1998), 6.8 or 6.3 for *A. flavus* (Chou *et al.*, 1999; Yu *et al.*, 1999). All the alkaline serine proteinases are either slightly acidic or slightly basic, none of the alkaline serine proteinases were very acidic as for *A. versicolor*.

A. versicolor belongs to subphylum Ascomycotina which undergo at least one sexual production stage, so it is possible that *A. versicolor* has gone some gene exchange during their sexual stage in evolution and therefore share slightly different gene contents for the similar enzyme alkaline serine proteinase as *A. fumigatus*, *A. flavus* and *A. oryzae*.

Table 8 – Comparison of the isoelectric point for the proteins

Species	pI
A. versicolor	4.5
A. fumigatus	7.9
A. flavus	6.3 or 6.8
A. oryzae	7.0

10.3 Comparison of N-terminal sequences between *A. versicolor* and *Penicillium* species

Aspergillus and *Penicillium* are thought to be very closely related species found in damp indoor conditions (Bouwer, 1996). Chou *et al.* (1999), Shen *et al.* (1998) and Yu *et al.* (1999) have identified the major allergen for airborne *Aspergillus* species is the 34kDa alkaline serine proteinase protein found in *A. fumigatus* and *A. flavus*. However, serine proteinase is a very common protein found in various fungi other than *Aspergillus* species, such as *P.citrinum, P. chrysogenum* and *P. notatum* (Lin *et al.*, 2000). The comparison of the N-terminal sequence of *A. versicolor* with three *Penicillium* species is shown in **Error! Not a valid bookmark self-reference.**.

Table 9 – The N-terminal amino acid sequence result and blast search comparison with *Penicillium* species

Protein	N-terminal amino acid sequence	identity with AVS 41kDa
A. versicolor 41kDa	ALTTQSDAPP-GLGAIS--HQGDAS(/A)SSYI	100%
P. citrinum 34kDa	ANVVQSNVPSWGLARISSKRTGTTS YTYD	42.3%
P. chrysogenum 34kDa	ANVVQSNVPSWGLARISSKRTGTTS YTYD	42.3%
P. notatum 34kDa	ANVVQRNAPS	40.0% (4/10)

All the 34kDa proteins from *Penicillium* species were identified to be alkaline serine proteinase. However, the N-terminal amino acid only shared approximately 40% identity with *A. versicolor*. The total number of amino acid and their cleavage site are quite different. For example, the pre-proprotein for *P. chrysogenum* was predicted to be 397 amino acids with a mature protein starting from amino acid 115. Shen *et al.* (1999) have reported the pH for *P. notatum* was approximately 6.7-7.2 which is also close to neutral as other species from *Aspergillus*, different from *A. versicolor*.

10.4 The cross reactivity test against various mAbs

In this study, a total of 5 different mAbs were produced against AVS 41kDa *in vivo* in mouse ascites. From the culture supernatant, three mAbs 2H12, 6E2 and 8A10 showed the high response against AVS 41kDa on Western blotting. However, there are some other proteins from the culture supernatant in different species showed to be positive to the mAbs indicating that these proteins have similar epitopes as AVS 41kDa at denatured condition and these epitopes could be recognized by the mAbs on Western blot (Figure 23, 24, 25). However, these positive bands would not have the same properties as AVS 41kDa at native conditions when despite sharing similar epitopes.

The three mAbs 2H12, 6E2 and 8A10 were chosen to test the reactivities against different strains of AV spores. They all showed positive against AVS 41kDa except strain 1-1a indicating that AVS 41kDa is a very common protein produced by various *A. versicolor* strains in spores. There was another protein found in culture supernatant for *A. versicolor*

possibly the mature protein for AVS 41kDa at 33kDa showed positive response against human sera and mAbs (Figure 17, 18). However, this protein is not found in spores (Figure 20, 21, 22). *A. versicolor* species use spores to reproduce. This protein found in spores probably was still in its non-activated form, so the peptide that would be cleaved off during protein activation in cells was still attaching to the protein, and the activated/matured protein AVS 33kDa was not found. An antigen is a substance that can cause a hypersensitivity of the immune response when enters the body. When this antigen is associated with an expression of disease, the antigen is called an allergen (Horner *et al.*, 1995). Benndorfl, *et al.* (2008) tried to identify allergens found in *A. versicolor* spores. However, they failed to find any specific allergens to *A. versicolor*. They used extraction buffers to extract the proteins from the spore fragments after the spores were lysed. However, only the major allergens and those can be extracted sufficiently by their extraction buffers from the spores can be identified such as glyceraldehyde-3-phosphate dehydrogenase, an unnamed protein with homology to a sorbitol/xylose reductase, catalase A, enolase and malate dehydrogenase by their method. None of their allergens showed similarities with AVS 41kDa. Their results showed that the IgE response from human sera does not correlate with mold exposure in individuals and an increase in IgE response from human sera does not correlate with the increase concentration of total extracted allergens.

Alkaline serine proteinase-specific monoclonal antibodies against *Aspergillus* and *Penicillium* species were produced by Lin *et al.* (2000). They tried to inject the mice with different fungal extracts from *Aspergillus* and *Penicillium*. The results showed very little

or no ELISA reactivities against mAb been produced. However, the 5 mAbs produced in this study showed very good activity against *A. versicolor* crude culture extracts and two mAb 6E2 and 8A10 showed only two possible cross reactive fungal species (*A. sydowii* and *S. brevicaulis* for 6E2 in Figure 24 and *P. crustosum* and *S. brevicaulis* for 8A10 in Figure 25 among 17 tested crude fungal culture extracts.

The study to produce specific mAbs against *A. versicolor* was also done by Schmechel *et al.* (2005). They injected the mouse with crude spore extracts containing both fragments and intact *A. versicolor* spores. However, all the mAbs showed different degree of cross reactivity among different fungal species, especially species from *Penicillium*. ELISA was used to test the cross reactivity against different fungal spores in this experiment. The data have shown that it has a very good activity against different AV spores, but only *P. spinulosum* showed >1 OD values among 12 different tested species (Figure 58). *P. spinulosum* is also an indoor fungus that is found in moisture damaged buildings although it is not as common as *A. versicolor*.

Murtoniemi *et al.* (2001) have shown that the *P. spinulosum* spores could induce inflammatory and toxic responses in mouse macrophages *in vitro* at a concentration of 5×10^6 spores, but they are not cytotoxic even at a high dosage. Therefore, it is very unlikely that *P. spinulosum* could cause acute respiratory inflammation. It is necessary to note that the OD response is a log based response, so OD 1.8 for *A. versicolor* is approximately 8 times more than 1.1 for *P. spinulosum*. The cross reactivity showed very little or no OD values against various *Penicillium* species as well as *A. flavus* and *A.*

fumigatus indicating that the produced mAb 6E2 does not react on the common sites found in various alkaline serine proteinases.

10.5 Various purification techniques for IgM from mouse ascites

The isotype of the mAbs produced was IgM. IgM consists of 5 IgGs with a very large molecular weight. IgM is not usually a desired mAb isotype for antigens, since it is very susceptible for degradation under high salt conditions and the titre of IgM in mouse ascites is generally much lower than IgGs (Stein, 1998). The common purification methods are ion exchange chromagraphy or hydrophobic chromatography followed by gel filtration, but these are not good methods for bulk production of mAbs, since the recovery is generally very low. The supply of the ascites was limited (only 6mL in total for 6E2) for this study, so a one-step purification method is desired in this experiment to achieve a high recovery and purity.

Two different affinity purification methods were used to purify IgM antibody: HiTrap IgM purification HP from GE healthcare and IgM purification kit from Pierce. Some studies have showed very good IgM purification results from IgM purification kit (Nethery et al., 1989; Nevens et al., 1992; Ohta et al., 1990). However, they did not show very good reactivity after purification on Western blot in this study. IgM is very susceptible to high salt concentration for affinity purification, so the IgM could undergo degradation at 1.0M AS, resulting in a loss of antibody (Figure 26, 27, 29). However, the

IgM antibodies could not bind to the affinity column under low salt conditions (Figure 28).

The second purification method was 50% AS pellet followed by desalting using a 100kDa centrifuge filter, since the results (Figure 29) showed that there was no major loss of IgM antibody in the 50% AS pellet. However the semi-purified IgM antibodies still had a high background against AVS 41kDa pure protein on ELISA (Figure 32, 33). High background could be attributed by various factors such as blocking time, purity of the antigen, the antibody incubation time as well as the purity of the primary antibody. The blocking time was over night, so it should be sufficient to block all the active sites on the 96 well plate. The antigen was only 80% pure, but there was no Western response against other proteins. The ELISA signal for the semi-purified protein was very weak (OD 1.0 for 5C9 and OD 0.7 for 6E2), so by reducing primary or secondary antibody incubation time could potentially reduce the background as well as the signal.

The majority of the purification methods of IgM use gel filtration columns at the last step, since IgM has the high molecular weight, far apart from other proteins found in mouse ascites such as transferrin and albumin (Feldhoff *et al.*, 1985). Also low salt concentration is required to elute antibody from the column. Gel filtration would be a desired method to purify IgM from mouse ascites. The expected IgM elution time for HiPrep 16/60 Sephacryl S-300 HR gel filtration column was between fraction volume of 37.835 mL and 45.973 mL. The gel filtration curves for all 5 mAbs showed very similar curve and they all present a small peak between 35mL – 41mL which falls into expected

fractionation range. Once the small peaks were concentrated separately, the resultant antibody was relatively pure with a good Western response (Figure 37, 38, 43, 45 and 47) as well as a low background on ELISA (Figure 40, 41, 48, 49 and 50).

The ELISA of purified 6E2 against AV 41kDa pure protein showed the best activity, so 6E2 was used to produce a linear calibration curve (Figure 51). The linear detection range was from 0.02μg – 0.2μg/well, which is relatively low sensitivity by this method of ELISA. There are several reasons for this outcome. The AVS protein purified was only approximately 80% pure, since some 28kDa protein and small molecular proteins (MW ~ 10kDa) co-eluted with our target protein in gel filtration. Gel filtration is a method that can effectively separate proteins that differ by at least twice of the molecular weight. The small molecular weight proteins could interfere with the antigen and antibody binding in the well. IgM has a very large molecular weight and it has 10 potential antigen binding sites, but it is very difficult to have an effective binding with small antigens due to their small flexibility.

However, since there are good monoclonal antibodies available in terms of relative sensitivity and selectivity, there are a number of possible approaches for ELISA development. It would be useful to biotinylate one of these two mAbs and to develop a capture ELISA assay using two different mAbs. This would increase the sensitivity to well within the adequate range without damage to the antibodies discussed in this thesis.

11 Future research

The full protein sequence needs to be generated in the future to confirm that this AVS 41kDa is an alkaline serine proteinase, and the AVS 43kDa protein and AVS 33kDa protein are in fact the same enzyme but presented in the different forms in biological system. More studies need to be done to investigate how the protein is cleaved and become active in fungi.

The mAbs were generated successfully in this study with little cross reactivity against different fungal culture supernatant and different fungal spores. Two of the best mAbs can be selected to develop a capture ELISA assay which is a more sensitive and specific assay to detect fungal spores for indoor air.

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