Extraction and Partial Characterization of a Lipophilic Fungicidal Molecule Associated with Serum Albumins

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By

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Abstract

Vulvovaginal candidiasis (VVC) is a mucosal infection caused by *Candida* species and represents one of the most common clinical problems in women of reproductive age (68,71). Annually in the United States there are approximately 13 million cases of VVC, resulting in 10 million gynecologic office visits per year (38). It is estimated that 75% of women will experience at least one episode in their lifetime, with a projected 50% of all women experiencing multiple episodes (23). *Candida albicans* is a dimorphic commensal organism of the urogenital and gastrointestinal tracts and has been identified as the main pathogenic agent in VVC, accounting for approximately 85-90% of patients with positive cultures (52).

Despite extensive research, the invasive mechanism of vaginal yeast infections is not well understood. Traditionally it has been assumed that changes in the host vaginal environment promote the dimorphic transition from blastospore to hyphae, resulting in a shift from asymptomatic colonization to symptomatic vaginitis (28). In contrast to the normal, systemic immune response, which confers an aseptic environment for tissue and organs, immune responses at the mucosal level are designed to prevent tissue invasion and local disease while maintaining an indigenous flora that could be both beneficial and pathogenic (28).

Since fungi are eukaryotic, the vital cellular mechanisms that are usually targeted by modern pharmacologic agents, such as DNA replication and protein translation, are either conserved or have a strong homology to their human orthologs. Obtaining a better understanding of natural fungal suppression mechanisms and molecules at the mucosal

level may pave the way for the development of more efficacious drugs or preventative regiments.

The mechanism by which the human immune system is able to resist fungal invasion at the vaginal mucosa is unknown. Our research was aimed at finding any host factors that might play a role in the suppression of or prevention of a fungal infection at the vaginal mucosa. In order to screen candidate molecules that might be important in this type of vaginal defense, we chose a pathogenic *C. albicans* strain, SC5314, to test fungal cell viability upon introduction of the candidate molecules.

We have identified a host factor that exhibits strong fungicidal activity when organically extracted from both human and bovine serum albumins. Characterization of this factor through organic extractions and acetone separations reveal that this molecule is a non-polar lipid. Serum samples that have been thoroughly stripped of fatty acids and other lipophilic molecules show no apparent fungicidal activity in cell viability assays. Since the factor is extractable from both human and bovine serum albumins, it may be conserved among mammals. Identification and characterization of this molecule may play a pivotal role in understanding host-*Candida* interactions at the mucosal membrane interface. Due to its human origin, the use of this factor as an antifungal would be extremely advantageous in regards to FDA (Food and Drug Administration) guidelines and ADMET (Adsorption, Distribution, Metabolism, Excretion, Toxicology) properties.

Acknowledgements

I would like to start by thanking Dr. Mitchell Sanders, my professional and academic mentor, and ECI Biotech for financing why graduate level education. Without their contributions none of this would have been possible. I would also like to thank my committee members, especially Reeta Prusty, for their continual support and guidance during the course of my research. I am also indebted to Professor Pins and his laboratory, especially Katie Bush, for the use of their microscopy equipment as well as their assistance in the operation of the instruments. It would be unthinkable that I would forget to thank both Eileen Dagostino and Carol Butler for their friendship and their professional excellence in the acquisition of the supplies needed for the execution of this project. Last but not least, I would like to thank my lab mates for turning the hard aggravating times into fun times, and my family, friends, coworkers, and my girlfriend, Tammy Lambert, for their love and support which has helped me overcome the hard times and greatly enhanced the good times during the last couple of years.

This thesis is dedicated to my father and mother who have always stressed the importance of education and hard work since I was a little boy. Without the qualities and morals that they have instilled in me, none of what I have done in my life would have been possible.

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1 Introduction

1.1 Objectives

Since *Candida albicans* is routinely found in the vaginal flora as a benign commensal, I hypothesize that there is a host factor present in the cervical vaginal fluid that inhibits *C. albicans* from initiating an infection. The primary objective of this masters' thesis is to investigate the possibility that there's a host factor responsible for preventing *C. albicans* from starting an infection. Since serum albumins are utilized as molecular transports and are present in high quantities in the vaginal fluid, I hypothesize that a previously unidentified inhibitory molecule may be associated with them. In order to separate these molecules from the albumins, organic extractions and separations were performed. From these extracts I isolated a lipophilic component that exhibited strong fungicidal activity against a pathogenic *Candida albicans* strain, SC5314. All extracts that exhibited activity were further characterized through LD_{50} (Lethal Dose 50%) experiments and lipid separations.

The eventual identification of the active biomolecule(s) and the elucidation of its fungicidal mechanism may help reveal how the human immune system prevents *C. albicans* from establishing an infection of the urogenital tract. The identified molecule could also lead to the development of a new class of antifungals or a novel prophylactic therapy.

1.2 Background

1.2.1 Pathogenesis and Virulence of *Candida albicans*

Candida albicans is the most frequently isolated invasive fungal pathogen in humans, with the majority of infections being localized to the urogenital or orapharangeal tracts of the patient (Fidel, 1996). In addition to localized infections, *C. albicans* is also able to establish a systemic infection in its host. Once the organism crosses into the bloodstream of the patient it is readily able to invade and flourish in major organs such as the liver, kidney, heart, and brain. Infection of these major organs can very easily cause a life-threatening infection. Systemic fungal infections are most common in patients with compromised immune systems, such as AIDS patients or those that are currently on immuno-suppression therapies.

C. albicans is a dimorphic fungus that primarily exists and propagates via its blastospore phenotype (also called blastoconidia). Blastospores are characterized by their oval-shapes, mono-nucleated cells and propagation through cellular budding (Braun, 1997). Upon perception of environmental signals, *C. albicans* is able to transform into one of two filamentous forms: psuedohyphae and hyphae (refer to Figure 1). Elongated, ellipsoidal cells that are attached to one another are referred to as psuedohyphae, while cells that are considered to be true hyphae are characterized by a cylindrical cellular morphology and are separated by perpendicular septal walls. These hyphal forms are comprised of conjoined cells that are divided by septal walls and are not syncytial in composition. Both the psuedohyphal and hyphal morphologies are routinely referred to as filamentous.

Throughout the last couple of decades, the filamentous form was commonly associated with pathogenicity, but recent genetic and animal studies have shown that both forms are needed for *C. albicans* virulence (Braun, 1997; Gow; Hogan). The hypothesis that the free transition between the organism's two phenotypes (phenotypic switching) is necessary for *C. albicans'* virulence is partly based upon the following research findings:

1. Histological evidence from tissues infected with *C. albicans*, reveals the presence of filaments invading deep into the infected tissues (Gow; Hogan).
2. *C. albicans* mutants that were filamentation-deficient exhibited a reduced capacity to infect mouse models. The infected mice also showed a reduction in candidiasis-related mortality (Hogan; Lo; Mitchell; Saville).
3. *C. albicans* mutants that are deficient in Tup1, a repressor of filamentation, were locked in the filamentous form but exhibited no virulence in mouse models (Braun 1997; Braun 2000).

C. albicans has evolved into a commensal organism as well as an opportunistic pathogen, implying that it is routinely present in what is considered to be a healthy mucosal microflora while retaining the ability to establish an infection in its host if circumstances allow it to do so. The ability of a pathogenic microorganism to detect and respond to environmental cues that signal an invasion opportunity is a potent survival mechanism. *C. albicans* is able to control its filamentation via its ability to respond to a variety of environmental signals, some of which are listed below (Refer to table 1).

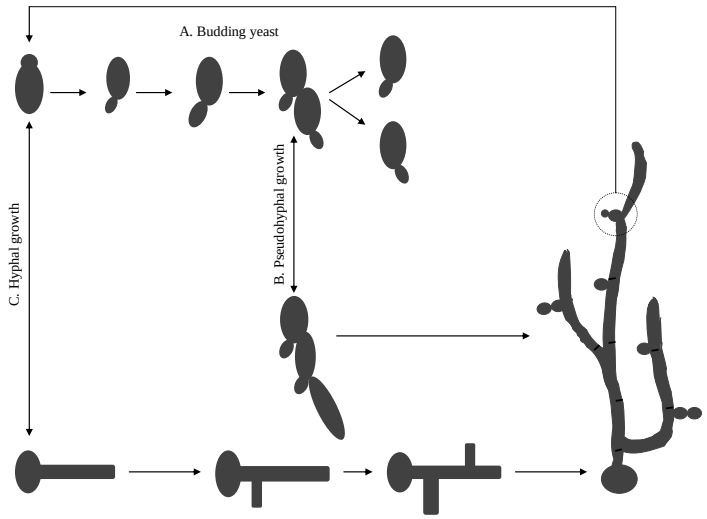

Figure 1: *Candida albicans* dimorphism. (A) Blastospores are unicellular forms of the fungus that divide by budding. (B) In the presence of some environmental factors, cylindrical growth of a germ tube will begin. (C) Germ tubes extend and septa are laid down behind the extending tip to form a hypha. The main factors that favor filamentation (yeast → hypha) are: temperature 37°C, pH 7.0, an inoculum of 1 X 10^6 blastospores/ml, and the presence of different compounds, such as N-acetylglucosamine, proline or serum. (Adapted from Molero)

Inhibition	Stimulation
Low growth temperatures	High growth temperatures (37°C/98.6°F)
Acidic pH (4-6)	High CO_2/O_2 ratio
Enriched growth media	Neutral pH
Farnesol (quorum sensing molecule)	Nitrogen starvation
Indole acetic acid (plant hormone)	Tyrosol (quorum sensing molecule)

Table 1: Environmental and Chemical Signals for the Stimulation or Inhibition of Filamentation. A variety of changes in environmental conditions as well the presence or absence of specific chemicals, are able to induce a hyphal change in *C. albicans*.

(Am. Soc. Microbiol. News; Braun 1997; Enjalbert; Ernst; Gow; Odds 1998; Shephard)

A couple of the environmental conditions that promote hypha formation are consistent with the environment presented during an infection opportunity. The switch to its filamentous form allows the pathogen to increase its ability to adhere to and invade the

vaginal epithelial cells as well as upregulate its own production of virulence factors (Naglik). Virulence factors expressed by *Candida albicans* vary depending on the type of infection (i.e, mucosal or systemic), the site and stage of infection, and the nature of the host response (Naglik). It has become apparent that an array of virulence factors are involved in the infection process, but no single factor accounts for its pathogenicity and not all expressed virulence factors may be necessary for a particular stage of infection (Naglik; Cutler; Odds 1994).

1.2.2 Host Factors That Affect *Candida albicans'* Filamentation Response

The most prominent induction signal observed between the host and *C. albicans* is the serum-induced filamentation response. When *C. albicans* is introduced into serum or a medium containing serum, typically at 5-10% concentrations, a very strong hyphal response is observed (Hornby). The serum component responsible for the hyphal response is currently unknown.

Another fascinating interaction between the fungal pathogen and its host involves an ingenious macrophage evasion mechanism. Upon being engulfed by macrophages, phagocytized cells undergo a dramatic reprogramming in their transcription (Lorenz). The engulfed cells quickly turn on their oxidative and starvation stress responses that ultimately leads to the production of osmoprotectants and hypha formation. The hyphae become too large for the macrophages to contain and subsequent macrophage lysis occurs (Figure2).

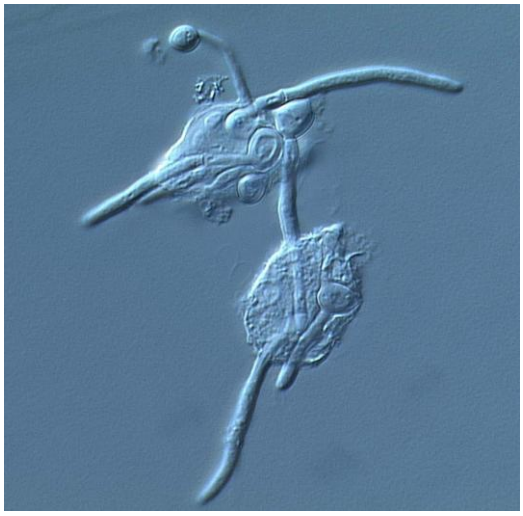

Figure 2: Once *C. albicans* is engulfed by murine macrophages, intracellular cascades are activated, causing phenotypic switching and the eventual lysis of the host cells.

(http://biology.uark.edu/dmcnabb.html)

Estrogen has also been linked to the induction and elongation of hyphae. Experiments performed by Cheng *et al.* show a dose-dependent, hyphal elongation response by *C. albicans* to 17-β-estradiol (Cheng). Interestingly, vaginal epithelial cells have been shown to possess fungistatic properties. Upon contact between the vaginal epithelial cells and *Candida albicans* via an acid-labile protein bound to its extra-cellular membrane (Barrouse, 2005). In order for the fungistatic activity to occur, the epithelial cell does not even need to be viable (Barrouse 2005). This activity is evident *in vitro* but does not contribute to any appreciable level of protection against *Candida* colonization (Barrouse, 2005).

With the exception of the slight fungistatic activity possessed by vaginal epithelial cells, no physiologic inhibitor of *Candida albicans* mucosal infections has been reported from mammalian sources. Our current understanding of the vaginal immune defense indicates that there are still large gaps in our knowledge concerning the protective mechanisms of the vaginal environment. The discovery of a novel host molecule with

fungicidal activity would help elucidate the host-pathogen interactions responsible for vaginal defense. This thesis investigates one such factor.

1.2.3 Establishment and Dissemination of Vaginal Candidiasis

Colonization of the epithelial cells is the first step in the establishment of vulvovaginal candidiasis. Adherence to the host epithelial cells is accomplished either through the use of adhesins or the involvement of proteases, which do not necessarily have to be enzymatically active in order to be involved in a pathogen-host docking event (Naglik). Although hyphal forms are commonly associated with disease and blastospores with benign colonization, blastospores have also been isolated from disease sites (Sobel 1988).

In order to cross tissue planes and establish an invasive infection, *Candida albicans* must invade cells that are not normally phagocytic, such as epithelial and endothelial cells. A common histopathologic finding in all types of candidiasis is the presence of fungal cells within these tissues (Filler). The mechanism by which this invasion occurs has been the focus of intense investigation for many years (Filler; Pizarro-Cerda). Two methods of cellular invasion have been established *in vitro*; invasion through hydrolytic degradation of the targeted cell wall and the induction of normally nonphagocytic cells to engulf the pathogen (Park; Stingaro; Ray). Recent work by Filler *et al* showed that the yeast adhesin Als3 binds to either a N-cadherin or an E-cadherin of the host epithelial cell resulting in the fungal cell being phagocytized (Stingaro; Ray; Hobbs). Invasion into the primary layer of cells via enzymatic digestion is more characteristic of filamentous *Candida* due to their upregulation of SAPs (secreted aspartyl proteases),

while blastospore entry appears to primarily occur through the induction of phagocytosis (Stingaro; Ray; Hobbs). *Candida albicans* contains a myriad of proteases, of which only the aspartyl protease group is secreted (Naglik). These SAPs exhibit a broad range of pH optima and substrate specificities thereby making them potent virulence factors (Koelsch; Naglik). Once the pathogen has entered the primary infection cells, it is the hyphal form that is predominantly responsible for tissue penetration and deeper tissue invasion. Via its filamentous forms, *C. albicans* is able to progress into the host tissue through further cell lysis and secondary blastospore budding (refer to figure 1).

If the pathogen is able to cross through the vaginal tissue, entry into the bloodstream can occur and a disseminated infection can be established. Once in the host bloodstream, *C. albicans* is readily able to migrate to other tissues and organs and establish a widespread systemic infection. The stages of infection and the resulting establishment of systemic infection are summarized below (refer to figure 4). It should be noted that a systemic *Candida albicans* infection poses a significant health risk with a mortality rate of approximately 50% (Hugonnet).

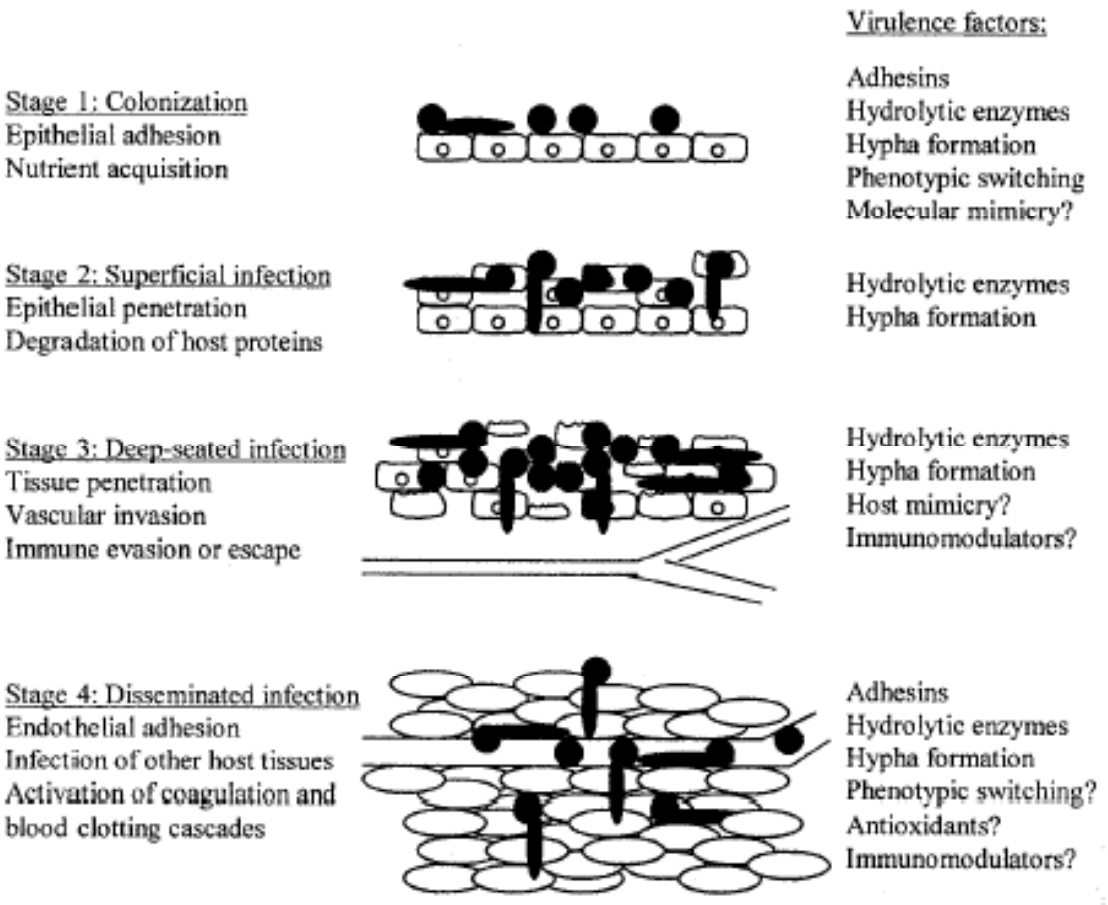

Figure 3: A schematic diagram illustrating the progression of infection as well as the virulence factors that are known or are thought to be associated with each stage of infection. *Candida albicans* commonly colonizes the epithelial surface (stage1) and causes a superficial infection (stage 2). Under certain conditions when the host is compromised, the fungus is able to establish a deep-seated infection (stage 3) through deeper penetration of the host tissues. Occasionally the fungus is able to establish a disseminated infection of the host (stage 4), which allows the pathogen to spread to other systemic tissues and organs via the host's circulatory system.

(Naglik)

1.2.4 Vulvovaginal Candidiasis: Epidemiology

Vulvovaginal Candidiasis (VVC) is a mucosal infection of the urogenital tract of women and is primarily caused by *Candida albicans* (Sobel, 1998). VVC is characterized by itching, burning, soreness, abnormal vaginal discharge, dysparunia, and phenotypical signs such as vaginal and vulvar erythema and edema (Sobel, 1990). Some exogenous factors

that have been linked to idiopathic VVC include changes or imbalances in reproductive hormones, as a result of oral contraception, pregnancy, or hormone replacement therapy (HRT), as well as antibiotic usage, and diabetes mellitus (Sobel, 1998). Most episodes of VVC respond well to treatment with currently available antifungals.

While acute VVC is largely treatable with current chemotherapeutics, there remains a subset of the population (5%-10% of women diagnosed with VVC) that exhibit recurrent VVC (RVVC). RVVC is defined as having three or more episodes per annum (Sobel, 1992). RVVC can be further broken down into two subgroups: primary and secondary RVVC. Primary infections are idiopathic and do not correlate to any known predisposing factors identified with acute VVC (Sobel, 1992). Secondary infections are defined as frequent episodes of acute VVC brought on by unavoidable predisposing factors such as diabetes mellitus or hormone replacement therapy (Vanden-Bossche). Women who are diagnosed with RVVC usually respond favorably to antifungal therapies with very little resistance. It has widely been accepted that women with primary RVVC are missing an important protective immune factor. Following a typical antifungal regimen for a symptomatic episode, if the pathogen was not fully eradicated, it could easily increase in population and cause recurrent infections through relapse (Sobel, 1992). A summary of the epidemiology of VVC and RVVC is provided in Table 2.

Percentage of healthy individuals asymptomatically colonized	5-20%
Lifetime occurrence of at least one episode of vaginal candidiasis in healthy women	50-75%
Candida albicans as causative agent	85-90%
Predisposing factors for infection	
Antibiotics	+++
High-estrogen contraceptive therapy	++
Steroids	+
Chronic mucocutaneous candidiasis	+/-
Chemotherapy	
Lymphoma/hematologic malignancy	+/-
Transplantation (allogeneic)	+/-
AIDS	+/-
Prevalence of Primary RVVC in healthy women	5%
Antifungal resistance	rare
+++, strong role; ++, intermediate role; +, weak role; +/-, little if any role	

Table 2: A brief summary of the epidemiology and factors affecting vaginal *C. albicans* infection. (+++, strong role; ++, intermediate role; +, weak role; +/-, little if any role)

(Adapted from Fidel, 2007)

1.2.5 Host Defense against Vaginal Candidiasis

As a result of the staggering numbers of *Candida albicans* infections per year, the majority of all healthy adults have developed a *Candida*-specific adaptive immunity. This adaptive was immunity is demonstrated by the presence of serum/mucosal antibodies, *in vitro* T-cell responses, and delayed skin test reactivity (Fidel, 2007; Calderone). What role these adaptive immune responses play in the prevention of or protection against *Candida* infections of the urogenital tract is not fully known, but the latest experimental findings show that their contributions appear to be nominal (Calderone).

Over the past 20 years, there have been innumerable studies pertaining to host-pathogen interactions and the mechanisms of urogenital infections by *Candida albicans*. Despite the large number of studies undertaken to identify the protective host

mechanisms against vaginitis we still do not have a complete understanding of how our immune system deals with potentially pathogenic commensals of the urogenital tract.

Mucosal candidiasis includes orapharyngeal, esophageal, gastrointestinal, and vaginal infections (Fidel, 2007). Prior to the AIDS epidemic, researchers thought that all mucosal membranes were equally susceptible to *Candida* infection through the same mechanism (Fidel, 2007). A large percentage of AIDS patients suffering from T-cell immuno-suppression developed mucosal candidiasis (mainly orapharyngeal in nature), and experimental models showed a strong role for T cells against *Candida* infection (Fidel, 2007). Clinical studies and animal models investigating RVVC showed no role for systemic or local cell-mediated immunity (CMI) or a shift in a *Candida*-specific Th1 to a Th2 response at the vaginal mucosa (Fidel,2007; Clift; Romani; Klein; Samaranayake; Sohnle; Fidel, (1994),Saavedra; Fidel, 1999). The lack of a systemic immune response at the urogenital tract against *Candida* is further backed by the observation that although female AIDS patients are commonly infected with oral candidiasis, vaginal candidiasis was no more prevalent than in the healthy population (Ferrante; Fidel, 1995; Fidel, 1993).

Puzzled as to why there was a lack of a T-cell response at the vaginal mucosa although a *Candida*-specific Th1-type immunity was evident in the blood and draining lymph nodes in mice, Fidel *et al.* began to research the mechanism that was preventing CMI from protecting against vaginal candidiasis. Their resulting experiments revealed that a strong downregulatory cytokine, TGF-β, was constitutively present at the vaginal mucosa and its expression and secretion by vaginal epithelial cells was transiently increased in response to either an infection or estrogen (Hobbs). Other cytokines that affect Th1/Th2 cells were extremely low during infection (Hobbs). From this new study, the

current paradigm of immunoregulation emerged as an explanation for the apparent lack of CMI protection against vaginal infection. Fidel has proposed that since *Candida albicans* is a vaginal commensal, the evolution of immunoregulation to avoid chronic inflammation at the reproductive tissue of the host, would only strengthen their symbiotic relationship (Fidel, 2003).

Investigations into the possible role of innate immunity at the vaginal mucosa showed that there was no detectable correlation between the presence of polymorphonuclear neutrophils (PMNs) and natural killer cells (NKs) and a response to an infection (Fidel, 2007; Fidel, 1999; Saavedra; Sohnle; Steele 1999a; Parr; Black; Carlsten). However, during these experiments, Fidel et al. discovered that vaginal epithelial cells from mice, humans, and macaques have the ability to inhibit *C. albicans* growth (Fidel, 2007; Steele, 1999a; Steele1999b, 1999b). This inhibitory activity was elucidated to be an acid-labile protein bound to the vaginal epithelial cell wall that upon contact with *Candida albicans* causes an unknown intracellular event resulting in the inhibition of its growth (Barrouse, 2005; Nomanbhoy). This activity was also evident in oral epithelial cells (Barrouse, 2005; Nomanbhoy). Since this activity is fungistatic and not fungicidal, it may provide evidence of a host mechanism to control commensalism. Clinical studies comparing the role of this fungistatic mechanism in healthy and RVVC afflicted women revealed that there was only a minor reduction in fungistatic activity in those with RVVC (Nomanbhoy). Since the reduction of activity was minor, researchers believe that there are other undiscovered host factors that are responsible for protection against fungal vaginitis Barrouse, 2005 ; (Fidel, 2007). This research project aims to identify and characterize such host factors.

1.2.6 Composition of Cervical-Vaginal Fluid

The mucosal fluid covering the vaginal epithelium is primarily made up of endometrial and oviductal fluids, secretions from cervical vestibular glands, and plasma transudate (Wagner). The molecular components of the fluid include inorganic salts, urea, amino acids, proteins, and a number of fatty acids; the fatty acids primarily provided by commensal organisms, of which *Lactobacillus spp.* predominate (Tang). Recently, the proteome of the cervical-vaginal fluid (CVF) of lavages obtained from healthy and *Candida*-colonized vaginas has been elucidated (Tang). Tang *et al*. discovered that there were surprisingly high levels of normal serum proteins present in the cervical-vaginal fluid. The serum proteins, albumin, immunoglobulin chains, and transferrin were found in relative abundance and accounted for 47% of all proteins identified within the fluid (Tang). Another startling discovery was the absence of proteins associated with vaginal commensal bacteria. Out of the 147 proteins identified, only one protein, an oligopeptide/dipeptide ABC transporter of *Lactobacillus reuteri* (Accession #Q1U7T2), was identified.

A further examination of the protein maps generated by Tang *et al*. showed a clear increase in the amounts of serum proteins in lavages obtained from *Candida*-infected patients compared to that of healthy patients (Tang). Although the levels of serum proteins found in the lavages from colonized patients increased, the proportions of other non-serum protein components were not significantly altered (Tang). In comparison to fluids from other mucosal membranes, such as the oral and nasal passages, only 16 proteins are conserved (Hu; Tang). If these proteins, or factors associated with them, play a

role in host-commensal/pathogen regulation it may account for the difference seen in the human immune response against *Candida albicans* between these two mucosal environments.

1.2.7 Serum Albumins as Small Molecule Transporters

Human serum albumin (HSA) is the most abundant protein in blood plasma and serves as a transport and depot protein for numerous endogenous and exogenous compounds (45). One of the primary transport roles of HSA is to carry fatty acids, which are poorly soluble in an aqueous environment (45). HSA binding to ligand results in an increased solubility in plasma, decreased toxicity, and protection against oxidation of the bound ligand (Kragh-Hansen, 2002).

HSA is a single chain, 66.5 KDa protein synthesized in and secreted from liver cells (Peterson). It is normally a simple, heart-shaped protein lacking prosthetic groups and covalently bound carbohydrates and lipids. X-ray diffraction has shown that the protein has three homologous domains (I-III), and that each of these is comprised of two subdomains (A and B) (Figure 3) (Barrouse, 2005). In addition to HSA's noncovalent binding properties, it also retains the ability to covalently bind ligands via its free cysteine residue (^{34}Cys).

The ground-breaking work of Sudlow *et al*. revealed that most small molecules bind with high affinity to one of two sites, called Sudlow site I and Sudlow site II (Sudlow). Site I is formed as a pocket in subdomain IIA and involves the lone tryptophan of the protein (Hu; Sugio; Tang). Site I's pocket is recognized as being large and flexible and is formed from hydrophobic side chains while its entrance is surrounded by positively

charged amino acids (Kragh-Hansen, 1983; Kragh-Hansen, 1985; Tang; Yomasaka). Dicarboxylic acids and bulky hetrocyclic molecules with a negative charge localized in the middle of the molecule comprise the usual site I ligands (Tang).

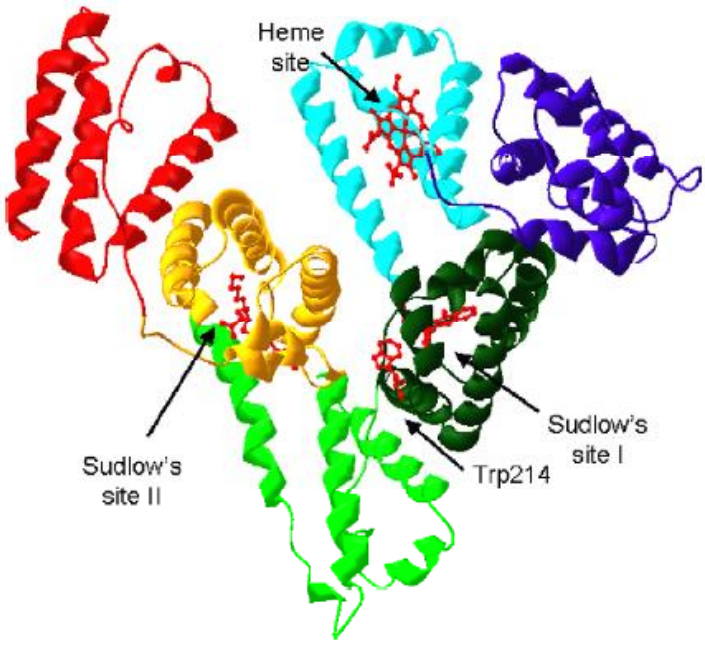

Figure 4: HSA structure. The six subdomains of HSA are colored as follows: subdomain IA, blue; subdomain IB, cyan; subdomain IIA, dark green; subdomain IIB, light green; subdomainIIIA, orange; subdomain IIIB, red.

Adapted from Ascenzi *et al*

Sudlow site II (also known as the indole-benzodiazepam site) is much smaller, narrower, less flexible, and is located in subdomain IIIA (Figure 5) (Ascenzi). Ligand binding affinities can be greatly altered by stereoselectivity or by substitution of ligands with a relatively small group (Tang). This is confirmed by the complete inhibition of binding of the serum ligand tryptophan by the simple replacement of a methyl group on the α-hydrogen (Chuang; McMenamy; Peter; Tang).

In addition to these two selective binding sites, albumin also contains multiple binding sites of varying affinities for non-esterified fatty acid anions and locations for

many saturated fatty acids binding sites (Bhattacharya; Tang). These sites are located within subdomains, but there are also others that are formed by adjacent subdomains or via the external protein residues. Binding of anions of medium-chain fatty acids and monooleoyl-glycerol occur at Sudlow Site II (Kragh-Hansen, 1988; Tang; Thumser).

Researchers currently do not have a lucid understanding of the host immune mechanisms of the cervical-vaginal tract. Investigation into the roles of the adaptive and innate immune response of the human body has shown that at the vaginal mucosal level, they offer no substantial immunity to *C. albicans* (Fidel 2000). The low level of protection offered by the combined actions of the innate and adaptive immune responses, as well as the fungistatic activity exhibited by vaginal epithelial cells, leads researchers to conclude that there might be an undiscovered factor responsible for the majority of the protective immunity exhibited by a healthy cervical-vaginal tract (Fidel, 2007).

An unknown immune factor responsible for comprising the bulk of vaginal immunity, doesn't necessarily have to be vaginal in origin. Serum proteins found in the cervical-vaginal fluid might be directly or indirectly involved in vaginal protection. Transporting an immune factor responsible for vaginal immunity is only one possible role that serum proteins may be fulfilling. Of the identified serum proteins found in cervical-vaginal fluid, albumin would most likely be the transporter, since it is already well established as a transport protein.

2 Materials and Methods

2.1 *Candida albicans* Culture Conditions

The *C. albicans* wild type strain, SC5314 was used for all fungicidal screening assays (Gillum). SC5314 culture stocks were stored in10% glycerol/YPD (0.5% Yeast Extract, 1.0% Peptone, and 2.0% Glucose) at -80°C. Cultures were streaked on YPD agar plates and grown overnight at 30°C. Each plate was utilized for 2 weeks, at which time a new YPD agar plate was streaked for use as an inoculum source for the experiments. 10ml of YPD media was inoculated with a single colony of a plated SC5314 culture. The culture was then grown up overnight at 30°C with 280 rpm agitation. Cells were harvested by centrifugation and washed twice with 10ml of sterile dH_20. The washed cells were resuspended in 1ml of dH_20 and an OD_{600} was taken. Cell density was calculated from the following equation: 1 OD_{600} = 2.0 X 10^7 cells/ml. The cell suspension was then normalized to 1.0 X 10^8 cells/ml.

2.2 Extraction Protocol/Sample Preparation

200 mg of albumin was dissolved into 10 ml of deionized H_20. Three, 40% (v/v) extractions with N-butanol (Fischer Scientific A383-1) were performed on the albumin solutions as stated below. 4 ml of N-butanol was added to each 10 ml sample and allowed to mix by rotation at room temperature for 15 minutes. After rotation the sample was centrifuged for 30 minutes at 4°C in a Beckman J6 centrifuge (TY JS5.2 rotor) at 2000 rpm. The organic layer was separated and the process repeated for a total of three extractions. The pooled organic phase was then frozen in liquid nitrogen and lyophilized

overnight. The lyophilized samples were reconstituted in a minimal volume of N-butanol (200µl N-butanol per sample volume), transferred to reaction tubes (1.5ml microcentrifuge tubes), and were dried on low heat.

2.3 GPP Media Formulation

The assay media chosen to carry out the fungicidal screening was a Glucose Phosphate Proline (GPP) media that was adapted from Hornby, 2004 (Hornby). The defined liquid medium contained the following per liter of distilled water: glucose (or sucrose), 20 g; KH_2PO_4, 4 g; Na_2HPO_4, 3.2 g; $MgSO_4$ • 7 H2O, 0.5 g; biotin, 20 g; thiamine • HCl, 200 g; pyridoxine • HCl, 200 g; $ZnSO_4$ • 7 H_2O, 1 mg; $MnCl_2$ • $4H_2O$, 1 mg; CuSO4 • $5H_2O$, 1 mg; $FeCl_3$, 1mg; 10 mM L-proline or 10 mM L-arginine • HCl (pH 6.0 ±0.2). The vitamins were prepared as a 1,000 x stock mixture in 20% aqueous ethanol, and the trace elements were prepared as a 5,000 x stock solution (5 mg/ml in 0.1 N hydrochloric acid). The vitamin and trace elements stock solutions were then .22µm filtered. The medium ingredients, except carbon and nitrogen sources, were dissolved in distilled water, dispensed into culture flasks, and autoclaved at 121°C for 15 min. The nitrogen source, proline, was prepared as 100 mM stock solutions, autoclaved separately, and added aseptically to the medium to give a final concentration of 10 mM. Similarly, glucose (or sucrose) was prepared as a 20% stock solution, autoclaved separately, and added aseptically to the growth medium to give a final concentration of 2%.

2.4 Acetone Separation

The processed sample was combined with 1 ml of ice-cold acetone (Sigma179124), vortexed vigorously for 3 minutes, and left to incubate at 20°C for 1 hour. The incubated samples were then centrifuged at 14,000Xg for 10 minutes on a benchtop microfuge. The supernatant was aspirated off and saved. Both the acetone pellet and the acetone supernatant then dried.

2.5 Assay Protocol

The processed samples that had been dried in the reaction tubes were brought up in 10µl tissue culture tested DMSO (SigmaD2438), combined with 170µl of GPP filamentation media, and inoculated with 20µl of the normalized SC5314 cell suspension. Reaction tubes were allowed to incubate at 37°C, with rotation for 3 hours. After incubation, serial dilutions were performed, and 15µl of each solution was plated onto YPD agar and grown overnight at 30°C. The following morning CFU counts were performed on serial dilutions that yielded manageable colony numbers. Each assay was performed in quintuplicate. Approximately 50-200 colonies were counted per plate.

3 Results

3.1 Effects of DMSO on Fungal Viability

DMSO was added to the screening media to help improve substrate solubility and to bypass any rate limiting step such as transmembrane transport in order to enhance the substrate's intracellular bioavailability. In order to determine the maximum final

concentration of DMSO that could be used in the assay that does not interfere with *C. albicans* cell viability, increasing percentages of DMSO were incubated with SC5314 cells using the established assay procedure stated in the materials and methods section above. Figure 5 demonstrates that a final concentration of 5.0% DMSO is acceptable to use in the screening assay since this is the highest concentration tested at which the SC5314 cells remain viable.

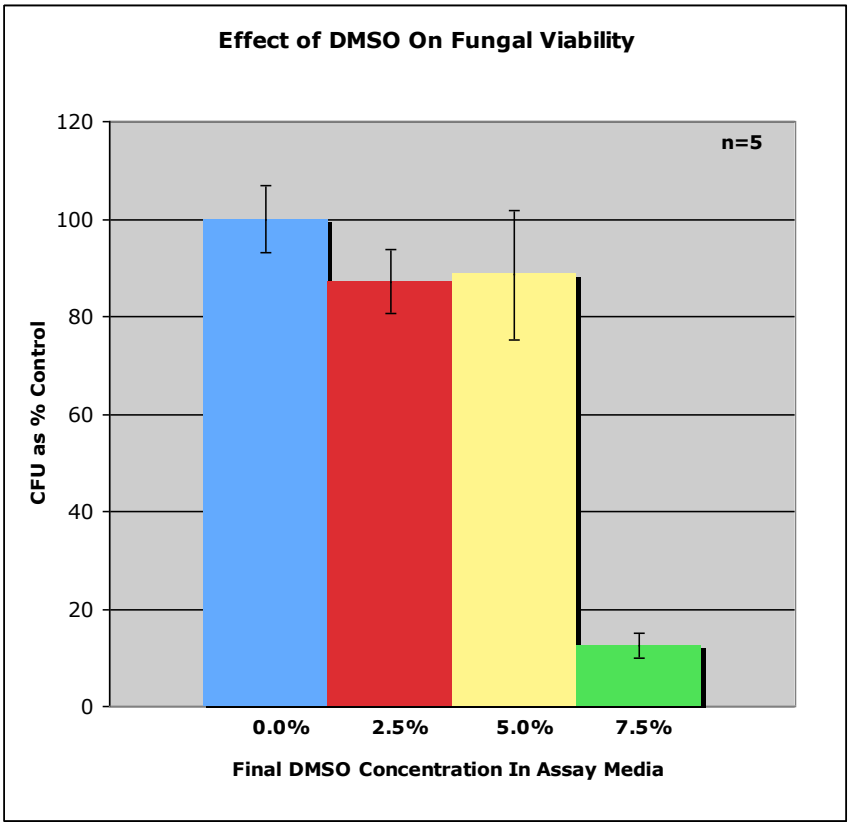

Figure 5: Increasing percentages of DMSO was incubated with SC5314 cells in order to ascertain the maximum DMSO concentration that did not affect cell viability. N=5. Error bars represent Standard Error of the Mean (SEM).

3.2 Fungicidal Activity Is Present in BSA Extracts

Since any albumin-associated fungicidal molecule involved in vaginal immunity against *Candida albicans* may very well be conserved among mammalian species, process validation and preliminary studies were performed on bovine serum albumin (BSA) as a surrogate for human serum albumin (HSA). Fungicidal activity was represented by a decrease in SC5314 cell viability. Cell viability was measured as the percent of viable cells cultured on YPD agar after the assay as compared to that of the control cells. The control utilized for these experiments was the addition of SC5314 cells into an aliquot of pure GPP/5%DMSO media with no extract added. There was no change in SC5314 viability when N-butanol by itself was dried in the reaction tubes and a control assay was performed (data not shown). Therefore any decrease in cell viability cannot be attributed to the extract solvent. For this experiment, increasing aliquots of stock extract were dried and their affects on cell viability was assayed. Figure 6 shows that approximately 75 μg (40 μl) of extract exhibited a complete fungicidal effect against the SC5314 strain of *Candida albicans*. This effect was also seen at subsequent higher concentrations.

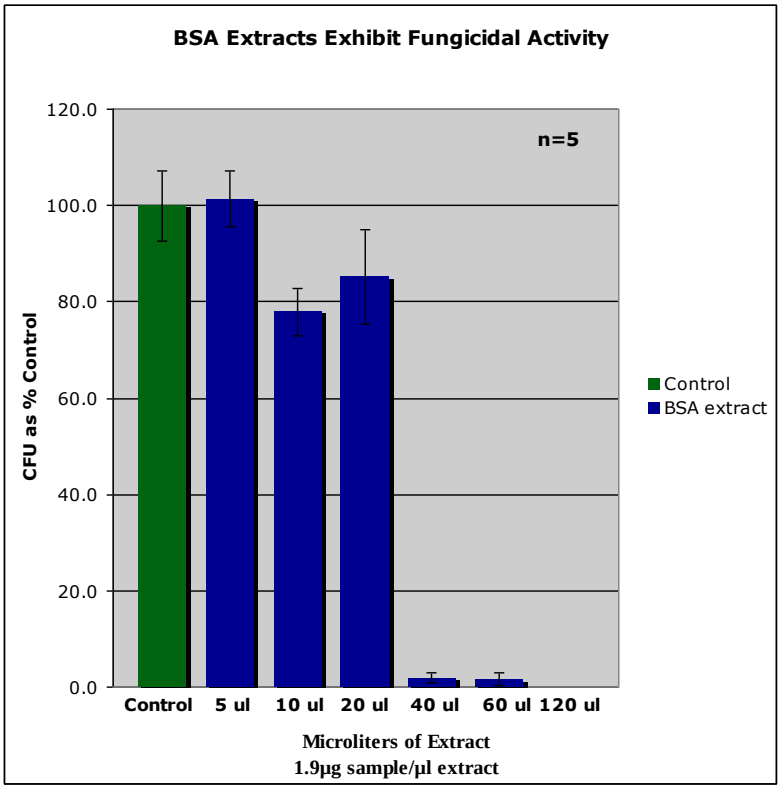

Figure 6: The effects on cell viability from increasing aliquots of BSA extracts were performed. N=5. Error bars represent Standard Error of the Mean (SEM).

3.3 Fungicidal Activity Is Present in HSA Extracts

Once process and assay validation was completed utilizing BSA samples, HSA samples were analyzed in order to link the fungicidal activity recovered from the serum albumin extracts directly to a human system. Figure 7 shows a dose-dependent fungicidal effect caused by the extracts of the human serum albumin. The same assay procedures and controls were used as in Experiment 3.2. At the highest tested dose, 200µl (375 µg), there remains only 18% cell viability when compared to the control samples. Due to extract batch limitations, higher doses were not analyzed.

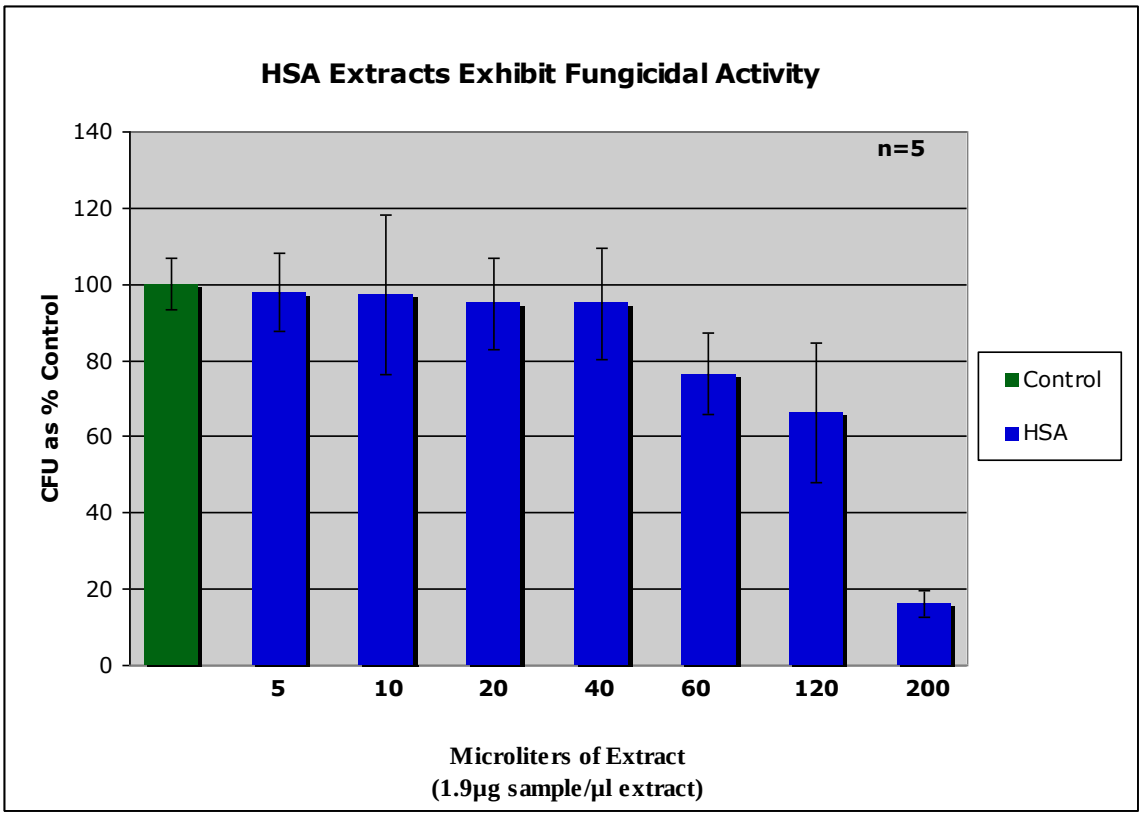

Figure 7: The effects on cell viability from increasing aliquots of HSA extracts were performed. N=5. Error bars represent Standard Error of the Mean (SEM).

3.4 Activity Comparison of BSA and HSA Extracts

Dosing studies using bovine and human serum albumins were compared side by side to evaluate the difference in specific activity. Since extractions were performed on the same amount of starting material and the lyophilized extract for each sample was resuspended in the same volume of N-butanol used to comprise the stock solution, the difference in activity can be directly attributed to the difference in sample sources. Figure 6 shows that BSA extractions yield approximately a 5 fold higher specific activity over the HSA samples.

It can be concluded that there is a difference in fungicidal activity depending on the source of the serum albumin (HSA or BSA). The reason for the difference in

fungicidal effect per µl of sample cannot be ascertained from these experiments; however since these extractions and experiments were carried out using the same protocol at the same time, a processing variation can be ruled out. The difference in activity may be due to differences in physiologic levels of the fungicidal factor associated with serum albumins from species to species, or a difference in the predominant isoform of the factor between species. Batch variation cannot be ruled out as the source of the difference in activity, but is considered unlikely since consistent activity was obtained from three different BSA extractions over the course of approximately one year (data not shown)

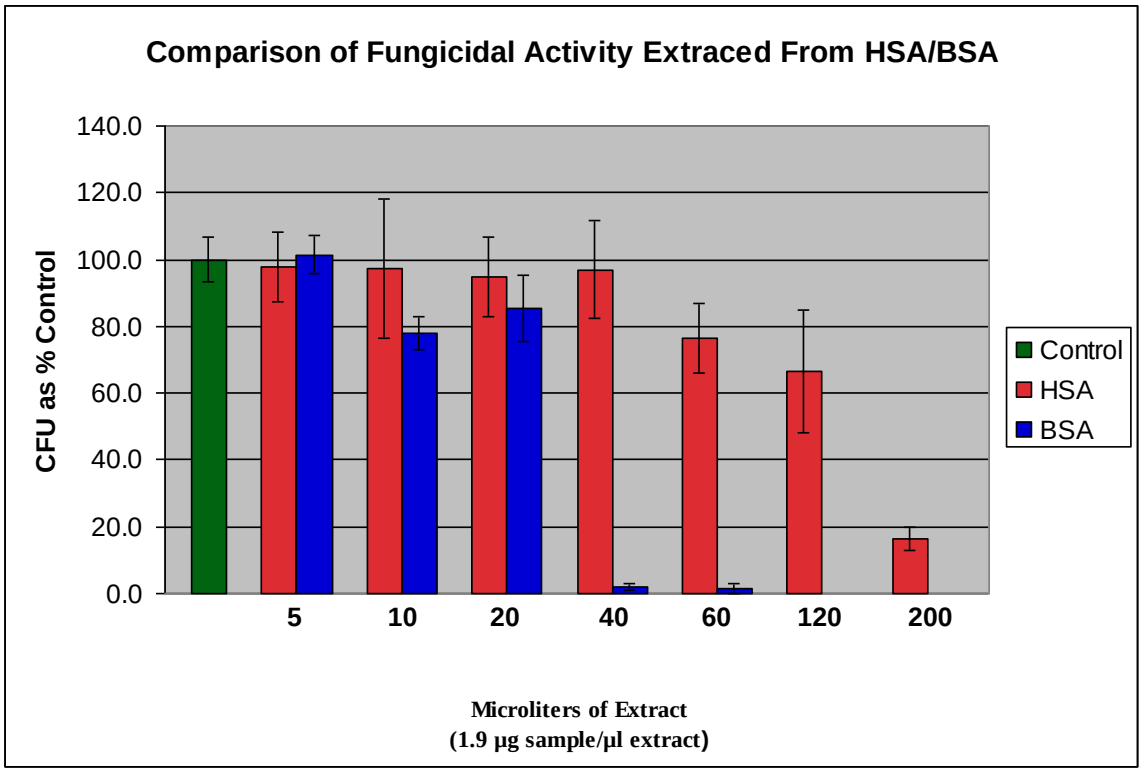

Figure 8: The effects on cell viability from increasing aliquots of BSA and HSA extracts were performed and compared. N=5. Error bars represent Standard Error of the Mean (SEM).

3.5 Fatty Acid Free (FAF) BSA Extracts Exhibit No Fungicidal Activity

Since the agent responsible for the fungicidal activity was extracted from the albumin using organic solvents, the molecule(s) containing this activity are most probably lipids or other hydrophobic small molecules. To test this theory, albumin samples that were stripped of their fatty acids via charcoal treatment under acidic conditions were purchased. The fatty acid free albumin samples were purchased from the same vendor and had undergone the same pre-purification regiment that the untreated albumin samples had undergone. Both extractions started from the same amount of starting material and were processed in the same manner as stated previously. The control represents fungal cells that had no serum extract added to the assay media.

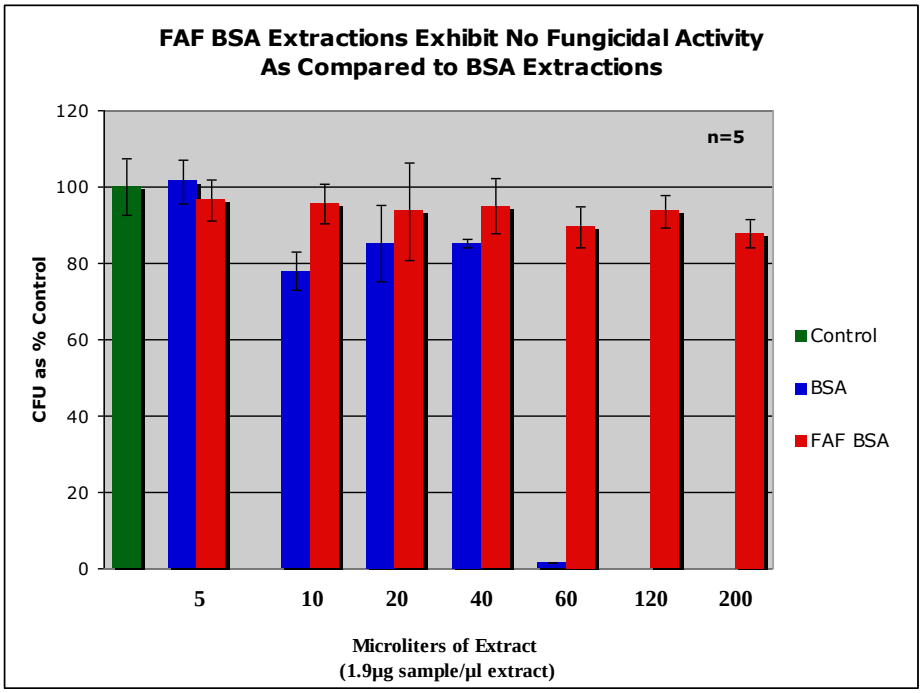

Figure 9: The effects on cell viability from increasing aliquots of fatty acid free BSA extracts were performed and compared against regular BSA extracts. N=5. Error bars represent Standard Error of the Mean (SEM).

It is evident from the results in Figure 9, that the BSA samples that been stripped of their lipophilic molecules exhibit no fungicidal effect. From these results we are able to conclude that the fungicidal factor associated with the BSA is lipophilic in nature.

3.6 Fatty Acid Free (FAF) HSA Extracts Exhibit No Fungicidal Activity

Based upon the results from experiment 3.5, the same study was performed on the fatty acid free human serum albumin samples. The same processing, extraction, and assay procedures were followed as in Experiment 3.5. Figure 10 shows that there was no loss in cell viability as compared to the HSA extracts indicating that by stripping the albumins of lipophilic molecules we see a complete loss of the fungicidal activity exhibited in the non-stripped samples. This is further evidence that the factor in question is indeed a lipophilic molecule and that the factor responsible for the fungicidal activity from the BSA and HSA extracts is the same molecule or closely related to one another.

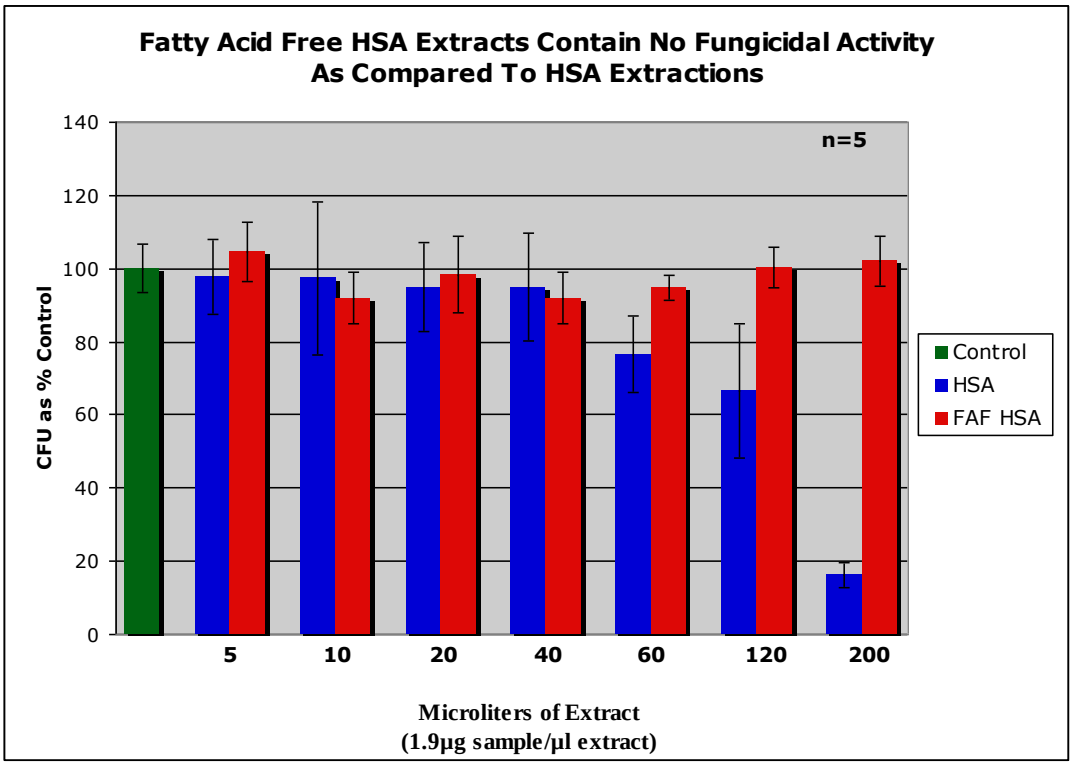

Figure 10: The effects on cell viability from increasing aliquots of fatty acid free HSA extracts were performed and compared against regular HSA extracts. N=5. Error bars represent Standard Error of the Mean (SEM).

3.7 The Fungicidal Factors from BSA and HSA Extracts Are Non-polar Lipophilic Molecules

The complete loss of fungicidal activity from the BSA and HSA samples that had been stripped of their lipophilic molecules is consistent with the hypothesis that the fungicidal factor is lipophilic in nature. In order to further classify these lipophilic molecules, an acetone separation was performed as outlined in section 2.4 of the Materials and Methods. In this separation, all polar lipids precipitate from the acetone solution while neutral and non-polar lipids remain soluble. Sample volumes of 60µl of extract were utilized for the experiment. This value was used since it represents the fungicidal dose associated with non-stripped BSA extractions. A positive and negative

control was run alongside the separated sample. To ensure that the dried acetone did not exhibit any fungicidal effects, sample volumes of acetone were dried and their reaction tubes were utilized in a cell viability assay. In these control experiments, no change in cell viability was observed (data not shown).

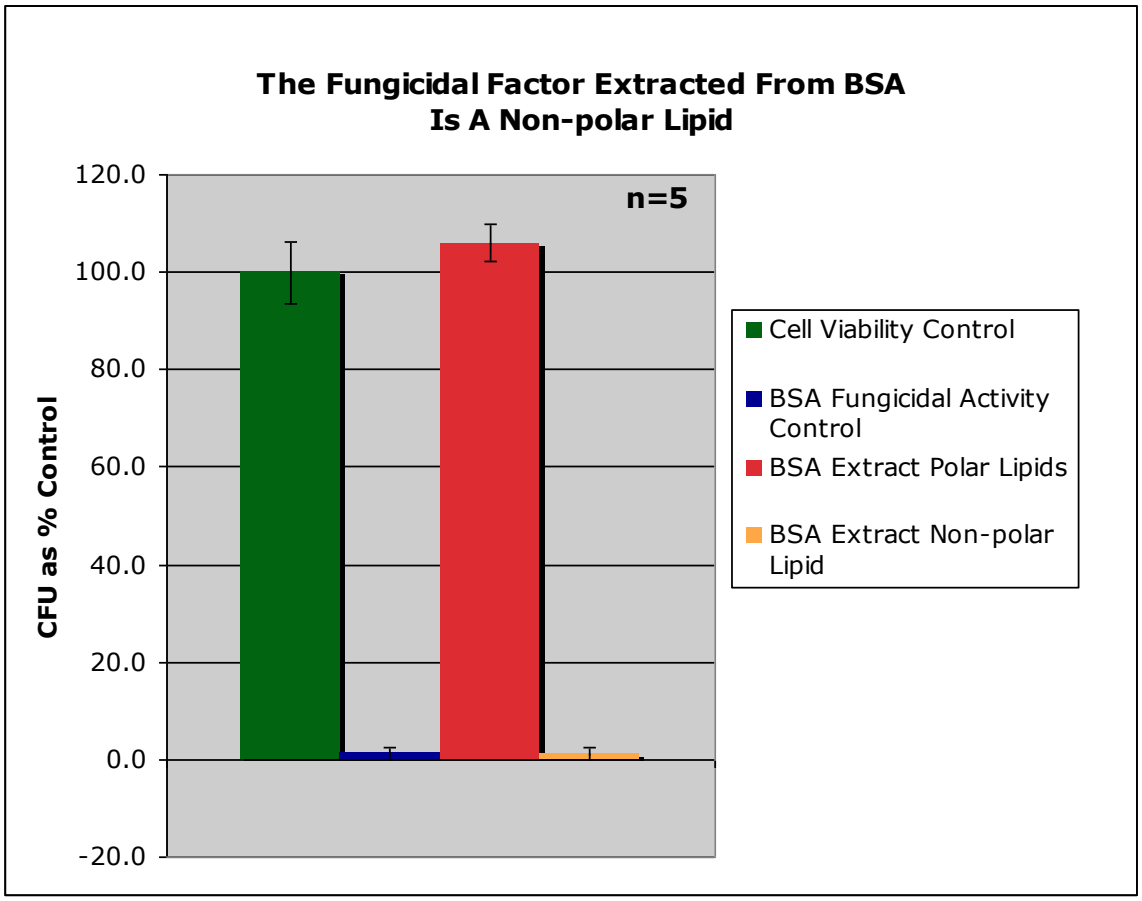

Figure 11: Lipid Separation was performed on the BSA extracts and a subsequent cell viability assay was performed. A non-fractionated BSA extract sample was also run to determine the maximum amount of activity that should be recovered from the two fractions. N=5. Error bars represent Standard Error of the Mean (SEM).

As seen in Figure 11, 100% recovery of the fungicidal activity was contained in the acetone soluble (non-polar lipid) fraction of the separation. The complete retention of activity in a single fraction may imply that there is a single factor responsible for the

fungicidal activity or if complementary factors are responsible, then they are very closely related in composition.

The same acetone separation procedure was performed on HSA extracts. For the cell viability assay the same controls were utilized as stated previously for the BSA lipid separation cell viability assay.

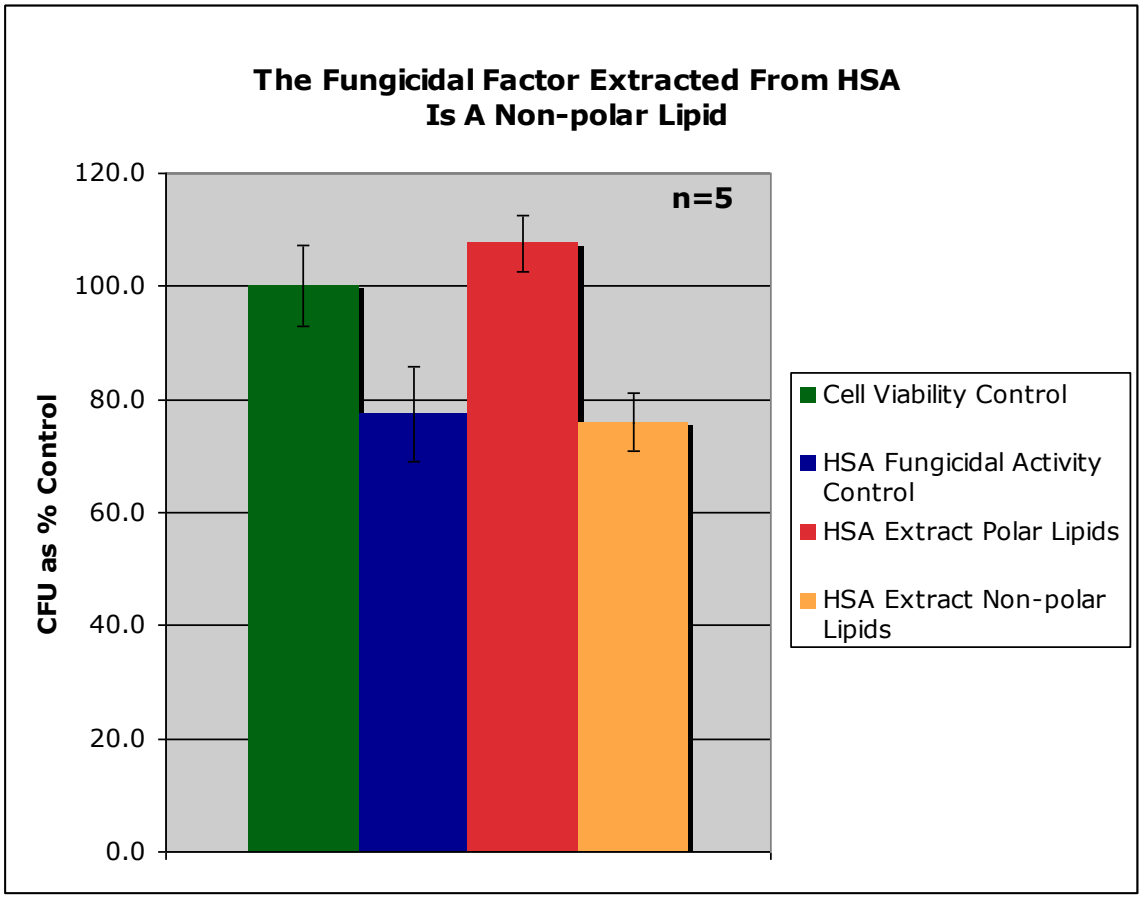

Figure 12: Lipid Separation was performed on the HSA extracts and a subsequent cell viability assay was performed. A non-fractionated HSA extract sample was also run to determine the maximum amount of activity that should be recovered from the two fractions. N=5. Error bars represent Standard Error of the Mean (SEM).

The results obtained from the HSA lipid fractionation experiment mimicked those of the BSA lipid separation experiment. However, only 60 µl of the extract was utilized

per sample and there was only a 24% loss of cell viability in the HSA fungicidal activity control. This loss in cell viability is consistent with the values obtained during the HSA extract dosing studies (refer to Figure 7). It is important to note that all of the fungicidal activity was retained within the non-polar lipid fraction of the separation. This result matches the results obtained from the BSA lipid separation experiments and further supports the hypothesis that the same molecule is responsible for the fungicidal activity in both extracts.

In order to eliminate the possibility that the process of the acetone separation was conferring the fungicidal effect exhibited by the samples. Acetone separations and fungal cell viability assays were performed on fatty acid free albumin samples of human and bovine origin (Appendix B, Figures 13 and 14).

4 Future Directions

4.1 Identification of the Fungicidal Factor

The further purification and identification of the fungicidal agent should be the first task undertaken in the progression of this project. This would provide both the identity of the molecule responsible and would also elucidate whether or not it is the same factor responsible for the activity in both human and bovine species. Since the cell viability assays were carried out using GPP media, the factor seems to be somewhat tolerable to hydrophilic conditions, and purification utilizing normal aqueous buffers should be feasible. Solubility studies will need to be performed in order to determine the extent of the factor's aqueous solubility. Preliminary attempts to purify this molecule using size-exclusion, ion exchange, and hydrophobic chromatography on a Biorad Duoflow FPLC machine proved unsuccessful (data not shown). It is possible that the factor was precipitating under these buffer conditions and was getting trapped in the pre-purification filtration step or was subsequently being retained on the column via precipitation or non-ideal interactions.

Solvent-based separation on a HPLC chromatography system should prove useful in the further purification of this molecule. In addition to this, mass-spectrometry would determine the approximate molecular weights of the components of the non-polar lipid fraction and might prove a useful starting point for HPLC purifications.

4.2 Interspecies Conservation

Since this factor is present in both bovine and human serum albumin samples, it would be interesting to see if this factor could also be obtained from various other mammalian species. Some of the commercially available serum albumins from mammalian species include: canine, guinea pig, porcine, rabbit, rat, and sheep. Additionally, it would also prove beneficial to test albumins from non-mammalian sources, such as ovalbumin. Preliminary testing of ovalbumin showed no fungicidal factor associated with it (data not shown), but more extensive testing should be performed to validate these results.

4.3 Clinical Samples

Once the factor responsible for the fungicidal effect is identified, the analysis of clinical samples from healthy, *Candida*-colonized but non-symptomatic, and VVC afflicted women should be performed. This analysis would reveal the physiologic levels of the fungicidal factor in relation to host-pathogen interactions. It would be interesting to see if the levels of the factor increase in the cervico-vaginal fluid in women afflicted with VVC. The cervico-vaginal fluid proteome mapped by Tang *et al* shows an increase in albumin concentrations of the fluid in response to *Candida* colonization (Tang). Since the fungicidal factor associates with serum albumins and albumin concentrations in the cervical-vaginal fluid increase in response to *Candida* colonization, experiments investigating the reason for the increase in albumin transcytosis across the vaginal epithelium should be performed.

4.4 Mechanism of Fungicidal Activity

Revealing the mechanism by which this factor is able to cause *Candida albicans* cell death would prove pivotal in ascertaining if this factor is suitable to be used in antifungal regimens. The modes of fungicidal action are cell lysis and induction of apoptosis. Microscopy should be able to reveal which cidal event is occurring since each event has different phenotypic characteristics. Molecular studies would also be required in order to ascertain the details of the mechanism or the pathways involved in the fungicidal event. Further characterization of the fungicidal activity against various *Candida* and *non-Candida* species would provide insight into whether or not this agent is *Candida albicans* specific or if it is a broad spectrum antifungal.

One common way that lipids are able to affect cell viability is through membrane disruption. Since cell membranes are composed of a lipid bilayer, extracellular lipids are able to insert themselves into the cell membrane and disrupt membrane stability, biophysics, and various membrane-bound processes (Odds 2003). Since DMSO affects membrane solubility, the fungicidal studies presented here in this thesis should be repeated with a nominal percentage of DMSO (0.5-1.0%) or in the absence of DMSO altogether.

4.5 Cytotoxic Effects against Host Cells

In order to evaluate whether or not it would be feasible to utilize this factor as a pharmacologic agent against *Candida albicans*, host cell toxicity studies must be performed. Even though the factor is produced by the host, the concentrations of the factor needed to elicit an antifungal effect may prove cytotoxic to host cells.

5 Discussion

The original hypothesis that there is a small molecule, or molecules, associated with serum albumin that affect *Candida albicans* is supported by the results obtained in this thesis project. The fact that the fungicidal activity can be extracted from serum albumins obtained from both bovine and human sources may implicate that this type of fungicidal effect is conserved in mammalian species. A rudimentary extraction and screen using ovalbumin was performed and showed no traces of fungicidal activity (data not shown). This result gives further support for the possibility that this activity resides only in mammalian species. Before any definitive conclusions about the conservation of this activity are made, experiments utilizing serum albumin samples from other mammalian species need to be performed.

An interesting observation is that the specific activity exhibited by the samples obtained from the two separate sources varied. Two possible causes for this variation are: a slight divergence of the fungicidal molecule's composition between species or a difference in the albumin orthologs that either alters the number of binding sites per albumin molecule or the protein's affinity to bind the fungicidal molecule. Since the albumin samples were purchased, any molecule that is found in the samples that is not albumin is essentially considered a contaminant. This raises an important question concerning the consistency of the lipid content associated with the albumin as the source and the batch of the albumin is changed. In order to address this possibility, the data obtained from assays utilizing three separate batches of BSA that were purchased over the period of 14 months were compared. The fungicidal activity seen in all three batches were identical to one another (data not shown). These ideas concerning the cause of the

variation in activity are only a few of the possible causes for the change in activity. The future identification and characterization of this factor should help elucidate the cause of the variation in fungicidal activity.

One possible identity of this molecule is indole-acetic acid (IAA). IAA is naturally produced in the tissue and organs of mammals as a natural byproduct from the catabolism of the amino acid tryptophan and can also be obtained from a diet rich in vegetable stems (De Melo, 2004). IAA has also been demonstrated to be fungistatic (Prusty) as well as cytotoxic to eukaryotic cells at concentrations of 1mM (De Melo, 1997). The fungistatic event is caused by a cell cycle arrest and is transient in nature (Prusty). In order to reduce the toxicity of IAA within the organism, it is usually conjugated to an amino acid and transported via serum albumins (Tomasic).

From research performed in the Prusty laboratory, IAA is soluble in organic solvents (Prusty). Whether or not IAA or its related conjugates are soluble in N-butanol would have to be addressed by performing solubility studies. The properties of the conjugated amino acid or prosthetic group would greatly influence the overall solubility of the molecule. Experiments utilizing commercially available IAA and IAA/amino acid conjugates can easily be performed to elucidate the extent of solubility of the molecules in both N-butanol as well as the assay medium. Fungal viability assays could also be performed to either validate or eliminate the possibility that the factor might be IAA or one of its physiologic conjugates.

Analysis and comparison of fatty acid free samples gave the first insight into the characterization of the fungicidal molecule. Since the serum albumin samples that were stripped of their lipophilic molecules exhibited no activity, we can deduce that this

molecule is a fatty acid or lipophilic in composition. Since the primary role of serum albumins is to act as a transport protein for lipids and fatty acids (Kragh-Hansen 2000), it is not surprising that the fungicidal factor extracted from them is lipophilic in nature. Although extracts of fatty acid free albumin samples yield a substantial pellet, the absence of any fungicidal activity gives reassurance that the fungicidal activity seen in the unstripped samples is not the result of nonspecific interactions.

The cell viability assays performed on the acetone-fractionated samples were utilized to further characterize the fungicidal activity. Solvent fractionation is the simplest, and sometimes most efficient, way for separating a group of lipids of interest. The use of ice-cold acetone as the fractionating solvent allows a complete, one-step separation of polar lipids, such as phospholipids and glycolipids, from all neutral or non-polar lipids such as triglycerides and cholesterol. In this fractionation, all the polar lipids are present in the precipitate, while all neutral or no-polar lipids are contained in the acetone layer. In fractionation assays all activity possessed in the original un-fractionated sample were recovered in the acetone layer of the fractionation, implying that the fungicidal molecule is a neutral or non-polar lipid.

Samples of acetone by itself were also processed and assayed in order to ensure that the fungicidal effect is not due to chemical byproducts that may be present in the solvent. No adverse effect on cell viability was seen in this assay (Data not shown). For a negative control, the extracts obtained from fatty acid free albumins were also acetone fractionated and assayed for the presence of any fungicidal effect. As was expected, no decrease in cell viability was observed from these experiments.

The suggestion that this factor is a conjugate of IAA is only one possibility that was based off of literature reviews and needs more extensive experimental validation. The factor may very well be a simple or modified non-polar lipid. Non-polar lipids that are typically found dissolved in this organic layer are: glycerides, sterols, sterol esters, carotenoids, and lipid soluble vitamins.

The fungicidal mechanism is completely unknown and further research will be needed in order to establish a working model system. It is also unclear whether or not this fungicidal activity is directly attributed to the factor itself or if it is being induced through downstream processes affected by the binding of the lipid to a receptor. It is possible that the factor itself is not fungicidal but after entry into the yeast cell enzymatic processes convert the factor into a fungicidal agent. If enzymatic conversion of the factor is the source of the effect, and the process is *Candida* specific, the factor might prove to be highly selective and effective as a pharmacologic agent.

Further analysis by mass spectrometry and extensive purification will be required to further purify and identify the responsible entity. Identification of this molecule and its fungicidal mechanism may be pivotal in the understanding of how the human immune system interacts with fungal species that are both commensal and pathogenic organisms of the vaginal tract.

6 Conclusions

The significant reduction of SC5314 cell viability upon incubated with N-butanol extracts from bovine and human albumins, provides a solid basis for the presence of a fungicidal molecule associated with serum albumins. Furthermore, the absence of this

fungicidal activity in fatty acid free albumin samples provides preliminary evidence that this factor is lipophilic in composition. Fungal viability assays upon the acetone separation fractions further characterize this lipophilic molecule as a neutral lipid. Although fungal cell viability assays show a difference in activity between human and bovine extracts, the fact that upon lipid separation, both extracts retained all of their fungicidal activity in the non-polar lipid fraction suggests that factor responsible for both extracts are of the same general classification. Further research will need to be carried out in order to identify the molecule responsible as well as its fungicidal mechanism.

Appendix A: Abbreviations

VVC	Vulvovaginal Candidiasis
RVVC	Recurrent Vulvovaginal Candidiasis
FDA	Food and Drug Administration
ADMET	Absorption Distribution Metabolism Excretion Toxicology
LD_{50}	Lethal Dose 50%
LD_{100}	Lethal Dose 100%
AIDS	Acquired ImmunoDeficiency Syndrome
SAP	Secreted Aspartyl Protease
HRT	Hormone Replacement Therapy
CMI	Cell-Mediated Immunity
Th1	T helper cells 1
Th2	T helper cells 2
TGF-β	Transforming Growth Factor Beta
NKs	Natural Killer Cells
PMNs	Polymorphonuclear Nuetrophils
CVF	Cervical-Vaginal Fluid
BSA	Bovine Serum Albumin
HSA	Human Serum Albumin
FAF	Fatty Acid Free
YPD	Yeast Peptone Dextrose
dH_2O	Deionized Water
OD	Optical Density
Rpm	Rotations Per Minute
ml	Milliliter
mg	Milligram
µl	Microliter
µg	Microgram
GPP	Glucose Phosphate Proline Media
mM	Millimolar
DMSO	Dimethyl Sulfoxide
CFU	Colony Forming Units
FPLC	Fast Protein Liquid Chromatography
HPLC	High Performance Liquid Chromatography
IAA	Indoleacetic Acid

Appendix B: Acetone Separation Controls

In order to eliminate the possibility that the acetone separation is conferring a fungicidal property to the non-polar lipid fraction, that is not attributed to the separation of an albumin associated factor, the acetone separations and subsequent cell viability assays were performed on the extracts obtained from BSA and HSA samples that had been stripped of their lipophilic molecules (Figures 15 and 16).

As predicted, there was no fungicidal activity observed in either the BSA and HSA fungicidal controls or the fractions obtained from them through acetone separation. This indicates that the fungicidal activity recovered in Figures 13 and 14 represents a recovery of fungicidal activity caused from the purified albumin factor.

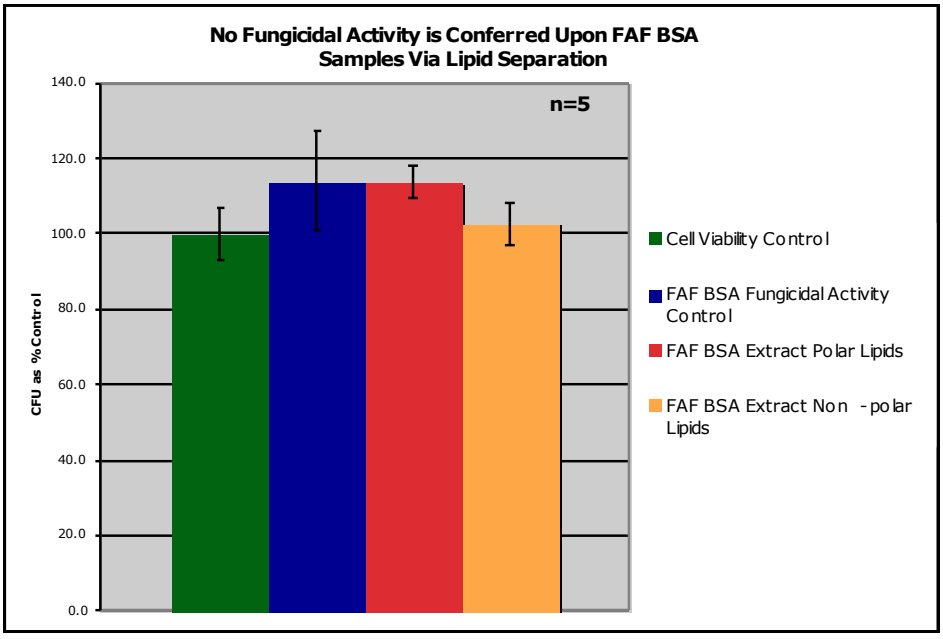

Figure 13: Lipid Separation was performed on the FAF BSA extracts and a subsequent cell viability assay was performed. A non-fractionated FAF BSA extract sample was also run to determine the maximum amount of activity that should be recovered from the two fractions. As expected no fungicidal effect was seen in any of the samples. N=5. Error bars represent Standard Error of the Mean (SEM).

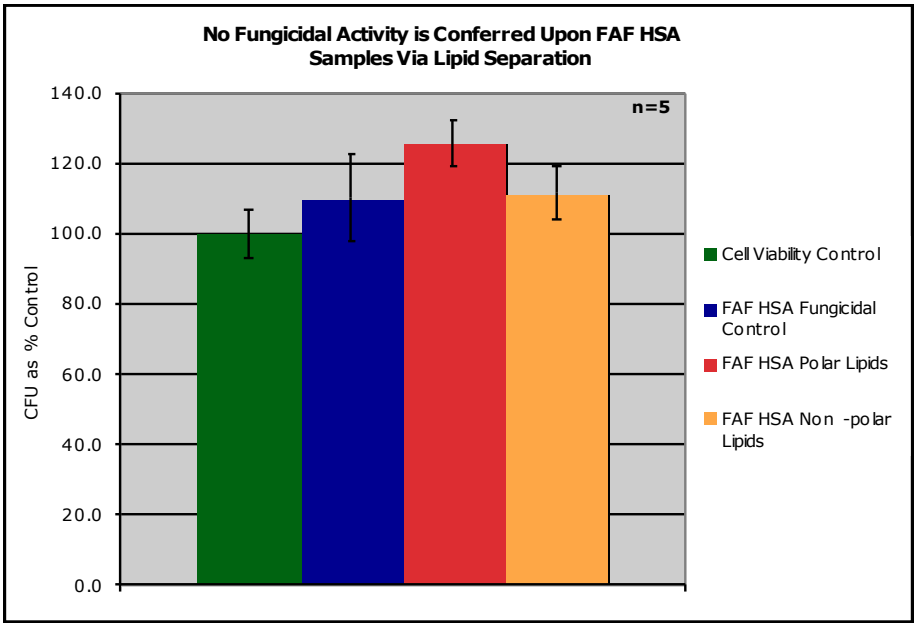

Figure 14: Lipid Separation was performed on the FAF HSA extracts and a subsequent cell viability assay was performed. A non-fractionated FAF HSA extract sample was also run to determine the maximum amount of activity that should be recovered from the two fractions. As expected no fungicidal effect was seen in any of the samples. N=5. Error bars represent Standard Error of the Mean (SEM).

Appendix C: Foundation for the Thesis Hypothesis

The original aim of my graduate research was to isolate and characterize a secreted factor of a cervical-vaginal commensal that prevents *Candida albicans* hyphal formation. Identification of this factor would help explain how *Candida albicans*, a natural commensal of the cervical-vaginal tract as well as a pathogen, is prevented from establishing an infection of its host. The commensal bacteria were investigated for secreted factors due to research conducted by J.D. Sobel that showed that vulvovaginal candidiasis is marked by an ablation of the normal bacterial flora (sobel 1988). Whether or not the loss of the normal vaginal flora occurs before, after, or during the establishment of an infection is unknown. If the loss of the healthy vaginal flora occurs before the onset of vulvovaginal candidiasis, then it is possible that the loss of the normal flora and its secreted products may give *Candida albicans* an infection opportunity. Clinical studies also revealed that with the onset of *Gardnarella vaginallis* induced bacterial vaginitis, there is a significant reduction in the concentration of *C. albicans* in the vaginal flora (Sobel 1988).

A collection of clinical commensal isolates provided by ECI Biotech was cultured in aliquots of a Simulated Vaginal Growth Media (Geshnizgani and Onderdonk). This collection of isolates was, for the most part, comprised of *Lactobacillus spp.*, the predominate commensals of a healthy vaginal flora, and *Gardnarella vaginallis* strains. The spent media provided from these cultures were than divided and processed for either protein purification or organic extractions. Protein purification experiments were utilized to isolate any proteins or peptides that might affect the *C. albicans* filamentation response,

while organic extractions were used to extract any small molecules that might affect the filamentation response.

In preliminary experiments, it was revealed that the organic extraction control (an N-butanol extraction of the Simulated Vaginal Growth Media, SVGM) exhibited fungicidal activity when introduced into a *Candida albicans* viability assay (data not shown). Upon testing the individual components of the SVGM, it was discovered that the fungicidal activity was only present in the albumin component of the media (results not shown). Searches of the literature showed that there was no known antifungal naturally produced by mammals and associated with serum albumins. Investigation and characterization of the molecule developed into the research presented in this thesis.

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