2012

Characterization of terrelysin, a potential biomarker for *Aspergillus terreus*

Ajay Padmaj Nayak
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Characterization of terrelysin, a potential biomarker for *Aspergillus terreus*

Ajay Padmaj Nayak

Dissertation submitted to the School of Medicine

at West Virginia University

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in

Immunology and Microbial Pathogenesis

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2012

Keywords: terrelysin, biomarker, *Aspergillus terreus*, monoclonal antibodies, diagnostics

ABSTRACT

Characterization of terrelysin, a potential biomarker for *Aspergillus terreus*

Ajay Padmaj Nayak

Exposure to fungi can lead to diverse conditions from allergy to colonization to invasive and disseminated infection, depending on the status of the host immune system. Recent data suggests that fungal infections and especially *Aspergillus* species has been on the rise. Though mostly affecting immunocompromised individuals, more recent reports have identified infections in immunocompetent populations as well. *Aspergillus terreus* is of particular interest, since it causes rapid disseminating infections with high mortality rates, owing to its natural resistance to antifungal drugs and production of vegetative aleurioconidia during infection. These attributes coupled with poor current diagnostics and increasing costs involved with controlling the infection, have generated considerable interest in developing novel strategies for diagnosis of *A. terreus* infections. Fungal hemolysins have been proposed as biomarkers of exposure because of an association between exposures to the black mold *Stachybotrys chartarum* and an outbreak of idiopathic pulmonary hemorrhage. Using polyclonal antibodies, the hemolytic antigen was detected at concentrations of 371 ng/ml in patient sera. Our initial experiments were designed to develop monoclonal antibodies (mAbs) towards a stachylysin hemolytic preparation. IgM mAbs were developed with limited cross-reactivity within *Stachybotrys* species. ELISA and FHIA analysis showed that most stachylysin was present in hyphae. Using proteomic analysis, we could not confirm the identity of stachylysin because the genome of *S. chartarum* has not been sequenced. To better characterize hemolysins as biomarkers, the clinically relevant fungus (*A. terreus*) with a sequenced genome was chosen for analysis. Initial attempts at the characterization of a hemolytic fraction from *A. terreus* resulted in development of multiple *A. terreus*-specific IgG₁ mAbs to proteolytic enzymes including, leucine aminopeptidase and dipeptidyl peptidase V. We were unable to identify the hemolysin of interest using this mAb approach. Alternatively, expression of recombinant terrelysin (rTerrelysin) using the pASK-IBA6 vector in *Escherichia coli* yielded a protein with secondary structure similar to other Aegerolysin family proteins. Highly specific mAbs (10G4, 15B5 and 13G10) to the recombinant protein consistently recognized native terrelysin in different strains of *A. terreus*. Expression kinetics suggested that terrelysin is produced in high concentrations immediately after germination but levels decrease with increasing fungal biomass, and not present at the later time points when the previous hemolytic preparations were made. These data suggest interpretation of previous studies on detection of ‘hemolysins’ in exposed individuals and experimental animals are likely an error. The mAbs developed in this study for terrelysin, leucine aminopeptidase, and dipeptidyl peptidase V have potential for development of rapid and specific diagnostic assays. Tools developed in this study could be used for analysis in various animal models to determine combinatorial assays using mAbs developed to different proteins of *A. terreus*.

ACKNOWLEDGEMENTS

One individual cannot achieve success of this nature on his own accord and neither was mine. Many people have put their time and effort in contributing towards this achievement and no one has sacrificed more than my parents. This marks an end of a long struggle of their aspirations for me. I am also very thankful to the roles played by many individuals without which I would not be here today. First of all I would like to thank Dr. Fred Minnear, Clair Noel and the rest of the Office of Research and Graduate Education for providing me with an opportunity to receive education at West Virginia University. I would also like to thank my aunt Sheela and my uncle Manohar for their generosity, which helped me pursue this opportunity.

Although receiving the opportunity was special, sustaining this was always going to be challenging and many people contributed towards helping me achieve my goals. None other has contributed more to this than my advisor Dr. Donald Beezhold. I am very thankful to Don for his constant encouragement and his belief in me. Thank you very much for challenging me to think and I will never forget the lessons I learned from our Monday afternoon meetings. Dr. Brett Green and Dr. Detlef Schmechel have imparted a tremendous wealth of knowledge to me and to this I express my gratitude. Dr. Green's commitment and dedication to his work has always inspired me to work hard. I would also like to thank my committee members Dr. John Barnett, Dr. Slawomir Lukomski and Dr. Michael Miller for their valuable contributions and constant encouragement. Some people have also contributed towards this success by filling up a lot of paperwork for this international student. My thanks to Brooke from WVU and Kathy from NIOSH for their dedication and promptness which ensured that I could perform my research duties uninterrupted.

Through last 6 years, I had the opportunity to make some great friends at work and outside of it. I would like to thank Abhijeet for being a great roommate. I wish him the best in his future endeavors as he too looks forward to life after his recent graduation. I would also like to thank EJ and Ashley for being very kind to me and I will always cherish my time at your wedding. I would also like to thank my American mothers Toni and Erika for their tremendous patience with me in the lab. I would like to thank my other lab mates Amanda, Bill, Itai and Steve for their valuable inputs.

I also express my gratitude to my fiancé Nedra for her love and support during the toughest times of my graduate life. Thank you for making me the better person that I am today. I am looking forward to spending the rest of our lives together working through many challenges and enjoying great times.

Last but not least I am very thankful to the taxpayers of United States. Their generous contributions have changed my life in more ways than they can imagine.

So many people have touched my life in so many different ways that it is hard to list all of them. I would like to thank all those who made my doctoral education one of the best times of my life.

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LIST OF ABBREVIATIONS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
ABPA	allergic bronchopulmonary aspergillosis
AHTC	anhydrotetracycline
ATCC	American Type Culture Collection
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
cAtp	cytolytic <i>A. terreus</i> preparation
CBS	Centraalbureau voor Schimmelcultures
CCB	carbonate coating buffer
CD	circular dichroism
CE	conidial extract
CF	culture filtrate
CSN	culture supernatant
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfonic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FDA	Food and Drug Administration
FGSC	Fungal Genetics Stock Center
FHIA	Fluorescent Halogen Immunoassay
HEA	hyphal exoantigens
HEPA	high efficiency particulate air
IA	invasive aspergillosis

IBT	Instituttet for Bioteknologi
mAb	monoclonal antibody
MALDI-TOF	Matrix assisted laser desorption/ionization- time of flight
MCE	mixed cellulose ester
ME	mycelial extract
MEA	malt extract agar
NBT	nitro blue tetrazolium
NIOSH	National Institute for Occupational Safety and Health
NRRL	Agricultural Research Service Culture Collection
OD	optical density
pAb	polyclonal antibody
PBST	phosphate-buffered saline pH 7.4, 0.05% (v/v) Tween 20
PBSTM	PBST, 1% non-fat dry milk
PBSTB	PBST, 5% BSA
PCR	polymerase chain reaction
RT	room temperature
SBA	sheep red blood agar
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SRBC	sheep red blood cells
TSB	tryptic soy broth
UAMH	University of Alberta Microfungus Collection and Herbarium

CHAPTER 1

General Introduction/Literature Review

1.1 INTRODUCTION

Fungi are one of the most ubiquitous groups of eukaryotic organisms in nature. Kingdom Mycota includes an estimated 1.5 million species of eukaryotic organisms including yeasts, molds and mushrooms. They characteristically lack chlorophyll and are saprophytes i.e. they depend on external sources of organic material for nutrition. In nature, they play an important role in recycling elements in soil. They are used in industry as a source of enzymes and important secondary metabolites that have tremendous economic and therapeutic value. Exposure of human population is ubiquitous and a growing number of individuals with impaired immune systems are susceptible to infections from these microbes, thus making them clinically relevant.

Within the Kingdom Mycota, fungi are divided based on distinguishing characteristics, with specific emphasis on the different reproductive stages. Kingdom Mycota is further divided into seven Phyla (1); Phylum **Glomeromycota** consists of fungi that have an important role in the ecosystem and form mutualistic symbiotic relationship with plants; fungi belonging to Phyla **Chytridiomycota**, **Blastocladiomycota** and **Neocallimastigomycota** are characterized by flagella and are present mostly in aquatic environment; Phylum **Microsporidia** consists of spore-forming unicellular obligate parasites; and Phyla **Basidiomycota** and **Ascomycota** represent the largest number of fungal species known and have been grouped together under the Sub Kingdom Dikarya.

Ascomycota, the largest and most diverse Phyla consists of approximately 64,000 species (2). Members of Ascomycota are present in most natural and industrial environments. They have also been surprisingly isolated from extreme environments (3-5). Ascomycetes include fungi that are important in industry for their enzymes and secondary metabolites that have nutritional and

therapeutic value. Ascomycetes also include of large number of species that can cause opportunistic infections in humans, animals and plants particularly *Aspergillus* and *Penicillium*.

1.2 MORPHOLOGICAL CHARACTERISTICS

Stachybotrys and *Aspergillus* spp, two filamentous fungal species that are the focus of this work, are molds that exist in the form of non-motile conidia or hyphae. Their perseverance is highly dependent on the availability of moisture and nutritive material. Typically, filamentous fungi exhibit the following morphological structures (6);

1.2.1 Conidium- Conidium (pl. conidia) also referred to, as a spore, mitospore or conidiospore is the dormant form of the fungus that acts as a secure ‘house’ for survival of the fungus in presence of adverse environmental conditions. The conidium has a highly structured external cell wall composed of α or β (1-3) glucan-proteins and chitin fibrils. In addition, pigments such as melanin are also associated with the conidial cell wall. Small hydrophobic proteins, called as hydrophobins coat the outer surface of conidia and this coating protects the conidia from dehydration and allows for attachment of conidia to various surfaces (6, 7). Ergosterol is present in the membrane and functions similarly to cholesterol in mammalian cells.

On access to nutrients and water, various signaling molecules initiate the process of germination, resulting in swelling of the conidia, differential expression of proteins, and changes in surface properties (8-10). The first evidence of hyphal growth is observed in the form of a developing germ tube. An important feature of germinated conidia is its increased adhesive property and a polarized cellular growth. At this stage, the outer coat that existed on the conidia is completely shed and is identified as a germling.

1.2.2 Hypha- Hypha (Pl. hyphae) extends from germinating conidia and continues expansive cellular growth. These structures are identified as the vegetative form of the fungus, usually identified during infections. The hyphal cell wall is thinner than the conidial cell wall and this allows for an easy flow of nutrients into the cell. Hyphae are multicellular and may (*Aspergillus* species) or may not (*Mucor* species) be divided into compartments by septa. Septa are usually incomplete and do not lead to permanent compartmentalization i.e. they allow the transport of ribosomes, mitochondria, nuclei and other organelles between compartments.

1.2.3 Mycelium- Mycelium (Pl. mycelia) is an aggregate of an extensive network of intertwining hyphae. Mycelia play a critical role in the environment in cycling of elements by degrading organic material. The mycelial stage also allows for aggressive utilization of organic materials available in the environment.

1.2.4 Foot cell, conidiophore and vesicle- As nutrient and water availability decreases, the cell resorts to producing conidia for promoting survival. Along the hypha, a stalk-like structure emerges to form a conidiophore. The basal cell from which the conidiophore arises is identified as the foot cell. The conidiophore produces conidia on a funnel-like structure called a vesicle. Conidia are formed as chains at the apex of the vesicle and are dispersed back into the environment.

1.3 FUNGAL DIAGNOSTICS

Diagnosis of fungal infections and specifically detection of filamentous fungal species has been challenging (11-14). Treatment of symptoms including prolonged fever and a condition of neutropenia with unresponsiveness to antibacterial therapy is usually followed by antifungal agents. This lag period can result in severe recalcitrant fungal infections, making it imperative to diagnose fungal infections early. The following methods have been used either singly or in combination to diagnose the fungal infection etiology (14);

1.3.1 Culture methods- Isolation of fungi has been possible from cutaneous and superficial infections (15), however fungal recovery from invasive infections is inconsistent and unreliable (16-23). Fungemia may not occur even though mycological evidence is clear in infected tissues (23-25). Detection of aleurioconidia (conidia produced by vegetative hyphae) can be definitive for identification of *A. terreus* (26) however, other fungi such as *Fusarium*, *Paecilomyces*, *Scedosporium* and *Acremonium* species may release morphologically similar vegetative conidia in tissues and blood during infection, thus making differentiation complicated (23). The overall low sensitivity and inconsistency of isolation and differentiation make macroscopic and microscopic determination difficult at species level.

1.3.2 Molecular methods- DNA based methods are rapid and definitive and the presence of fungal DNA from sterile sites such as blood or other affected tissues (except lung) could possibly hold diagnostic value. A few studies have reported success from using PCR based techniques at qualitative as well as quantitative levels (27, 28). PCR methods have been developed using specific genes such as ribotoxin (29), tRNA (30), cytochrome b (31) and 18S rRNA (32, 33) as

primer targets. Notwithstanding the popularity and demand for PCR based diagnostics, currently there is no FDA-approved fungal diagnostic PCR testing systems available. Certain experimental and methodological limitations hinder the use of PCR methods. Isolation of fungal DNA from clinical samples is unreliable and success rate varies between various methods (34). Detailed information on advantages and disadvantages of using PCR based methods has been reviewed extensively elsewhere (14, 35).

1.3.3 Radiological techniques- Radiological scanning techniques used for diagnosis of invasive aspergillosis may be suggestive of an active infection but are not species specific for developing targeted therapeutics (36, 37). Chest radiograph can help in identification of aggregated fungal mass in the lungs (38). Computed tomography exhibits higher sensitivity compared to other radiological techniques especially during early stages of infection (39). Collectively, these observations in collaboration with clinical manifestations of aspergillosis have been used successfully in improving the outcomes of invasive aspergillosis, but limitations hinder the use of these techniques especially in pediatric populations (40).

1.3.4 Histology- The lung is not considered a sterile site for etiological determination during fungal infections, since many different fungi may also be isolated. This makes clinical samples such as bronchial lavage and sputum less than ideal for the diagnostic detection of fungal material. However, histological confirmation of invasive vegetative hyphae or positive culture from biopsies of sterile sites such as pleural fluid may hold diagnostic value (41). Commonly used staining procedures stain for carbohydrate moieties that are shared by most fungi aid in ascertaining fungal growth but are not species specific (14).

1.3.5 Serology- One of the biggest advantages of developing serological diagnostics for invasive aspergillosis is that it obviates the need for invasive procedures (41). Historically however, serological immunodiagnosis has been difficult due to unreliable antibody responses and insensitive results (42-45). Humoral antibody responses to *Aspergillus* species during invasive infection can be weak due to the immunocompromised nature of these patients. Measurement of serum galactomannan (46) and β -D-glucans, which constitute the fungal cell wall have been widely used with some success in diagnosis of invasive fungal disease (35). Galactomannan is a polysaccharide with a mannose backbone and galactose side chains, while β -D-glucans are polysaccharides with D-glucose monomers linked by β -glycosidic bonds. While these assays are currently used for diagnosis of invasive fungal diseases, they lack sensitivity and detection is not species specific, which results in inconsistent determinations (14, 47, 48).

1.3.6 Biomarkers for immunodiagnostics- Detection of circulating antigens expressed by vegetative hyphae using species-specific monoclonal antibodies holds potential for developing ideal immunodiagnostics (49). Some have reported the presence of circulating protein antigens in infected patients (50-52). To have diagnostic value, prospective biomarkers should be expressed in the vegetative hyphae and preferably secreted by the growing hyphae. Detection of the biomarker should be rapid and species specific. The development of monoclonal antibodies for detection of the biomarker is essential to maintain species specificity in detection. This also provides an opportunity to develop standardized quantitative assays.

1.4 FUNGAL HEMOLYSINS

Fungal hemolysins have been suggested as specific biomarkers for fungal exposure (53-55), as they have been detected in sera and tissues of patients and experimental animals (53, 56). Hemolysins are pore-forming toxins that can lyse membranes of red blood cells. Though they are typically called hemolysins because of their ability to lyse red blood cells, many are capable of lysing other eukaryotic cells as well. Hemolysins and their role as virulence factors have been extensively studied in bacteria (57, 58); however less is understood about fungal hemolysins and their role in pathogenesis.

Hemolytic activity was first identified in fungi by Henrici in 1939 in mycelial extracts of *A. fumigatus* and was later reported in other fungal species by Salvin in 1951 (59, 60). Subsequently, hemolytic activity has been reported for many fungal species and the hemolysins have been identified and are summarized in **Table 1.1**. The first fungal hemolysin to be purified was from *A. fumigatus*; called asp-hemolysin. Similar hemolysins have been identified in other fungi and are currently identified as a family of proteins called Aegerolysins. Increasing interest in these proteins has led to improved understanding of the mechanisms underlying their hemolytic activity, role as virulence factors, binding to lipoproteins, and diagnostic potential as biomarkers of fungal exposure (61).

FUNGI	AEGERLOYSIN IDENTIFIED	REFERENCES
ASCOMYCETES		
<i>Aspergillus clavatus</i>	YES	(62)
<i>Aspergillus flavus</i>	YES	(59, 63, 64)
<i>Aspergillus fumigatus</i>	YES	(56, 59, 62, 65-75)
<i>Aspergillus nidulans</i>	YES	(76, 77)
<i>Aspergillus niger</i>	YES	(78-81)
<i>Aspergillus oryzae</i>	YES	(82, 83)
<i>Aspergillus terreus</i>	YES	(84)
<i>Alternaria tenuis</i>	NO	(85)
<i>Blastomyces dermatidis</i>	YES	(60, 86-88)
<i>Candida albicans</i>	NO	(60, 89, 90)
<i>Candida glabrata</i>	NO	(91)
<i>Coccidioides posadasii</i>	YES	(92)
<i>Cordyceps militaris</i>	YES	(93)
<i>Cryptococcus neoformans</i>	NO	(60)
<i>Fusarium oxysporum</i>	YES	(94)
<i>Histoplasma capsulatum</i>	YES	(60, 92, 95-97)
<i>Metarhizium acridum</i>	YES	(98)

<i>Metarhizium anisopliae</i>	YES	(98)
<i>Nectria haematococca</i>	YES	(99)
<i>Neurospora crassa</i>	YES	(100)
<i>Neurospora tetrasperma</i>	YES	(101)
<i>Paracoccidioides brasiliensis</i>	YES	(102-104)
<i>Penicillium chrysogenum</i>	YES	(105, 106)
<i>Sordaria macrospora</i>	YES	(107)
<i>Stachybotrys chartarum</i>	NO	(53, 108-112)
<i>Trichophyton species</i>	NO	(113)
BASIDIOMYCETES		
<i>Moniliophthora perniciosa</i>	YES	(114, 115)
<i>Agrocybe aegerita</i>	YES	(116, 117)
<i>Pleurotus eryngii</i>	YES	(118, 119)
<i>Pleurotus ostreatus</i>	YES	(117, 120-132)
<i>Pleurotus nebrodensis</i>	NO	(133)
<i>Amanita phalloides</i>	NO	(134-142)
<i>Amanita rubescens</i>	NO	(139, 143-146)
<i>Hypholoma fasciculare</i>	NO	(139)
<i>Laetiporus sulphureus</i>	NO	(147-149)

<i>Rhodophyllus rhodopolius</i>	NO	(150)
<i>Flammulina velutipes</i>	NO	(151-156)
<i>Volvariella volvacea</i>	NO	(157)
<i>Termitomyces clypeatus</i>	NO	(158)
<i>Schizophyllum commune</i>	NO	(159)
<i>Wallemia sebi</i>	NO	(160)
ZYGOMYCETES		
<i>Rhizopus nigricans</i>	NO	(161, 162)
<i>Rhizopus arrhizus</i>	NO	(161)

Table 1.1: Hemolytic activity of different fungal species. *This strain is now identified as *Stachybotrys chlorohalonata* (163).

1.5 AEGEROLYSINS

1.5.1 General characteristics- Current information on the Aegerolysin family proteins (Pfam: PF06355; InterPro: IPR 009413) is largely based on studies of asp-hemolysin (*A. fumigatus*) and ostreolysin (*P. ostreatus*) (61). The Aegerolysin protein domain is currently identified in 114 sequences spread across fungi, bacteria, plants and a virus (**Appendix A2**). The Pfam protein database provides information on species distribution of the aegerolysins (164). Aegerolysin proteins are typically 15-20 kDa in size and are rich in negatively charged residues (61, 117,

165). The isoelectric point of aegerolysins is acidic and has been experimentally determined for ostreolysin (pI 5.0), and aegerolysin (pI 4.85) (117).

1.5.2 Hemolytic and cytolytic activity- Fungal hemolytic activity was initially reported from crude or partially purified mycelial extracts (63, 166-168). Improvements in biochemical techniques contributed to purification of asp-hemolysin from *A. fumigatus* (169). Asp-hemolysin was identified to form distinctive pores on the surface of cell membranes to induce lysis by the colloid-osmotic mechanism (170). Detection of asp-hemolysin *in vivo* during infection of *A. fumigatus* has led to suggestions that the protein may be important for pathogenesis (49, 171). The hemolytic activity is stable in a wide range of pH (65, 119, 123, 169, 170, 172). Other studies have characterized the ability of these proteins in lysing red blood cells and other eukaryotic cells from different animal species (**APPENDIX A3**).

Pore forming toxins bind to diverse receptors on the surface of target cells (173-175). Membrane lipid receptors for aegerolysins were identified by partitioning of ostreolysin to detergent resistant membranes (DRMs) in natural and artificial systems (122). Ostreolysin did not bind pure cholesterol (117, 121). Binding occurred when cholesterol was combined with sphingomyelin (122, 126). In further studies, removal of cholesterol from membranes of CHO-K1 cells by methyl β -cyclodextrin inhibited binding of ostreolysin (176).

1.5.3 Studies in experimental animals- Several studies have characterized the effects of fungal hemolysins *in vivo* (59, 61, 63, 125). The intravenous (i.v.) LD₅₀ value for asp-hemolysin in mice is reported to be 750 μ g/kg (169) and that for ostreolysin was higher at 1170 μ g/kg for mice (125). For ostreolysin, concentrations higher than LD₅₀ value resulted in death of

experimental animals within 20 mins. In an interesting study, injecting asp-hemolysin i.v. simultaneously with non-lethal dose of *A. fumigatus* conidia increased the mortality rates in experimental mice (56). Injecting asp-hemolysin also aided in improved survival of *A. fumigatus* conidia within the animals. More interestingly, mice immunized i.p. with anti-asp-hemolysin antibodies before *A. fumigatus* conidial challenge promoted survival of the animals.

Asp-hemolysin has also been detected in infected tissues of mice challenged with *A. fumigatus* conidia (177). Intravenous administration of experimental animals with asp-hemolysin showed that it can bind to arterial walls in the kidneys, heart, liver and the brain. Binding resulted in thickening of epithelial cells in the kidney, degeneration and necrosis of the cardiac muscles in heart, necrosis of liver cells with pyknosis and focal lesion and hemorrhage in brain. Asp-hemolysin also induced contraction of guinea pig ileum.

Studies using ostreolysin have also yielded similar results in terms of toxicity. Intravenous administration of ostreolysin resulted in hyperkalaemia, which resulted from lysis of host cells (125). This observation was further supported in another study where increasing doses of ostreolysin lead to development of tension in isolated rat aortic rings and diminished the relaxation of endothelium (129). This in combination with cytotoxicity towards human umbilical vein endothelial cells suggests a possible role in cardiotoxicity for these proteins.

1.5.4 Role of aegerolysin proteins in biology of the organisms- Initial studies identified that expression of hemolysins is specific for the vegetative hyphae and suggest a role during the early stages of immature fruiting bodies (116). Studies with ostreolysin and aegerolysin highlighted that the expression of these proteins varies with the morphological changes occurring in developing mushrooms (117). Ostreolysin is expressed by the growing hyphae and it may play a

role in aggregation and compaction of hyphae to form primordia (117, 124). Addition of ostreolysin externally enhanced hyphal aggregation and formation of fruiting bodies (127). A clearer understanding of the role of these proteins in filamentous fungi is needed.

1.6 FUNGAL HEMOLYSINS AS BIOMARKERS

Interest in use of hemolysins as specific biomarkers for fungal exposure developed first in cases of infant idiopathic pulmonary hemorrhage (IPH) reported from water damaged homes in Cleveland, OH which resulted in mortality (178, 179). Exposure to water-damaged homes resulted in 8 infants with IPH and 1 mortality and further investigation suggested the black mold, *Stachybotrys chartarum* was the etiological agent responsible for IPH (180-182).

S. chartarum is a dematiaceous fungus that has been associated with a number of human and veterinary health problems (108). *S. chartarum* is particularly adept at growing on water infiltrated cellulose-based building materials. The morphological features of *S. chartarum* are characterized by septate hyphae and conidiophores that bear clusters of phialides from which chains of dematiaceous conidia emerge. Identification of *Stachybotrys* conidia in tape lift or air samples in indoor environments is considered a biomarker of indoor fungal contamination by various federal, state, and academic institutions. *Stachybotrys* conidia and hyphae contain mycotoxins, allergens, proteases, and other immunostimulatory molecules (183, 184). Personal exposure to *S. chartarum* is also considered an etiological agent for respiratory disease (180-182).

Initial investigation of the water-damaged homes in Cleveland, Ohio led to identification of mold contamination and isolation of multiple strains of *S. chartarum* (108, 185). Toxicity studied in strains isolated from water damaged homes and control homes suggested additional

factors might be responsible for the pathogenicity. *S. chartarum* strains isolated from water damaged homes exhibited hemolytic activity *in vitro* when grown on wet wallboards at 37°C (108).

Hemolytic activity has often been associated with pathogenesis of various microorganisms (58, 186-190). Three strains of *S. chartarum* isolated from the homes in Cleveland where infants became sick (Cleveland strains) and from the lung of an infant patient in Houston (Houston strain) suffering from IPH showed high toxicity and hemolytic activity, suggesting a possible correlation between the hemolysin and IPH (108, 109, 111, 185, 191). Cleveland and Houston strains of *S. chartarum* exhibited higher levels of hemolytic activity compared to control strains (109). In a later study it was reported that when all *S. chartarum* strains were grown in medium containing blood, culture supernatant (CSN) concentrate from all the strains exhibited hemolytic activity (111). These observations have been reported in a prior study (109).

Further studies led to partial purification of the hemolytic protein of *S. chartarum*, stachylysin and its initial biochemical characterization (110). The authors emphasized that purification of the hemolytic principle from the fungi and generation of antibodies could help develop diagnostic assays to determine fungal exposure. Detection of the hemolysin would in principle act as a surrogate biomarker for *S. chartarum* exposure. Initial characterization of stachylysin suggested it could be an aegerolysin including; 1) Using MALDI-TOF, stachylysin was identified as an ~12 kDa protein, which is the typical size of aegerolysin proteins. 2) Stachylysin contains large number of negatively charged amino acids also typically associated with aegerolysin proteins. 3) Kinetics of hemolysis was similar to that of ostreolysin, presenting an initial lag prior to hemolysis, suggesting aggregation of monomers on the surface of target

cells. 4) The hemolytic activity of stachylysin was compromised on heat treatment at 60°C for 30 min. Aegerolysin proteins are heat-labile and lose their hemolytic activity at temperatures above 65°C.

Previously, the role of hemolysins in causing hemorrhagic lesions in experimental animals exposed to asp-hemolysin from *A. fumigatus* was established (169). The possible involvement of stachylysin in IPH was suggested based on studies performed in an earthworm (*Lumbricus terrestris*) model (111). Stachylysin injected into *L. terrestris* resulted in release of erythrocyte hemoglobin and lethality. Furthermore, lung tissues from mice and rat exposed to *S. chartarum* conidia via intra-tracheal instillation developed granuloma and stachylysin was immuno-stained surrounding the conidia in the mouse lung suggesting its diffusion and possible role in damage to the tissue (112).

In a critical study in identifying this hemolysin as a biomarker for detection of exposure to *S. chartarum*, stachylysin was detected in sera of rats exposed to *S. chartarum* conidia using anti-stachylysin polyclonal antibodies raised in rabbits (53). In the same study, the data suggested that stachylysin could be detected in sera from human subjects exposed to *S. chartarum* in a water-damaged environment. Since, the levels recorded were extremely high (371 ng/ml), stachylysin was proposed as a potential quantifiable biomarker for personal exposure to *S. chartarum*.

1.7 CONCLUSIONS

Fungal hemolysins have been characterized for their role in fungal development and have been suggested as putative biomarkers for pathogenesis. Studies have identified that hemolysins are expressed in high concentrations in mycelium, which is the infective stage of fungi. There is

limited evidence that these hemolysins might also be present in fungal conidia. Some studies have also used polyclonal antibodies to detect fungal hemolysins in sera of patients. Collectively, these observations substantiate the utility of hemolysins as putative biomarkers for fungal exposure.

We hypothesize that hemolysins are secreted during initial stages of germination and that high concentrations could be present in mycelium. Since mycelium is the invasive stage of infection, high concentration of hemolysins could be present during active infection. Hemolysins may be released into the surrounding tissues including blood and thus may be available for identification as a biomarker. In the following research we have characterized the production of hemolysins from *Stachybotrys* and *Aspergillus terreus* to determine their suitability as potential biomarkers. A previously uncharacterized hemolysin (terrelysin) from *A. terreus* was identified and further characterized by producing a recombinant protein expressed in *Escherichia coli*. Recombinant terrelysin was used to develop monoclonal antibodies (mAbs) as research tools for characterization of terrelysin production and expression in the fungi. Finally, we explore the potential use of these antibodies as diagnostic tools to test for terrelysin as a biomarker of fungal disease.

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CHAPTER 2

**Characterization of monoclonal antibodies developed against a
hemolytic preparation (stachylysin) from *Stachybotrys***

2.1 INTRODUCTION

Filamentous fungi belonging to *Stachybotrys* species are occasionally isolated as contaminants of indoor and outdoor air. *Stachybotrys* are widely present and currently, 55 species of *Stachybotrys* including *Memnoniella* are known (1). *Stachybotrys* species produce trichothecene mycotoxins called as satratoxins, which bind to eukaryotic ribosomes and act as inhibitors of protein synthesis (2, 3). *Stachybotrys* species also produce proteinases that contribute to lung inflammation and injury through degradation of host collagen and can induce activation of a pro-inflammatory response (4).

S. chartarum exhibits hemolytic activity, which has been attributed to its hemolysin, stachylysin (5-8). Moreover, the detection of stachylysin in sera of experimental animals and patients with known exposure to *S. chartarum* in indoor environment provided an impetus for development of specific immunodiagnostic methodologies (9). However, polyclonal antibodies (pAbs) used in this study can lack specificity and are cross reactive as reported in previous studies in our laboratory (10-13). pAbs are also prone to batch-to-batch variability, which may influence the development of standardized assays. In order to overcome these limitations, we aimed to develop monoclonal antibodies (mAb) to stachylysin that were species-specific. Compared to pAb-based methodologies, mAbs are mono-specific, can be generated as a constant resource, and therefore useful for development of standardized immunoassays. In our laboratory, we have previously successfully developed a species-specific mAb against *S. chartarum* that recognizes conidia but not the hyphae (11, 13, 14).

To characterize stachylysin, we used *S. chlorohalonata*, a newly identified species that has the same phenotypic features as *S. chartarum* chemotype A, but morphologically is characterized by green extracellular pigmentation on Czapek yeast autolysate (CYA) medium (1,

15). The strain of *S. chlorohalonata* (ATCC 201863; IBT 9825) was chosen because it was used in previous studies to produce the stachylysin (7). It was originally designated *S. chartarum* and isolated from the home of an infant diagnosed with IPH (5, 7). Nearly all strains of *S. chlorohalonata* produce *S. chartarum* chemotype A toxins, atranones and dolabellanes (15). Atranones also contribute to a variety of immunotoxic, inflammatory and pathological changes on exposure of animals to conidia from *S. chartarum* chemotype A (16). Dolabellanes are C₂₄ atranones that also have been previously reported to be cytotoxic (17).

In this chapter, we report the generation of IgM mAbs to a stachylysin preparation and characterize their reactivity to purified stachylysin and extracts of *S. chlorohalonata*. In addition, we characterize the mAbs for cross-reactivity to other fungal species including multiple *Stachybotrys* species.

2.2 METHODS AND MATERIALS

2.2.1 Semi-purified cytolytic *Stachybotrys* preparation- Stachylysin from *Stachybotrys chlorohalonata* ATCC 201863 (American Type Culture Collection, Manassas, VA) was semi-purified from tryptic soy broth (TSB, Becton Dickinson, Sparks, MD) CSN as previously described (7). Briefly, *S. chlorohalonata* conidia (1×10^5) were used to inoculate 500 ml of TSB in a 1 liter flask placed on an incubator shaker for 7 days (7). Cellular debris was removed from the TSB CSN by centrifugation for 15 minutes at 5000 g. The supernatant was then centrifuged in a Millipore Centricon plus 80 filter apparatus (Millipore, Bedford, MA) with a molecular mass cutoff of 50 kDa at 4000 g for 15 minutes. The concentrate was then subjected to gel filtration and fractions were collected and plated onto sheep blood agar to determine hemolytic activity as previously described (7). The five most hemolytic fractions were pooled, desalted, and

lyophilized as previously described (7). The lyophilized pellet was resuspended in sterile water and stored at -20°C for further analysis and for immunization of animals for antibody production.

2.2.2 Preparation of fungal hyphal extracts- Fungi were grown in standard unsealed Petri plates containing 5 ml of malt extract agar (MEA; 2% dextrose, 0.1% peptone, 2% malt extract, 2% agar; Difco, Becton Dickinson, Sparks, MD). After 2 weeks of incubation at room temperature (RT), conidia were collected from sporulating cultures into TSB and 10 ml of the conidial suspension (1×10^6) were transferred into a 125 ml Corning flask containing 50 ml of TSB. The flasks were rotated at 120 rpm at 37°C for 3-4 days before the hyphae were harvested by filtration using a cell strainer (70 μ m, Becton Dickinson). The collected hyphae were washed two times in 50 ml phosphate-buffered saline pH 7.4 containing 0.05% (v/v) Tween 20 (PBST) before being homogenized in the cell strainer using the plunger from a 10 ml syringe. The homogenate was centrifuged at 4100 g for 5 min at 4°C and aliquots of the supernatants were stored at -80°C. The total protein concentration in the hyphal extracts was determined using a BCA™ protein assay kit (Pierce, Rockford, IL).

2.2.3 Production of monoclonal and polyclonal antibodies against stachylysin- Five 10-14 week old BALB/c female mice were housed under controlled environmental conditions in HEPA-filtered ventilated polycarbonate cages on autoclaved hardwood Beta-chip bedding and were provided Teklad 7913 rodent chow (Harlan Laboratories, Madison, WI) and autoclaved tap water ad libitum. Sentinel mice were free of viral pathogens, parasites, mycoplasma, and *Helicobacter* spp. The National Institute for Occupational Safety and Health (NIOSH) animal

facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Mice were immunized intraperitoneally at bi-weekly intervals. Mice were primed with 50 µg of stachylysin emulsified in equal volumes of TiterMax® (TiterMax USA Inc., Norcross, GA). The antigen concentration was reduced by half for each of 5 subsequent booster immunizations. A final boost of 50 µg was given three weeks after the sixth immunization and mice were sacrificed 3 days later for hybridoma production.

Hybridomas were produced by standard polyethylene glycol-based cell fusion techniques using SP2/0-AG14 myeloma cells (ATCC# CRL-1581). Cell cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Rockville, MD), supplemented with 1 mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.292 mg/ml L-glutamine, 100 mM sodium hypoxanthine, 16 mM thymidine, 10% fetal calf serum (HyClone, Logan, UT), and 100 units/ml IL-6 (Boehringer, Mannheim, Germany). DMEM medium was also supplemented with azaserine for selective propagation of hybridomas. Positive clones were identified using 1µg/ml of the stachylysin in an indirect ELISA (see below). Positive colonies were cloned twice by limiting dilution and the stable hybridomas were grown in bulk, aliquoted, and stored in liquid nitrogen. Hybridoma CSN containing anti-stachylysin mAbs were stored at 4°C and used for various experimental analysis.

Rabbit pAbs against stachylysin were custom-produced and affinity-purified by Bethyl Laboratories (Montgomery, TX) using standard laboratory protocols.

2.2.4 Screening ELISA format for the analysis of hybridoma culture supernatants-

Hybridoma CSN were screened using an indirect ELISA as previously described (11). In brief, ELISA plate wells were coated with 100 μ l of the stachylysin (1 μ g/ml) in carbonate coating buffer (CCB, 60 mM sodium carbonate, 140 mM sodium bicarbonate, pH 9.6) and incubated at RT overnight. Wells were washed 3 times with 200 μ l/well of phosphate buffered-saline containing 0.5% Tween-20 (PBST) for 10 min. The plates were then blocked for 1 hr at RT with 200 μ l/well of PBST containing 1% non-fat dry milk powder (PBSTM). Hybridoma CSN were incubated for 1 hr at 37°C with 100 μ l of mAb CSN diluted 1/5 (v/v) in PBSTM. Bound antibodies were detected with 100 μ l of Biotin-SP-conjugated AffiPure goat anti-mouse IgG and IgM secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) for 1 hr at 37°C at a dilution of 1/5000 (v/v) in PBSTM. Bound biotin was detected with 100 μ l of alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) by incubation for 1 hr at 37°C at a dilution of 1/5000 (v/v) in PBSTM. The reaction product was produced by incubating 100 μ l per well of 4-nitrophenyl phosphate (Sigma, St. Louis, MO) in alkaline phosphatase substrate buffer (1 M diethanolamine, pH 9.5; 5 mM MgCl₂) at RT and the optical density (OD) was determined at 405 nm after 30 min using an UltraMicroplate Reader, Model EL_x800 (BIO-TEK Instruments, Inc., Winooski, Vermont). Negative control values were obtained by substituting plain culture supernatant for mAb culture.

2.2.5 Capture ELISA format for the analysis of hyphal extracts- The specificity of mAbs was tested against hyphal extracts of 7 *S. chartarum* isolates, 8 isolates of other *Stachybotrys* species including *S. chlorohalonata* as well as 39 related and non-related fungi commonly found in indoor environments. All extracts were tested in a capture ELISA using rabbit anti-stachylysin pAbs as solid-phase capture reagent. In brief, ELISA plate wells were coated with 100 µl of rabbit anti-stachylysin pAbs (1 µg/ml) in CCB overnight at RT. The plates were washed and blocked as described above and 100 µl/well of hyphal extract from each fungus (50 µg/ml) in PBST were added and allowed to react for 1 hr at 37°C. After washing the plates, mAb CSNs diluted 4 fold in PBST were incubated for 1 hr at 37°C and the plates were processed as described for the screening ELISA. The ODs of supplemented DMEM that served as negative control ranged from 0 to 0.06. An OD $\geq$ 0.2 (negative control + 3 standard deviations) was considered to be a positive result. The sensitivity of the mAbs was measured with the same capture ELISA format except that the pAb was used at 2 µg/ml and the mAb CSN were diluted 5 fold.

2.2.6 Western Blot analysis of mAb reactivity- Stachylysin preparation was separated by SDS-PAGE on a 10% acrylamide gel and transferred to nitrocellulose membranes (BioRad, Hercules, CA). The membranes were blocked with 3% bovine serum albumin (BSA) for 1 hr at RT and incubated with mAbs (diluted 1/5 in PBST) for 1 hr at RT. After incubation for 1 hr with a 1/5000 (v/v) dilution of biotin-conjugated goat anti-mouse IgM antibodies (Jackson Immuno Research Laboratories Inc., West Grove, PA) the membranes were incubated with alkaline phosphatase-conjugated streptavidin (1/5000) (v/v) for 1 hr at RT. Blots were developed

with the nitroblue tetrazolium and bromo-chloro-indolyl phosphate substrate (NBT/BCIP) (Promega, Madison, WI).

2.2.7 Fluorescent halogen immunoassay- *S. chartarum* conidia, phialides, and hyphae were aerosolized by directing a jet of air across 2-week old sporulating cultures grown on MEA medium. Aerosolized particles were collected by suction onto mixed cellulose ester (MCE) protein-binding membranes (0.45 μ m pore size; Millipore Corporation, Bridgewater, MA) and immunostained using the fluorescent halogen immunoassay (FHIA) as previously described (13). Briefly, a clear adhesive and glass coverslip was used to laminate samples and the antigens were extracted in 0.2 M borate buffer, pH 8.2 at RT for 3 hrs. The samples were blocked with 5% BSA in PBS pH 7.4 for 90 mins and then incubated overnight at 4°C with the mAb 6D4 diluted 1/50 (v/v) in PBST containing 5% BSA (PBSTB). Negative control treatments were processed in parallel by substituting the mAbs with supplemented DMEM medium diluted 1/50 (v/v) or isotype control mAb 1B9 (IgM) diluted 1:50. The membranes were rinsed 3 times in PBST and incubated for 90 mins with Alexa Fluor® 488-conjugated goat anti-mouse IgM (Molecular Probes Inc., Eugene OR) diluted 1/500 (v/v) in PBSTB. The membranes were rinsed 3 times in distilled H₂O and mounted on a microscope slide in ProLong® Gold antifade reagent (Molecular Probes Inc.). Confocal laser scanning images were captured using a Zeiss LSM 510 laser scanning confocal system (Carl Zeiss Inc., Thornwood, NY). The images of mAb-labeled fungal particles were captured using 488-nm excitation and a narrow emission filter bandwidth (505-550 nm). Fluorescent and differential interference contrast images (DIC) were captured using Zeiss software version 3.2 (Carl Zeiss Inc., Thornwood, NY). All settings on the confocal laser microscope remained constant in the analysis.

2.3 RESULTS

2.3.1 mAb reactivity to *S. chlorohalonata* stachylysin and extracts- Immunization with stachylysin resulted in the production of eight murine IgM isotype antibodies (3C3, 6D4, 7D11, 9G6, 24D11, 27C10, 27E2, and 29E5). The mAbs 3C3, 6D4, 7D11, and 9G6 were selected based on preliminary studies of reactivity to *S. chlorohalonata* extracts. These antibodies showed varying degrees of reactivity towards stachylysin preparation (**Fig. 2.1a**). mAbs 7D11, 3C3, and 6D4 showed highest reactivity, while mAb 9G6 showed the lowest reactivity. The same pattern of mAb reactivity was also found against the hyphal extract of *S. chlorohalonata* when analyzed by capture ELISA (**Fig 2.1.b**). Reduced reactivity was observed to conidial extracts compared to hyphal extracts.

2.3.2 Localization of stachylysin in *S. chlorohalonata*- To determine the localization of stachylysin in morphological structures of *S. chlorohalonata*, we used the FHIA to ultrastructurally locate the antibody binding sites of mAb 6D4 in *S. chlorohalonata* conidia and hyphae (**Fig. 2.2**). mAb 6D4 immunostaining was primarily localized around phialides and sterigmata (**Fig. 2.2b**) with highest staining primarily localized around hyphal septal junctions and hyphal branch points (**Fig. 2.2c**). In contrast, the staining of *S. chlorohalonata* conidia was less intense and mostly restricted to the surface of the conidia (**Fig 2.2a**). We did not observe any specific staining with DMEM medium control or 1B9 IgM isotype control antibody staining (data not shown).

Fig. 2.1a. mAb reactivity against the stachylysin protein preparation. Stachylysin from S. chlorohalonata was diluted in PBSTM and the reactivity of mAbs 3C3, 6D4, 7D11 and 9G6 were analyzed by capture ELISA. Optical densities (OD) were measured at 405 nm after 30 mins incubation with substrate.

Fig. 2.1b. Reactivity of the 4 mAbs against S. chlorohalonata conidial and hyphal extracts. Extracts from conidia or hyphae of S. chlorohalonata were analyzed with mAbs 3C3, 6D4, 7D11 and 9G6 using the capture ELISA. Optical densities (OD) were measured at 405 nm after 30 mins incubation with substrate.

Fig. 2.2. Fluorescent halogen immunostaining of S. chlorohalonata. a) Conidia b) Phialides c) Hyphae. Green dots represent AlexaFluor®488 staining to identify the localization of mAb 6D4 specific antigens in the distinct morphological structures of S. chlorohalonata. White arrows highlight the regions of hyphal branch bifurcation where maximum staining was observed for stachylysin. Staining was also observed at septate junctions.

2.3.3 Cross-reactivity- Hyphal extracts obtained from 48 different fungal species were tested in the capture ELISA for cross-reactivity with the 8 different mAbs. This included hyphal extracts from 7 different strains of *S. chartarum* as well (**Table 2.1**). All mAbs exhibited varying reactivity to most of the *S. chartarum* strains tested here. However, mAb 29E5 failed to react with 4 of the 7 (57%) *S. chartarum* strains. All mAbs demonstrated limited cross-reactivity with different species of the genus *Stachybotrys*. The most frequent cross-reactivity was observed with *S. chartarum*, *S. bisbyi* and *S. parvispora*. However, none of the mAbs reacted with hyphal extracts of *S. albipes*, *S. kampalensis* or *S. oenanthae*. mAbs 27E2 and 29E5 did not react with hyphal extracts derived from *S. chlorohalonata*. mAb 7D11 strongly reacted to hyphal extract of *S. cylindrospora* while mAbs 27E2 and 6D4 exhibited cross-reactivity to *S. nephrospora*. mAbs 7D11, 9G6, and 27E2 also displayed minimal cross-reactivity with *Memnoniella echinata*; a species that is phylogenetically related to *S. chartarum*. mAbs 9G6 and 27E2 also cross-reacted with *M. subsimplex*. No cross-reactivity was observed with any of the other 39 tested fungal species.

2.3.4 Western blot- Western blot analysis following SDS–PAGE of *S. chlorohalonata* hyphal extracts indicated that the mAbs recognized multiple bands. mAbs 3C3, 7D11 and 6D4 recognized two doublet bands at ~ 30 kDa and single band at ~ 48 kDa (**Fig 2.3**), while mAb 9G6 recognized a streak of high molecular bands. Since, the antibodies were cloned by limiting dilution; the antigens identified by these antibodies may be highly processed proteins or possibly be components of protein complexes of varying molecular weight.

Fungal species	Strain I.D.	mAb 3C3	mAb 6D4	mAb 7D11	mAb 9G6	mAb 24D11	mAb 27C10	mAb 27E2	mAb 29E5
<i>Stachybotrys chartarum</i>	IBT 7711	1.886	1.806	2.411	1.841	1.419	1.285	0.519	0.187
<i>Stachybotrys chartarum</i>	IBT 9460	1.888	1.636	2.185	1.717	1.302	1.511	0.437	0.241
<i>Stachybotrys chartarum</i>	IBT 9466	1.572	1.484	0.307	1.649	0.925	1.932	0.736	0.399
<i>Stachybotrys chartarum</i>	IBT 9631	1.307	1.149	1.537	1.499	0.598	1.241	0.591	0.356
<i>Stachybotrys chartarum</i>	IBT 9633	1.113	1.139	1.244	1.134	0.694	1.208	0.382	0.211
<i>Stachybotrys chartarum</i>	IBT 14915	2.305	2.244	0.930	1.993	1.541	1.736	0.862	0.335
<i>Stachybotrys chartarum</i>	IBT 14916	1.702	1.799	2.013	2.520	0.786	1.649	0.655	0.207
<i>Stachybotrys chlorohalonata</i>	ATCC 201863 (IBT 9825)	1.856	1.761	1.951	0.795	1.333	1.565	0.043	0.017
<i>Stachybotrys albipes</i>	ATCC 18873	0.031	0.014	0.041	0.025	0.031	0.050	0.023	0.018
<i>Stachybotrys bisbyi</i>	ATCC 18825	2.790	2.575	0.320	2.618	1.783	2.052	0.938	0.578
<i>Stachybotrys cylindrospora</i>	ATCC 16276	0.010	0.009	1.039	0.068	0.026	0.410	0.012	0.003
<i>Stachybotrys kampalensis</i>	ATCC 22705	0.084	0.285	0.000	0.007	0.009	0.025	0.136	0.077
<i>Stachybotrys nephrospora</i>	ATCC 18839	0.027	0.952	0.047	0.042	0.033	0.064	0.455	0.022
<i>Stachybotrys oenanthos</i>	CBS 252.76	0.024	0.016	0.236	0.046	0.059	0.121	0.039	0.034
<i>Stachybotrys parvispora</i>	CBS 100155	2.156	2.332	3.355	0.017	1.528	2.768	0.008	0.004
<i>Memnoniella</i>	NRRL	0.217	0.265	1.623	1.185	0.054	0.033	0.439	0.199

<i>echinata</i>	2373								
<i>Memnoniella subsimplex</i>	ATCC 32888	0.107	0.103	0.043	1.099	0.057	0.090	0.346	0.130
<i>Aspergillus chevalieri</i>	NRRL 78	0.130	0.052	0.091	0.126	0.094	0.132	0.069	0.062
<i>Asp42ergillus clavatus</i>	NIOSH 6-22-78	0.024	0.016	0.067	0.062	0.058	0.098	0.042	0.035
<i>Aspergillus flavus</i>	ATCC 24689	0.026	0.013	0.069	0.059	0.060	0.116	0.047	0.041
<i>Aspergillus fumigatus</i>	FGSC A1100	0.023	0.011	0.031	0.029	0.038	0.053	0.016	0.013
<i>Aspergillus nidulans</i>	NIOSH 15-22-08	0.016	0.013	0.029	0.042	0.057	0.018	0.031	0.021
<i>Aspergillus niger</i>	FGSC A1144	0.014	0.003	0.044	0.054	0.065	0.060	0.019	0.010
<i>Aspergillus parasiticus</i>	ATCC 26691	0.023	0.014	0.053	0.087	0.021	0.054	0.027	0.015
<i>Aspergillus repens</i>	NRRL 13	0.061	0.061	0.097	0.137	0.091	0.181	0.097	0.080
<i>Aspergillus sydowii</i>	ATCC 9507	0.102	0.053	0.098	0.120	0.094	0.109	0.073	0.067
<i>Aspergillus terreus</i>	ATCC 1012	0.019	0.016	0.056	0.058	0.041	0.095	0.028	0.025
<i>Aspergillus ustus</i>	NRRL 275	0.018	0.000	0.043	0.052	0.036	0.019	0.005	0.000
<i>Aspergillus versicolor</i>	ATCC 44408	0.023	0.017	0.037	0.054	0.067	0.106	0.043	0.034
<i>Penicillium aurantiogriseum</i>	NRRL 971	0.029	0.010	0.052	0.099	0.029	0.068	0.040	0.031
<i>Penicillium expansum</i>	NRRL 973	0.015	0.012	0.038	0.027	0.039	0.060	0.025	0.013
<i>Penicillium fellutanum</i>	NRRL 746	0.032	0.005	0.045	0.054	0.025	0.023	0.012	0.002
<i>Penicillium purpurogenum</i>	NRRL 1062	0.104	0.026	0.017	0.084	0.003	0.043	0.012	0.000

<i>Penicillium roqueforti</i>	NRRL 844	0.014	0.012	0.026	0.016	0.051	0.034	0.012	0.011
<i>Alternaria alternata</i>	ATCC 11612	0.065	0.044	0.097	0.095	0.070	0.156	0.084	0.085
<i>Alternaria brassicicola</i>	ATCC 96836	0.046	0.039	0.064	0.091	0.034	0.088	0.053	0.044
<i>Wallemia sebi</i>	NIOSH 26-41-01	0.015	0.017	0.040	0.027	0.026	0.049	0.027	0.023
<i>Acremonium strictum</i>	ATCC 46646	0.011	0.005	0.027	0.017	0.032	0.030	0.009	0.007
<i>Stemphylium botryosum</i>	ATCC 26881	0.014	0.028	0.069	0.052	0.056	0.093	0.060	0.050
<i>Trichoderma viride</i>	ATCC 16640	0.026	0.009	0.037	0.021	0.031	0.033	0.017	0.009
<i>Ulocladium chartarum</i>	UAMH 5703	0.016	0.007	0.039	0.031	0.031	0.053	0.019	0.009
<i>Cladosporium herbarum</i>	ATCC 6506	0.018	0.006	0.027	0.014	0.028	0.021	0.020	0.013
<i>Cladosporium sphaerospermum</i>	ATCC 11288	0.027	0.019	0.037	0.075	0.021	0.060	0.038	0.033
<i>Paecilomyces variotii</i>	ATCC 66705	0.104	0.064	0.102	0.093	0.081	0.141	0.082	0.073
<i>Chaetomium globosum</i>	ATCC 6205	0.009	0.025	0.015	0.035	0.003	0.036	0.018	0.007
<i>Botrytis cinerea</i>	ATCC 11542	0.026	0.022	0.042	0.031	0.032	0.048	0.017	0.010
<i>Geotrichum candidum</i>	UAMH 7863	0.018	0.012	0.041	0.031	0.036	0.085	0.035	0.017
<i>Epicoccum nigrum</i>	ATCC 34929	0.041	0.026	0.052	0.037	0.041	0.068	0.038	0.029
<i>Eurotium amstelodami</i>	NIOSH 19-23-3	0.004	0.003	0.013	0.000	0.000	0.002	0.010	0.013
<i>Exserohilum rostratum</i>	ATCC 26856	0.008	0.007	0.026	0.015	0.046	0.037	0.015	0.012
<i>Fusarium</i>	Penn State	0.025	0.034	0.040	0.059	0.072	0.098	0.027	0.018

<i>moniliforme</i>	M6131								
<i>Myrothecium verrucaria</i>	NRRL 2003	0.014	0.013	0.044	0.034	0.053	0.096	0.041	0.034
<i>Rhizopus stolonifer</i>	NIOSH 17-59-14	0.021	0.007	0.040	0.035	0.037	0.077	0.027	0.019
<i>Scopulariopsis brumptii</i>	ATCC 16278	0.083	0.047	0.087	0.057	0.102	0.131	0.076	0.060

Table 2.1. Cross-reactivity profiles of the eight IgM anti-stachylysin mAbs analyzed by the capture ELISA. Optical densities (OD) were measured at 405 nm after 30 mins incubation with substrate. Bolded values identify reactivity of mAbs (columns) against a particular species (rows). ATCC- American Type Culture Collection, NRRL- Agricultural Research Service Culture Collection, NIOSH- National Institute for Occupational Safety and Health, FGSC- Fungal Genetics Stock Center, UAMH- University of Alberta Microfungus Collection and Herbarium, Penn State- Pennsylvania State University, IBT- Institutet for Bioteknologi, Denmark, CBS- Centraalbureau voor Schimmelcultures, Netherlands.

Fig 2.3. Western blot reactivity patterns: Mycelial extract from S. chlorohalonata were developed in Western blot analysis using Lane 1- mAb 3C3, Lane 2- mAb 6D4, Lane 3- mAb 9G6 and Lane 4- mAb 7D10. Reactive bands are labeled with black arrows.

2.4 DISCUSSION

The fungal genus, *Stachybotrys*, is a tertiary colonizer of moisture infiltrated cellulose-based building materials (18) and an indoor air contaminant (19). Personal exposure to *Stachybotrys* conidia has been associated with respiratory disease (5, 20-25); however, the scientific basis is not fully understood.

The identification of *Stachybotrys* in indoor environments requires macroscopic and microscopic identification of conidia by a certified indoor air quality professional. A few studies have developed strategies aimed at detection of *Stachybotrys* and associated biomarkers to determine exposure (9, 11, 13, 14, 26-32). Often, mycological expertise is required to confirm the presence of *Stachybotrys* conidia in indoor environments, however, morphologically indiscernible hyphae and fragments that are equally important biomarkers of contamination, remain overlooked and are not quantified (33). Given the presence of *Stachybotrys* hyphae and hyphal fragments in indoor air samples and the potential health effects associated with personal exposure, the development of mAbs that recognize this overlooked fraction is an important step that will improve the quantification of these particulates. Fragments derived from hyphae and conidia also contain mycotoxins and other immunostimulatory antigens (34), however, the health effects associated with personal exposure remain uncharacterized. Recent developments in molecular and immunodiagnostic detection methodologies have improved the detection and quantification of *S. chartarum*. These studies have provided new insight into potential biomarkers of personal exposure (11, 20, 26, 27, 29) including the cytolytic protein, stachylysin (9, 30, 35, 36).

Recently, several laboratories have developed mAbs towards *S. chartarum* (32, 37). The utility of antibody-based immunoassays for the quantification of personal exposure to *S.*

chartarum has been explored elsewhere (9). A pAb has been utilized in an indirect ELISA for the quantification of stachylysin in environmental samples. Stachylysin has been detected in the serum of *S. chartarum* exposed animals. Similarly, stachylysin has also been detected in pooled serum derived from *S. chartarum* exposed workers but not in control subjects that had no *a priori* *S. chartarum* exposure (9). Although polyclonal antibodies have been developed against other *S. chartarum* exoantigens for indoor environment exposure assessment studies, pAbs often lack specificity and cross-react with other fungal species (9). To the best of our knowledge, mAbs that recognize *Stachybotrys* hyphae have yet to be developed.

Though the protein sequence of stachylysin is currently unavailable, the amino acid composition was reported using an amino acid analysis service at Yale University (7). Amino acid composition similarity search using AAccompIdent with ‘hemolysis’ as a key word identified amino acid composition similarities to hemolytic proteins from different species (**APPENDIX A1**).

In the present study, we immunized mice with stachylysin preparation purified from *S. chlorohalonata* hyphal CSN. Eight anti-stachylysin IgM isotype mAbs were produced that exhibited reactivity to the original stachylysin preparation used for immunization. Four mAbs, that consistently showed greater reactivity toward hyphal extracts derived from *S. chlorohalonata* and *S. chartarum* species, were selected for further characterization. Previously, it was shown that hyphae contain higher levels of this hemolytic protein than the conidia and this observation was consistent for all strains studied (9). We have experimentally confirmed this earlier observation in this study where we found high concentrations of stachylysin in hyphae compared to conidia.

Immunolocalization studies utilizing the FHIA, confirmed that the greatest reactivity of the mAbs were toward antigens released from phialides and hyphae compared to conidia. Almost all reactivity in hyphae was observed at septal junctions and branch points of hyphal bifurcation. At this point in time it is difficult to suggest any possible role for stachylysin located at these points of growth. mAb 1B9, which served as an IgM isotype control in this study, was previously developed in our laboratory against particulate fraction of homogenized *Aspergillus versicolor* conidia (12).

Recent taxonomic studies have segregated *S. chartarum* into two separate chemotypes, based on the production of metabolites (15, 38). Chemotype A comprises atranone and dolabellane producing strains (IBT 9466, IBT 9633, IBT 14915) and chemotype S consists of satratoxin and other macrocyclic trichothecene producing strains (IBT 7711, IBT 9460, IBT 9631, IBT 14916). In the present study, all mAbs with the exception of mAb 29E5, reacted to antigens released from both chemotype A and S strains. mAbs 7D11, 9G6, and 27E2 that were developed in this study identified *S. chlorohalonata* and did cross-react with closely related species belonging to the genus *Memnoniella*. Also, our mAbs could not clearly differentiate between *S. chartarum* and *S. chlorohalonata*. Recent studies showed that other commercially available detection methodologies could also not differentiate between *S. chartarum* and *S. chlorohalonata* (39). Antibodies of IgM isotype can show high cross-reactivity due to the pentameric nature of the antibody, which contributes to increased avidity to the epitope. Development of IgG isotype antibodies to stachylysin may help overcome some of the cross-reactivity problems. The reduced cross-reactivity observed for anti-stachylysin mAbs compared to the mAbs previously produced in our laboratory against *Penicillium brevicompactum* and *A. versicolor* could be due to the source of the antigen. (10, 12) Conidial antigens are fewer and

may be shared between various fungal species and may show less antigenic variation compared to hyphal antigens, which are diverse and present in larger quantities. We have observed similar results in *Aspergillus versicolor*, where mAbs produced against conidia were cross-reactive (12).

The stachylysin preparation used in these studies was previously characterized as a homogenous protein preparation of ~ 12 kDa which migrates as ~ 30 kDa protein in SDS-PAGE (7). Interestingly, we observed immunoreactivity of the mAbs to multiple bands in Western blot including a doublet band at ~ 30 kDa. This suggests that mAbs recognize the epitopes on oligomers or higher molecular weight protein complexes covalently bound to stachylysin. Since hemolysins conceptually aggregate after binding to their target and not in solution, it is possible that the mAbs may have been generated against higher molecular weight protein complexes present in *S. chlorohalonata*.

In this study, four IgM monoclonal antibodies have been developed directed against stachylysin derived from *S. chlorohalonata* and partially characterized. Although the mAbs showed limited reactivity with conidia, the results demonstrate the potential utility of these mAbs for the development of immunodetection methods for the quantification of *Stachybotrys* hyphae in indoor environments.

In this initial attempt to study fungal hemolysins as potential biomarkers we chose stachylysin due to its association to IPH and clinical disease exposure. Collectively, our data suggest the possibility of multiple proteins in the stachylysin preparation. This makes it difficult to develop mAbs to study the role of a single protein using these preparations. Additionally, the genome for *Stachybotrys* has not been sequenced, which would have aided in better characterization of stachylysin using recombinant techniques. Also, IgM antibodies are not ideal

for developing immunoassays. Overall, the study was disappointing in that we were unable to identify the stachylysin protein with biochemical features of a hemolysin.

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CHAPTER 3

***Aspergillus terreus* and first approach at development of mAbs to
putative hemolysin and its characterization**

3.1 INTRODUCTION

Aspergillus terreus was chosen because the genome was sequenced and a putative hemolysin was identified (1). *A. terreus* is one of the 4 major species including *A. fumigatus*, *A. flavus* and *A. niger* that are involved in invasive aspergillosis in humans. *A. terreus*-related invasive infections have been reported with highest mortality rates compared to other *Aspergillus* species. One of the contributing factors for high mortality is the resistance of *A. terreus* to the first line of treatment. Currently, there is a need for species-specific diagnostics for improved treatment strategies.

Previously, asp-hemolysin of *A. fumigatus* was detected in tissues of exposed experimental animals (2). This generated an interest in characterization of the hemolysin of *A. terreus* and developing it as a biomarker. Observations on hemolytic activity of 50 different fungal species cultivated on sheep blood agar (SBA) were reported in the former study. Thirty-five of 50 different fungal species tested exhibited hemolytic activity between days 1-5 of growth. Interestingly, *Aspergillus terreus* was one of the only 2 fungi and the only member of genus *Aspergillus* to show hemolysis after 1 day of culture.

Our aim was to develop monoclonal antibodies (mAbs) to hemolytic protein of *A. terreus* by purifying it with methods described earlier for purification of stachylysin (3). Our goal was to develop species-specific mAbs recognizing *A. terreus* hemolysin for development of standardized rapid detection assays. This chapter describes *A. terreus*, its role in our environment, industrial uses and its emergence as a pathogen. Later we characterize the development of mAbs to purified hemolytic proteins from *A. terreus*.

3.2 ASPERGILLUS SPECIES

Aspergillus species were first identified by a Roman Catholic clergyman and biologist Pier Antonio Micheli in the year 1729 (4). He identified microscopic structures bearing spores as being similar to ‘asperges’, which is a device used to sprinkle holy water. *Aspergillus* species are very common and pervasive. At present, over 600 species of *Aspergillus* have been identified however, with recent developments in molecular identification techniques, new species are routinely identified (5-8). *Aspergillus* species are divided into 8 different sub genera (**Appendix A2**).

Aspergillosis is the disease state identified by infection with members of *Aspergillus* species. Infections involving fungi have been reported more frequently and *Aspergillus* species are the most common fungi associated with invasive fungal diseases in immunocompromised patients (9-12). Though more than 40 species of *Aspergillus* have been identified as medically relevant; *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus flavus* are most frequently isolated (13). Additional species including *Aspergillus clavatus* and *Aspergillus nidulans* are increasingly being identified in invasive infections (14). Although immunosuppression is a major prerequisite to invasive infection, some conditions in otherwise immunocompetent individuals may predispose them to *Aspergillus* infections (15). Recent reports have also emerged on pulmonary invasive aspergillosis after H1N1 infection (16, 17).

Aspergillus species cause diverse infections inversely associated to the status of host immune system (18). Pulmonary infection is often initiated by inhalation of metabolically dormant conidia. Owing to their small size, conidia can reach deep within the lungs in the alveoli. If the conidia germinate, the hyphae grow and demonstrate vasculotropic growth

resulting in invasion of local blood vessels, causing obliteration of host tissue and leads to pulmonary hemorrhage (13).

3.3 ASPERGILLUS TERREUS

Aspergillus terreus was first described by Thom and Church in 1918 (19). *A. terreus* gets its name from the cinnamon brown (terrestrial) color of the colonies. Though colonies are predominantly brown, slight variations may occur between strains and may be attributed to differences in sporulation by individual strains (20). It plays an important role in nature in recycling of nutrients by decomposition of compost and plant material (21). In industry, it is used as a source of mevinoic acid (lovastatin) the first statin approved by FDA, which helps in lowering serum cholesterol (22-24). *A. terreus* is also a valuable source for various industrial chemicals and enzymes such as itaconic acid, gluconic acid, xylanases and proteases (25-29). *A. terreus* produces enzymes that are useful in biodegradation of lignocellulosic waste, plastics etc. (29-35).

A. terreus conidia are 1.5-2.4 μm in diameter and spherical in shape with striations on the surface (20). Conidiophores are smooth hyaline structures with vesicles at the apex bearing conidia on phialides. *A. terreus* produces accessory conidia that form laterally on hyphae. These accessory conidia, called aleurioconidia, are produced by the vegetative hyphae under submerged conditions and have been observed *in vitro* as well as *in vivo* (20). Some have speculated that aleurioconidia are involved in dissemination of the fungus during infection (36-38). In comparison to phialidic conidia, aleurioconidia are larger in size, metabolically more active, possess enhanced adherence, and germinate very rapidly (37). All strains of *A. terreus*, clinical as well as environmental produce aleurioconidia, however variations in number and size exist (37). There is also a difference in the number of aleurioconidia borne at a single locus (37,

39, 40). Other *Aspergillus* species that are closely related to *A. terreus*, such as *A. flavipes*, *A. carneus* and *A. niveus* have also been reported to also produce aleurioconidia (36, 38).

An increasing number of cases of aspergillosis involving *A. terreus* have been reported in recent years (20, 41-43). The same growth competences that make *Aspergillus* species excellent agents of decomposition of organic materials also make them formidable opportunistic pathogens. The ability of the pathogen to grow optimally at 35-37°C and its ability to tolerate higher temperatures (40-42°C) allows it to grow at internal body temperature that is inhibitory to many fungi (44, 45).

A. terreus can cause wide range of diseases from superficial infections to invasive and disseminated aspergillosis. *A. terreus* has been identified as the causative agent in otomycosis (46-48), onychomycosis (49-51), endophthalmitis (52-54), cutaneous infection (55-57), peritonitis (58-60), osteomyelitis (61-63), allergic bronchopulmonary aspergillosis (64-66), aspergilloma (67, 68), and invasive aspergillosis (IA) (41, 42). *A. terreus* pathogenesis is poorly understood, however secondary metabolites that may have toxic effects on host cells have been reported (69-71).

Species-specific diagnosis of *A. terreus* opportunistic infections is clinically important due to the pathogen's resistance to the primary antifungal therapeutic amphotericin B (72-74). To date, the identification of *A. terreus* infections has challenged the most seasoned clinicians (75). Clinical diagnosis of *A. terreus* infection is subjective and has been restricted to macroscopic and microscopic characterization of tissue samples (76), computed tomography imaging (77), and detection of serum galactomannan or (1,3)- β -D-glucan (78, 79). These diagnostic methods are not specific and prevents the identification of the causative agent (79). Other more specific molecular methods such as PCR have been recently developed but are limited by a number of

confounding factors such as problems with consistent DNA recovery and interference by inhibitors of PCR process (79, 80). Due to increasing *A. terreus* infections reported in the literature, its resistance to amphotericin B, and the high mortality rate associated with infection, it is critical to develop sensitive and specific diagnostic tests (41).

In this chapter, we describe the production and characterization of species-specific mAbs to a partially purified cytolytic *A. terreus* preparation (cAtp). Using the mAbs, we characterized the cross-reactivity profiles, kinetics of antigen expression, and detection of antigens in presence of human serum proteins. Collectively, these results suggest that anti-cAtp mAbs may be useful for immunodiagnostic assays to detect invasive *A. terreus* disease.

3.4 MATERIALS AND METHODS

3.4.1 Preparation of cAtp and polyclonal antibodies- *A. terreus* ATCC 1012 (American Type Culture Collection, Manassas, VA) conidia were inoculated in Tryptic Soy Broth (TSB), grown in liquid culture for 7 days. cAtp was partially purified from TSB using molecular sieve and gel filtration steps to isolate the cytolytic fraction as previously described (3). Polyclonal antibodies (pAbs) to cAtp were generated in rabbits by Bethyl Laboratories (Montgomery, TX) and affinity purified using cAtp immobilized on activated sepharose columns.

3.4.2 Production of monoclonal antibodies (mAbs) to cAtp- Four 10-14 week old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were housed under controlled environmental conditions in HEPA-filtered ventilated polycarbonate cages on autoclaved hardwood beta-chip bedding. Mice were provided Teklad 7913 rodent chow (Harlan Laboratories, Madison, WI) and autoclaved tap water *ad libitum*. Sentinel mice, housed in the animal quarters were free of viral

pathogens, parasites, mycoplasma, and *Helicobacter* spp. The animal protocol was approved by the National Institute for Occupational Safety and Health (NIOSH) Animal Care and Use Committee (ACUC) and the NIOSH animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Mice were immunized (6 times) intraperitoneally at bi-weekly intervals using 25 µg of cAtp emulsified in TiterMax® adjuvant (TiterMax USA, Norcross, GA). Mice received a final boost (7th immunization) of 25 µg of HEA without adjuvant 3 days before hybridoma production. Pre- and test-bleed mouse IgG-specific titers to HEA were tested using an indirect ELISA method. Briefly, 96-well Nunc Immuno MaxiSorp microplates (Thermo Fisher Scientific, Rochester, NY) were coated with 0.1 µg/well cAtp in carbonate coating buffer (CCB, 60 mM sodium carbonate, 140 mM sodium bicarbonate, pH 9.6). Antibody binding from mouse sera was determined using biotin-SP goat anti-mouse IgG Fcγ (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and alkaline phosphatase (AP)-conjugated Streptavidin (Jackson ImmunoResearch Laboratories Inc.). ELISA plates were developed with 4-nitrophenyl phosphate substrate (Sigma, St. Louis, MO) and read at 450 nm after 30 mins as previously described (81).

Three days following the final boost, the immunized mice were euthanized by CO₂ asphyxiation, the spleens were surgically removed, and splenocytes were fused with SP2/0-Ag 14 ATCC myeloma cells (ATCC# CRL-1581). Hybridomas were selected by growing the cells in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Rockville, MD) supplemented with 1 mM sodium pyruvate, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.292 mg/mL L-glutamine, 100 mM sodium hypoxanthine, 16 mM thymidine, 10% fetal calf serum (FCS) (HyClone, Logan, UT), and 100 U/mL IL-6 (Boehringer, Mannheim, Germany).

DMEM medium was also supplemented with azaserine for selective propagation of hybridomas. After 10-14 days of hybridoma cell growth, the medium from individual wells was replenished with fresh medium. The tissue culture supernatant (CSN) fluid from individual hybridoma colonies was tested using a modification of a previously described sandwich ELISA. CSN from individual hybridoma clones was tested in duplicate to confirm the presence of cAtp-specific mAbs. Each positive hybridoma clone was further cloned twice by limiting dilution and single positive clones were screened and selected for bulk mAb production. Hybridoma cell lines of individual clones were frozen in FCS containing 10% dimethyl sulfoxide (DMSO) and stored at -80°C for 2 weeks, and then transferred to a liquid nitrogen tank for long term storage.

3.4.3 cAtp capture ELISA- In brief, 96-well Nunc Immuno MaxiSorp microplates (Thermo Fisher Scientific, Rochester, NY) were coated with 100 µl/well rabbit anti-cAtp polyclonal antibody (pAb) (1 µg/ml) in CCB and incubated overnight at RT. Wells were washed three times by incubating with 200 µl/well of PBS containing 0.05% Tween 20 (PBST) for 10 min. The plates were then blocked for 1 h at RT with 200 µl/well of PBSTM (PBST containing 1 % non-fat dry milk powder). Wells were then incubated for 1 h at 37°C with 100 µl/well cAtp (1 µg/ml) to bind cAtp. Plates were then processed with hybridoma CSN, followed by goat anti-mouse IgG antibody as described above. Negative control values were obtained by substituting hybridoma CSN with supplemented DMEM.

3.4.4 Isotyping and quantification of IgG antibodies- Isotyping of individual mAbs was determined by a direct ELISA. Plates were coated with CSN from *A. terreus* diluted in CCB (1 µg/ml) and incubated overnight. Next day plates were blocked with PBSTM and incubated with

mAb solutions from individual hybridomas. mAbs bound to cAtp antigens were detected using Biotin SP-conjugated AffiniPure goat anti-mouse IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) at a dilution of 1:5000 in PBSTM. ELISA plates were developed using methods described earlier.

For quantification, mAbs were serially diluted and captured on ELISA plates coated with AffiniPure goat anti-mouse IgG, Fc fragment of either subclass 1, 2a, 2b or 3 specificity at 1 µg/ml concentration depending on the isotype of the mAb to be quantified (Jackson ImmunoResearch Laboratories Inc.). IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ standards (Sigma) were used to set up a standard curve for quantification purposes. AP-conjugated goat anti-mouse secondary antibodies (1:5000) diluted in PBSTM were used for quantification experiment.

3.4.5 Preparation of *A. terreus* extracts for characterization of mAbs- *Conidial extracts*.

Conidia were collected from 10-14 days old *A. terreus* cultures grown on Malt Extract Agar (MEA) by rolling approximately 1 gm of 0.5 mm glass beads (BioSpec Products Inc., Bartlesville, OK) over the plate. Glass beads with conidia were collected into a 2 mL screw cap microcentrifuge tube and processed in a mini bead beater (BioSpec Products Inc.). Mechanical bead beating was carried out for 2 mins to disrupt the outer cell wall of the conidia. Conidial proteins were collected in 50 mM ammonium bicarbonate buffer pH 8.0 containing 0.5 M ethyldiaminetetraacetic acid, 0.1 M phenylmethylsulfonyl fluoride, and cOmplete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). The suspension was centrifuged at 4100 g for 10 mins and the supernatant fluid was collected and lyophilized overnight. Lyophilized conidial protein extract was resuspended in PBS pH 7.4 and stored at -80°C.

Hyphal extracts and culture supernatants. Viable *A. terreus* conidia (2.5×10^7 conidia) were inoculated in 50 mL minimal medium consisting of 1% glucose, nitric salts and trace elements (82). Viability of conidia was determined by LIVE/DEAD® BacLight™ viability kit (Invitrogen, Carlsbad, CA) previously used for determination of viability of fungal conidia (83). Cultures were grown at RT or 37°C with shaking (200 rpm) for varying intervals of time. For mAb reactivity assays, cultures were grown for a period of 6 days and for the kinetics of expression studies, cultures were grown for up to 12 days with samples collected every 24 hrs. Mycelial cultures were harvested by centrifugation at 4100 g for 5 mins and the hyphae (pellet) and CSN were collected and concentrated by lyophilization. The lyophilized CSN was reconstituted in 5 mL PBS pH 7.4 containing complete Mini Protease Inhibitor Cocktail and stored at -80°C for further analysis. Lyophilized hyphae were macerated in a mortar containing liquid N₂, suspended in cold PBS containing complete Mini Protease Inhibitor Cocktail, and incubated overnight on a rocker at 4°C to extract proteins. The mycelial extract (ME) was centrifuged at 4100 g for 5 mins and the supernatant fluid was collected, aliquoted and stored at -80°C for further analysis. Protein concentrations in all fungal extract preparations were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE).

3.4.6 Characterization of cAtp expression in fungi- For cross-reactivity analyses, 56 fungal isolates representing 46 fungal species (**Table 1.**) were grown on MEA medium for 7-10 days. The conidia (1.25×10^8) were collected, and inoculated into 50 mL TSB medium. Cultures were grown for 4 days at RT and centrifuged at 4100 g for 5 mins to collect the mycelial pellet. The pellet was washed 3 times with cold PBS pH 7.4, macerated, and then centrifuged at 4100 g for 5

mins as previously described. The supernatant fluid was collected, protein concentration determined by NanoDrop spectrophotometer, and mAb reactivity tested using the previously described capture ELISA. Positive reactivity was determined by an Optical Density (OD) of ≥ 0.2 (negative control value + 3 standard deviations). The OD values of the negative controls ranged from 0 to 0.18 for different fungal extracts.

The kinetics of antigen expression during culture was also examined. Individual mAbs were tested against *A. terreus* conidial, hyphal, and CSN extracts used at 100 $\mu\text{g/ml}$ of total protein to determine the level of antigen expression during different phases of *A. terreus* growth. The pAb-based capture ELISA was used for this analysis and all mAbs were normalized to 500 ng/ml in PBSTM. Plates were developed using secondary antibodies and reagents as previously described.

3.4.7 cAtp detection in spiked human serum- Detection of cAtp antigens by the mAbs in presence of human serum was studied using the ELISA methods described earlier. CSN was collected from a 6 day culture of *A. terreus* and was diluted to 100 $\mu\text{g/ml}$. Pooled human serum (Sigma) was spiked with dilutions of *A. terreus* CSN and assayed in the capture ELISA at a final concentration of 50% total volume using 100 μl of sample. Additionally, samples were also incubated in PBS and served as control for this experiment. All samples including controls were incubated at 37°C for 1 hr before analysis in capture ELISA method.

3.4.8 Western blot assay- *A. terreus* CSN (100 $\mu\text{g/ml}$) obtained from a 6 day TSB culture was separated on a 12% polyacrylamide gel using SDS-PAGE under reducing conditions. Proteins were transferred overnight at 30V to a 0.2 μm nitrocellulose membrane. The membrane was

blocked with containing 3% bovine serum albumin (BSA) in PBST (PBSTB) for 2 hrs. The membrane was washed in PBST and then processed using a Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad Laboratories, Hercules, CA). Each lane on the membrane was incubated with an individual mAb (500 ng/ml) for 1 hr with shaking. The membrane was washed with PBST and incubated with AP-conjugated goat anti-mouse IgG (H+L) (Promega, Madison, WI) diluted 1/5000 (v/v) in PBST for 1 hr with shaking. The membrane was then washed with PBST and developed using 1-step NBT/BCIP substrate (Promega). The substrate reaction was developed for 15-20 mins and stopped by washing the membrane with distilled water.

3.4.9 Immunoprecipitation of cAtp antigens using Protein G- Immunoprecipitation of antigens recognized by mAbs 13E11, 12C4 and 19B2 was performed using Dynabeads® Protein G (Invitrogen Dynal AS, Oslo, Norway) per the manufacturer's instructions. Briefly, Protein G magnetic beads were washed twice with W&B buffer (0.1M Na-phosphate, 0.01% Tween 20, pH 8.2) and incubated with 5 µg of the individual mAbs diluted in W&B buffer for 10 mins with rotation at RT. Antigen capture was performed after washing the Protein G magnetic beads with PBS and incubating with *A. terreus* hyphal extract overnight at 4°C. The beads were washed thoroughly in PBS and the immunoprecipitate was eluted with Laemmli Sample Buffer (BioRad, Hercules, CA) containing 2-mercaptoethanol (5% v/v). Immunoprecipitate samples were analyzed by SDS-PAGE and stained with Imperial™ Protein Stain (Thermo Scientific, Rockford, IL). Bands of interest as determined by a parallel Western blot analysis were excised from the SDS-PAGE gels and processed for proteomic analysis.

3.4.10 Proteomic analysis- *In gel reduction/alkylation/digestion.* All reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted. All incubations were performed at 37°C with shaking (300 rpm). Protein bands of interest were excised, reduced, alkylated, and digested with porcine trypsin according to published methodologies (84, 85). Protein bands were transferred to low retention, siliconized 0.5 ml microcentrifuge tubes and destained with two washes of 200 µl 200 mM NH_4HCO_3 in 40/60 acetonitrile/water for 30 mins and the supernatant removed. The gel bands were dehydrated 30 mins in a vacuum concentrator and rehydrated with 200 µl 0.5 mM tributylphosphine in 25 mM NH_4HCO_3 , followed by 15 min incubation. The supernatant was removed and replaced with 200 µl 0.4 mM iodoacetamide, followed by 30 min incubation. The gel bands were washed twice with 200 µl 25 mM NH_4HCO_3 for 15 mins followed by one wash with 200 µl 200 mM NH_4HCO_3 in 40/60 acetonitrile/water. Gel bands were dehydrated 30 mins in a vacuum concentrator and rehydrated with 1 µg porcine trypsin in 100 µl of 50 mM NH_4HCO_3 . The samples were allowed to digest overnight and the supernatant was removed to a clean siliconized microcentrifuge tube. The gel bands were washed once with 0.1% trifluoroacetic acid in 50% acetonitrile for 15 min. The wash and digest supernatants were combined and concentrated for 1 hr in a vacuum concentrator and reconstituted in 20 µl 0.1% trifluoroacetic acid prior to LC-MS/MS analysis.

Ultra-performance liquid chromatography/Tandem mass spectrometry. Enzymatic peptides were separated on a Waters (Milford, MA) nanoACQUITY ultra-performance liquid chromatography (UPLC) system. Aliquots (3 µl) of the digest mixture were injected and trapped/desalted on a 5 µm SymmetryC₁₈ (180 µm x 20 mm) trapping column with 99.5/0.5 A/B (A:0.1% formic acid; B:0.1% formic acid in acetonitrile) at a flow rate of 15 µl/min for 1 minute. Separation was performed on a 1.7 µm BEH130 C₁₈ (75 µm x 100 mm) analytical column

utilizing gradient elution at a flow rate of 300 nl/min and a gradient of 99/1 to 60/40 A/B over 60 min. The eluent from the UPLC system was directed to the nanoelectrospray source of a Waters SYNAPT MS quadrupole time-of-flight (qTOF) mass spectrometer. Positive ion nanoelectrospray was performed utilizing 10 μ m PicoTip (Waters) emitters held at a potential of +3.5 kV. The cone voltage was held constant at +40 V for all experiments. Dry N₂ desolvation gas was supplied to the instrument via a nitrogen generator (NitroFlowLab, Parker Hannifin Corp., Haverhill, MA). [Glu]¹-Fibrinopeptide B (100 fmol/ μ l in 75/25 A/B) was supplied to an orthogonal reference probe and the [M+2H]²⁺ ion (m/z = 785.84265u) measured as an external calibrant at 30 sec intervals. Ultra-high purity (UHP) argon was used as collision gas. Spectra were acquired in an “MS^e” fashion (86). Alternating one-second mass spectra were acquired. The collision energy was set to 6 eV (1 sec low energy scan) and a 15-30 eV ramp (1 sec high energy scan).

Homology searching. Data were analyzed with ProteinLynx Global Server v. 2.4 (Waters) using the default PLGS search engine to query a custom *Aspergillus terreus* database downloaded from UniProtKB/Swiss-Prot and UniProtKB/TrEMBL (<http://www.uniprot.org/uniprot/?query=terreus>). A minimum of 2 peptides, with a minimum of three fragment ions each was required for identification. Trypsin was specified as the digestion enzyme and 1 missed cleavage was allowed. Carbamidomethyl cysteine was specified as a fixed modification and oxidized methionine and phosphoryl serine, threonine, and tyrosine were allowed as variable modifications. Protein assignments were confirmed via manual inspection of tandem mass spectra.

3.4.11 Confocal Scanning Laser Microscopy (CSLM) analysis for localization of putative leucine aminopeptidase and probable dipeptidyl-peptidase V of *A. terreus*-

Immunolocalization of *A. terreus* leucine aminopeptidase and dipeptidyl-peptidase V was studied using methods previously described with slight modifications (87, 88). Briefly, *A. terreus* FGSC 1156 cultures were grown on alcohol-sterilized cover slips in 6-well tissue culture plates containing minimal medium. Cultures were incubated at 37°C without shaking for 24 hrs. Cover slips were fixed with 8% formalin buffered saline containing 50 mM PIPES (pH 6.7), 25 mM EGTA, 1% dimethyl sulfoxide (DMSO) and 5 mM MgSO₄. Cells were fixed for 1 hr at RT and later rinsed with MTSB (50 mM PIPES (pH 6.7), 5 mM EGTA and 5 mM MgSO₄). Cell wall digestion was carried out for 1 hr at RT with an enzyme solution containing 2.5% Driselase® (Sigma, St. Louis, MO), 1% lysozyme from chicken egg white (Sigma) and 2 mM EGTA. Cells were rinsed with H₂O and were extracted with 0.1% Triton X-100 in Tris Buffered Saline (TBS) pH 7.4 for 10 mins. Cells were quickly rinsed in MTSB and TBS once each. Cells were blocked with 3% bovine serum albumin in TBS (TBSB) overnight at 4°C with gentle shaking. Next day, cover slips were incubated with mAb 9B4, 13E11 or 19B2 at 3 µg/ml in TBSB for 3 hrs with gentle shaking. mAb 9B4 was previously developed in our laboratory against an unidentified *Stachybotrys chartarum* conidial surface protein and is of IgG₁ isotype (81). Cells were washed thoroughly in TBS containing 0.05% Tween 20 (T-TBS) and stained with AlexaFluor® 594 conjugated goat anti-mouse IgG (H+L) (Molecular Probes Inc., Eugene, OR) diluted 1:50 (v/v) in TBSB for 1 hr at RT. Cells were washed thoroughly in T-TBS and cover slips were placed on clean slides with ProLong® Antifade Reagent with DAPI (Molecular Probes Inc.). Cells were observed with a Zeiss LSM-510 Meta Confocal Microscope System (Axioplan 2 Stand) (Carl Zeiss, Thornwood, NY). The images of AlexaFluor® 594 labeled secondary antibodies bound to

anti-HEA mAbs were acquired with Zeiss software version 3.2 (Carl Zeiss). All settings on the confocal laser microscope remained constant throughout the analysis.

3.5 RESULTS

3.5.1 Characterization of mAb reactivity to fungal extracts- All 4 mice immunized with cAtp, developed detectable IgG response to specific antigens after 3-4 immunizations, which mostly stabilized by the 4th immunization. Twenty-three murine IgG₁ isotype hybridomas were developed against cAtp (**Table 3.2**). Since the mAbs were developed against a partially purified cytolytic extract, reactivity of the mAbs to *A. terreus* culture extract antigen was tested. The mAb reactivity was the highest to mycelial extracts (ME); however, weak reactivity to conidial extracts was also observed (**Fig. 3.1a**). As expected, the mAbs also showed significant reactivity to 6 day CSN from *A. terreus* cultures grown at RT and greater reactivity to CSN grown at 37°C (**Fig. 3.1b**). Collectively these results show that the mAbs react predominantly with hyphal antigens that are possibly actively secreted into the CSN.

3.5.2 Cross-reactivity of mAbs towards different fungi- Fifty-six different isolates of 46 fungal species representing 20 different genera were tested for cross-reactivity in the capture ELISA (**Table 3.1**). Positive reactivity was defined as a value ≥ 0.2 OD₄₀₅. All mAbs showed reactivity to ME tested from each *A. terreus* strain tested in this study (**Table 3.1**). Of the 23 mAbs that react with cAtp, 16 (70%) did not cross-react with any of the fungal species tested here (**Table 3.2**). Seven mAbs cross-reacted with other fungi. Most of the cross-reactivity observed in these 7 mAbs was limited within the genus *Aspergillus* except mAb 22D9 that also

cross-reacted with ME derived from *Acremonium strictum*. Five mAbs (22D9, 38B6, 40C6, 52G7 and 64B3) cross-reacted with ME from at least 1 other fungus, while 24D7 cross-reacted with 2 different fungi (**Table 3.2**). The mAb 67G7 exhibited the greatest cross-reactivity, reacting to hyphal extracts derived from 4 different *Aspergillus* species (*A. flavus*, *A. parasiticus*, *A. repens* and *A. sydowii*). Of the fungal species tested, *A. sydowii* and *A. repens* ME showed highest cross-reactivity.

3.5.3 Western blot analysis of cAtp- Western blot analysis was performed with CSN from *A. terreus* culture using the 16 species-specific mAbs and the cross-reactive mAb 67G7 (**Fig. 3.2**). The results demonstrated differences within the mAbs in their reactivity to antigens in *A. terreus* CSN. The mAbs 13E11, 19B7, 24C8, 29C9 and 61E5 showed a similar pattern with strong reactivity to bands at ~18 kDa, 45 kDa, and ~70 kDa bands. The mAb 19B2 identified unique high molecular weight bands at ~ 100 kDa and ~ 150 kDa. It is possible that the immune reactivity to the 10 kDa and the 45 kDa antigens is due to binding of antibodies to shared epitopes or carbohydrate antigens.

Fig. 3.1. mAb reactivity to *A. terreus* extracts. a) mAb reactivity to *A. terreus* conidial and hyphal extracts. Conidial and mycelial extracts of *A. terreus* were tested against 16 specific mAbs. CE- Spore extract, RT ME- Mycelial extract collected from an *A. terreus* culture grown at room temperature, 37°C ME- Mycelial extract collected from an *A. terreus* culture grown at 37°C. b) mAb reactivity to *A. terreus* culture supernatants. Culture supernatant (CSN) collected from RT CSN- room temperature and 37°C CSN- 37°C cultures of *A. terreus* were tested against 16 specific mAbs. Error bars represent the standard deviations of duplicate determinations from three independent experiments.

Fungal Species	Culture collection I.D.	Fungal Species	Culture collection I.D.
<i>Aspergillus terreus</i>	ATCC 1012	<i>Myrothecium verrucaria</i>	NRRL 2003
<i>Aspergillus terreus</i>	FGSC A1156	<i>Paecilomyces variotii</i>	ATCC 66705
<i>Aspergillus terreus</i>	ATCC 16794	<i>Penicillium aurantiogriseum</i>	NRRL 971
<i>Aspergillus terreus</i>	SRC 2174	<i>Penicillium expansum</i>	NRRL 973
<i>Aspergillus chevalieri</i>	NRRL 78	<i>Penicillium fellutanum</i>	NRRL 746
<i>Aspergillus clavatus</i>	NIOSH 6-22-78	<i>Penicillium purpurogenum</i>	NRRL 1062
<i>Aspergillus flavus</i>	ATCC 24689	<i>Penicillium roqueforti</i>	NRRL 844
<i>Aspergillus fumigatus</i>	FGSC A1100	<i>Rhizopus stolonifer</i>	NIOSH 17-59-14
<i>Aspergillus nidulans</i>	NIOSH 15-22-08	<i>Stachybotrys albipes</i>	ATCC 18873
<i>Aspergillus niger</i>	FGSC A1144	<i>Stachybotrys bisbyi</i>	ATCC 18825
<i>Aspergillus parasiticus</i>	ATCC 26691	<i>Stachybotrys chartarum</i>	IBT 7711
<i>Aspergillus repens</i>	NRRL 13	<i>Stachybotrys chartarum</i>	IBT 9290
<i>Aspergillus sydowii</i>	ATCC 9507	<i>Stachybotrys chartarum</i>	IBT 9460
<i>Aspergillus ustus</i>	NRRL 275	<i>Stachybotrys chartarum</i>	IBT 9466
<i>Aspergillus versicolor</i>	ATCC 44408	<i>Stachybotrys chartarum</i>	IBT 9631
<i>Acremonium strictum</i>	ATCC 46646	<i>Stachybotrys chartarum</i>	IBT 9633
<i>Alternaria alternata</i>	ATCC 11612	<i>Stachybotrys chartarum</i>	IBT 14915
<i>Alternaria brassicicola</i>	ATCC 96836	<i>Stachybotrys chartarum</i>	IBT 14916
<i>Botrytis cinerea</i>	ATCC 11542	<i>Stachybotrys chlorohalonata</i>	ATCC 201863

<i>Chaetomium globosum</i>	ATCC 6205	<i>Stachybotrys cylindrospora</i>	ATCC 16276
<i>Cladosporium herbarum</i>	ATCC 6506	<i>Stachybotrys kampalensis</i>	ATCC 22705
<i>Cladosporium cladosporioides</i>	ATCC 11288	<i>Stachybotrys nephrospora</i>	ATCC 18839
<i>Epicoccum nigrum</i>	ATCC 34929	<i>Stachybotrys oenanthae</i>	CBS 252.76
<i>Exserohilum rostratum</i>	ATCC 26856	<i>Stachybotrys parvispora</i>	CBS 100155
<i>Fusarium moniliforme</i>	PS M6131	<i>Scopulariopsis brumptii</i>	ATCC 16278
<i>Geotrichum candidum</i>	UAMH 7863	<i>Stemphylium botryosum</i>	ATCC 26881
<i>Memnoniella echinata</i>	NRRL 2373	<i>Trichoderma viride</i>	ATCC 16640
<i>Memnoniella subsimplex</i>	ATCC 32888	<i>Wallemia sebi</i>	NIOSH 26-41-01

Table 3.1. Fungal species tested for cross-reactivity of cAtp-mAbs. Designation of sources for the 54 species representing 20 different genera of fungi. ATCC- American Type Culture Collection, NRRL- Agricultural Research Service Culture Collection, NIOSH- National Institute for Occupational Safety and Health, FGSC- Fungal Genetics Stock Center, UAMH- University of Alberta Microfungus Collection and Herbarium, Canada, PS- Pennsylvania State University, IBT- Institutet for Bioteknologi, Denmark, CBS- Centraalbureau voor Schimmelcultures, The Netherlands.

cAtp-IgG₁ mAbs	Cross-reactivity
2G3, 7C9, 8G7, 12C4, 12G9, 13D9, 13E11, 14C8, 15B11, 15D3, 19B2, 19B7, 24C8, 29C9, 34F8, 61E5	No cross-reactivity to any other species tested.
22D9	<i>Acremonium strictum</i> ATCC 46646
24D7	<i>Aspergillus sydowii</i> ATCC 9507, <i>Aspergillus repens</i> NRRL 13
38B6, 40C6, 52G7, 64B3	<i>Aspergillus repens</i> NRRL 13
67G7	<i>Aspergillus flavus</i> ATCC 24689, <i>Aspergillus parasiticus</i> ATCC 26691, <i>Aspergillus repens</i> NRRL 13, <i>Aspergillus sydowii</i> ATCC 9507

Table 3.2. Cross-reactivity profiles of cAtp-mAbs analyzed by capture ELISA.

Fig. 3.2. Western blot reactivity of proteins in A. terreus culture supernatant extracts using anti-cAtp mAbs. 1- mAb 2G3, 2- mAb 7C9, 3- mAb 8G7, 4- mAb 12C4, 5- mAb 12G9, 6- mAb 13D9, 7- mAb 13E11, 8- mAb 14C8, 9- mAb 15B11, 10- mAb 15D3, 11- mAb 19B2, 12- mAb 19B7, 13- mAb 24C8, 14- mAb 29C9, 15- mAb 34F8, 16- mAb 61E5, 17- mAb 67G7.

3.5.4 Kinetics of *A. terreus* exoantigen expression- Four mAbs (13E11, 15D3, 19B2 and 19B7) were chosen for further study based on differences in patterns of reactivity in ELISA and Western blotting. **Fig. 3.3** illustrates the time course of cAtp expression in *A. terreus* cultures. For mAbs 13E11, 15D3, and 19B7, the concentration of the cAtp rapidly increased in ME during days 3 to 5 and peaked at day 6 (**Fig. 3.3a**). Reactivity to cAtp in the CSN followed a similar pattern but appeared to lag behind ME by 24 hrs (**Fig. 3.3b**). At 37°C, cAtp mAb reactivity was readily detectable by 24 hrs in both ME (**Fig. 3.3c**) and CSN (**Fig. 3.3d**) and peaked by days 3 and 4, respectively. Interestingly, cAtp mAb reactivity could be detected in both ME and CSN beyond day 6 but appeared to decline in CSN after 10 days at 37°C. The mAb 19B2 antigen, while having a similar pattern as the other mAbs, appeared to lag in expression suggesting that this mAb may recognize a different antigen compared to other mAbs used in this study. Also, we observed a more rapid degradation of mAb 19B2 antigen after day 10 in CSN of *A. terreus* cultures grown at 37°C. This could probably be due to proteolytic degradation of the antigen.

3.5.5 Human serum detection assay- To determine the ability of these mAbs to be used in immunodiagnostic assays, we tested 10 mAbs for reactivity to *A. terreus* antigens spiked into human serum (**Fig. 3.4**). CSN grown at 37°C was mixed with human serum and assayed using a capture ELISA. Overall, there was a slight reduction in the detection of cAtp when spiked into human serum as compared to PBS alone for all mAbs tested, however, there did not seem to be any significant binding to serum components for any of the antibodies.

Fig. 3.3. Time point kinetic assay of *cAtp* in *A. terreus* hyphae and CSN. a) ME collected from *A. terreus* cultures grown at RT. b) CSN collected from *A. terreus* cultures grown at RT. c) ME collected from *A. terreus* cultures grown at 37°C. d) CSN collected from *A. terreus* cultures grown at 37°C. All samples were collected at 24 hr intervals for 12 days and tested in capture ELISA.

Fig 3.4. Lack of interference by serum proteins in the detection of cAtp by mAbs in presence of serum. A. terreus CSN was spiked into 50% human serum or PBS and detected using capture ELISA. PBS served as a control in the experiment.

3.5.6 Immunoprecipitation and determination of protein IDs- Three mAbs (13E11, 19B2 and 12C4) were selected for further analysis based on exhibition of differential patterns of reactivity to *A. terreus* extract (**Fig. 3.2**). The mAbs were incubated with the *A. terreus* hyphal extract, and protein-antibody complexes bound to the protein G magnetic beads were eluted and separated using SDS-PAGE. Using mAb 13E11 to stain the immunoblot showed immunoprecipitation of an ~ 65 kDa band, slightly higher than the antibody heavy chain band. Interestingly, mAb 13E11 also showed similar reactivity to immunoprecipitate of mAb 12C4 but not to that of mAb 19B2 (**Fig. 3.5**). This suggests that mAb 13E11 and mAb 12C4 recognized similar antigens. In contrast, mAb 19B2 demonstrated immunoreactivity to an antigen localized at ~ 100 kDa. While mAb 12C4 precipitated a 66 kDa band that was recognized by mAb 13E11, this mAb does not recognize this band when used to stain the Western blot, indicating that it may recognize a conformational epitope.

Specific bands were excised from a parallel SDS-PAGE gel on which immunoprecipitated samples from each mAb were separated. Samples were subjected to UPLC tandem (MS/MS) analysis to determine the identity of the proteins. By comparing peptide masses of recovered peptides *in silico* to the generated database for *A. terreus*, we identified peptides for a putative uncharacterized protein (Q0CAZ7) in immunoprecipitates from both mAbs 13E11 and 12C4. The protein Q0CAZ7 has (> 60%) sequence homology to a leucine aminopeptidase found in other *Aspergillus* species such as *A. fumigatus*, *A. nidulans*, *A. oryzae* and *A. flavus*. Peptides for a probable dipeptidyl peptidase V (Q0C8V9) were also identified following UPLC MS/MS analysis of mAb 19B2 immunoprecipitates (**Table 3.3**). Both of these proteolytic enzymes consist of putative *N*-glycosylation sites and are secreted after processing of a signal peptide.

Fig. 3.5. Immunoprecipitation of cAtp antigens. mAbs 13E11, 19B2 and 12C4 were used for immunoprecipitation of their respective antigens from A. terreus CSN. Immunoprecipitates were analyzed using individual mAbs to identify cross-identification of antigens between mAbs. IP: Immunoprecipitate and IB: Immunoblot.

3.5.7 Immunolocalization of cAtp antigens- The immunolocalizations of *A. terreus* leucine aminopeptidase (mAb 13E11) and dipeptidyl peptidase V (mAb 19B2) in the hyphae were determined using confocal scanning laser microscopy. Interestingly, the leucine aminopeptidase was localized in extracellular matrix (ECM) structures (**Fig 3.6**). Immunostaining was also observed within the hyphae. mAb 19B2 immunostaining for a probable dipeptidyl peptidase V was diffuse but uniform over the entire hypha, suggesting that this protein might be present in the cytoplasm of *A. terreus* hyphae. No staining was observed in the extracellular matrix for mAb 19B2. The mAb 9B4 served as a isotype control for these studies and did not stain any structures.

Monoclonal Antibody	cAtp antigen ID	Sequence coverage	N-glycosylation sites	Signal Peptides prediction
13E11 and 12C4	Putative uncharacterized leucine aminopeptidase (Q0CAZ7)	10%	232, 349, 432, 435, 466	Between 17 and 18 (NN), between 21 and 22 (HMM)
19B2	Probable dipeptidyl-peptidase V (Q0C8V9)	52%	37, 79, 97, 154, 255, 339, 381, 451, 509, 608	Between 19 and 20. (NN and HMM)

Table 3.3 Proteomic analysis of cAtp antigens. Numbers for N-Glycosylation and signal peptides sites are amino acid sites on pre-protein. NN- Neural Networks, HMM- Hidden Markov Models.

Fig. 3.6. Immunolocalization of cAtp antigens. Immunolocalization of HEA antigens was determined by AlexaFluor® 594 –labeled goat anti-mouse IgG secondary antibodies (Red) and nuclear staining was identified by DAPI (Blue). mAb 9B4 acted as an IgG₁ isotype control antibody in this study.

3.6 DISCUSSION

Invasive aspergillosis develops in the lungs of immunocompromised patients following the inhalation of viable *Aspergillus* conidia from the environment (13, 41, 89). Upon germination, the conidia differentiate into a vegetative hyphal form that can cause damage to the host tissue and may allow the fungus to disseminate to other parts of the body (15). To date, the diagnostic methods have been limited to identifying macroscopic and microscopic characteristics in lung biopsies (18, 79). Serological diagnostics have also been developed but are limited to the detection of galactomannan and (1,3)- β -D-glucan (18, 79). Although this methodology may confirm a fungal infection, it does not identify the specific pathogenic species involved. Previous studies have detected *Aspergillus* antigens in the sera and urine of patients diagnosed with invasive aspergillosis (90-93). These results suggest that during infection, *Aspergillus* species secrete proteins into the blood stream that could be detected serologically. Therefore it is critical to identify biomarkers that could be used serologically for the identification of individual pathogenic *Aspergillus* species.

Although *A. fumigatus* is the most widely known etiological agent of invasive aspergillosis, *A. terreus* has emerged as a pathogen and can cause a variety of infections including fatal disseminated aspergillosis (20). To our knowledge, no immunodiagnostics have been developed for the specific detection of *A. terreus* in clinical samples. In this study, 23 IgG₁ mAbs were produced using a partially purified cytolytic cAtp preparation using a methodology previously used to purify stachylysin from *Stachybotrys chartarum* (3). The mAbs developed in the present study specifically detect antigens localized in conidia and hyphae, but more importantly, these antigens were detected in the CSN fluid. These findings demonstrate that these hemolytic antigens may be actively secreted during hyphal differentiation and growth and

may function as candidate biomarkers for immunodiagnostic assays similarly to stachylysin. Detection of hemolytic antigen in higher concentrations in hyphae is consistent with our previous studies of stachylysin described in the previous chapter and in other studies as well (3, 94-96). Sixteen mAbs were found to be species-specific, while seven cross-reacted with other species. The species-specific mAbs did not cross-react with ME from other important *Aspergillus* pathogens including *A. fumigatus*, *A. flavus*, *A. niger* and *A. nidulans*. No cross-reactivity was observed with other fungal species belonging to the genera *Penicillium* and *Fusarium*. Most cross-reactivity was minimal with OD₄₅₀ values ≤ 0.5 .

Previously, differences were reported in metabolic activities, growth rate and virulence capabilities of different *A. terreus* strains depending on their environmental source (44). We were curious to see if any of these differences reflected in altered expression of cAtp antigens. All mAbs reacted with the mycelial extracts from the 4 *A. terreus* strains used in this study, however, comprehensive testing with additional clinical strains of *A. terreus* strains will be critical prior to the development of diagnostic methods for use in the clinical setting. Moreover, other species that are closely related to *A. terreus* such as *A. carneus*, *A. niveus* and the newly identified *A. alabamensis* (5) as well as other unrelated species including *Scedosporium* and *Rhizopus stolonifer* (11) should be tested for cross-reactivity.

A. terreus growth is accompanied by conidial germination during favorable nutrient and environmental conditions. This process involves the swelling of conidia, initiation of primary metabolism, and eventually hyphal extension and aggregation. In this study, we observed that the antigens were detected earlier in hyphal growth than in CSN suggesting an active secretion of these proteins. The concentration of these antigens appeared to correlate with an increase in the total biomass of the fungus as measured by the mycelial pellet size and protein concentration

(data not shown) during cAtp kinetic experiments. mAb reactivity to cAtp was also observed to increase progressively with increases in pellet size. Most importantly, cAtp was continuously detected in CSN at 37°C, emphasizing the relative stability of these antigens to proteolytic degradation for longer period of time.

Detection of antigens in CSN may not fully reflect antigen production during invasive disease. Furthermore, secreted antigens may bind to serum proteins or other factors that alter the conformation of the protein and subsequently reduce the availability of the epitope for mAb detection. Certain fungal proteins are known to bind serum components *in vitro* (97). In tests with pooled human serum, there was only a slight reduction in detection of the epitopes using our mAbs. This suggests that the epitopes detected by our mAbs do not interact with serum components and this may have potential use for serodetection of invasive *A. terreus* disease.

Leucine aminopeptidase and dipeptidyl-peptidase V are both predicted to possess putative *N*-glycosylation sites as determined by N-Glycosite (98). These proteins are secreted with putative signal peptides as determined by SignalP, version 3.0, *in silico* analysis (99-101). This has been confirmed experimentally by us in this study and previously by others for *A. terreus* and other fungal species (102, 103). Homologous dipeptidyl-peptidase V in other fungal species have been reported as a potential virulence factor or allergen and as important for tissue invasion and modulation of host immune responses (103-109).

Immunolocalization studies demonstrated that the putative leucine aminopeptidase identified by mAb 13E11 was localized to extracellular structures containing DNA. Similar structures containing extracellular DNA have been very recently reported *in vitro* and *in vivo* and have been identified as the extracellular matrix of fungi (110-112). ECMs have been only recently identified and there is little information on their role in pathogenesis of fungal

infections. mAb 13E11 may be a useful tool in studying ECM and more importantly, the putative leucine aminopeptidase may function as a biomarker of invasive *A. terreus* disease. In contrast, mAb 19B2 recognized a probable dipeptidyl peptidase V and immunostaining was primarily localized within the cytoplasm. These mAbs also have the potential to be used for the immunofluorescent detection of *A. terreus* in bronchoalveolar lavage samples.

In conclusion, we observed that cAtp antigens were released from vegetative hyphae and into CSN in a time-dependent manner. The mAbs developed in this study recognized these antigens and the binding was not inhibited by human serum components in spiking experiments. Collectively, the data suggest that mAbs developed to cAtp may have potential diagnostic value in cases of *A. terreus* invasive aspergillosis. Serological detection of *A. terreus*-specific antigens in patient serum would obviate the need to obtain clinical specimens by invasive methods to identify the causal agent. Previously, dipeptidyl-peptidase V has been reported as one of the two major antigens with the greatest serodiagnostic potential for detecting aspergillosis due to *A. fumigatus* (107, 108, 113, 114). These methodological developments may aid in the development of standardized immunoassays for rapid identification of pathogenic species in clinical samples.

Our goal in these experiments was to develop mAbs to the hemolysin of *A. terreus* (terrelysin) as a potential biomarker of *A. terreus* infection. In characterizing these mAbs, we identified these antigens to be other proteolytic proteins, which appear to be major constituents of the cAtp preparation. These proteins may have ‘hemolytic’ activity owing to their general proteolytic activity. The mAbs we developed to HEA recognize secreted proteins and have potential diagnostic value. Our interest in developing mAbs to the terrelysin to study its expression and potential as a biomarker were not achieved and required a different approach. We propose to develop terrelysin using recombinant techniques in order to develop highly purified

form of the hemolysin. This will also assist in developing standardized reagents for development of consistent methodologies.

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CHAPTER 4

Cloning, Expression and purification of recombinant terrelysin

4.1 INTRODUCTION

The aegerolysin family (Pfam: PF06355; InterPro: IPR009413) consists of low molecular weight, acidic cytolytic proteins from both prokaryotes and eukaryotes. The fungal hemolysins, aegerolysin (*Agrocybe aegerita*), ostreolysin (*Pleurotus ostreatus*), asp-hemolysin (*Aspergillus fumigatus*) and more recently the hemolysin from the basidiomycete *Moniliophthora perniciosa* have been characterized (1-6). The role of bacterial hemolysins as virulence factors has been well established (7, 8) and a similar putative role for the fungal hemolysins has been suggested (9, 10). *In vitro* studies have shown that asp-hemolysin causes lysis of erythrocytes and is cytotoxic to macrophages, fibroblasts, leukocytes, and endothelial cells *in vitro* (11-13). Ostreolysin and aegerolysin have been suggested to play an important role in the development of fruiting bodies in the basidiomycete fungi (2, 5).

As noted previously, rapid identification of fungi at the species level could aid in early and improved treatment of fungal infections. Fungal hemolysins have been hypothesized as candidates for use as biomarkers for species identification thus aiding in their early detection (10, 14, 15).

In characterization of hemolytic preparations from *A. terreus* culture, we identified the presence of multiple proteins. However, we could not detect any mAbs to proteins in the expected size range of the specific hemolytic protein, terrelysin. Also, proteomic analysis of the hemolytic preparations did not yield any peptides belonging to terrelysin.

Recently, the genomes of a number of fungi including *A. terreus* were completed and the sequences of aegerolysin-like proteins were reported (16-21). Using homology search, we identified the sequence of a putative terrelysin, based on homology to asp-hemolysin (*A. fumigatus*), as a prototype of the aegerolysin family. This chapter describes the development of a

recombinant terrelysin by expressing it in *Escherichia coli*, its purification and the development of mAbs to terrelysin.

4.2 MATERIALS AND METHODS

4.2.1 Culture of *A. terreus* and cloning of rTerrelysin- Conidia of *A. terreus* ATCC 1012 (1 x 10⁶) were inoculated in TSB medium (50 ml). Cultures were grown for 3 days at RT after which the mycelium was harvested and washed thoroughly in phosphate buffered-saline (PBS) pH 7.4. Total RNA was extracted from mycelium with RNAqueous kit (Ambion Inc. Austin, TX). cDNA was synthesized using a High Capacity RNA-to-cDNA Master Mix (Applied Biosystems Foster City, CA) according to the manufacturer's instructions. Terrelysin encoding open reading frame was amplified by PCR using cDNA as the template and a PCR SuperMix High Fidelity kit (Invitrogen Carlsbad, CA). The following primers were used: forward primer (F1) 5'_ATGGTAG**GTCT**CAGCGCATGGACGACTCTCAATGGGTTTC_3' and reverse primer (R1) 5'_ATGGTAG**GTCT**CATATCAGGCAGCAGAAATATCAATGAA AACA_3' (BsaI restriction sites are indicated in bold). PCR was performed with initialization at 94°C for 30 secs followed by 32 cycles of denaturation at 94°C for 30 secs, annealing at 68°C for 30 secs and extension at 72°C for 1 min; with final holding at 68°C for 1 min. The PCR product was ligated into the BsaI-digested pASK-IBA6 vector (IBA-GmbH, Göttingen, Germany), transferred into One Shot® TOP 10 chemically competent *Escherichia coli* cells (Invitrogen). Clones were screened by PCR and analyzed on 1.2% agarose gel stained with ethidium bromide (200 µg/ml). Bands were observed using a MultiImage™ Light Cabinet (Alpha Innotech Corporation, San Leandro, CA). Clones were verified for orientation of the insert by DNA sequencing with primers (designated F2 and R2) recommended by IBA.

4.2.2 Expression and purification of rTerrelysin- The terrelysin construct was introduced into One Shot® BL21 StarTM (DE3) chemically competent *E. coli* cells (Invitrogen) for protein expression. Transformed *E. coli* were grown in LB medium (50 ml) supplemented with 100 µg/ml ampicillin overnight at 37°C. The overnight culture was inoculated into flasks containing LB medium (500 ml) supplemented with ampicillin. Cultures were incubated at 37°C until they reached an OD₆₀₀ of 0.5-0.7. rTerrelysin expression was induced by the addition of 100 µg anhydrotetracycline (AHTC) per 500 ml culture, and growth was continued at 30°C for 3 hrs. *E. coli* were harvested by centrifugation at 12,000 g for 15 mins at 4°C and the pellet was re-suspended in 10 ml CelLytic B solution (Sigma, St. Louis, MO). Phenylmethylsulfonyl fluoride (PMSF) (100 µl) was added to inhibit endogenous protease activity. Avidin (100 µl, 2 mg/ml) was added to sequester endogenous biotin carboxyl carrier protein (BCCP) from *E.coli* that would otherwise bind to the *Strep*-Tactin column (IBA) and thus interfere with the purification of rTerrelysin. The suspension was centrifuged at 12,000 g (4°C for 15 mins) to remove lysed *E. coli* and cellular debris. The supernatant was applied to an affinity column packed with 10 ml *Strep*-Tactin-sepharose (IBA) and unbound proteins were removed with washing buffer (100 mM TRIS, 150 mM NaCl, 1 mM EDTA, pH 8) (IBA). rTerrelysin was then eluted with washing buffer containing 2.5 mM desthiobiotin, pH 8 (IBA) and the concentration of the purified rTerrelysin was determined using a NanoDrop ND1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Recombinant protein was then dialyzed in PBS pH 7.4 and was stored at -20° C.

4.2.3 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

(MALDI-TOF MS)- Samples were prepared for MALDI-TOF MS analysis by mixing equal volumes of rTerrelysin (500 µg/ml) and α -cyano-4-hydroxycinnamic acid (10 mg/ml). Aliquots of 1 µl were air-dried on the gold sample stage (Bio-Rad, Hercules, CA) and MALDI-TOF mass spectra were acquired using a Ciphergen PBS-IIc linear TOF mass spectrometer (Bio-Rad, Hercules, CA) with a flight path of 0.8 m. Spectra were acquired over the m/z range of 0 to 30 kDa, with the delayed extraction parameters set to optimally focus the 15 to 20 kDa range. Protein calibrants between 1 to 66 kDa were used for external calibration.

4.2.4 Circular Dichroism Spectroscopy-

Circular Dichroism (CD) spectra were recorded using a JASCO J-810 spectro-polarimeter (JASCO Inc., Easton, MD) under the following conditions: Temperature 20°C; pathlength 0.1 cm; protein concentration 0.2-0.35 mg/ml; scanning range between 300-180 nm (Far-UV). For pH effect studies, rTerrelysin was dialyzed into the following buffers: pH 3: 20 mM citrate buffer; pH 6-8: 20 mM phosphate buffer; pH 9-10: 20 mM borate buffer. CD spectra were recorded for rTerrelysin (pH 8) between temperature 20°C-90°C with a 5°C interval to study the thermal denaturation of rTerrelysin. Baseline correction was performed with respective buffers without protein and an average spectrum was obtained from 3 scans. Spectra were fit using CDPro over the range of 260-185 nm. The fitting procedure can be used to predict the tertiary class of the protein assuming it is all α -helix, all β -sheet, a mixture of α -helix and β -sheet, or a denatured protein (22).

4.2.5 SDS-PAGE and protein blot analysis- SDS-PAGE electrophoresis was performed using 12% acrylamide separating gel and 4% stacking gels. SDS-PAGE was carried out using constant voltage of 100 V for 90 mins and the separated proteins were stained with ImperialTM protein staining solution (Pierce, Rockford, IL). For protein blots, gels were transferred to a nitrocellulose membrane at a constant voltage of 15 V overnight at 4°C and the membranes blocked for 1 hr in 3% bovine serum albumin (BSA) in PBST (PBSTB). After washing with PBST, the membrane was incubated for 1 hr with horseradish peroxidase-conjugated *Strep-Tactin* diluted 1/100,000 (v/v) (IBA). The blots were subsequently developed with tetramethylbenzidine solution (TMB) (KPL Inc., Gaithersburg, MD).

4.2.6 Hemolysis assay- Hemolysis assay was performed by incubating serial dilutions of rTerrelysin (5 mg/ml-0.02 mg/ml) in PBS pH 7.4 containing sodium citrate (0.57%). Each rTerrelysin dilution (100 µl) was incubated with 100 µl of sheep red blood cells (SRBC) at several concentrations ranging from 2 to 5% in PBS pH 7.4 containing 0.57% sodium citrate in a 96-well microtiter plate. Samples were incubated for 30 mins at RT. SRBC incubated with Tween (1%) acted as a positive control and SRBC incubated with PBS acted as a negative control. After incubation, plates were centrifuged at 4100 g for 5 mins. The supernatant was collected and measured at OD₅₄₀ nm.

4.3 RESULTS

4.3.1 Sequence alignment of aegerolysin-like proteins- The putative sequence for terrelysin was obtained by performing a BLAST search with asp-hemolysin (ASPH_ASPFU) as a search query. The search yielded results for homologues present in different Ascomycetes, Basidiomycetes, bacteria and plants. A putative uncharacterized protein sequence from *Aspergillus terreus* ([Q0CRX8](#)) with 26% identity to Asp-hemolysin was identified. Sequences of putative uncharacterized proteins from *A. fumigatus*, *A. terreus*, *A. flavus*, *A. clavatus*, *A. oryzae*, *Penicillium chrysogenum*, *Agrocybe aegerita*, and *Pleurotus ostreatus* were analyzed using Clustal W multiple sequence alignment software (23) (**Fig. 4.1**). Significant homology was observed in the C-terminal region of aegerolysin-like proteins. Terrelysin (Q0CRX8) shares > 50% identity with putative hemolysins from *A. niger* ([A2RBK6](#)-55%) and *A. nidulans* ([Q5BD27](#)-69%) and $\geq 30\%$ identity with 13 other aegerolysin-like proteins. Terrelysin Q0CRX8 contained a unique sequence DCSRSGLPLLFR at AA 29-40 that was not found in the other aegerolysins.

4.3.2 Cloning and expression of rTerrelysin- Total RNA was isolated from *A. terreus* and reverse transcribed to cDNA. The open reading frame encoding the putative terrelysin (**Fig. 4.1**) was amplified using specific primers F1 and R1, which amplified a 390 bp PCR product that was cloned into the pASK-IBA6 vector (**Fig. 4.2**). The open reading frame did not contain the sequence for the peptide AA29-40 found in Q0CRX8, which instead was replaced by a glutamic acid (AA29).

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cDNA      -----MDDSQWVSIHIRDRLAQGNITIRESFLEYEQFHSPEDKKKALTEDDIDQLI-51
rTerrelysin -----MDDSQWVSIHIRDRLAQGNITIRESFLEYEQFHSPEDKKKALTEDDIDQLI-51
ASPH_ASPFU -----MASVQAYAQWVTVHLINSMSETLSIQNASLSWGKWKDGDKDAEITSEDVQOKT-55
A4DA65_ASPFU -----MASTTGQQFLDIEIKDDMKYD-IRIENAHIESGEFYREGDQNDTLTTDDIEDMI-53
B8NXA7_ASPFL ---MDTDLNKNYSKWVEFHIRDHLKDGEISVRHTVIEDGEFQDPNNRRKSIHEDVIDEIV-57
B8N7M1_ASPFL IHRQVDMPADAYGQYVDIKIEDGMKYD-LRIENAHLD SGQFYRQGDQGDIMTADDIDDMI-119
A1CGD5_ASPCL -----MAPTSAHQYLDMEIKDDMQYD-IRIENARIDSGEFHSA SDENDTLTTDDVEDLM-53
A2QA29_ASPNG -----MAERAEAQWVHIRVNSLSFDTLVRNTWLGWKGFKHKNKSAEIPVSDINALR-54
A2RBK6_ASPNG -----MPAAGPMDDQWVSFHIRDHLEKGEITVRHTVIEGGEFHD PNNRRQSLTEDEIDEIT-56
Q2U8X3_ASPOR MALCLCQQSKISGAQELIRLLFDIDLHLLID-LNIRSRKIPKGNMKRTKKYASQVNGKE-59
B6GXR4_PENCH ---MDNMDKYNYSQWVSFHIRDHLKDGEILVQNTVIEGGEFADPNNRRSLTEEEIDEIK-56
B6HE31_PENCH -----MASSLQKSLQIQIQDDLKYD-IRIENAKLESGKFYNEDDQSDILTS EDVDDMV-53
AAPR1_AGRAE --MDSNKDERAYAQWVIIILHN-VGSSPFKIANLGLSWGKLYADGNKDKEVYPSDYNGKT-57
Q56QW9_PLEOS -----AYAQWVIIILHN-VGQQNVKIKNLNASWGKLYADGDKDTEVPASKYEGMV-49

cDNA      IPS-EGIGEV CARGR----RGSEGWMDFD---GEKKICELHWDNR-TKRPSNEFEVIDG-102
rTerrelysin IPS-EGIGEV CARGR----RGSEGWMDFD---GEKKICELHWDNR-TKRPSNEFEVIDG-102
ASPH_ASPFU   APP-GGSVNVNSCGRSDASSGTTGGFDLYD---GNTKIGRVHWD CP-WGSKTND FVGER-110
A4DA65_ASPFU IRHNGGLRHVCSGEEKGFGKLGQTIDLID-DVKDAKICTLEWNA PMEPGKRNTFMLRDQ-112
B8NXA7_ASPFL IPS-DGIGEICAHGR----RGSEGRDLDFH---GNDKICELHWD DR-DGRENLVEMLDE-108
B8N7M1_ASPFL IRHNGGIREVCSGGETDSMSGTQGTIDLID-DVKDTRICTLWASAPMQSGRKNRFSMLNH-178
A1CGD5_ASPCL IRHNGGTRHVCSSGEEGFGKLGQTIDLVD-DVRDARICTLAWNAPME PGKRNLMMHHH-112
A2QA29_ASPNG AAP-GGSFNVFACGRAHSPSGTEGSFDVYN---GDVRVAYIYWDCP-WGSKRNQFRVDRI-109
A2RBK6_ASPNG IPS-YGIGEICARGR----RGSEGRDLDFH---DEEKICELHWDNR-QGNPVNIVEM LDS-107
Q2U8X3_ASPOR IIP-GTSQLETCGRSSAASGTEGTLELWD---KTQRICKLYWDCP-WGSKVNKFKVQEK-114
B6GXR4_PENCH ISP-DGVGEIGASSR----RGSEGRDLDFH---DNDKICELHWENR-GGEHANLVEVL DK-107
B6HE31_PENCH IRHNGGIRSVCS-----MDGSEGSIDLVD-DVRDAKICNLTW RASPEGGERNEIKKLNE-106
AAPR1_AGRAE VGP-DEKIQINSCGRENASSGTEGSFDIVDPNDGNKTIRHFYWECP-WGSKRNTWTPSGS-115
Q56QW9_PLEOS IAP-GDQVQINACGREDAAGETTGTDFLVDPNDS DKQVRHISWDCP-WGTKANSWVVGGS-107
          : :      * * : : .      : . *      *

cDNA      DKDYKIECSGWSPOAG-PLGHVFIDISAAKKKAAAAAAAK--141      --      --
rTerrelysin DKDYKIECSGWSPOAG-PLGHVFIDISAA-----130      100      100
ASPH_ASPFU   NKNYWVEIGTWNKYGG-AIGTVDVVEVGRKR-----139      29      55
A4DA65_ASPFU DPRYHIDIGQWN-ESG-ILGKVPVTISDE-----139      34      53
B8NXA7_ASPFL SDKYRIEHGGWSPEANGPLGHVYVDVWAKDKSK-----141      53      74
B8N7M1_ASPFL DPRYKVDIGKWQ-ESG-TMGTVNVAIKDE-----205      33      52
A1CGD5_ASPCL DPQYNVEIGKWN-ESG-VMGTVPITISNQ-----139      32      51
A2QA29_ASPNG ADDYWVETGYWNQSGG-AIGSVTVEIGRRDRAIRSSL-----145      28      51
A2RBK6_ASPNG NSKYRVEHGGWSPEAG-PLGHVYIDILEQQKKKNSK-----142      60      78
Q2U8X3_ASPOR YKTYRVEVGPWNSYGG-ALGNVDVEISRKRA-----144      31      57
B6GXR4_PENCH SDKYTIEHGGWSSEAG-PLGHVFVDVAEKKK-----137      48      69
B6HE31_PENCH DTRYMVDIGSWN-ESG-TVGSVLVTVKDQE-----134      31      54
AAPR1_AGRAE NTKWMVEWGSQNLDSG-ALGTITVDVLRKGN-----145      24      41
Q56QW9_PLEOS NSKWMIEYTGQNLDSG-ALGTITVNTLKIGN-----137      N.A.      N.A.
          : :      . . : * : :

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Fig 4.1. Sequence alignment of putative aegerolysin family proteins produced by various fungi. Alignment was performed using Clustal W. Proteins were identified by their database accession numbers. Abbreviations of organisms from which the proteins were identified. PENCH: *Penicillium chrysogenum*, ASPOR: *Aspergillus oryzae*, ASPFL: *Aspergillus flavus*, ASPFU: *Aspergillus fumigatus*, ASPCL: *Aspergillus clavatus*, ASPNG:

Aspergillus niger, AGRAE: *Agrocybe aegerita*, PLEOS: *Pleurotus ostreatus*. The full-length sequence of terrelysin identified by translation of the cDNA sequence is identified as cDNA, while the recombinant sequence without the Strep-tag II is identified as rTerrelysin. The % identities and % similarities were reported from BLAST results on EBI website. ‘*’ Fully conserved residues, ‘:’ strongly conserved residues, ‘.’ weakly conserved residues. N.A. is defined as “not determined” using the BLAST tool on EBI website with terrelysin (Q0CRX8) as a query. The query did not yield results for identities and similarities for ostreolysin (Q56QW9). Sequence analysis was performed on August 12, 2009.

Using the primers F2 and R2 the ligation of the insert in the pASK-IBA6 vector was verified. The terrelysin insert was not present in unligated pASK-IBA vector (Lane 2) but was present after ligation into the vector (Lane 3) as observed by an approximately 390bp difference in bands on the gel. After transferring the plasmid into competent *E. coli* by heat-shock transformation, positive clones were selected on the basis of ampicillin resistance and the correct orientation of the insert was confirmed by sequencing primers F2 and R2. Clones expressing the terrelysin sequence in the proper reading frame were identified and chosen for further study. The presence of terrelysin cDNA was demonstrated in the TOP 10 cloning strain (Lane 5) and the BL21 (DE3) expression strain (Lane 6) of *E. coli*, but not in the original *E. coli* DH5 α strain bearing the plasmid with no insert (Lane 4) (**Fig. 4.3**).

Fig 4.2. Cloning strategy for recombinant terrelysin in pASK-IBA6 vector and expression in *Escherichia coli*.

4.3.3 Characterization of rTerrelysin- The pASK-IBA6 vector is designed for expression of the recombinant protein into the periplasmic space through a signal peptide OmpA inserted at the N-terminal of the recombinant protein. The signal is cleaved by endogenous signal peptidase during the protein translocation resulting in the release of the protein into the periplasmic space. Immediately downstream of the OmpA signal the pASK-IBA6 vector contains a *Strep*-tag II and Factor Xa recognition sequence at the N-terminus that is a part of the rTerrelysin polypeptide.

Protein preparations from uninduced *E. coli*, AHTC induced *E. coli*, and *Strep*-Tactin column purified rTerrelysin were analyzed by SDS-PAGE (**Fig. 4.4a**). An ~ 17 kDa band representing rTerrelysin was observed only in induced cultures (Lane 3) and the purified fraction (Lane 4), but was absent in the uninduced cultures (Lane 2).

Protein blots were performed and rTerrelysin was identified using HRP-conjugated *Strep*-Tactin and TMB as a substrate (**Fig. 4.4b**). A band corresponding to rTerrelysin was present in AHTC induced cultures (Lane 5) and in the *Strep*-Tactin column-purified fraction (Lane 6), but absent in uninduced (Lane 4) or induced *E. coli* cultures without the insert (Lane 3). The bands observed in Lanes 4 and 5 at ~ 17 kDa just above the rTerrelysin band could possibly be BCCP from *E. coli*. This band is absent in purified preparation (Lane 6) suggesting the removal of BCCP after addition of avidin prior to purification on the *Strep*-Tactin column.

The mass of rTerrelysin was determined using MALDI-TOF MS (**Fig. 4.5**). The single charged $[M+H]^+$ ion of rTerrelysin was observed at m/z 16428, in good agreement with SDS-PAGE results (**Fig. 4.4**). The peak observed at m/z 8197 represents the doubly charged $[M+2H]^{2+}$ ions of rTerrelysin, respectively.

Fig 4.3. Agarose gel electrophoresis. Lane 1: DNA ladder, Lanes 2, 4: pASK-IBA6 vector with no insert (PCR products of primers F2 and R2), Lanes 3, 5, 6: pASK-IBA6 vector with terrelysin gene insert (PCR products of primers F1 and R1). Lane 5: Cloning strain (OneShot Top10) and Lane 6: expression strain (DE3).

Fig 4.4a. SDS-PAGE for terrelysin. Lane 1: Molecular weight marker, Lane 2: Uninduced pASK-IBA6 vector (with terrelysin insert), Lane 3: Induced pASK-IBA6 vector (with terrelysin insert), Lane 4: Recombinant terrelysin purified on Strep-Tactin column.

Fig 4.4b. Protein blot for rTerrelysin. Lane 1: Molecular weight marker, Lane 2: Uninduced pASK-IBA6 vector (no insert), Lane 3: Induced pASK-IBA6 vector (no insert), Lane 4: Uninduced pASK-IBA6 vector (with terrelysin insert), Lane 5: Induced pASK-IBA6 vector (with terrelysin insert), Lane 6: Recombinant terrelysin purified on Strep-Tactin column.

Fig 4.5. MALDI-TOF MS of rTerrelysin. $[M+H]^+$: 16428.4 Da. $[M+2H]^{2+}$: 8196.7 Da.

4.3.4 Secondary structure of rTerrelysin- The secondary structure of rTerrelysin was determined using far-UV (185-240) CD spectroscopy analysis (**Fig. 4.6**). The lowest ellipticity at 216 nm combined with the highest ellipticity at 195 nm, identified rTerrelysin as a predominantly β -sheet protein (**Fig. 4.6a**). This result was further supported by fitting the data with the prediction that the protein was completely in the β -sheet conformation. The effect of different pH (3-10) on rTerrelysin unfolding was studied. The optimum β -sheet conformation was observed at pH 8. With decreasing pH, a decrease in β -sheet conformation population was observed, with the loss of almost all of the β -sheet conformation at pH 3 (**Fig. 4.6b**). A similar effect was observed with the change in pH from 8-10 (data not shown). With the increase in alkalinity a gradual decrease in β -sheet conformation was observed.

Thermal denaturation studies with rTerrelysin and its effect on its secondary structure was studied using CD spectroscopy with monitoring the spectra of rTerrelysin from 20°C-90°C with a 5°C interval. The spectra revealed a gradual shift from a predominantly β -sheet conformation to a α -helical structure with the increasing temperature (**Fig. 4.6c**). Spectrum measurement in the far UV range identified three different states of secondary structure conformation for rTerrelysin. Between 20°C-65°C, the CD spectrum revealed predominantly β -sheet structure. Between 65°C-75°C, the spectrum showed a possible intermediate structure with β -sheet as well as α -helix elements in the secondary structure. Above 75°C, the structure was predominantly α -helix.

4.3.5 Hemolysis assay- The rTerrelysin preparation showed hemolytic activity when tested on agar plates containing SRBC with a zone of clearance around the site of inoculation (data not shown). Dose-dependent hemolysis was analyzed by incubating dilutions of rTerrelysin with varying concentrations of SRBC. Hemolysis was measured spectrophotometrically as release of hemoglobin at 540 nm (**Fig. 4.7**). Hemolysis, with a steep dose-response curve was observed at relatively high concentrations of rTerrelysin higher than 313 µg/ml.

Fig 4.6. Secondary structure analysis of rTerrelysin. a) CD analysis of rTerrelysin. Negative signal at 216 nm and a positive signal of similar magnitude at 195 nm identify the protein as a predominantly β -sheet protein.

b) rTerrelysin was dialyzed into various buffers of differing pH. Optimum β -sheet conformation was observed at pH 8 with a reduction in β -sheet conformation with decreasing pH. A disordered secondary structure is observed at pH 3. c) Thermal denaturation of rTerrelysin was studied between temperatures 20°C-90°C. rTerrelysin

retained β -sheet structure up to 65°C and beyond that the structure included α -helix elements. Above 75°C, the structure was predominantly α -helix.

Fig 4.7. Hemolysis assay. Hemolysis was observed at high concentrations of purified rTerrelysin. Increased hemolysis was observed on increasing the concentration of SRBC. Hemolysis was measured at OD540 nm.

4.4 DISCUSSION

Hemolysins are proteins that lyse red blood cells as well as various other eukaryotic cells. Here we report the cloning, expression and purification of a recombinant fusion protein of a putative hemolysin produced by *A. terreus* called terrelysin. Terrelysin belongs to the aegerolysin family of proteins that consists of cytolysins produced by other filamentous fungi, bacteria, and plants (9). A search using the terrelysin sequence as a template in the NCBI-BLAST2 – Protein Database Query yielded sequence similarities to 40 other proteins from different fungi, bacteria and certain members of kingdom Animalia such as *Schistosoma japonicum* and *Ciona intestinalis*. Terrelysin is a 141 AA protein with a predominantly β -sheet structure and a predicted molecular weight of 15,805 Da and a pI of 5.23.

Recombinant terrelysin was expressed as a *Strep*-tag II fusion protein in the pASK-IBA6 vector. An apparent intron sequence (DCSRSGLP LLFR) found in the genomic sequence Q0CRX8 was not found in the cDNA transcribed from *A. terreus* mRNA. In addition, we truncated the recombinant protein at AA 130 to better correspond with other aegerolysins. The recombinant also contained the linker, *Strep*-tag II and Factor Xa recognition sequences (ASWSHPQFEKIEGR) at the N-terminus giving the fusion protein a predicted MW of 16,290 Da and pI of 4.91.

The benefit of using the *Strep*-tag II system rather than other, more common systems such as histidine tag (His₆), is that it is less likely to interfere with protein folding or function (24-28). For example, asp-hemolysin has been cloned and expressed as a Maltose Binding Protein (MBP) fusion construct, however, this protein was not functionally active (3, 4). Other proteins from the aegerolysin family (Cbm 17.1 and Cbm 17.2 produced by *Clostridium bifermentans*) were produced as His₆-tag fusion proteins (29) that failed to be hemolytic.

Similarly, His₆-tagged PA0122 protein from *Pseudomonas aeruginosa* was also successfully expressed in *E. coli* but found to be non-hemolytic (30). His₆-tag constructs of terrelysin were also made in our lab and we did not observe any hemolytic activity with them. With the MBP-tagged protein, the large size of MBP (42.7 kDa) may affect the conformation and function of asp-hemolysin (14.2 kDa) (4), but to date recombinant hemolysins have been functionally inactive.

In preliminary experiments, we observed the rTerrelysin *Strep*-tag II fusion protein preparation to have hemolytic activity against SRBC. However, given the rather steep dose response and the significant doses required, the possibility of surfactants or detergents being present in rTerrelysin preparation could not be ruled out. The CelLytic B solution used for lysing *E. coli* cells contains a proprietary formulation with detergents, which could not be completely removed by dialysis. Recent research has identified the requirement of a co-factor for the functional activity of pleurotolysin (31, 32). Whether a co-factor is required for terrelysin activity will require further work but this helps to further explain the lack of functional activity of recombinant hemolysins.

Sequence alignment of multiple aegerolysin family proteins demonstrated that most conserved residues were present in the C-terminal region of the protein sequences. The hemolysins of *A. oryzae* and *A. flavus* in the genomic database were observed to share the exact same sequence. These two species belong to the *Aspergillus* section *Flavi*, and are phylogenetically closely related (20, 33, 34). Interestingly, it was also observed that members of the aegerolysin family share some structural homology with a family of proteins that contain an E-set domain (Conserved Domain Database: cl09101) for sugar utilizing enzymes (35, 36).

Although aegerolysins are typically characterized as hemolysins, they may have other enzymatic functions not yet identified.

Differences in mass of fungal hemolysins have been reported (1, 2, 37-39). Aegerolysin family proteins are typically low molecular weight (~14-17 kDa) proteins. Our experimentally determined rTerrelysin mass is in good agreement with those of other members of the aegerolysin family (2, 4, 29, 30). The secondary structure is very important for functionality of native hemolysins since they undergo conformational changes once inserted into the target membrane, to induce pore formation (40). We expressed rTerrelysin in the periplasmic space of *E. coli* to allow for proper folding of the protein.

The secondary structure of aegerolysin family proteins has been predicted to be predominantly β -sheet (41, 42). The secondary structures of nigerlysin and ostreolysin have been reported (38, 41). While nigerlysin was reported as α -helical structure, ostreolysin was identified as a β -sheet protein. β -sheet structures have also been identified in bacterial hemolysins such as alpha toxin of *Staphylococcus aureus* and perfringolysin-O of *Clostridium perfringens* (43, 44). We have identified rTerrelysin as a predominantly β -sheet protein and it may be similar in function to some of the bacterial hemolysins that play a role in pathogenesis.

Our findings on terrelysin unfolding as a result of pH changes confirm the observations of Berne *et al.* on ostreolysin (41). These authors suggested that ostreolysin might exist in conformationally distinct states as a function of pH. The authors identified the optimum β -sheet conformation at ~ pH 8, an observation that has been confirmed for several hemolysins (45, 46). Berne *et al.* also reported that as the pH is shifted to the acidic or alkaline range, the conformation of the protein changes. The disordered conformation of rTerrelysin at pH 3 may

provide the protein flexibility that it lacked in the β -sheet conformation. This flexibility may increase the formation of oligomers and in pore formation.

A. terreus is a thermotolerant organism capable of growing at elevated temperatures of up to 50° C. Our thermal denaturation studies on the secondary structure of rTerrelysin indicate that the β -sheet conformation of the recombinant protein is stable up to 65°C. Presence of a thermostable protein may provide the fungus an advantage in colonizing in elevated soil temperatures or within human body.

The aegerolysin family of proteins is an interesting new group of proteins that may have multiple functions. In basidiomycetes (mushrooms), they have been found to accelerate fruiting body formation (2, 47) and may be critical during germination of spores of filamentous fungi. Their role in pathogenesis appears to be related to their cytolytic activities (12, 13, 48). Interestingly, ostreolysin and other aegerolysins may bind microheterogeneous domains within the membrane that are rich in cholesterol (49). Some have also characterized the binding of aspholysin to low density lipoproteins (50-52). Preliminary studies have suggested the possibility of the use of fungal hemolysins as diagnostic markers (10, 53).

Future studies will be aimed at generating monoclonal antibodies to rTerrelysin and developing inhibition ELISA methods using rTerrelysin as reference standard to quantify terrelysin in human serum samples or environmental samples. These experiments will help to further understand the biological significance of terrelysin, its expression, localization and potential use as a biomarker for exposure to *A. terreus*.

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CHAPTER 5

**Development of monoclonal antibodies to recombinant terrelysin
and characterization of terrelysin expression in *Aspergillus terreus***

5.1 INTRODUCTION

Hemolysins have been suggested to play a key role in pathogenesis by lysing host cell membranes, which assists in causing infection (1-3). However, less is understood about the biological relevance of these hemolytic proteins in fungi. Fungal hemolysins have been studied for a few decades for their role as virulence factors (4-7), their lipoprotein-binding properties (8, 9) and utility as unique markers of micro-domains on eukaryotic cell membranes (10, 11). Plants, animals and humans are opportunistic hosts of the fungus and these hemolytic proteins may have distinct functions in fungal biology in nature. Their hemolytic activity and other cytotoxic properties associated with host factors may only be coincidental. Some studies have earlier reported involvement of these hemolysins in the morphology and development of fungi especially mushrooms (12, 13). However, limited information is currently available on expression and localization of these hemolysins in filamentous fungi, which may be essential in ascertaining a role for these proteins in fungal biology.

In our previous work we attempted to develop mAbs to fungal hemolysins using partially purified hemolytic preparations. This led to identification of multiple proteins in the preparation and we identified some of these proteins to be proteolytic enzymes. In our effort to study the specific hemolysin of *A. terreus*, terrelysin, we cloned the gene for terrelysin and expressed the protein in *E. coli*. The recombinant rTerrelysin was purified and used in production of monoclonal antibodies (mAbs) for characterization of expression of terrelysin by *A. terreus* and development of immunoassays for detection of terrelysin as a surrogate biomarker for exposure to *A. terreus*.

In this report we describe the generation of monoclonal antibodies (mAbs) to terrelysin, their purification and characterization of kinetics of native terrelysin expression by *A. terreus*.

We will also use these tools to study the localization of terrelysin within the fungal hyphae and conidia. This will help in suggesting putative functions for these proteins in fungi.

5.2 METHODS AND MATERIALS

5.2.1 Production of monoclonal antibodies against rTerrelysin- Three 8-10 week old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were housed in the animal facility at the National Institute for Occupational Safety and Health (NIOSH), Morgantown, WV. The facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and animals were free of viral pathogens, parasites, mycoplasma, and *Helicobacter* spp. Animals were housed together in a HEPA-filtered ventilated polycarbonate cages on autoclaved hardwood Beta-chip bedding with cotton fiber nesting material. The animals were provided with Teklad 7913 rodent chow (Harlan Laboratories, Madison, WI) and autoclaved tap water *ad libitum*. All animal procedures and immunizations were reviewed and approved by the NIOSH Animal Care and Use Committee (ACUC).

Prior to immunization, blood samples were collected from the tail vein and the serum was stored at -20°C. Each animal was immunized 3 times, every other week with 25 µg of purified rTerrelysin emulsified (50% v/v) in TiterMax® (TiterMax USA, Norcross, GA) adjuvant. The mice were monitored for adverse health effects post immunizations. Between immunizations, serum was collected from the tail vein to monitor for rTerrelysin-specific IgG antibody response.

Following the development of sufficient IgG titers, the animals were euthanized by CO₂ asphyxiation. The spleen was aseptically removed from each mouse and single cell suspensions of the splenocytes produced. Fusion of splenocytes with SP2/0-Ag 14 myeloma cells (ATCC# CRL-1581) was performed as previously described (14). Hybridomas were selected by growing

cells in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Rockville, MD) supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.292 mg/ml L-glutamine, 100 mM sodium hypoxanthine, 16 mM thymidine, 10% fetal calf serum (FCS) (HyClone, Logan, UT), and 100 U/ml IL-6 (Boehringer, Mannheim, Germany). DMEM was also supplemented with azaserine for selective propagation of hybridomas. After 10-14 days of growth, medium from individual wells with growth of hybridoma cells was replenished with fresh DMEM medium. The supernatants from individual hybridoma colonies were tested in ELISA to detect presence of antibodies specific to rTerrelysin.

Supernatants of individual clones were tested twice to confirm reactivity of antibodies. Hybridomas from each positive well was further cloned, twice by limiting dilution analysis and single positive clones were screened and selected for production of large quantities of mAbs. Positive clones were frozen in 10% dimethyl sulfoxide (DMSO) and stored at -80°C for 2 weeks and later transferred to liquid nitrogen facility for long-term storage.

5.2.2 Production, isotyping and quantification of rTerrelysin mAbs- All rTerrelysin mAbs were concentrated and partially purified by ammonium sulfate precipitation as described previously (15). Briefly, mAb supernatant collected from individual hybridomas was centrifuged at 20,000 g for 30 min at 4°C. The supernatant was collected and saturated ammonium sulfate was slowly added to the supernatant to 45% (v/v) and incubated for 8-10 hrs at 4°C. The tubes were centrifuged for 45 mins at 20,000 g at 4°C and the precipitate was collected and resuspended in PBS, pH 7.4. Concentration of purified antibodies and their isotype were determined using methods described earlier.

5.2.3 Fungal cultures and extracts- For time point assay, fungal cultures were grown by inoculating 50 ml minimal medium containing glucose, nitric salts and trace elements with 2.5×10^7 *A. terreus* viable conidia. Viability was determined using LIVE/DEAD® BacLight™ Viability kit (Molecular Probes, Inc., Eugene, OR) as described previously (16). Following inoculation, flasks were incubated at either RT or at 37°C depending on the specific experimental design. *A. terreus* cultures were grown for 12 days with an individual flask representing a 24 hr time point were collected each day. *A. terreus* culture and mycelial pellets were collected in 50 ml tubes and centrifuged at 4100 g for 10 mins. The culture supernatant (CSN) and mycelial pellets were collected and stored at -80°C and the lyophilized CSN residue was resuspended in PBS, and mycelial pellets were processed using a mortar and pestle with PBS containing Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). Mycelial slurry was then collected into 15 ml polypropylene tubes and incubated at 4°C overnight on a shaker to facilitate the release of intracellular proteins into the lysis solution. The next day, mycelial extracts (ME) were centrifuged at 4100 g for 10 mins and the supernatant collected and stored at -20°C until analysis.

For cross-reactivity studies, ME were prepared from 29 different fungal species including 12 different *Aspergillus* species using the same method (**Table 1.**) Fungi were grown till mycelial pellets had formed (3-4) days. Protein concentrations of CSN and ME were estimated using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE).

5.2.4 ELISA methods- Screening mAbs. Hybridomas producing anti-rTerrelysin mAbs were identified by indirect ELISA. In brief, 96-well Immuno MaxiSorp microplates (Nunc, Rochester, NY) were coated with rTerrelysin (1 µg/ml) in 0.05 M carbonate coating buffer pH 9.6 (CCB), and blocked with phosphate buffered saline (PBS) containing 0.5% Tween-20 and 5% nonfat dry milk (PBSTM) for 1 hr. CSN from each hybridoma was incubated in duplicate wells for 1 hr at 37°C, washed with PBS containing 0.5% Tween-20 (PBST), and detected using alkaline phosphatase conjugated goat-anti mouse IgG antibody (H+L) (Promega, Madison, WI) diluted 1:5000 in PBSTM for 1 hr at 37°C. The wells were then washed in PBST and developed for 30 min using 4-paranitrophenyl phosphate substrate (Sigma). Reactivity was determined by measuring the optical density (OD) at 405 nm. TO compare the reactivity of identified mAbs, each mAb was purified and used at a concentration of 1 µg/ml and assayed with various dilutions of rTerrelysin bound to the assay plate. The plates were washed 3 times between individual steps of incubation with PBST.

Inhibition ELISA for analysis of kinetics of terrelysin expression. An inhibition ELISA was performed over a period of 2 days. On day 1, the ‘assay plate’ was coated with 0.1 µg/ml of rTerrelysin and a second plate ‘inhibition plate’ was blocked overnight with PBSTM to prevent protein binding. On day 2, both plates were washed with PBST and the ‘assay plate’ was blocked with PBSTM at RT for 2 hrs. In the inhibition plate, serial dilutions of ME and CSN samples (protein concentration range 5 mg/ml-0.078 mg/ml) and serially diluted rTerrelysin standard time points (days 1-12). Standards were set up by serial dilution of rTerrelysin (protein concentration range 1 mg/ml-78 µg/ml) were incubated with 40 ng/ml of anti-terrelysin mAb 10G4. Negative control wells were incubated with PBS and positive control wells were incubated with mAb 10G4 alone. The inhibition plate was incubated on a shaker at RT for 1 hr at 200 rpm and for 30

min without shaking. Next, the assay plates were washed and incubated with 100 µl of reaction mixture from the inhibition plate at 37°C for 1 hr. Plates were washed and incubated with alkaline phosphatase conjugated goat anti-mouse IgG antibody (H+L) diluted 1:5000 in PBSTM for 1 hr at 37°C. Plates were washed and developed using 4-paranitrophenyl phosphate substrate. Reactivity was measured at OD at 405 nm and terrelysin concentrations were determined for each sample by comparing to the standard curve using regression analysis.

5.2.5 Western blot analysis of rTerrelysin and fungal ME- Reactivity to rTerrelysin and native terrelysin. Pooled polyclonal sera collected each immunized mouse was tested using Western blot with rTerrelysin and *A. terreus* ME. *A. terreus* ME was collected from 3 days old cultures grown in TSB for 72 hrs at RT. rTerrelysin (50 ng) and *A. terreus* ME (25 µg) were separated using a 12% acrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked for 1 hr in PBST containing 3% bovine serum albumin (BSA) (PBSTB) and incubated with anti-rTerrelysin pooled polyclonal sera (1/2000 v/v) in PBSTB for 1 hr at RT. The membrane was washed 3 times with PBST and then incubated with alkaline phosphatase conjugated goat anti-mouse IgG. The membrane was washed again and immunoreactive proteins were detected using the chromogenic substrate NBT/BCIP (Promega, Madison, WI).

Western blot analysis was performed for screening individual mAbs reactivity to rTerrelysin, native terrelysin (*A. terreus* CSN and ME extracts), and for cross-reactivity analysis. rTerrelysin (500 ng/ml) and ME (2.5 mg/ml) collected from day 4 culture of *A. terreus* were individually separated by performing SDS-PAGE on a 12% polyacrylamide gel. For cross-reactivity testing, ME (2.5 mg/ml) from 29 different fungal species were separated on 12% polyacrylamide gels. Proteins were transferred overnight to nitrocellulose membranes (0.22 µm,

BioRad) and the membranes were blocked using Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and 3% bovine serum albumin (blocking buffer). Membranes were washed with TBST and transferred to a BIO-RAD Multi Screen apparatus (BioRad, Hercules, CA). Individual lanes were incubated with 1 µg/ml of mAbs diluted in blocking buffer and incubated on a rocker for 1 hr. Membranes were washed 3X with TBST, and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (H+L) diluted 1:5000 in blocking buffer for 1 hr on a rocker. Membranes were then washed with TBST and developed for 15-20 mins using 1-Step NBT/BCIP (Promega, Madison, WI) substrate solution. The reaction was stopped by washing the membranes with distilled water.

5.2.6 Epitope mapping- Epitope mapping was performed using synthetic peptides synthesized by Sigma Genosys (JPT Peptide Technologies GmbH, Berlin, Germany) using methods previously described (17). For peptide scans, 68 peptides spanning the entire rTerrelysin (including the Strep-tag II sequence) were synthesized as linear decapeptides overlapping by 2 amino acids. Peptides were covalently bound to a Whatman 50 cellulose support (PepSpots membrane) at the C-terminus and N-terminus of peptides was acetylated for higher stability. The membranes were processed for epitope mapping per the manufacturer's instructions.

In brief, the PepSpots membrane was rinsed in methanol for 5 mins, washed 3X with TBS for 10 mins, blocked overnight at 4°C on a shaker with TBS containing 3% BSA. The membrane was incubated with 5 µg/ml of rTerrelysin mAbs for 3 hrs at RT on a shaker. The mAb 9B4, an IgG₁ isotype, which recognizes a *Stachybotrys chartarum* conidial surface protein served as a negative control. The membrane was washed in TBST 3X for 5 min each and then incubated with goat anti-mouse IgG horseradish peroxidase (HRP) conjugated antibody

(Promega, Madison, WI) diluted 1:50,000 in blocking buffer for 1 hr at RT on shaker. The membrane was washed thoroughly in TBST 3X for 5 mins each and developed with ECL Western blotting substrate (Promega) as per manufacturer's instructions. After a brief incubation, excess substrate was discarded and the membrane was exposed to CL-XPosureTM clear blue X-Ray film (Thermo Scientific, Rockford, IL) and developed using a SRX-101A tabletop processor (Konica Minolta, Ramsey, New Jersey). For regeneration, the PepSpots membrane was washed twice with water for 10 mins each and then incubated with regeneration buffer I (62.5 mM TRIS containing 2% SDS, pH 6.7; 100 mM 2-mercaptoethanol) at 50°C using four 30 mins incubations. The membranes were washed 3X for 20 mins with PBS (10X), 3X with TBST for 20 mins and 3X with TBS for 10 mins at RT. The membrane was analyzed to ensure efficient removal of bound primary and secondary antibodies prior to analysis of new mAbs.

5.2.7 Microscopic examination of *A. terreus* morphological changes- In order to correlate the expression of terrelysin to associated growth changes in *A. terreus*, we cultivated *A. terreus* in 24-well plates containing 2 ml of minimal medium. Wells were incubated with 1×10^6 viable *A. terreus* conidia and incubated on a shaker at either RT or 37°C. Growth of *A. terreus* was monitored at various time points using Olympus IX70 microscope (Olympus Corporation, Tokyo, Japan) and images were captured using a QImaging Retiga 2000R Fast camera (QImaging, Surrey, Canada) and processed using the SimplePCI6 software (Hamamatsu Corporation, Sewickley, PA).

5.2.8 Confocal Scanning Laser Microscopy (CSLM) analysis for localization of native

terrelysin in *A. terreus*- Immunolocalization of terrelysin was studied using a slight modification of previously described methods (18, 19). Briefly, *A. terreus* FGSC 1156 cultures were grown on alcohol-sterilized cover slips in 6-well tissue culture plates that contained minimal medium. Cultures were incubated at 37°C under static conditions for 24 hrs. Cover slips were fixed with 8% formalin buffered saline containing 50 mM PIPES (pH 6.7), 25 mM EGTA, 1% dimethyl sulfoxide (DMSO) and 5 mM MgSO₄ for 1 hr at RT and the cover slips were then rinsed with MTSB (50 mM PIPES (pH 6.7), 5 mM EGTA and 5 mM MgSO₄). Cell wall digestion was carried out for 1 hr at RT with an enzyme solution containing 2.5% Driselase® (Sigma, St. Louis, MO), 1% lysozyme from chicken egg white (Sigma) and 2 mM EGTA. Cells were rinsed with H₂O and then treated with 0.1% Triton X-100 in TBS pH 7.4 for 10 mins. Cells were then rinsed in MTSB and TBS once each. Cells were blocked with 3% bovine serum albumin in TBS (TBSB) overnight at 4°C with gentle shaking. Next, the cover slips were incubated with mAb 15B5 at 3 µg/ml in TBSB for 3 hrs with gentle shaking. mAb 9B4 (*S. chartarum* conidial surface protein) served as a negative control (20). mAb 13E11 described earlier in chapter 3, and reacts with hyphal exoantigen leucine aminopeptidase from *A. terreus* served as a positive control. Cells were washed thoroughly in TBS containing 0.05% Tween 20 (TBST) and stained with AlexaFluor® 594 conjugated goat anti-mouse IgG (H+L) (Molecular Probes Inc., Eugene, OR) diluted 1:50 in TBSB for 1 hr at RT. Cells were washed in TBST and cover slips were placed on clean slides with ProLong® Antifade Reagent with DAPI (Molecular Probes Inc.). Cells were observed with a Zeiss LSM-510 Meta Confocal Microscope System (Axioplan 2 Stand) (Carl Zeiss, Thornwood, NY) and the images were acquired with Zeiss

software version 3.2 (Carl Zeiss). All settings on the confocal laser microscope remained constant throughout the analysis.

5.3 RESULTS

5.3.1 Isotyping and sensitivity screening of rTerrelysin mAbs- Mice immunized with rTerrelysin developed detectable specific IgG after the first immunization that increased with subsequent immunizations. Thirty-two hybridomas recognized rTerrelysin during the initial screening. Sixteen clones survived the multiple cloning steps and were further analyzed in screening, cross-reactivity, and immunolocalization experiments. Nine clones (2G4, 3B2, 6D2, 7D8, 13G10, 15B5, 16C7, 9F4, 19E3) produced IgG₁ isotype mAbs, 4 (3B7, 10G4, 15C5, 15E4) produced IgG_{2a} isotype mAbs and 3 (6E4, 10G7, 2D3) produced IgG_{2b} isotype mAbs. The mAbs exhibited variable reactivity to immobilized rTerrelysin in ELISA (**Fig. 5.1**). mAbs 2G4, 9F4, 19E3, 6E4 and 15E4 showed least reactivity (≤ 0.25 OD₄₀₅ value) to rTerrelysin (data not shown). mAbs 3B2, 6D2, 7D8 and 16C7 showed moderate reactivity (**Fig. 5.1a**), while mAbs 13G10, 15B5, 3B7, 10G4, 15C5, 10G7 and 2D3 showed highest reactivity to rTerrelysin (**Fig. 5.1b**).

Fig 5.1. Reactivity of mAbs to rTerrelysin. Moderate reactivity is defined as ≤ 1.0 (OD_{405} value) in assay with 1 µg/ml of mAb concentration. High reactivity is defined as ≥ 1.0 (OD_{405} value) in assay with 1 µg/ml of mAb concentration.

5.3.2 Western blot reactivity of pooled sera and mAbs to rTerrelysin and native terrelysin- Pooled sera from mice immunized with rTerrelysin was used to confirm the presence of immunoreactive terrelysin in extracts from the mycelia of *A. terreus* (**Fig. 5.2**). Western blot analysis revealed reactivity to denatured rTerrelysin (16.5 kDa) for 11 of the 16 mAbs (**Fig. 5.3a**). The higher molecular weight bands observed in the lanes with purified rTerrelysin are likely to be aggregates formed during the storage of the protein at -20°C. For native terrelysin, 4 of the 11 mAbs (3B2, 6D2, 16C7 and 2D3) did not show any reactivity with *A. terreus* ME (**Fig. 5.3b**). Seven mAbs (7D8, 13G10, 15B5, 3B7, 10G4, 15C5, and 10G7) reacted to a putative native terrelysin in ME and were chosen for further study.

Fig 5.2. Western blot analysis with pooled sera. Lane 1: Molecular weight marker, Lane 2: rTerrelysin and Lane 3: mycelial extract from 72 hr old culture of A. terreus.

5.3.3 Cross-reactivity and strain variations- Five strains of *A. terreus* including the pathogenic strain FGSC 1156 and the commonly used environmental strain ATCC 1012 were analyzed with each mAb by Western blot. All mAbs, except 7D8, exhibited reactivity to a protein of the same molecular weight in ME derived from all strains (**Table 5.1**). Interestingly, the mAb 7D8 reacted to ME from 3 strains but 2 strains (NIOSH 17-30-31 and NIOSH 35-08-06) did not react.

ME from 11 other *Aspergillus* species including the pathogens *A. fumigatus*, *A. flavus*, *A. nidulans* and *A. niger* were also tested. mAb 15C5 showed maximum cross-reactivity among the mAbs tested, exhibiting cross-reactivity to ME from 6 different fungi, including *A. nidulans* and *A. parasiticus*. mAb 10G7 did not cross-react with any tested *Aspergillus* species tested here, but it did react to ME from *E. rostratum*, *P. variotii* and *T. harzianum*. mAb 3B7 cross-reacted to ME from *C. cladosporioides*. No cross-reactivity was observed with any mAbs against ME from 4 different *Penicillium* species. 4 mAbs (7D8, 13G10, 15B5 and 10G4) did cross-react with any of the tested fungal species.

Fig. 5.3a. Western blot reactivity of rTerrelysin-mAbs with rTerrelysin. Lane 1: Molecular weight marker, 2: mAb 2G4, 3: mAb 3B2, 4: 6D2, 5: mAb 7D8, 6: mAb 13G10, 7: mAb 15B5, 8: mAb 16C7, 9: mAb 9F4, 10: mAb 19E3, 11: mAb 3B7, 12: mAb 10G4, 13: mAb 15C5, 14: mAb 15E4, 15: mAb 6E4, 16: mAb 10G7, 17: mAb 2D3. Positive reactivity is identified by presence of an immunoreactive band at ~ 17 kDa.

Fig. 5.3b. Western blot analysis of A. terreus mycelial extract with terrelysin mAbs. Lane 1: Molecular weight marker, 2: mAb 2G4, 3: mAb 3B2, 4: 6D2, 5: mAb 7D8, 6: mAb 13G10, 7: mAb 15B5, 8: mAb 16C7, 9: mAb 9F4, 10: mAb 19E3, 11: mAb 3B7, 12: mAb 10G4, 13: mAb 15C5, 14: mAb 15E4, 15: mAb 6E4, 16: mAb 10G7, 17: mAb 2D3. Positive reactivity is identified by presence of an immunoreactive band at ~ 17 kDa.

FUNGI	7D8	13G10	15B5	3B7	10G4	15C5	10G7
<i>As. terreus</i> FGSC 1156	+	+	+	+	+	+	+
<i>A. terreus</i> ATCC 1012	+	+	+	+	+	+	+
<i>A. terreus</i> NIOSH 17-30-31	-	+	+	+	+	+	+
<i>A. terreus</i> NIOSH 35-08-05	+	+	+	+	+	+	+
<i>A. terreus</i> NIOSH 35-08-06	-	+	+	+	+	+	+
<i>A. candidus</i> NIOSH 17-28-24	-	-	-	-	-	-	-
<i>A. chevalieri</i> NRRL 78	-	-	-	-	-	-	-
<i>A. clavatus</i> NIOSH 6-22-78	-	-	-	-	-	-	-
<i>A. flavus</i> NIOSH 15-41-07	-	-	-	-	-	-	-
<i>A. fumigatus</i> FGSC A1100	-	-	-	-	-	-	-
<i>A. nidulans</i> NIOSH 15-22-08	-	-	-	-	-	+	-
<i>A. niger</i> FGSC A1143	-	-	-	-	-	-	-
<i>A. parasiticus</i> ATCC 26690	-	-	-	-	-	+	-
<i>A. penicilloides</i> ATCC 16910	-	-	-	-	-	-	-
<i>A. repens</i> NRRL 13	-	-	-	-	-	-	-
<i>A. ustus</i> NRRL 275	-	-	-	-	-	-	-
<i>Ac. strictum</i> ATCC 46646	-	-	-	-	-	-	-
<i>Al. alternata</i> ATCC 11612	-	-	-	-	-	+	-
<i>Ch. globosum</i> NRRL 1870	-	-	-	-	-	-	-
<i>Cl. cladosporioides</i> NIOSH 17-28-17	-	-	-	+	-	-	-
<i>Cl. sphaerospermum</i> ATCC 11288	-	-	-	-	-	-	-
<i>Ex. rostratum</i> ATCC 26856	-	-	-	-	-	+	+
<i>Fu. moniliforme</i> NIOSH 32-40-16	-	-	-	-	-	-	-
<i>Fu. oxysporum</i> NIOSH 32-40-14	-	-	-	-	-	-	-
<i>Ne. fischeri</i> NIOSH 29-53-20	-	-	-	-	-	-	-
<i>Pa. variotii</i> ATCC 66705	-	-	-	-	-	+	+

<i>Pe. chrysogenum</i> NRRL 1951	-	-	-	-	-	-	-
<i>Pe. expansum</i> NRRL 973	-	-	-	-	-	-	-
<i>Pe. melinii</i> NIOSH 32-46-01	-	-	-	-	-	-	-
<i>Pe. purpurogenum</i> NRRL 1062	-	-	-	-	-	-	-
<i>Sc. brumptii</i> ATCC 16278	-	-	-	-	-	-	-
<i>Tr. harzianum</i> NIOSH 29-32-13	-	-	-	-	-	+	+
<i>Tr. viride</i> ATCC 16640	-	-	-	-	-	-	-

Table 5.1- Cross-reactivity profiles of terrelysin-mAbs analyzed by Western blot. As: Aspergillus, Ac: Acremonium, Al: Alternaria, Ch: Chaetomium, Cl: Cladosporium, Ex: Exserohilum, Fu: Fusarium, Ne: Nesorartorya, Pa: Paecilomyces, Pe: Penicillium, Sc: Scopulariopsis, Tr: Trichoderma. +: Positive reactivity.

5.3.4 Epitopes for rTerrelysin-mAbs- Owing to the high specificity determined in cross-reactivity studies, 3 mAbs (15B5, 13G10 and 10G4) were tested to determine their epitopes using overlapping decapeptides that span the entire sequence of rTerrelysin. For mAb 15B5, 2 spots were recognized at positions 20-21 (**Fig. 5.4**). The sequence of spots 20 and 21 correspond to 'SFLYEGQFHS' and 'LYEGQFHSPE' respectively. These data suggest that the epitope recognized by mAb 15B5 is 'LYEGQFHS'.

After ensuring complete removal of bound mAb 15B5 and regeneration of the SPOTs membrane, the membrane was then scanned using mAb 13G10. Four consecutive spots (52-55) reacted with mAb 13G10 and the resultant epitope was 'PSNEFE'. Subsequently, mAb 10G4 was found to recognize the same epitope as mAb 13G10 based on SPOTscan data. SPOTscan of membrane using control mAb 9B4 and secondary antibodies did not result in reactivity to any spots (data not shown).

Fig. 5.4 Epitope mapping of anti-rTerrelysin mAbs. SPOTs membrane scans for mAbs 15B5, 13G10 and 10G4. Each spot represents a decapeptide of rTerrelysin sequence. Decapeptides were sequential with an overlap of 2 amino acids and spanned the entire sequence of rTerrelysin including the N-terminal purification Strep-tag II and the Factor Xa cleavage site. mAb 15B5 recognizes the epitope 'LYEGQFHS', while mAbs 13G10 and 10G4 recognize the epitope 'PSNEFE'.

5.3.5 Time-point kinetics of expression of terrelysin- The expression of native terrelysin was examined using an inhibition ELISA to quantify the amount of terrelysin in CSN and ME at 24 hr intervals (**Fig. 5.5a**). In ME, the highest relative concentrations of terrelysin were observed during the first few days of growth ranging between 10-12 $\mu\text{g}/\text{mg}$ of total fungal protein. A 10-fold reduction was observed by day 4-5 (1 $\mu\text{g}/\text{mg}$ of total protein) and after day 6, very little terrelysin was quantified in ME. Lower levels of terrelysin were detected in the CSN with a maximum at day 6 and then declining to nearly baseline by day 12.

The morphological features that were observed at the same time intervals are depicted in **Fig. 5.5b**. The early time points when expression of terrelysin was the highest, involved the initial stages of conidial germination and hyphal extension (12 hrs to 24 hrs). Hyphal aggregation was observed up to 96-120 hrs, when quantities of terrelysin were observed to decline. Beyond this time interval, the morphological changes could not be differentiated. Collectively, these results suggest that terrelysin expression occurs during initial stages of fungal growth between days 0-4. When hyphal aggregation is complete, a concurrent reduction in the production of terrelysin was observed. Terrelysin was detected in the CSN at each measured time interval with peak concentrations observed on day 6.

As *A. terreus* is thermotolerant, this experiment was repeated at 37°C to further compare morphological stages with terrelysin production. Again, cultures were grown for 12 days and ME and CSN was collected at 24 h intervals. Overall the growth of *A. terreus* was accelerated and the morphological stages of conidial germination and hyphal extension occurred earlier compared to RT treatments (**Fig. 5.6b**). Terrelysin expression was highest on day 1 (12 $\mu\text{g}/\text{mg}$ of total protein) and decreased 3-fold by day 2 (4 $\mu\text{g}/\text{mg}$ of total protein) (**Fig. 5.6a**). The concentration of terrelysin was further reduced 8-fold by day 3 (0.5 $\mu\text{g}/\text{mg}$ of total protein). In

CSN, comparatively higher proportion of terrelysin was detected during initial growth at 37°C compared to RT; however, there was a decline in the concentration of terrelysin in the CSN at 37°C.

In summary, we observed that terrelysin expression is concurrent with conidial germination, hyphal extension and aggregation. Terrelysin concentration declined on formation of the mycelial pellets. Lower levels of terrelysin were detected in the CSN.

Fig. 5.5a. Time point kinetics of expression of terrelysin at RT. Analysis performed using mAb 10G4.

Fig. 5.5b. Morphological changes and progression of A. terreus culture growth at RT. Black arrows point to germinating conidia and hyphal extension in early cultures. Bar sizes correspond to 10 µm for 0-24 hrs and 100 µm for 48-120 hrs.

Fig. 5.6a. Time point kinetics of expression of terrelysin at 37°C. Analysis performed using mAb 10G4.

Fig. 5.6b. Morphological changes and progression of A. terreus culture growth at 37°C. Black arrows point to germinating conidia and hyphal extension in early cultures. Bar sizes correspond to 10 μm for 12-24 hrs and 100 μm for 36-72 hrs.

5.3.6 Immunolocalization of terrelysin- The cellular localization of terrelysin was examined by immunohistochemical staining methods using the terrelysin-specific mAb 15B5. Terrelysin was widely distributed within the hyphae (**Fig. 5.7**), with staining often of greater intensity near the hyphal tips. This pattern was distinctly different than the positive control (mAb 13E11) that stained a leucine aminopeptidase, a secreted protease within the extracellular matrix of the hyphae. No staining was observed in the extracellular matrix with mAb 15B5.

Fig. 5.7. Immunolocalization of terrelysin in A. terreus hyphae. Immunolocalization of the antigens was determined AlexaFluor® 594-labeled goat anti-mouse IgG secondary antibodies (Red) and nuclear staining was identified through DAPI staining (Blue). mAb 13E11 served as a positive control and stains specifically for A. terreus leucine aminopeptidase while mAb 9B4 served as a negative control.

5.4 DISCUSSION

Aspergillus terreus is an emerging opportunistic pathogen reported to be an etiological agent of fatal disseminated infections in immunocompromised populations (21-23). Due to the ability of this species to grow optimally at internal body temperature and produce accessory conidia, this filamentous fungus is capable of initiating an infection and disseminating within the body. Resistance to antibiotic treatment such as amphotericin B allows for a longer infection time until effective treatment. A combination of these factors facilitates rapid dissemination of the fungus and can result in mortality. To date, very little is known about the overall pathogenesis and involvement of specific virulence factors of *A. terreus* during infection.

In *A. fumigatus* infections, asp-hemolysin was reported in tissues of experimental animals in an infection model and a role for hemolysin as potential virulence factors had been previously suggested (4, 7, 24, 25).

Previously, the gene for terrelysin was cloned and used to produce a recombinant protein as described in chapter 4. In this study, rTerrelysin was used as the antigen for the development of terrelysin-specific mAbs, which were then utilized to characterize terrelysin expression during *A. terreus* growth. Four of the 11 mAbs (3B2, 6D2, 16C7 and 2D3) did not react to native terrelysin in *A. terreus* ME. Two possibilities for this include; a) the mAbs recognized the N-terminal tag associated with rTerrelysin or b) their epitopes may be modified by post-translational processes under natural conditions as there is putative *N*-glycosylation (N-X-S/T) sites in the terrelysin sequence. One antibody (mAb 2D3) reacted with purified recombinant proteins (rEnolase from *Chaetomium globosum* and rHev b 5 an allergen from *Hevea brasiliensis*) expressed in the same pASK-IBA6 vector (data not shown). It was concluded that mAb 2D3 recognizes an epitope in the N-terminal tag expressed by this vector. Epitope mapping

showed that mAb 2D3 recognizes the *Strep*-tag II sequence ‘WSHPQFEK’ (data not shown). The other 3 mAbs did not show reactivity to the other recombinant proteins, thus it is possible that their epitopes are conformational or blocked by post-translationally modifications to the native protein.

In order to be useful as a diagnostic tool for the serological detection of terrelysin, it is essential that the mAb be species-specific. We tested 5 *A. terreus* strains, including the strain used for genome sequencing (NIH 2624/FGSC A1156). All mAbs tested detected terrelysin expressed in this strain. Interestingly, 7D8 showed reactivity towards 3/5 strains of *A. terreus* tested and while low affinity might explain this inconsistent detection, genetic diversity within the epitope for this mAb is also a possibility. Although additional strains from different clinical and environmental isolates were not tested, the data suggests consistent expression of terrelysin across strains. Recently, a new species, *A. alabamensis*, which is phenotypically homologous but genetically different to *A. terreus* was isolated from immunocompetent patient populations (26). It will be important to test the reactivity of these mAbs to *A. alabamensis* as well as other fungal species within Section *Terrei* such as *A. carneus* and *A. niveus*, which can also cause infections under rare circumstances (27, 28).

The terrelysin mAbs did not cross-react to ME from other clinically relevant *Aspergillus* species such as *A. fumigatus*, *A. flavus* and *A. niger*. Weak reactivity was observed for mAb 15C5 with *A. nidulans* and *A. parasiticus* in Western blots. This reactivity was restricted to high molecular weight proteins of ~ 50 and ~ 60 kDa and probably represents non-specific staining. Using cross-reactivity data, we identified 3 mAbs (13G10, 15B5 and 10G4) that did not cross-react with any of the tested species. These 3 highly specific mAbs are important candidates for immunodiagnostic detection of *A. terreus* in clinical samples. Based on epitope mapping studies,

we identified 2 different epitopes recognized by these antibodies. mAb 15B5 (IgG₁) recognized the epitope 'LYEGQFHS' while both mAbs 13G10 (IgG₁) and 10G4 (IgG_{2a}) recognized the epitope 'PSNEFE'. These epitope sequences were not identified in homologous hemolysins derived from other fungal species with known aegerolysin sequences (**Fig. 4.1**).

Expression of hemolysin in relation to growth has been studied previously for *Pleurotus ostreatus* and *Pseudomonas aeruginosa* (12, 13, 29). For *P. ostreatus*, a basidiomycete, the greatest expression of ostreolysin was observed during the initial stages of fungal fruiting. For the bacteria *P. aeruginosa*, it was observed that the expression of its hemolysin, PA0122 is highest in the stationary phase of growth (29). In contrast, limited information is available for the expression of aegerolysins in ascomycetes. Recent studies have reported high asp-hemolysin transcript levels in developing hyphae of *A. fumigatus* (30). In the present study, the expression of terrelysin was associated with morphological stages of *A. terreus* growth. The presence of terrelysin was highest during initial growth stages that encompassed conidial germination, hyphal extension and hyphal aggregation ('log phase'). During stationary growth phases, (lack of increase in mycelial pellet size) detection of terrelysin was significantly reduced. This was observed at earlier time points when cultures were grown at 37°C and the presence of terrelysin consistently correlated with the morphological changes. These findings are consistent with studies by Swedish mycologist Lars Rutqvist which showed that the hemolytic principle could be purified from *A. fumigatus* mycelia only during a limited period of incubation at room temperature and that this period was earlier and shorter at 37°C (31).

Using an inhibition ELISA, terrelysin was detected in CSN even though we did not identify a signal peptide on the terrelysin sequence using SignalP 3.0 (32). This is in contrast to an earlier report where terrelysin was not identified in the secretome of *A. terreus*. Whether the

detection of terrelysin in CSN a result of active secretion, hyphal degradation, fragmentation or due to involvement of unique secretory processes is currently not known and is the focus of future research. Recently, asp-hemolysin was identified as one of the most abundant protein in the *A. fumigatus* secretome even though the protein does not possess a signal peptide as noted earlier (33). Other studies have highlighted secretion of proteins with no predicted signal sequence and have proposed non-conventional secretory mechanisms (34-36). At day 6 in RT cultures and day 4 at 37°C, small increases in levels of terrelysin were observed in CSN. Hyphal fragmentation was observed at these time points and this could, in part, explain the presence of terrelysin in CSN. Our immunolocalization studies demonstrate a uniform distribution of terrelysin within *A. terreus* hyphal cytoplasm with greater reactivity localized at the hyphal tips. The hyphal tips are a region of extensive metabolic activity and consist of higher concentrations of proteins compared to regions trailing the hyphal tip. Based on these morphological and immunohistochemical observations, it appears that terrelysin is expressed during early stages of growth, probably during the emergence of hyphae from conidia and active hyphal growth.

In summary, 7 terrelysin mAbs were characterized and 3 were identified to be highly specific for *A. terreus*. These mAbs were used to show that terrelysin is expressed during conidial germination and early growth of *A. terreus* hyphae. Since terrelysin was detected in CSN, it is possible that it could be detected in the serum of infected patients; however, this aspect has not been confirmed and remains the focus of future research. Serologic detection of terrelysin would make terrelysin useful as a biomarker, however the early expression of terrelysin in *A. terreus* growth may limit its usefulness for detecting infection. The kinetics of terrelysin production *in vivo* is unknown and more studies are required.

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CHAPTER 6

GENERAL DISCUSSION

6.1 INTRODUCTION

Recent interest in fungal hemolysins was stimulated by an association of *Stachybotrys chartarum* with an outbreak of idiopathic pulmonary hemorrhage (IPH) in Cleveland (1). *Stachybotrys chartarum* in particular was isolated from the homes of infants with IPH. Fungal proteins capable of hemolysis (hemolysins) were postulated as the causative agent and stachylysin was proposed as a potential biomarker for exposure to *Stachybotrys chartarum* (2-4). Detection of hemolysins in sera and tissues of experimentally exposed animals and in some humans working in chronic exposure environments further fueled an interest in utilizing fungal hemolysins as biomarkers for fungal exposure (4, 5). Thus methods of detecting and quantifying stachylysin were necessary to better understand this association.

Stachylysin was first proposed as a potential biomarker for detection of exposure to *Stachybotrys chartarum* based on immunoassays using a poorly characterized polyclonal antiserum (4). In order to verify stachylysin as a biomarker, we developed mAbs to stachylysin preparation in collaboration with Dr. Steve Vesper at the Environment Protection Agency in Cincinnati, OH. The initial goal of this project was to develop *S. chartarum*-specific mAbs and the development of rapid detection assays for stachylysin from environmental and clinical samples. However, the hemolytic antigen preparation contained multiple proteins making a monoclonal antibody to stachylysin difficult to obtain. Since, the genome of *S. chartarum* has not been sequenced, we then shifted the focus to studying similar Aegerolysins from a well characterized fungal species where the genome has been sequenced. We chose to study the hemolysin from *A. terreus*, an emerging pathogen, especially for immunocompromised patients (6, 7). As the first step of this strategy, we developed mAbs to a similar hemolytic preparation purified from *A. terreus* culture medium. We successfully obtained IgG₁ mAbs that were highly

specific for *A. terreus*, however, on initial characterization of the mAbs; we again identified reactivity to multiple proteins with little evidence of reactivity to a hemolysin. On further characterization, we elucidated that the mAbs recognized proteolytic enzymes secreted by *A. terreus*.

Since, the genome of *A. terreus* had been sequenced, our next approach involved identifying terrelysin, using homology to the sequence information available for asp-hemolysin, the Aegerolysin of *A. fumigatus*. We subsequently produced a recombinant terrelysin using the pASK-IBA6 vector in an *E. coli* host. The recombinant protein then served as the antigen and murine mAbs of IgG₁, IgG_{2a} and IgG_{2b} isotypes were generated against rTerrelysin. These mAbs are highly specific to *A. terreus* and have the potential for developing diagnostic assays for detection of *A. terreus* in environmental and clinical samples.

6.2 STACHYLYSIN

As noted above, a goal of this project was to develop mAbs to stachylysin, a molecule that had been previously characterized in multiple studies using a polyclonal antiserum (2-4, 8). The data published using this polyclonal reported there were very high levels of stachylysin in the lungs and sera of exposed animals (10.9 ng/ml and human (371 ng/ml) suggesting the assay was subject to interferences or inaccuracy. It was reasoned that a mAb would produce a more accurate and reproducible assay and also improve the specificity of the assay. Using the hemolytic preparation developed by Dr. Vesper's group. (2), we developed murine mAbs to this partially purified hemolytic fraction. Although the extract was purified from *S. chartarum*, the specific strain was later identified and characterized as a related species called *S. chlorohalonata* (9). The obtained mAbs showed consistent reactivity to hyphal extracts from multiple strains of

both *S. chartarum* and *S. chlorohalonata* and the reactivity were notably weaker to conidial extracts. This also confirmed an earlier report that stachylysin was expressed in higher concentrations in the hyphae (4). We also observed significant reactivity of the mAbs to hyphae in immunohistochemical localization studies using the halogen immunoassay. Interestingly, most reactivity to hyphal structures was concentrated at septal junctions and hyphal branch points. Repeated attempts to confirm that stachylysin was the antigen recognized by the mAbs were not successful undoubtedly due to the fact that these hemolytic preparations used as an antigen source, do not appear to contain the Aegerolysins.

One of the goals was to develop IgG mAbs for better utility in immunoassays; however, we only identified IgM mAbs to the *S. chlorohalonata* hemolytic preparation. The screening procedure used antibodies to IgG and IgM isotypes to ensure identification of the greatest number of clones. This enabled us to detect IgM mAbs, but the nature of the immune response appears to have been primarily IgM after 6 immunizations with the hemolytic preparation. This is likely due to the presence of glycoproteins in the hemolytic preparation. Previously in our lab, we have reported difficulty in generation of detectable IgG titers in animals immunized with conidial, hyphal or secreted antigens, with similar issues (10).

The mAbs were developed to the hemolytic preparation with the primary purpose of identifying *S. chartarum* stachylysin. While we could not confirm the presence of stachylysin, the mAbs are species specific and react to hyphal extracts from both atranone as well as satratoxin producing chemotypes of *S. chartarum*. Although some cross-reactivity was observed with other closely related fungal species such as *Memnoniella* species, overall the IgM mAbs showed good specificity to *Stachybotrys* species. Reactivity to conidia was minimal but detectable using assays developed here. Since no mAbs are available for detection of

Stachybotrys hyphal antigens and these antigens are expressed in high concentrations in hyphae, these mAbs may be useful for the specific identification of *Stachybotrys* hyphae using ELISA or microscopic techniques.

6.3 TERRELYSIN

The genome of *Stachybotrys* species has not been sequenced which hampered our efforts to identify stachylysin and hence we focused on characterization of a hemolysin in a fungus whose genome had been sequenced. The genome of *A. terreus* was recently sequenced (11) and we identified the sequence for the hemolysin based on the homology to asp-hemolysin of *A. fumigatus*. In our first approach, Dr. Vesper's group at EPA, Cincinnati, OH, purified terrelysin using the previously used biochemical methods (2, 12). With the knowledge that this preparation was only partially purified, the preparation was identified as a cytolytic *A. terreus* preparation (cAtp) in our studies.

Using this preparation, we successfully developed highly specific IgG₁ mAbs to cAtp in mice. Initial analysis of the anti-cAtp mAbs using Western blot analysis showed multiple bands suggesting that the mAbs might react to different proteins that co-purified with the hemolysin. No mAbs exhibited reactivity to an 18 kDa protein, the expected molecular weight of terrelysin. However, we did observe reactivity to multiple high molecular weight bands, suggesting that the epitopes might be shared between different proteins or the high molecular weight proteins might be aggregates of terrelysin.

Using immunoprecipitation and subsequent proteomic analysis of the immunoreactive bands, we identified leucine aminopeptidase (LAP) and dipeptidyl peptidase V (DPP 5) as two antigens of cAtp. We did not identify any peptides with the terrelysin sequence in the cAtp

preparation. As previously noted for stachylysin, higher concentrations of LAP and DPP 5 were detected in the hyphae than the conidia. Also, we detected high levels of these proteins in *A. terreus* CSN, suggesting that these proteins might be secreted. Indeed, we identified putative signal peptides in the N-terminal domain of the sequences of these proteins. Also, on investigation of the kinetics of expression of these proteins, we observed a lag period between expression of the antigens within the hyphae and in the growth medium, suggesting involvement of active secretion processes.

In immunolocalization studies, we observed that LAP staining was predominantly outside the growing hyphae in extracellular structures. These extracellular structures also stained with DAPI for DNA, and genetic material has been reported as part of the extracellular matrix of the fungi (13, 14). Presumably, fungi use these structures to anchor to the substrate on which they colonize. Little is known about the clinical significance of fungal ECM and LAP. Interestingly, these proteins were detected in the fungal growth medium throughout the course of the expression studies. In previously reported studies, DPP 5 has been reported as an important serodiagnostic marker for *A. fumigatus* (15, 16) and recently identified as the 3rd most abundant protein among the secreted proteins of *A. fumigatus* (17). These results highlight the potential of mAbs developed in this study for serodiagnostic assays for detection of *A. terreus*.

6.4 RECOMBINANT TERRELYSIN

Our goal of identifying terrelysin using previously described biochemical methods was not achieved and required a different strategy. We utilized the *A. terreus* genome sequence information available in the database to identify a homologue to asp-hemolysin of *A. fumigatus* and used molecular techniques for cloning and expression of terrelysin. In our first attempt to

produce recombinant terrelysin, we expressed the clone in *E. coli*, and purified the protein using the His₆-tag at the N-terminus of the expressed protein. We did not observe any hemolytic activity with this construct and also encountered problems of impurities after purification on Ni-column. One of the reasons for the lack of hemolytic activity could be that the protein expressed using this system was localized within *E. coli* cytoplasm where reducing conditions may not permit proper folding of the protein. Folding of the hemolytic proteins and the associated conformational changes play a vital role in the mechanism of hemolysis (18).

To address this issue, we expressed the protein in the pASK-IBA6 vector, which utilizes a signal peptide at the N-terminus of the vector sequence that enables efficient expression of the protein in the periplasmic space. Here, the conditions are more favorable for the proper folding of the expressed recombinant protein. Also, the high affinity *Strep*-tag II allows for purification of the expressed recombinant protein under physiological conditions. Using recombinant protein produced in this system, we confirmed the secondary structure of terrelysin using CD techniques and also assessed the conformational changes resulting from changes in pH and temperature. The observations were similar to those previously reported for native ostreolysin, an Aegerolysin purified from the mushroom *P. ostreatus* (19).

Murine mAbs were raised to the rTerrelysin and characterized for reactivity to native terrelysin and also for cross-reactivity towards other fungal species. Except for one, all of the mAbs to recombinant terrelysin also recognized the native terrelysin in extracts from different strains of *A. terreus* including the pathogenic FGSC A1156 strain. Three of the mAbs reacted to hyphal extracts from other fungal species, while 3 mAbs did not show any cross-reactivity. These 3 *A. terreus*-specific mAbs, 13G10, 15B5, and 10G4 were used for further characterization of terrelysin. Epitope mapping results showed that mAb 15B5 recognizes a

different epitope than mAbs 13G10 and 10G4. The epitopes recognized by these mAbs were also absent in fungal species in which homologous Aegerolysin family hemolysins have been identified. Overall, these mAbs have the potential for use in developing double monoclonal antibody-based diagnostic assays for highly specific and sensitive assays for the early detection of terrelysin in clinical and environmental samples.

We also studied the kinetics of terrelysin expression and correlated it with the morphological changes during growth of *A. terreus*. We observed that terrelysin expression was high during the initial 3 days of growth but its concentration diminished with the increasing fungal biomass, and was essentially non-detectable in mycelium from the 7-day old cultures that were used by Dr. Vesper to make isolate the hemolytic antigen preparations. Also, we detected some terrelysin in the *A. terreus* growth CSN. Although terrelysin does not possess a putative signal peptide; it has been reported that fungi possess non-classical strategies for protein secretion (20-22). Interestingly, terrelysin was not identified in the *A. terreus* secretome (23), however, a recent study identified that although asp-hemolysin does not have a signal peptide, it was the 4th most abundant protein in the *A. fumigatus* secretome (17). These discrepancies suggest that although hemolysins may be secreted proteins they are differentially expressed and regulated in filamentous fungal species.

Our studies demonstrate the difficulties of using a biochemical approach for isolation and characterization of hemolytic proteins from fungi. Previously asp-hemolysin and ostreolysin were purified from *A. fumigatus* and *P. ostreatus* respectively, using similar biochemical methods (hemolytic fractions of separated proteins) (12, 24). This resulted in the purification of homologous Aegerolysins with similar biochemical and pathogenic properties. However, using similar methods for the purification of stachylysin and terrelysin did not result in purification of

Aegerolysin proteins. For *A. terreus* at least, we were able to determine that our mAbs detected the proteases, LAP and DPP 5. It is now apparent that the hemolytic activity that was followed during the purification of the “hemolysin” was likely contributed by these proteases. Proteases have been previously shown to have hemolytic activity (25, 26). To further corroborate this point, in a recently published report on asp-hemolysin, hemolysis and cytotoxicity was not altered using deletion mutants for asp-hemolysin (17). This work together with ours, demonstrates that Aegerolysin family proteins are not the major hemolytic factors in fungal extracts.

6.5 OTHER CONCLUSIONS

In our studies with *Stachybotrys*, Western blot staining with our mAbs identified a 30 kDa protein as a putative Aegerolysin using a monoclonal antibody developed after immunization with the hemolytic protein preparation. Previously, stachylysin was reported as a 12 kDa protein that migrates in SDS-PAGE as a 30 kDa protein (2). It was suggested that stachylysin may be a highly acidic protein and such proteins have been previously reported to demonstrate anomalous behavior in SDS-PAGE (27). However, based on our findings with *A. terreus*, we should reinterpret that data as it likely represents a protease from *Stachybotrys*.

In this study for the first time we have cloned and expressed terrelysin and to date it is the only Aegerolysin from *A. terreus* that has been identified and cloned. Although we did observe hemolytic activity with the purified recombinant protein, because of the possibility of contamination of the purified rTerrelysin preparation with surfactants, we could not be sure the hemolytic activity was due to rTerrelysin. Although we did not examine the role of terrelysin in pathogenesis, recent studies using asp-hemolysin mutants show that asp-hemolysin may not be

important for virulence and may be dispensable for pathogenesis (17). This challenges earlier observations regarding the role of asp-hemolysin in pathogenesis of *A. fumigatus* (5, 12, 28-30). The concentration of asp-hemolysin used in these studies may not necessarily be at levels achieved during an *in vivo* infection.

Reagents developed in these studies will be beneficial in developing standardized and rapid diagnostic tests for diagnosis of environmental and clinical exposure to *Stachybotrys* species and *A. terreus*. For *A. terreus*, mAbs developed to hyphal exoantigens such as LAP and DPP 5 used in combination with mAbs developed to terrelysin may be helpful in developing surveillance assays for *A. terreus*. Since terrelysin is expressed early during growth, its detection may suggest early infection, while detection of LAP and DPP 5 and reduced levels of terrelysin may suggest late infection. On treatment, levels of LAP and DPP 5 may be monitored to assess efficacy of treatment and clearance of *A. terreus* infection.

However, we have limited information regarding the expression and the stability of these proteins *in vivo* during infection. Studies utilizing animal models for invasive aspergillosis in immunocompromised mice will help in determining the presence of these proteins in various clinical samples. Some studies have reported presence of these proteins in urine samples collected from patients (31). Development of dip-stick based assay kits may also introduce portability.

Collectively these mAbs have tremendous potential in development of diagnostic assays for *Stachybotrys* species and *A. terreus* exposures. The improved methodologies described in these studies will help in better and consistent characterization of fungal growth and protein expression. Tools and methodologies developed in these studies will also be useful in developing highly specific double monoclonal antibody based assays.

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APPENDIX

A1. Amino acid composition similarity search. Search was performed using keyword ‘hemolysis’ and input of % amino acid composition of stachylysin. Swiss-Prot and TrEMBL constellation 0 was chosen for analysis.

Protein	Species	Accession #
Hydralysin-2	<i>Burkholderia glumae</i>	HLYS2_CHLVR
Hydralysin-1	<i>Burkholderia glumae</i>	HLYS1_CHLVR
Aerolysin	<i>Aeromonas sobria</i>	AERA_AERSO
Aerolysin	<i>Aeromonas salmonicida</i>	AERA_AERSA
Aerolysin	<i>Aeromonas trota</i>	AERA_AERTR
Aerolysin-4	<i>Aeromonas hydrophila</i>	AER4_AERHY
Aerolysin-5	<i>Aeromonas hydrophila</i>	AER5_AERHY
Aerolysin-3	<i>Aeromonas hydrophila</i>	AER3_AERHY
Alveolysin	<i>Bacillus alvei</i>	TACY_PAEAL
Hemolysin	<i>Vibrio cholerae</i>	HLYA_VIBCH
Thermostable direct hemolysin 1	<i>Vibrio parahaemolyticus</i>	HLY1_VIBPA
Echotoxin-2	<i>Cymatium echo</i>	ACTP2_CYMEC
Verrucotoxin subunit beta	<i>Synanceia verrucosa</i>	VTXB_SYNVE
Pneumolysin	<i>Streptococcus pneumoniae</i>	TACY_STRPN
Pneumolysin	<i>Streptococcus pneumoniae</i>	TACY_STRR6
Pneumolysin	<i>Streptococcus pneumoniae</i>	TACY_STRP2
Stonustoxin subunit alpha	<i>Synanceia horrida</i>	STXA_SYNHO
Toxin A	<i>Carybdea alata</i>	CTXA_CARAL
Thermostable direct hemolysin 2	<i>Vibrio parahaemolyticus</i>	HLY2_VIBPA
Cytolysin	<i>Vibrio vulnificus</i>	VVHA_VIBVU

A2. Sub divisions of Aspergillus species

Sub genus	Section (e.g. of representative species)
1. Aspergillus	a. Aspergillus- <i>Aspergillus glaucus</i> b. Restricti- <i>Aspergillus penicilloides</i>
2. Fumigati	a. Fumigati- <i>Aspergillus fumigatus</i> b. Clavati- <i>Aspergillus clavatus</i>
3. Circumdati	a. Circumdati- <i>Aspergillus ochraceus</i> b. Nigri- <i>Aspergillus niger</i> c. Flavi- <i>Aspergillus flavus</i> d. Cremei- <i>Aspergillus wentii</i>
4. Terrei	a. Terrei- <i>Aspergillus terreus</i> b. Flavipedes- <i>Aspergillus flavipes</i>
5. Nidulantes	a. Nidulantes- <i>Aspergillus nidulans</i> b. Usti- <i>Aspergillus ustus</i> c. Sparsi- <i>Aspergillus sparsus</i> d. Raperi- <i>Aspergillus raperi</i> e. Silvati- <i>Aspergillus silvaticus</i> f. Ochraceorosei- <i>Aspergillus ochraceoroseus</i> g. Bispori- <i>Aspergillus bisporus</i>
6. Ornati	a. Ornati- <i>Sclerocleista ornata</i>
7. Warcupi	a. Warcupi- <i>Warcupiella spinulosa</i> b. Zonati- <i>Aspergillus zonatus</i>
8. Candidi	a. Candidi- <i>Aspergillus candidus</i>

A3. List of species in which Aegerolysins have been identified

<i>Aspergillus terreus</i>	<i>Histoplasma capsulatum</i>
<i>Aspergillus fumigatus</i>	<i>Neurospora crassa</i>
<i>Aspergillus oryzae</i>	<i>Pleurotus ostreatus</i>
<i>Aspergillus clavatus</i>	<i>Agrocybe aegerita</i>
<i>Aspergillus niger</i>	<i>Spirosoma linguale</i>
<i>Aspergillus flavus</i>	<i>Pseudomonas aeruginosa</i>
<i>Aspergillus nidulans</i>	<i>Burkholderia glumae</i>
<i>Penicillium chrysogenum</i>	<i>Clostridium bifermentans</i>
<i>Paracoccidioides brasiliensis</i>	<i>Bacillus thuringiensis</i>
<i>Coccidioides immitis</i>	<i>Trichoplusia ni ascovirus 2c (TnAV-2c)</i>

A4. Cytotoxicity of Aegerolysin proteins on different cell lines. Modified from (1)

Protein	Cell Line (Source)	ED₅₀ (µg/ml)	References
Asp-hemolysin	Leukocytes (Human)	500	(2)
	Alveolar macrophages (Guinea pig)	60	(2)
	Peritoneal macrophages (Mouse)	25	(3)
	Umbilical vein endothelial cells (Human)	100	(4)
Ostreolysin	Fibrosarcoma HT 1080 (Human)	10	(5)
	Mammalian tumor MCF 7 (Human)	10	(5)
	Ovary cells (Chinese hamster)	1	(6)
	Articular chondrocytes (Human)	1	(7)
	Lung fibroblasts V-79-379A (Chinese hamster)	1.3	(8)
	Umbilical vein endothelial cells (Human)	2.2	(8)

A5. Protein A and G-based antibody immunoprecipitation

Species	Ig subclass	Protein A	Protein G
Human	IgG ₁ , IgG ₂ , IgG ₄	+++	+++
	IgG ₃	+	+++
	IgD	-	-
	IgD	+	-
	Fab	+	+
	ScF _v	+	-
Mouse	IgG ₁	+	++
	IgG _{2a} , IgG _{2b} , IgG ₃	+++	+++
	IgM	-	-
Rat	IgG ₁	+	++
	IgG _{2a}	-	+++
	IgG _{2b}	-	+
	IgG _{2c}	+++	+++
Goat/Sheep/Bovine	IgG ₁	+	+++
	IgG ₂	+++	+++
Horse	IgG(ab)	+	-
	IgG(c)	+	-
	IgG(T)	-	+++
Rabbit	Total IgG	+++	+++
Dog/Cat/Pig/Guinea Pig	Total IgG	+++	+
Chicken	Total IgG	-	-

+++ : strong binding, ++ : moderate binding, + : weak binding, - : no binding

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1. **Nayak, A.P.**, Green, B.J. and Beezhold, D.H. 2012. **Oral Presentation.** Van Liere Convocation and Research Day. West Virginia University, Morgantown, WV.
2. Buskirk, A.D., Templeton, S.P., Hettick, J.M., **Nayak, A.P.**, Green, B.J., Siegel, P.D. and Beezhold, D.H. 2012. Van Liere Convocation and Research Day. West Virginia University, Morgantown, WV.
3. **Nayak, A.P.**, Green, B.J. and Beezhold, D.H. 2012. Annual meeting of American Academy of Allergy, Asthma and Immunology (AAAAI), March 2-6, Orlando, FL.
4. **Nayak, A.P.**, Green, B.J., Hettick, J.M. and Beezhold, D.H. 2010. 110th General meeting of American Society for Microbiology (ASM), May 23-27, San Diego, CA.
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1. **Nayak, A. P.**, Blachere, F. M., Hettick, J. M., Lukowski, S., Schmechel D and Beezhold, D. H. 2011. Characterization of recombinant terrelysin, a hemolysin of *Aspergillus terreus*. *Mycopathologia* **171(1): 23-34**. PMID: 20632211.
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National Institute for Occupational Safety and Health (CDC/NIOSH)
Aerobiologia
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