ANTIMICROBIAL SPECTRUM DETERMINATION OF THE K5 TYPE YEAST KILLER PROTEIN AND ITS KINETICS OF CELL KILLING

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ABSTRACT

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Some yeast strains under certain conditions secrete into the medium polypeptide toxins which are inhibitory to sensitive cells. These yeast strains are termed as killer yeasts and their toxins are designated as killer proteins or killer toxins. Killer proteins are classified into 11 typical types (K1-K11). These toxins have different killing mechanisms on sensitive cells. Some of them hydrolyze major cell wall component β-1,3- glucans. As mammalian cells lack cell walls research and development of novel highly selective antifungals are mostly focused on the agents which target the components of the fungal cell wall. We have previously characterized the K5 type killer protein. This protein is an exo β-1,3-glucanase which is stable at pH's and temperatures appropriate for its medical usage. β-1,3-glucan hydrolyzing activity of the K5 type killer protein highlighted the potential use

of this protein as a selective antimycotic agent. Antifungal activity of the K5 type yeast killer protein was tested against 26 human pathogenic yeast and 9 dermathophyte strains and found to be affective on all of the tested strains. Toxin MIC_{50}, MIC_{100} and MFC values were found to be between 0.25-4, 0.5-8, 1-8 µg/ml respectively except *Candida krusei* isolates. Cell killing analysis revealed that toxin activity starts within first 2 hours and complete cell death time differs due to the susceptibility of strains to the K5 type yeast killer protein. K5 type yeast killer protein would be used as a novel and selective agents with the results obtained from this study.

Key words: Antimycotic agent, K5 type yeast killer toxin, human pathogenic fungi, MIC, MFC

ÖZ

K5 TİPİ ÖLDÜRÜCÜ MAYA PROTEİNİNİN ANTİMİKROBİYAL ETKİ SPEKTRUMUNUN TESPİTİ VE HÜCRE ÖLDÜRME KİNETİĞİ

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Bazı maya suşları belirli şartlar altında ortama duyarlı hücreleri inhibe edici polipeptid toksinler salgılarlar. Bu tip mantarlara öldürücü mantarlar ve salgıladıkları toksinlere öldürücü proteinler veya öldürücü toksinler adı verilmiştir. Öldürücü proteinler 11 değişik tip altında sınıflandırılmışlardır (K1-K11). Bu toksinlerin hassas hücreler üzerindeki etki mekanizması farklılıklar gösterir. Bazıları hücre duvarındaki β-1,3-glucan yapısını hidrolize eder. Memeli hücreleri hücre duvarına sahip olmadığı için yeni ve seçiciliği yüksek antifungalların araştırma ve geliştirilmesi mantarların hücre duvarı bileşenlerini hedef teşkil eden ajanlar üzerinde yoğunlaşmaktadır. Laboratuvarımızda yaptığımız çalışmalarda *P.anomala* suşunun öldürücü etkisinden sorumlu olan K5 tipi öldürücü toksinin biyokimyasal

karakterizasyonu sonucunda bu toksik proteinin tıpalanında kullanım için uygun sıcaklık ve pH şartlarında aktivitesini koruyan bir exo β-1,3-glukanaz olduğu bulunmuştur. K5 tipi öldürücü proteinin duyarlı hücrelerin, hücre duvarlarındaki β-1,3 glukan yapısını hidrolize etme özelliği, bu proteinin seçici bir antimikotik ajan olarak kullanımını önemli ölçüde arttırmaktadır. K5 tipi öldürücü maya proteininin antifungal aktivitesi NCCLS metoduna göre 26 insan patojenik maya ve 9 dematofit suşu üzerinde denenmiştir ve bütün test edilen suşlarda etkili olduğu bulunmuştur. Toksin $MİK_{50}$, $MİK_{100}$ and MFK'lari, MFK'nu 16 µg/ml' den fazla olan *Candida krusei* izolatları haricinde, sırasıyla 0.25-4, 0.5-8, 1-8 µg/ml arasında olduğu saptanmıştır. Hücre öldürme analizleri toksin aktivitesinin ilk 2 saat içinde başladığını ve hücre ölümünün gerçekleştiği zamanın suşların K5 tipi öldürücü maya proteinine duyarlılıklarına bağlı olduğunu ortaya koymuştur. Bu çalışma K5 tipi öldürücü maya proteininin medikal uygulamalar için kullanılmasının önünü açacaktır.

Anahtar kelimeler: Antimikotik ajan, K5 tipi maya öldürücü proteini, insan patojen mantarları, MİK, MFK

To My Family

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ATCC	American Type Culture Collection
bp	Base pair
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DSMZ	German National Resource Centre for Biological Material
DTT	Dithiothreitol
HPLC	High Performance Liquid Chromatography
kb	Kilo base
kDa	Kilo dalton
K_m	The Michaelis constant, is the substrate concentration required to reach half-maximal velocity ($V_{max}/2$).
L dsRNA	Large size double stranded RNA
MIC_{50}	Minimum concentration required to inhibit the growth of 50% of the cells
MIC_{100}	Minimum concentration required to inhibit the growth of all the cells
MFC	Minimum fungicidal concentration
MWCO	Molecular Weight Cut-Off
M dsRNA	Medium size double stranded RNA
NCCLS	National Comitee for Clinical Laboratory Standarts
NCYC	National Collection of Yeast Cultures
TEMED	N, N, N' N'- Tetramethylethylenediamine
%T	Ratio of the sum of the weights of the acrylamide monomer and the cross linker in the solution , expressed as %w/v
%C	The ratio of cross-linker to acrylamide monomer

CHAPTER I

INTRODUCTION

Fungi are eukaryotic microorganisms. They exist as yeasts, moulds, or as a combination of both forms. Yeast cells are typically single, small and oval, and mostly reproduce asexually by a process called budding. A bud is formed on the outer surface of the parent cell as the nucleus divides. One nucleus migrates into the elongating bud. Cell wall material forms between the bud and the parent cell and the bud breaks away (Figure 1.1.) [1]. Yeasts can also reproduce sexually by a process called mating. Sexual spores (a and α) called ascospores which result from the fusion of the nuclei from two cells followed by meiosis (Figure 1.2.). Sexual reproduction is much less common than asexual reproduction but does allow for genetic recombination [2].

The most well-known and commercially significant yeasts are the related species and strains of *Saccharomyces cerevisiae* [2].Strains of *Saccharomyces cerevisiae* are known as an ideal model of eukaryotic microorganisms for biological studies. The suitability of this organism is due to its ease of growth, non pathogenicity and also it can even be obtained in bulk as Baker's or Brewer's yeasts if necessary [3,4]. Other advantages of yeast cells used for biological studies are well defined genetic system and highly versatile DNA transformation system [3].

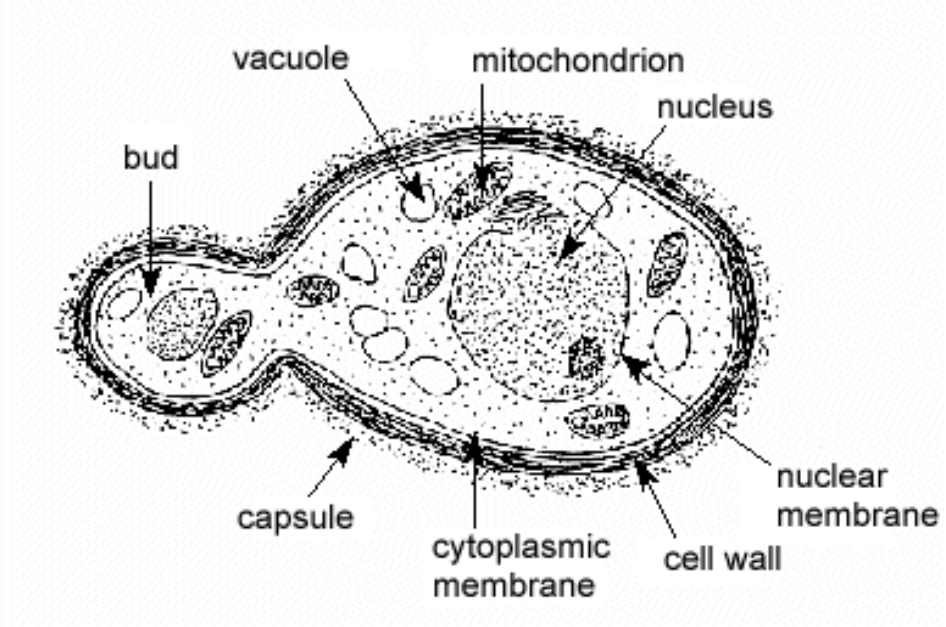

Figure 1.1. Budding of a Yeast Cell [5].

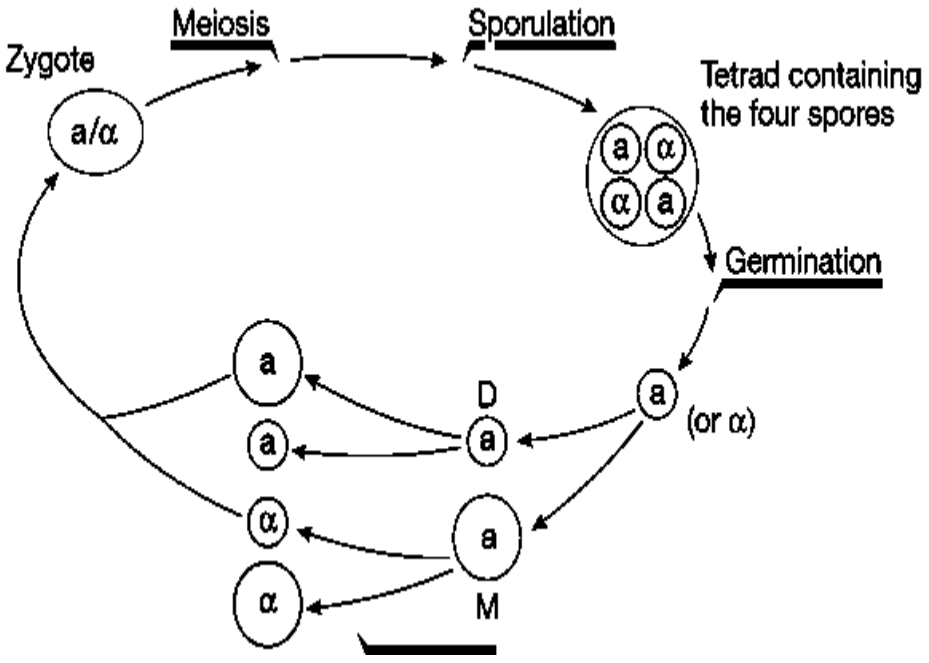

Figure 1.2. Sexual Reproduction of a Yeast Cell [6].

Yeast cells are widely used in industry other than biological studies (Figure 1.3.). They are either cultured for the cells themselves, for cell components or for the end products that are produced during the alcoholic fermentation [7]. Industrial usage of yeast cells includes bread making, food supplements, animal feed, alcohol and glycerol production, enzyme production, vitamin production, production of beer, whiskey, wine, brandy, vodka, rum along with environmental technologies [7,8].

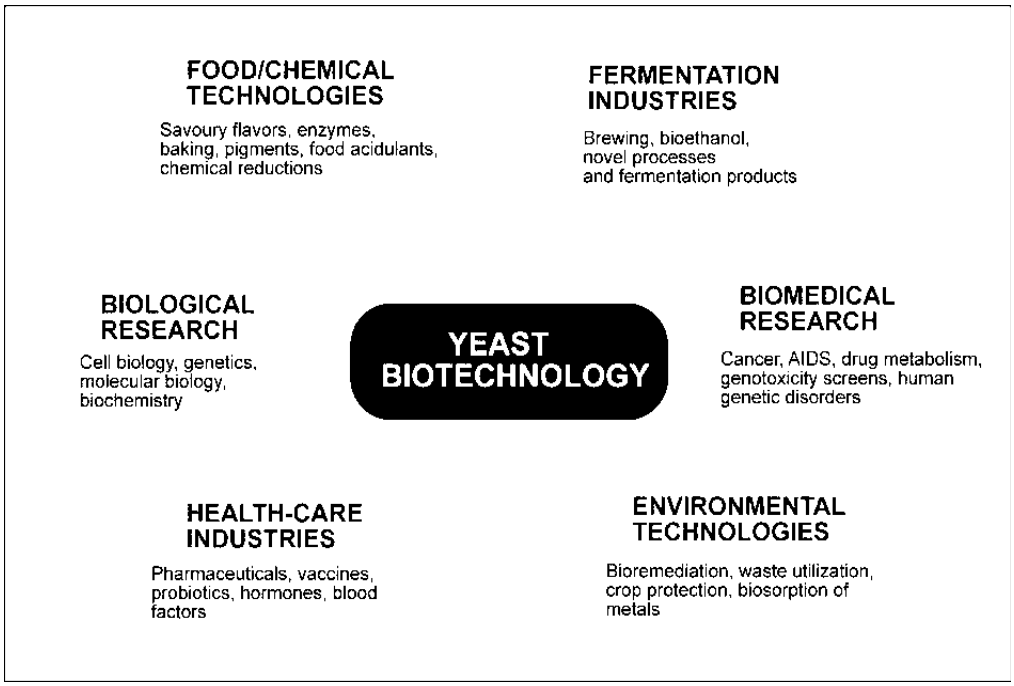

Figure 1.3. Yeast Biotechnology [8].

Moreover *S. cerevisiae* and other yeasts have been widely used in medical applications and production of therapeutics since their high level of protein expression is an advantage for the production of medically important proteins. These therapeutic proteins include hormones, growth factors, blood proteins, enzymes and interferons [8].

Powerful antifungal and antimycotic proteins are naturally produced by a diverse group of organisms including bacteria, fungi, insects, vertebrates and invertebrates as well as plants. Some yeast strains under certain conditions secrete polypeptide toxins into the medium. These yeast strains are termed as killer yeasts and their toxins are designated as killer proteins or killer toxins. These extruded killer proteins inhibit the growth of the same species or of different species of a single genus, and sometimes even representatives of other genera that are sensitive to them [9-11]. Also killer toxins of certain yeast strains have potential growth inhibitory affect on gram positive pathogenic bacteria and plant pathogenic fungi [12, 13]. Production of these protein toxins confers an advantage to the killer strains in competition with the sensitive cells for the available nutrients in the environment [14, 15]. Killer proteins are mostly glycosylated and their production strictly depends on the pH and temperature of the medium [16-20].

The killer phenotype (K+) was first discovered by Bevan and Makower in 1963 in *Saccharomyces cerevisiae* [21]. Further studies showed that this killing character is widely distributed among yeast genera including the species of the genus *Candida, Cryptococcus, Debaryomyces, Hansenula, Hanseniaspora, Kluyveromyces, Pichia, Rhodotorula, Torulopsis, Trichosperon, Ustilago, Williopsis* and *Zygowilliopsis* [22-29].

These proteins are first classified into 10 different types (K1-K10) by Young and Yagiu [14]. A new killer protein producing strain (K11) was later added to this classification [30]. This classification is based on the killing spectra and immunity cross reactions of the strains. Killer strains and their proteins are shown in Table 1.1. Other than killer yeasts, non-killer strains that do not secrete a killer protein but immune to a particular killer protein were also isolated.

Table 1.1. Killer Yeasts and Their Protein Toxins [14].

Killer Protein Producing Strain	Classification
Saccharomyces cerevisiae	K1
Saccharomyces cerevisiae	K2
Saccharomyces capensis	K3
Candida glabrata	K4
Pichia anomala	K5
Kluyveromyces fragilis	K6
Candida valida	K7
Hansenula anomala	K8
Hansenula mrakii	K9
Kluyveromyces drosophilarum	K10
Candida glabrata	K11

Other killer proteins that are not included in this classification are also produced by different killer yeast strains such as *Williopsis mrakii* and *Kluyveromyces lactis.*

1.1. Genetic Basis of Killer System

The entire genome sequence of *Saccharomyces cerevisiae* was released in April, 1996 and was the first eukaryotic genome to be sequenced. The haploid genome contains 16 chromosomes ranging in size from 200 to 2,200 kb with a total genome size of approximately 13,000kb [3,31].

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The 2 μm-plasmid and mitochondrial DNA are found as two additional sources of DNA in yeast. The 6.3 kb 2 μm-plasmid has no known function in

yeast other than to replicate. The 2 μm-plasmid accounts for about 5%, mitochondrial DNA 10 %, and chromosomes 85 % of the total yeast DNA [3, 31]. Yeast strains also carry as many as five non-homologous species of ds RNA, known as L-A, L-BC, T, W and M. All show non-Mendelian inheritance [32].

Table 1.2. Basic Features of the Yeast Genome [31].

Genome size	12,5 Mb + rDNA
Single copy DNA	12,0 Mb
Length of genetic map	>4300 cm
Number of chromosomes	16
Organelle DNA	Mitochondrial, 75 kb
Extrachromosomal elements	2-micron DNA, double stranded RNA

The genomes of the killer proteins have been mapped either on ds RNA molecules, a linear ds DNA plasmid or a chromosome. [33].

1.1. a. ds RNA Encoded Yeast Killer Proteins

Double stranded RNA viruses were the first investigated agents responsible for the killing activity. The infection of the yeast ds RNA viruses is only possible via cell to cell fusion. These ds RNA's are found in the cytoplasm and they are surrounded by a protein coat and thus termed as yeast viruses. M ds RNA virus like particles are responsible for the killer toxin production and immunity. There are 3 types of M ds RNA viruses; M1, M2 and M3 which are responsible for the production of K1, K2 and K3 killer proteins respectively [32]. Another ds RNA

named as L-A helper virus which is 3 times larger than M is responsible for the encapsidation and replication of M ds RNA [31].

1.1.b. Linear ds DNA Encoded Yeast Killer Proteins

In some yeasts killer character is encoded by the linear ds DNA plasmids. These linear ds DNA plasmids have been identified for various yeast genera such as *Debaryomyces, Wingea, Kluyveromyces* and *Saccharomyces*. The best characterized example for ds DNA plasmids are pGKL1 and pGKL2 which are found in *Kluyveromyces lactis*. The killer protein of *K. lactis* is encoded by the pGKL1 plasmid and its molecular mass is 180 kD. pGKL2 is thought to play a role in the replication of pGKL1 [34-36].

1.1.c . Chromosomally Encoded Yeast Killer Proteins

In some killer yeasts such as strains of *Williopsis, Pichia, Candida, Debaryomyces* and *Torulopsis* the killer character is not encoded by neither ds RNA viruses nor ds DNA plasmids. Thus it is suggested that genes responsible for killer character are located on a chromosome [37]. In one of the killer strains of *Saccharomyces cerevisiae*, the killer character was found to be encoded by two different genes which are mapped on chromosome V and chromosome IX [38].

The genetic basis for the expression of killer proteins in different killer yeast strains is given in Table 1.3.

Table 1.3. Genetic Basis for Killer Phenotype Expression [33].

Killer Yeasts	Genetic Basis
Saccharomyces cerevisiae	ds RNA
Hansenula uvarum	ds RNA
Zygosaccharomyces bailii	ds RNA
Ustilago maydis	ds RNA
Kluyveromyces lactis	Linear ds DNA plasmid
Pichia acaciae	Linear ds DNA plasmid
Pichia inositovora	Linear ds DNA plasmid
Pichia kluyveri	Chromosomal
Pichia farinosa	Chromosomal
Pichia anomala	Chromosomal
Williopsis mrakii	Chromosomal

1.2. Structure, Processing and Secretion of the Killer Proteins

Killer yeasts and their toxins have been used as model systems in fundamental research for studying the mechanisms of regulation of eukaryotic polypeptide processing, secretion and receptor binding. The killer system in yeast is thought to be closely parallel to the synthesis and action processes of mammalian hormones and neuropeptides [39].

Toxin secretion pathways are fully identified for K1 and K28 which are both secreted by *S. cerevisiae.* Although these two killer proteins have different amino acid compositions and mode of action; their synthesis, processing and secretion shows significant homologies. K1 and K28 toxins are both encoded by ds RNA viruses and composed of two subunits named as α and β. Killer toxins are initially translated as preprotoxin which undergoes post-translational

modifications within the endoplasmic reticulum, and the golgi complex until it is finally secreted as mature α/β heterodimeric protein toxin [33].

Preprotoxin enters the endoplasmic reticulum with the help of a highly hydrophobic signal peptide in the N-terminal region. In the endoplasmic reticulum cleavage of the leading hydrophobic sequence by a signal peptidase and glycolysation of γ subunit take place. In a late golgi compartment the Kex2p, which is the product of KEX2 gene, cleaves the proregion and removes the intramolecular γ sequence. A carboxypeptidase Kex1p, which is the product of KEX1 gene, removes the dipeptide region on the C terminal of the α subunit. Then mature toxin is transferred to a secretory vesicle and secreted out of the cell. Secretion process is possible with the products of SEC genes [31-33, 40].

At the same time, the killer cell is effectively protected against its own toxin. It is well known that the unprocessed toxin precursor is sufficient to confer protective immunity. However, the detailed mechanism of immunity is still largely unknown; it may be due to the binding of an immunity sequence to the toxin receptors of the producer cell. Thus the killer protein is inhibited from binding to the producer cell [33]. Secretion pathway of K28 is shown in Figure 1.4. and processing of K1 type yeast killer toxin is shown in Figure 1.5.

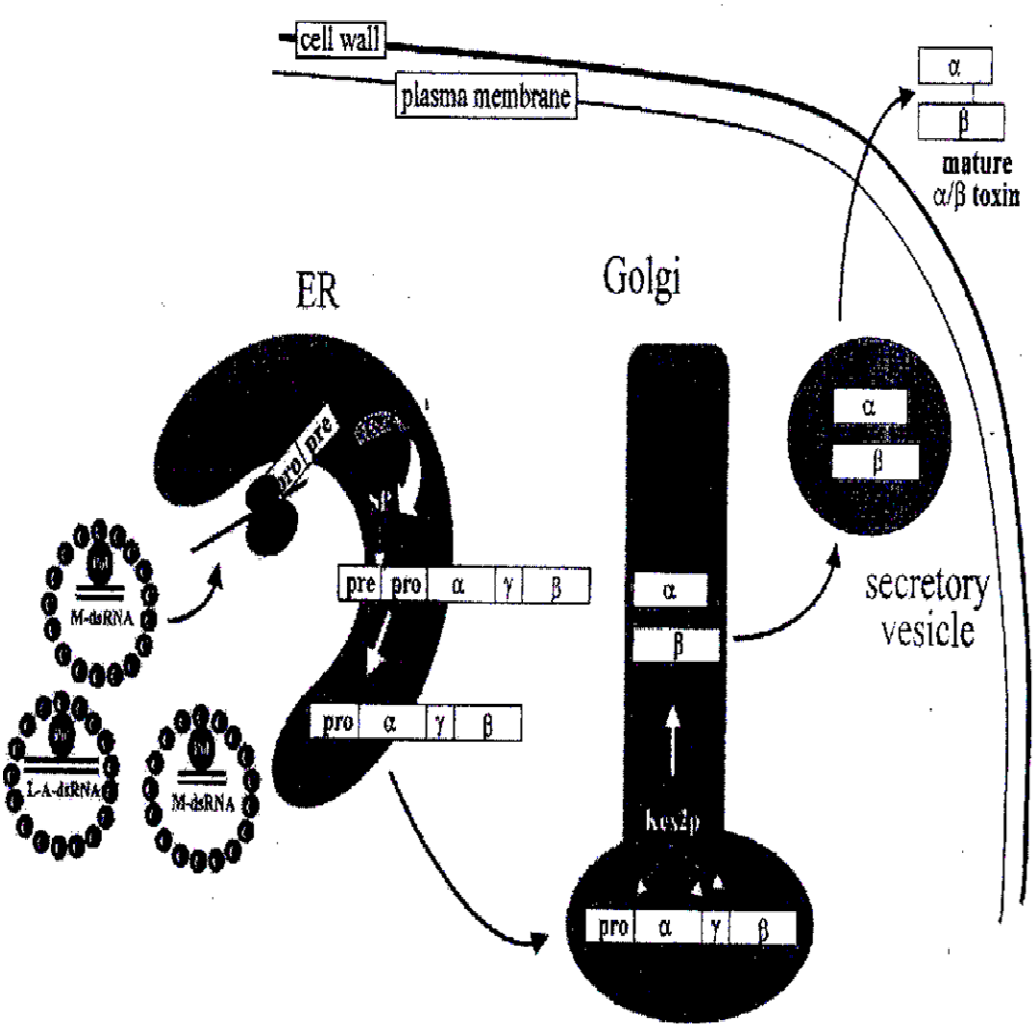


Figure 1.4. Secretory Pathway of Killer Toxin K28 in *S. cerevisiae* [33].

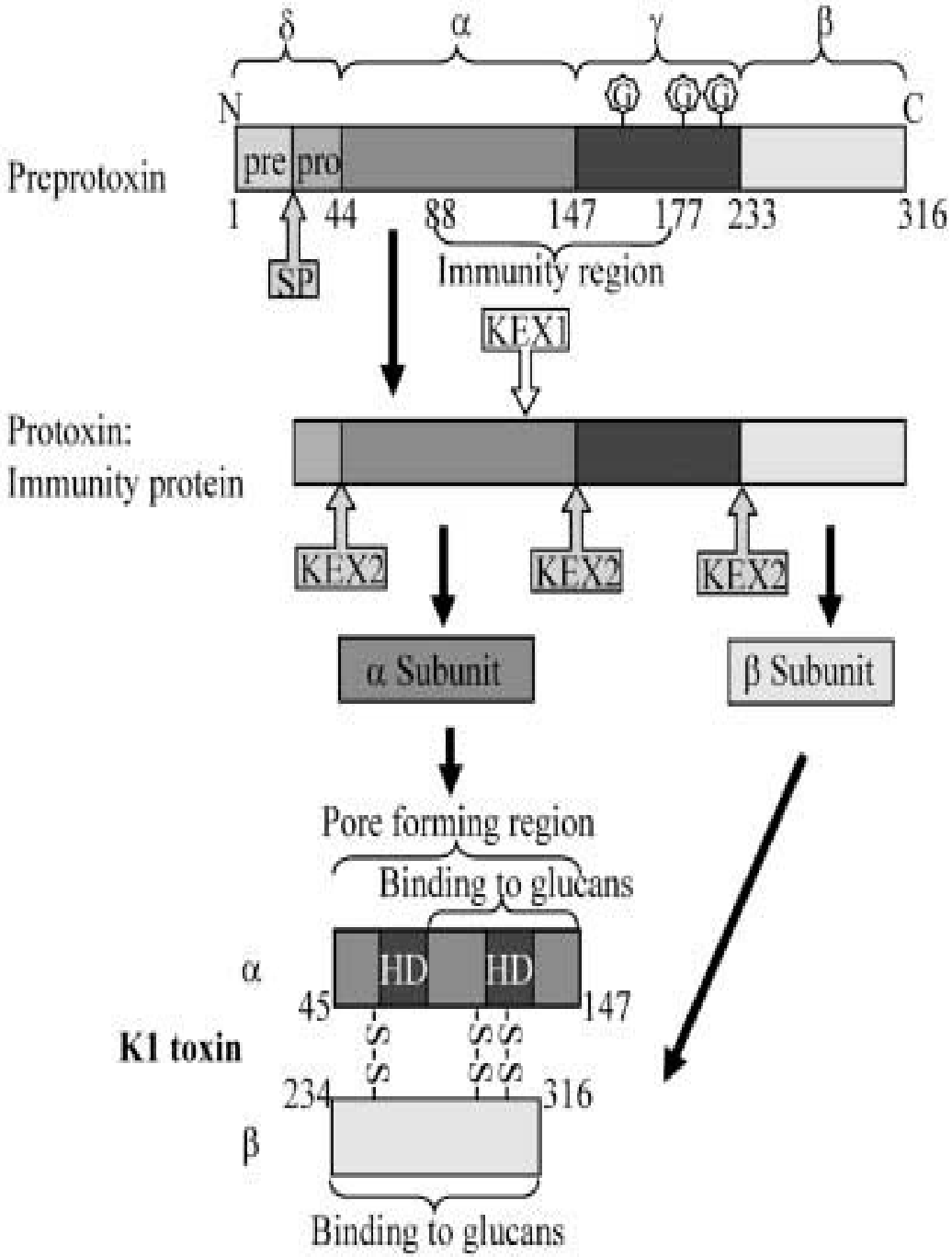


Figure 1.5. Structure and Processing of the K1 Type Yeast Killer Toxin [41].

1.3. Mode of Action of the Yeast Killer Proteins

Killing activity of the yeast killer proteins involves 2 steps. Although actual cell killing mechanisms of these toxins show significant differences, they all use the yeast cell wall components as receptors. The binding of the killer protein to a cell wall receptor is the first step of the killing activity. This step is fast and energy dependent [33].

Yeast cell wall (Figure 1.6.) is mainly composed of mannoproteins, β- glucan backbone containing linear or branched β-1,3 and β-1,6, glucans and some chitin [42]. It has been identified that these components serve as primary binding sites and cell wall receptors for the killer toxins [10, 43, 44].

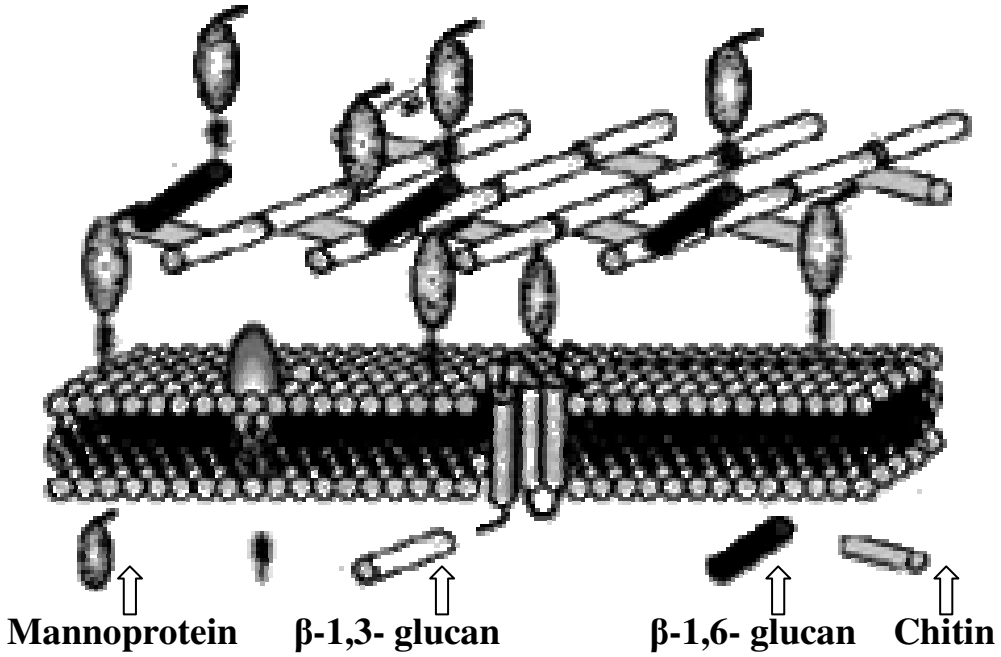


Figure 1.6. Yeast Cell Wall Structure [45].

Killer toxins of *Debaryomyces hansenii, Hanseniaspora uvarum, Pichia membranifaciens* and *Saccharomyces cerevisiae* (K1, K2) bind to β-1,6-glucan residues on the yeast cell wall as primary receptors whereas toxins of *Saccharomyces cerevisiae* (KT28), *Schwanniomyces occidentalis* and *Zygosaccharomyces bailii* bind to mannoproteins. Two other killer toxins secreted by *Pichia acaciae* and *Kluyveromyces lactis* use chitin residues as receptors [41].

Actual cell killing step takes place after binding to the cell wall receptors. Killer toxins kill the sensitive cells by different mechanisms.

K5 type yeast killer toxin produced by *Pichia anomala* inhibits the growth of sensitive microbial cells by hydrolyzing the major cell wall component β-1,3-glucan residues which results in cell burst [46]. Another mechanism that affects the cell wall of fungal cells is the inhibition of β-1,3-glucan synthase which is required for the synthesis of β-1,3-glucans. Killer protein of *Hansenula mrakii* causes pore formation by inhibiting the β-1,3-D-glucan synthesis occurring at a budding site which results in leakage of cell material and eventual cell death [33, 47].

The affect of the killer proteins on cell membrane is due to the formation of non-selective ion channels that leads to sudden K^+ and ATP leakage. These toxin induced channels alter the gradient of the cell membrane and result in sudden cell death. Killer toxin produced by *Pichia kluyveri* exerts its killing effect on sensitive microbial cells by ion-channel formation [48].

Yeast cells in their S phase when treated with certain killer toxins, such as killer toxin of *Kluyveromyces lactis,* do not divide although they complete their cell cycles. Thus they increase in size since the normal metabolic activities continue and they are arrested in G1 phase [49, 50].

Some other toxins inhibit the DNA synthesis as it has been observed that during cell division, DNA had not been transferred to the spores of the toxin exposed sensitive cells. K28 killer toxin produced by *Saccharomyces cerevisiae* causes arrest of the sensitive cells at G2 phase which results in the daughter cells lacking DNA molecules [51].

1.4. Applications of Yeast Killer Proteins

Several potential applications for the killer yeasts and their toxins have been studied and suggested. They can be used as antifungal agents against both human and plant pathogens because of their wide killing spectrum on sensitive cells. Also they are used in biotechnological processes such as fermentation, to combat with the contaminating sensitive strains, and recombinant DNA technology [44].

1.4.1. Biotyping of Pathogenic Yeasts

Killer yeasts have found applications in the biotyping of the pathogenic yeasts such as *Candida albicans* and *Cryptococcus* species by using their sensitivity or resistance to a wide range of killer toxins, especially those from *Saccharomyces, Pichia, Kluyveromyces* and *Candida*. The same methods are also approved applicable to classifying actinomycetes [52, 53].

1.4.2. Secretion Vectors

The killer trait can be used to produce large amounts of foreign proteins in yeast. Several heterologous genes, including genes coding biotechnologically important enzymes, have been expressed using killer plasmids as vectors. The toxin cDNA clone has been used to construct a secretion vector with the signal

region of the toxin. *Cellomonas fimi* yeast cells, constructed with the killer toxin cDNA, was used to secrete carboxymethylcellulase [54]. In this respect linear plasmids (pGKL1 and pGKL2) of *K. lactis* have some major advantages, when compared with circular plasmids such as their high copy number and extreme stability. Their cytoplasmic localization makes them independent of nuclear control in terms of replication and transcription. h-TPA (human tissue plasminogen activator) cDNA was fused with the leader sequence of *K. lactis* killer toxin in order to achieve high level expression of h-TPA in yeast [55].

1.4.3. Brewing Strains

Introduction of the killer trait into fermentation strains can prevent the spoilage of the product by other yeast strains such as *Candida, Hanseniaspora, Kloeckera* and *Pichia* in mixed culture. If the brewing strain is capable of mating, killer character can be introduced by cytoduction [56]. The transfection of the brewing strain with isolated viral particles is another alternative that does not require mating [57]. Wide cell killing spectrum of the killer proteins is a major advantage in protection of the fermentation products. Also introduction of the killer character to brewing strains does not affect the fermentation ability of the strain. Killer character was introduced to the strains of *Saccharomyces cerevisiae* which is used as the starter strain of wine production. It was observed that the resulting killer strain has a potential competitive advantage over other sensitive and killer strains of *S. cerevisiae* in wine fermentation [58].

1.4.4. Food Preservation

Growth of moulds in food and animal feed leads to reduced nutritional values and production of allergenic spores and hazardous mycotoxins. Killer yeasts are proposed as promising biocontrol agents, providing alternatives to chemical fungicides in the postharvest storage of fruits and vegetables. Killer

strains of *Pichia anamola* was found to be effective on *Debaryomyces hansenii* and *Saccharomyces cerevisiae* which are responsible for the most of the fungal infections in storage of wheat. Also this killer strain was found to be affective on 17 other food pathogenic fungal strains depending on the dose of inoculation [59]. *Botrytis cinerea* which is responsible for the infections in citrus and apple was also found to be susceptible to the killer toxin of *P. anomala* [60].

1.4.5. Medical Use

The incidence of fungal infections is increasing at a high rate along with the challenge in therapies. This increase is directly related to the growing population of immunocompromised patients due to intensive chemotherapy, immunosuppressive drugs, HIV and other diseases which also cause immunosuppression. These types of infections can be mortal in immunosupressed patients [61-65].

One of the most frequently seen infections is the superficial fungal infections. These infections occur at the outer most layer of the skin, nails, hair and mucous membranes. Dermathophytes; especially *Trichophyton* spp., *Microsporum* spp., and *Epidermophyton* spp. are responsible for most of the superficial fungal infections although yeasts and some non-dermathophyte moulds can also be causative agents [66-68]. Dermathophyte infections are named according to the site of infection such as tinea pedis, infection of the feet; tinea corporis, infection of the body surface; tinea capitis, infection of the head. These names are not related with the causative agent since for example tinea pedis can be caused by either *Microsporum* spp. or *Trichophyton* spp. [67]. Dermathophytes that are involved in the superficial infections and related diseases are given in Table 1.4.

Table1.4. Superficial Infections and Causative Pathogens [66].

Disease	Typical Causative Pathogen
Tinea pedis (feet)	*Trichophyton rubrum* *Trichophyton mentagrophytes*
Tinea capitis (scalp)	*Trichophyton tonsurans* *Microsporum audouinii*
Tinea unguium (nails)	*Trichophyton tonsurans* *Trichophyton mentagrophytes* *Candida* spp.
Mucosal candiasis (oral and genital candidiasis)	*Candida* spp Usually *Candida albicans*

These infections are initially treated with topical antifungal agents such as azoles or allylamines since they infect the outer layer of the skin [69].

Another group of fungal infections is the systemic fungal infections. These infections are primarily caused by opportunistic fungal pathogens especially when some aspects of the normal host defense are compromised. Such infections are life threatening and associated with high rates of death [66, 70]. *Candida albicans* among all the other species of the genus *Candida* is responsible for most of the systemic fungal infections. This pathogenic strain basically can attack nearly every organ in the body and is the most common yeast species isolated from blood. Non- *Candida albicans* isolates are considered as new and emerging pathogens. These emerging pathogens include *Candida parapsilosis, Candida glabrata, Candida krusei, Candida guilliermondii* and *Candida keyfr* [71]. Most of these pathogens are resistant to the conventionally used antifungal drugs which contribute to the arising incidence of related infections. Also mucosal colonization of the pathogenic *Candida* species

induces the risk of systemic candiasis [66]. The antifungal agents that are used in the therapy of these infections are limited to azole derivatives, amphotericin B and 5-fluorocytosine [71].

Many diversely produced natural peptides, as well as those produced semi-synthetically and synthetically, have been found to inhibit the growth or even be lethal to a wide range of fungi. Some of these have the potential to aid mankind in combating with mycoses caused by emerging pathogens or as a result of the increasing number of antifungal-resistant fungi. Killer proteins are proposed as novel antifungal agents. Especially toxins of the genus *Hansenula*, *Pichia*, and *Kluyveromyces* have a wide range of killing spectrum on human and animal fungal pathogens [13, 72-76]. Thus the use of killer toxins as antifungal agents gained considerable importance. Also some killer yeast strains have potential growth inhibitory activity on gram-positive pathogenic bacteria such as *Streptococcus pyogenes, Bacillus subtilis, Sarcina lutea* and *Staphylococcus aureus* [20]. Although cell killing activity of the yeast killer proteins highlighted the use of these proteins as novel antifungal agents, studies on killer proteins are limited to the interaction of pathogenic fungal cells with the killer protein producing strains. Further studies with the purified killer proteins are required for the potential use of these agents as novel antifungal agents.

The antimycotics which are currently used in the therapy of fungal infections such as amphotericin B, fluconazole, ketoconazole, itraconazole cause severe side effects on the patients because these agents disrupt the integrity of the eukaryotic cytoplasmic membranes and thus damage the infected host organism (Table 1.5.) [77]. Therefore development studies on novel antifungal drugs are focused on selective agents which target the components of yeast and fungal cell walls. These agents would not harm the host cells since mammalian cells lack these components. [46, 72]. Cell wall components of the fungal cells are composed of mannoproteins, β- glucan backbone and chitin [42] and these components serve as primary binding sites for the yeast killer toxins [10, 43, 78]. Thus these protein toxins are suggested as highly selective antifungal agents

[44, 79]. Also beside these side affects of the currently used antifungal drugs even in short term applications, microorganisms gain resistance to them and these resistances spread rapidly among other microorganisms which are pathogenic to man and cause problems in the therapy of those pathogens [80-82].

Table 1.5. Side Affects of the Currently Used Antifungals. [45]

Drugs	Side affects
Polyenes	
Amphotericin B	Nephrotoxicity, fever, chills, phlebitis, anaemia, GI disturbance
Fluorinated pyrimidines	
Flucytosine	Bone marrow suppression, hepatotoxicity, GI disturbance
Azoles	
Saperconazole	GI disturbance, hepatotoxicity
Imidazoles	
Miconazole	Headache, pruritus, thrombophlebitis,
Ketaconazole	Hepatotoxicity
Triazoles	
Itraconazole	Hepatotoxicity
Fluconazole	Hepatotoxicity, Stevens-Johnson syndrome

The need to develop new classes of antifungal agents never ceases to expand. It seems primarily from the continuous rise in the incidence of fungal infections and the limited number of effective side effect free antifungals. The development of resistance to antifungal drugs, currently appreciated as an emerging problem, makes the search for newer agents critical.

1.5. K5 Type Yeast Killer Protein

K5 type yeast killer protein is produced by *Pichia anomala* NCYC 434 according to the Young and Yagiu classification.

Pichia anomala is a fungi belonging to the phylum; *Ascomycetes*, class; *Hemiascomycetes*, order; *Saccharomycetales*, family; *Saccharomycetaceae*, genera; *Pichia* (*Hansenula*), species; *anomala* [83]. *P. anomala* is present in various environments and have been isolated from fruit and plant material, cereal grain, maize silage, and from high sugar food products. *P. anomala* is frequently isolated from wine and has been reported to be non-*Saccharomyces* wine yeast [83].

In several studies *P.anomala* toxins have been reported to have a wide-range intergeneric killing spectrum with relatively high stability in comparison to toxins of other killer yeasts [84, 85]. Among *P. anomala*, the strain NCYC 434 especially has been extensively studied for various applications [86- 88].

Production of K5 type yeast killer protein by *P. anomala* NCYC 434 cells is highly dependent on the composition of the medium. It is stimulated by the presence of β-glucan and amount of the toxin increases in correlation with the amount of the β-glucan and the type of its linkage. Glucose is also required to maintain the highest level of K5 type toxin production [89].

K5 type yeast killer toxin was previously purified and characterized in our laboratory. It is a glycosylated protein with a molecular mass of 49 kDa and with a pI value of 3.7. Temperature and pH stability testing of the toxin shows that the optimum pH value for the K5 type yeast killer protein is 4.5 and about 70 % of the activity remains even at pH 2.5. However toxin is inactivated at pH values above 6.5 (Figure 1.7). The optimum temperature for the toxin activity is 25 ºC. Toxin looses 10 % of its activity at 37 ºC and 50 % at 100 ºC (Figure 1.8.) [46].

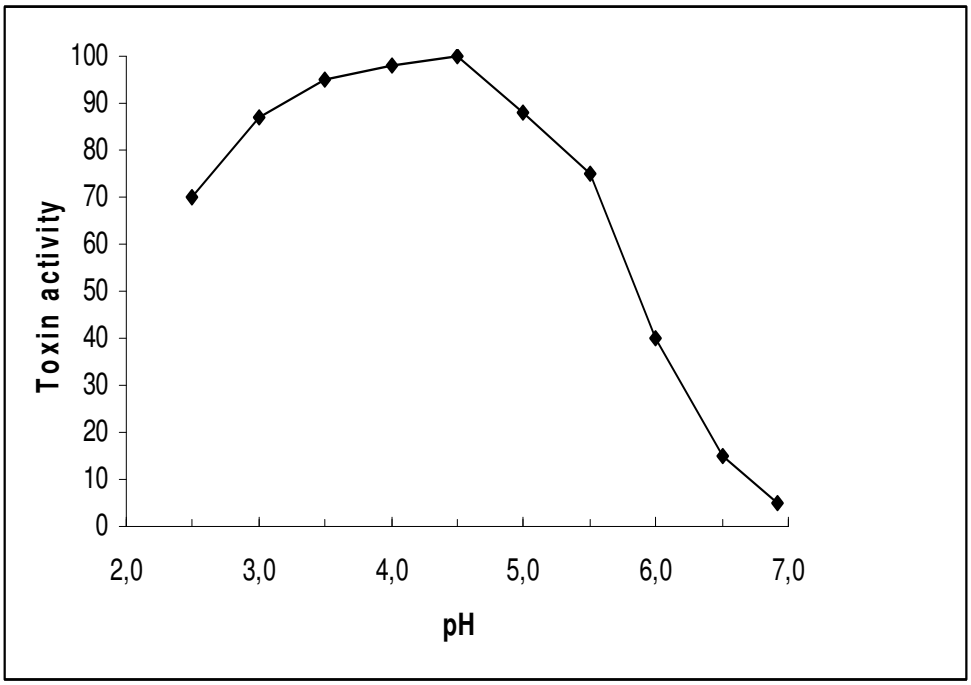


Figure 1.7. pH Stability of the K5 Type Yeast Killer Toxin [46].

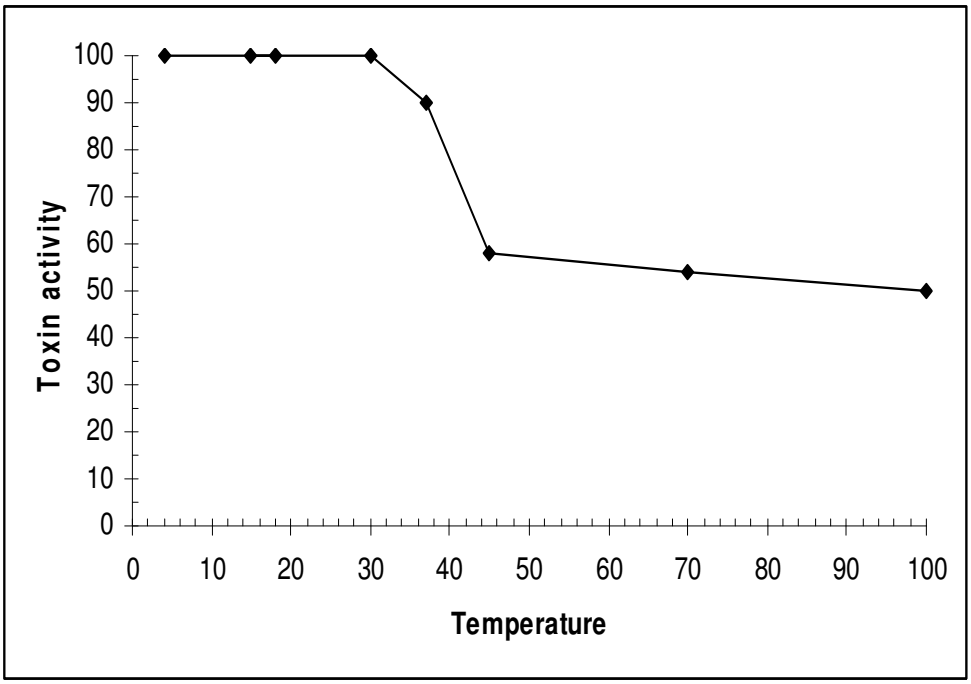


Figure 1.8. Temperature Stability of the K5 Type Yeast Killer Toxin [46].

Internal aminoacid sequences of the toxin shares 100 % homology with the exo-β-1,3-glucanase of *P. anomala* Strain K which is a glycoprotein of 45.7 kDa with a pI of 4.7. (Figure 1.9.) [46].

Mode of action of the K5 type yeast killer protein was also studied by İzgü et. al. K5 type yeast killer protein is mostly absorbed by laminarin which is mainly composed of β-1,3- glucans. This indicates that the toxin exerts its lethal affect by hydrolyzing β-1,3- glucan residues of the cell wall of sensitive fungal cells and causes loss of cell wall rigidity which leads to cell death due to the osmotic pressure. Further studies on its mode of action shows that K5 type yeast killer protein exerts hydrolytic activity on the β-1,3- glucans in an exo like fashion [89].

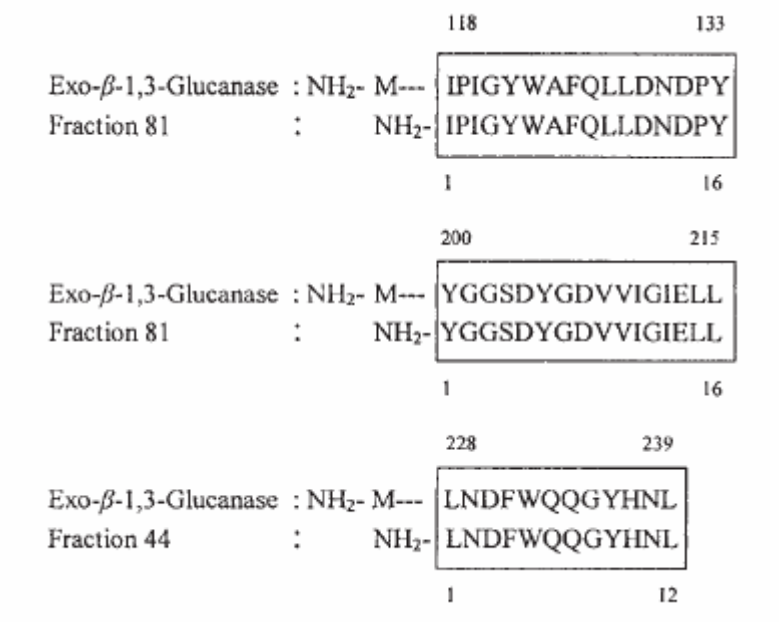


Figure 1.9. Internal Amino Acid Sequence Homology of the K5 Type Yeast Killer Toxin with the Exo-β-1,3-glucanase of *P.anomala* Strain K

Specific activity of the K5 type yeast killer toxin on laminarin is 120 U/mg and the Michaelis Menten constants K_m and V_{max} are 0.25 mg/ml and 370 µmol/min/mg respectively [89].

Affects of metal ions on the enzymic activity of the toxin was also studied. Activity is fully inhibited by Hg^{+2}, but increases with some other metal ions such as Ba^{+2}, Ni^{+2}, Cr^{+2}, Zn^{+2} ; most of all by Pb^{+2} (Table 1.5.) [89].

Table 1.6. Affect of Various Compounds on the Exo- β-1,3- glucanase Activity towards Laminarin.

Affecter	Relative Activity (%)
None	100
Ca^{2+}	115
Mn^{2+}	126
Mg^{2+}	120
Zn^{2+}	117
Pb^{2+}	163
Cu^{2+}	122
Cd^{2+}	134
Ba^{2+}	123
Cr^{2+}	119
Fe^{2+}	132
Ni^{2+}	127
EDTA	100
DTT	115
Hg^{2+}	-

Exo- β-1,3- glucanase activity of the K5 type yeast killer protein on sensitive cells highlighted the potential use of this protein as a highly selective antifungal agent since its hydrolyzing activity is specific to the fungal cell wall and does not affect the host cells in terms of side affects. Also high stability of the K5 type toxin at pH values between 3-5.5 and temperatures up to 37 ºC is appropriate for the use of this protein in medical and industrial biotechnology.

Although activity of the K5 type yeast killer toxin was determined against human pathogenic fungal cells by interaction of *P.anomala* NCYC 434

cells with the pathogenic strains of genus *Candida*, studies with the pure toxin are required to determine the actual antifungal spectrum of the K5 type yeast killer protein.

We have previously isolated and characterized the K5 type yeast killer toxin in our laboratory. K5 type yeast killer toxin was suggested as a novel antifungal agent. Antifungal susceptibility studies of the pure K5 type yeast killer toxin should be performed on human pathogenic fungal species for the use of this protein in medical industry as an antifungal agent with an appropriate formulation. In this study our aim was to determine the antifungal spectrum of the pure toxin along with analyses of its kinetics of cell killing. MIC (Minimum inhibition concentration) and MFC (minimum fungicidal concentration) of the toxin were determined for different *Candida* species and dermathophytes, causing either systemic or superficial fungal infections, to evaluate the cell killing activity of the K5 type yeast killer toxin. Cell killing patterns of the toxin were determined with the analyses of cell killing kinetics on representative strains of the genus *Candida.*

CHAPTER II

MATERIALS AND METHODS

2. 1. MATERIALS

2.1.1. Fungal Strains

The source of the K5 type yeast killer protein, *Pichia anomala,* (NCYC 434, K5) and killer toxin sensitive strain, *Saccharomyces cerevisiae* (NCYC 1006) were purchased from the National Collection of Yeast Cultures, Norwich, U.K. Pathogenic fungal strains which were used in antifungal susceptibility studies are given in Table 2.1. and 2.2.

Table 2.1. Dermatophyte Strains Used in Antifungal Susceptibility Studies.

STRAIN	**STRAIN NUMBER\ SOURCE**	**ISOLATION**
Microsporum audouinii	DSMZ 10649	Human
M. canis	DSMZ 10708	Human skin
M. gypseum	DSMZ 3824	Human
Trichophyton rubrum	DSMZ 4167	Human
T. mentagrophytes	DSMZ 4870	Tinea pedis
T. verrucosum	DSMZ 7380	Human skin
T. interdigitale	DSMZ 12283	Tinea pedis
T. equinum	DSMZ 12284	Human skin
T. tonsurans	DSMZ 12285	Human skin

DSMZ: German National Resource Centre for Biological Material

Table 2.2. Pathogenic Yeast Strains Used in Antifungal Susceptibility Studies.

STRAIN	**STRAIN NUMBER\ SOURCE**	**ISOLATION**
Candida albicans	ATCC MYA 2730	Patient with candidemia
C. albicans	ATCC 10231	Patient with bronchomycosis
C. albicans	ATCC 26555	Patient with mucocutoneus candidiasis
C. albicans	ATCC 36802	Human
C. albicans	ATCC 90028	Human blood
C. albicans	DSMZ 3454	Patient with vaginal candidiasis
*C. albicans**	A1	Patient with Flukanazol resistant bronchomycosis
*C. albicans**	A2	Patient with Azol resistant bronchomycosis
*C. albicans**	A3	Human blood
*C. albicans**	A4	Patient with vaginal candidiasis
*C. albicans**	A5	Patient with vaginal candidiasis
C. parapsilosis	ATCC 90018	Human blood
*C. parapsilosis **	A6	Human blood
C. krusei	ATCC 14053	Human blood
C. krusei	ATCC 6258	Patient with bronchomycosis
*C. krusei**	A7	Human blood
*C. krusei**	A8	Patient with bronchomycosis
*C. guilliermondii**	A9	Human blood
*C. guilliermondii**	A10	Human leg
*C. guilliermondii**	A11	Patient with bronchomycosis
*C. guilliermondii**	A12	Human blood
*C. guilliermondii**	A13	Patient with bronchomycosis
*C. pseudotropicalis**	A14	Cutoneus candidiasis
*C. tropicalis**	A15	Human abscess
C. glabrata	ATCC 90030	Human blood
*S. cerevisiae**	A16	Human

*Clinical isolates were collected either from German Hospital, İstanbul or Gülhane Military Medical Academy, Ankara.

ATCC: American Type Culture Collection, USA

A: Our laboratory collection number

2.1.2. Culture Media

Fungal cells were grown in YEPD medium at pH 5.5 consisting of 1 % Bacto-yeast extract, 1 % Bacto-peptone and 2 % dextrose along with 2 % Bacto-agar or Sabouraud medium at pH 6.0 containing 1 % Bacto-peptone and 2 % dextrose along with 2 % Bacto-agar for the maintenance and routine growth. *P. anomala* cells were grown in YEPD medium buffered to pH 4.5 with phosphate citrate buffer with the addition of 5 % glycerol for the production of the K5 type yeast killer protein. Killer activity was determined in YEPD medium with 2 % Bacto-agar buffered to pH 4.5 with phosphate-citrate buffer.

Prior to antifungal susceptibility studies all pathogenic fungal cells were subcultured in potato dextrose agar (0,4 % potato extract, 2 % dextrose and 5 % agar) plates to ensure purity and viability. Synthetic RPMI 1640 medium with phenol red and without sodium bicarbonate dissolved in pH 4.5 100mM Na_2HPO_4- citric acid buffer was used for all the susceptibility testing. Sabouraud broth buffered to pH 4.5 with 100mM Na_2HPO_4- citric acid buffer was used for the analysis of cell killing kinetics.

2.1.3. Chemicals

The chemicals and the suppliers are listed in the Appendix A.

2.1.4. Buffers

Buffers and solutions used in the experiments are given in Appendix B.

2.2. METHODS

2.2.1. Sterilizations

The glassware was sterilized on dry-cycle at 200^{o} C for two hours. The media for stock cultures and for routine growth of the yeast cells were sterilized at 121^{o} C for 15 minutes on liquid cycle. RPMI 1640 medium was filtered through 0.45µm and 0.22µm (Sartorius, AG, Germany) cellulose acetate filters respectively for sterilization. Buffers used for the chromatographic purification steps were filtered using 0.45µm cellulose acetate filters (Sartorius, AG, Germany) prior to sterilization on liquid cycle.

2.2.2. Maintenance of the Fungal Cultures

Freeze-dried cultures of *Pichia anomala* (NCYC 434, K5) and *Saccharomyces cerevisiae* (NCYC 1006) in glass ampoules were opened aseptically and 0.5 ml of YEPD medium was added to dissolve the dried culture using a sterile pasteur pipette. Dissolved yeast cells were plated onto petri dishes containing YEPD agar at pH 5.5 and incubated at 25 ºC until the colonies were formed [3].

Active cultures of dermatophyte strains in glass tubes were covered with adequate amount of sterile saline solution. Spores were scratched with the tip of a sterile pasteur pipette and 0.5 ml of the spore suspension was drawn under sterile conditions and plated onto YEPD pH 5.5 or SDA pH 6.0 agar plates and incubated at 28º C. Dermatophyte strains were sub-cultivated to PDA plates to promote spore formation prior to antifungal susceptibility testing.

Pathogenic yeast strains (*Candida* spp.) were cultivated on YEPD pH 5.5 agar plates and subcultured to PDA plates to ensure purity and viability prior to antifungal susceptibility testing.

2.2.3. Production of the K5 Type Yeast Killer Toxin

Production, concentration and isolation of the K5 type yeast killer protein were done as described previously by İzgü and Altınbay [46]. *Pichia anomala* NCYC 434 cells were cultivated into 10 ml of YEPD pH 5.5 medium and incubated for 24 hours at 25 ^{o}C. One ml of cell suspension was further inoculated into 100 ml of the same medium. After the yeast cells were incubated at 25 ^{o}C for 24 hours at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA), 10 ml of cell suspension was transferred to 1L of YEPD medium (containing 5 % glycerol) buffered to pH 4.5 with citrate-phosphate buffer and incubated until stationary phase at 20 ^{o}C for 36 hours at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA). Centrifugation (KR 22i, Jouan, France) at 5000 rpm for 10 min. at 4^{o} C was applied to obtain the cell free culture medium and supernatant was filtered through 0.45µm and 0.2µm cellulose acetate membranes (Sartorius, AG, Germany) respectively for the sterilization of the medium.

2.2.4. Determination of Killer Toxin Activity

Toxin activity at various stages of the study was tested according to Brown et al. [90] with an agar diffusion assay. Twenty five ml of molten YEPD agar (pH 4.5) was seeded with 1 ml of *Saccharomyces cerevisiae* (NCYC 1006) cells in sterile water at a density of 10^5 cells/ml and poured into petri dishes. Protein samples of 30µl were spotted onto petri dishes and incubated at 23 ^{o}C. The killer activity was determined by measuring the clear zone of growth inhibition after 48 hours of incubation. Killer toxin which gave a clear zone of 13 mm in diameter was defined as 1 arbitrary unit (AU).

2.2.5. Crude Toxin Preparation

Cell free culture medium containing the K5 type yeast killer protein was concentrated 100 fold by using 30 kDa molecular cut-off ultrafilters (Vivaspin VS2021, Sartorius ,AG, Germany) at 4200 rpm, 4^{o}C (MR23i, Jouan, France) [46].

2.2.6. Isolation of the Killer Toxin

Crude toxin obtained from the previous step was subjected to ion exchange and gel permeation chromatographies respectively by using a fully automated FPLC system (Biocad 700E Perseptive Biosystems, USA) including an automatic fraction collector (SF-2120 Super Fraction Collector, Advantec MFS, Japan). Detections were done with UV absorbance at 280 nm at 20 ^{o}C [46].

2.2.6. a. Anion Exchange Chromatography

Crude toxin was buffer exchanged to 30mM N-methylpiperazine-HCl (pH 4.8) by using 5 kDa molecular cut-off centrifugal filters (Vivaspin VS2021, Sartorius) at 4200 rpm, 4^{o}C (MR23i, Jouan, France). Buffer exchange step was repeated for 3 times to maintain YEPD free samples. Samples were filtrated through 0.22µm disk filters (Sartorius, AG, Germany) prior to injection to the FPLC column.

750µL of the buffer-exchanged protein sample was put on an anion-exchange column (POROS HQ/M 4.6 mmD/100mmL, Perseptive Biosystems, USA) that is previously equilibrated with N-methylpiperazine-HCl (pH 4.8). The column was washed to 20 column volumes (CV) with the same buffer. Elution was done with a linear gradient of 0 to 500mM NaCl in N-methylpiperazine-HCl

(pH 4.8) at a flow rate of 10 ml/min. Killer protein containing fractions (1600µL) that corresponds to 120mM NaCl were pooled. These eluted fractions were concentrated and buffer-exchanged to 100mM Na_2HPO_{4-} citric acid buffer, pH 4.5 by using 5 kDa molecular cut-off centrifugal filters (Vivaspin VS2021, Sartorius ,AG, Germany) at 4200 rpm, 4^{o}C (MR23i, Jouan, France).

2.2.6. b. Gel-Permeation Chromatography

The concentrated and buffer exchanged sample obtained from the previous anion exchange step was then subjected to gel permeation chromatography using a TSK G2000 SW (7, 5 mmD/300mmL TosoHaas, Japan) column. Prior to injection of the sample, column was equilibrated with 100mM Na_2HPO_{4-} citric acid buffer, pH 4.5, containing 100mM Na_2SO_4 at a flow rate of 1 ml/min. Ninety µl of sample was injected into the column and elution was done with the same buffer at a flow rate of 1 ml/min. Killer toxin containing eluted fractions (1300µl) that corresponds to 8.5 ml were pooled. These active fractions are then concentrated and buffer exchanged to the same buffer but the salt by using 5 kDa molecular cut-off ultrafilters (Vivaspin VS2021, Sartorius, AG, Germany). Thirty µl of the purified protein obtained from gel permeation chromatography was spotted on to YEPD (pH 4.5) agar plates seeded with killer toxin sensitive *S. cerevisiae* NCYC 1006 cells for the assessment of the killer toxin activity.

2.2.7. Assessment of Protein Concentration

Protein concentration was determined as described previously by Bradford [91] with some minor modifications. Bradford reagent used for the determination of protein concentration was prepared as follows. A hundered mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95 % ethanol then mixed with 100 ml of 85 % (w/v) phosphoric acid. Finally the solution was

completed to a total volume of 1 L with distilled water and filtered through Whatman #1 paper.

Bovine serum albumin (Fraction V) was used as the protein standard. Different concentrations of bovine serum albumin was prepared in 100 mM Na_2HPO_{4-} citric acid buffer ranging from 2.5 to 25 µg/ml in a total volume of 800µl. These standard solutions were mixed with 200µl Bradford reagent. 100µl protein sample was also diluted in 700µl 100 mM Na_2HPO_{4-} citric acid buffer and mixed with 200µl Bradford reagent. Absorbances were immediately measured at 590nm by using UV visible spectrophotometer (model 1208, Shimadzu, Japan). Protein concentration was calculated using a standard curve of absorbance versus protein amount.

2.2.8. SDS and Non-denatured SDS Polyacrylamide Gel Electrophoresis

Molecular weight determination of the pure toxin was done with a 5-20 % linear gradient SDS polyacrylamide gel in a discontinuous buffer system as described by Laemmli [92], using a vertical slab gel electrophoresis unit SE 600 (Hoefer, USA). The 5-20 % linear gradient gel was prepared with a gradient maker (Hoefer, USA).The density of the gel was 20 % at the bottom and decreased to 5 % towards the top of the gel.

Concentrated protein obtained from the gel permeation step was electrophoresed on a 15 % linear, 0.75 mm thick native polyacrylamide gel in a discontinuous buffer system using a vertical slab gel electrophoresis unit SE 600 (Hoefer, USA) to ensure the purity of the toxin. 2-β-mercaptoethanol was left out the standard Laemmli protocol for non-denaturing conditions.

Gradient (5%-20%) or 15 % separating gel was prepared and poured into the electrophoresis unit and covered with saturated n-butanol to avoid contact of the gel with air and left for polymerization for 1 hour. When the polymerization

was completed n-butanol was washed with water and stacking buffer respectively before the stacking gel was poured. Stacking gel was also left for polymerization for 1 hour. Separating and stacking gel components are given in Table 2.3 and 2.4.

Protein samples were subjected to acetone precipitation and resuspended in 125 mM Tris-Cl pH 6.8. They were heated at 100 °C for 5 min in equal volume of sample buffer (20 % (v/v) glycerol, 4 % (v/w) SDS, 0.02 % (v/w) bromophenol blue pH 6.8) for the non-denaturing SDS PAGE and 10 % 2-β-mercaptoethanol was added to the sample buffer for SDS PAGE. The samples were loaded onto the gel after the polymerization of the stacking gel was completed.

Electrophoresis was done at 15 mA/0.75 mm gel (Power supply PP4000, Biometra, Germany) at 15 ^{o}C using a circulating water bath (Heto Holten, Denmark).

The gels were visualized by silver staining. For the molecular weight determination the SDS gel was scanned using a GT9500 Color Image Scanner (Epson, Japan) and the data were processed with Gelworks 1D software (UVP Products, UK).

Table 2.3. Separating Gel Mixtures.

	5% Gel	15% Gel	20% Gel
Acrylamide-bisacrylamide (30:0.8)	3.34 ml	12.5 ml	13.2 ml
4X Seperating Gel Buffer (1.5M Tris-Cl , pH:8.8)	5 ml	7.5 ml	5 ml
10 % SDS	0.2 ml	0.3 ml	0.2 ml
ddH_2O	11.4 ml	9.6 ml	-
Sucrose	-	-	3 gr
10 % Ammonium persulfate*	66µl	150 µl	66µl
TEMED*	6.6µl	10 µl	6.6µl

*Ammonium Persulfate and TEMED were added after deaeration

Table 2.4. Stacking Gel Mixture (4%T).

Acrylamide-bisacrylamide (30:0.8)	1.33 ml
4X Stacking Gel Buffer (0.5M Tris-Cl , pH:6.8)	2.5 ml
10 % SDS	0.1 ml
ddH_2O	6 ml
10 % Ammonium persulfate*	50 µl
TEMED*	5 µl

*Ammonium Persulfate and TEMED were added after deaeration.

2.2.9 Protein Detection in Gels: Silver Staining

Protein bands were visualized by silver staining method. After the gel was removed, it was placed in Destain 1 (100 ml) solution and incubated for 30 minutes with gentle shaking. Then the solution was replaced with Destain 2 (100 ml). After 30 minutes Destain 2 was discarded and replaced with 100 ml of cross-linking solution. Gel was washed with several changes of water over 2 hours. After the final wash DTT solution was added and incubated for 30 minutes with slow shaking. DTT solution was replaced with 100 ml of Silver Nitrate Solution. Gel was washed with distilled water and developing solution respectively and 100 ml of fresh developing solution was added. When the bands appeared developing solution was replaced with Destain 2 [93].

2.2.10. Antifungal Susceptibility Testing

Pathogenic yeast (26 *in toto*) and dermatophyte (9 *in toto*) strains were tested for their susceptibility to the K5 type yeast killer protein. NCCLS (National Committee for Clinical Laboratory Standards) M 27-A2 (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts) [94] was used for the susceptibility testing of *Candida* strains and NCCLS M 38-A (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi) [95] was used for the dermatophyte strains; with modifications to ensure the stability of the K5 type yeast killer protein during testing.

2.2.10. a. Susceptibility Testing of Pathogenic Yeast Strains

Minimum inhibition concentrations (MICs) were determined by a broth microdilution test using 96 well microtitre plates. Two fold serial dilutions of the K5 type yeast killer protein was prepared in 100 mM Na_2HPO_4-citric acid buffer

ranging from 0.25 to 16µg/m. Test media, RPMI 1640, was prepared at twice its final concentration and buffered to pH 4.5 with the same buffer. Inocula were prepared by picking 5 colonies from 24 h old yeast cultures and suspending them in 5 ml of sterile saline solution. The resulting suspension was vortexed (Heidolph, Germany) for 15 seconds and the cell density was adjusted to 0.5 McFarland (DEN-1 McFarland Densitometer, HVD, UK) which yielded a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells/ml. Twice the final inoculum size of (1.5±1.0) x 10^3 cells/ml was achieved with a 1:50 dilution followed by a 1:20 dilution of the yeast stock cultures in RPMI (pH 4.5) medium. A hundred µl of cell suspension was added into the wells of a microtitre plate containing 100 µl of protein solution. This step diluted the protein and medium concentrations also inoculum densities to the desired test concentrations. Growth control well was prepared by mixing 100 µl of cell suspension with 100 µl of Na_2HPO_4-citric acid buffer. Each row also contained a sterility control well including medium and toxin free buffer. Microtitre plates were incubated at 25 ^oC for 48 hours and the wells were mixed with a multi-channel pipette at every 2 hours during 6 hours. MIC_{100} was defined as the lowest concentration of the K5 type yeast killer protein where there was no visual growth or 95 % reduction in the growth compared to the protein free control well which was determined by using an automatic microtitre plate reader at 590nm (Spectramax 190, Molecular Devices, USA) whereas MIC_{50} was defined as the lowest concentration where there was 50 % reduction in growth compared to growth control well which was determined both visually and spectrophotometrically.

Minimum fungicidal concentrations (MFCs) were determined as described by Canton et al [96]. After 48 h of incubation entire volume from all visually clear MIC wells was spotted on to PDA plates and incubated for 24 hours at 25 ^oC The minimum fungicidal concentration (MFC) was the lowest protein concentration that killed 99.9 % (with less than 5 colonies remaining) of the final inoculum.

2.2.10. b. Susceptibility Testing of Dermatophyte Strains

Minimum inhibition concentrations (MICs) of the K5 type yeast killer protein for dermatophyte strains were also determined by a broth microdilution test using 96 well microtitre plates. Two fold serial dilutions of the K5 type yeast killer protein was prepared in 100 mM Na_2HPO_4-citric acid buffer ranging from 0.25 to 16µg/ml. All dermatophyte strains were grown on PDA agar in glass tubes at 25 ^oC for 7 days in order to provide spore formation. Seven days old colonies were covered with 1 ml sterile saline solution and scratched with tip of a transfer pipette to suspend the spores. Resulting spore suspension was transferred into a sterile tube and allowed to settle down for 5 minutes. After the heavy particles such as hyphal filaments settled down, 1 ml of the upper suspension was transferred to a sterile tube and vortexed (Heidolph, Germany) for 15 seconds. Spores were counted with a haemocytometer to adjust the required cell density of 4 x 10^5 to 25 x 10^5 spores/ml. Adjusted spore suspensions were further diluted 1:50 in 2 fold concentrated RPMI (pH 4.5) to obtain the 2 x the density needed of 0.4 x 10^4 to 5 x 10^4 spores/ml. A hundred µl of the spore suspension was transferred to the wells of a microtitre plate containing 100 µl of protein solution. Each row on the microtitre plate also included protein free growth control and sterility control wells. Microtitre plates were incubated at 25^o C for 4 to 6 days and the wells were mixed with a multi-channel pipette in every 2 hours for the first 6 hours. MIC_{100} and MIC_{50} values were determined visually at the end of the incubation.

2.2.11 Cell Killing Kinetics of the K5 Type Yeast Killer Toxin

K5 type yeast killer toxin was tested on 14 representative strains of the pathogenic yeasts (*Candida* spp.) for its cell killing kinetics (Table 2.5). Selections of the strains were based on the sites of isolation. Analyses of cell killing kinetics were done according to İzgü et.al [89]. Every strain was tested in its two fold MIC_{100}. Cell suspensions with a density of 0.5 McFarland which

corresponds to 1 x 10^6 to 5 x 10^6 cells/ml were prepared and diluted 1:100 in 2 fold concentrated Sabouraud broth (pH 4.5) to a final volume of 0,5 ml. 4 x MIC_{100} concentrations were prepared in 100mM Na_2HPO_{4-} citric acid buffer by diluting the appropriate amount of concentrated toxin to 0,5 ml. These two solutions were mixed in sterile test tubes to obtain the desired cell density of approximately 10^4 cells/ml and the final toxin concentration of 2 x MIC_{100}. Growth control tube included yeast suspension with toxin free buffer. Tubes were incubated at 25 ^oC for 48 hours. Fifty µl sample was removed from test tubes at predetermined time points (0, 2, 4, 6, 8, 10, 12, 24, 36, 48 hours) and 10 fold diluted in pH 6.8 citrate-phosphate buffer to deactivate the toxin. Fifty µl of the diluted sample was spread on to PDA plates. Colony counts were determined after incubating at 25 ^oC for 24 to 48 hours. If the colony counts were suspected to be less than 1000 CFU/ml samples were diluted 2 fold in pH 6,8 citrate-phosphate buffer.

Table 2.5. Pathogenic Yeast Strains Selected for Analyses of Cell Killing.

STRAIN	**STRAIN NUMBER SOURCE**	**ISOLATION**
C. albicans	ATCC 26555	Patient with mucocutoneus candidiasis
C. albicans	ATCC MYA2730	Human blood
C. albicans	DSMZ 3454	Patient with vaginal candidiasis
C. albicans	A1	Patient with Flukanazol resistant bronchomycosis
C. krusei	ATCC 14053	Human blood
C. krusei	ATCC 6258	Patient with bronchomycosis
C. guilliermondii	A9	Human blood
C. guilliermondii	A10	Human leg
C. guilliermondii	A11	Patient with bronchomycosis
C. pseudotropicalis	A14	Cutoneus candidiasis
C. tropicalis	A15	Human abscess
C. glabrata	ATCC 90030	Human blood
C. parapsilosis	ATCC 90018	Human blood
S. cerevisiae	A16	Human

CHAPTER III

RESULTS

3.1. Production of the Killer Toxin

P. anomala cells were grown in YEPD pH 4.5 medium with the addition of 5% glycerol as toxin stabilizer at 20 ^{o}C to maintain the highest degree of killing activity [46] since the productions of the killer toxins are highly dependent on the pH of the cultivating medium and incubation temperature [97].

3.2. Isolation of the K5 Type Yeast Killer Toxin

P. anomala cells were removed from the culture liquid by centrifugation and concentrated 100 fold by ultrafiltration with 80% recovery of the killer toxin. Crude toxin obtained from ultrafiltration step was buffer exchanged prior to anion exchange chromatography. K5 type yeast killer protein was purified by anion exchange chromatography using POROS HQ/M column for the optimal resolution (Figure 3.1). Toxin was eluted in the fraction (indicated by arrow) corresponding to 120 mM of NaCl and the resulting elute contained 280- fold purified toxin. Killer protein obtained from anion exchange chromatography was concentrated and buffer exchanged then put on a gel permeation column TSK G 2000SW (Figure 3.2). Active fraction was eluted at 8.5 ml (indicated by arrow).

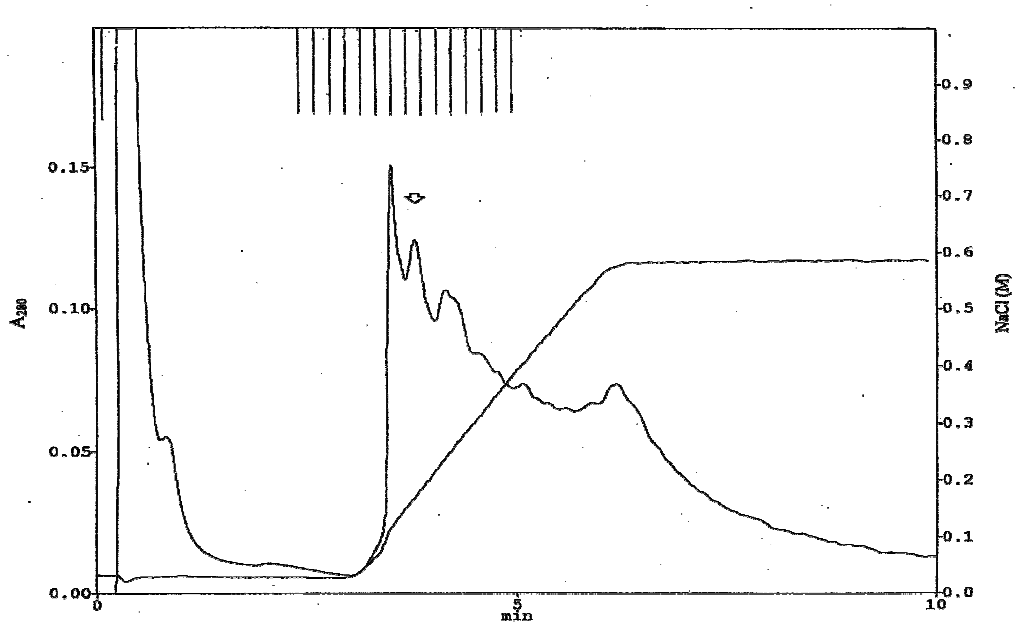


Figure 3.1 Elution Profile of the K5 Type Toxin on a POROS HQ/M Column. Column size: 4,6 mmD/100mmL; Sample: 750 µl; Starting buffer 30 mM N-methyl piperazine- HCl pH:4,8; Gradient: 0-500 mM NaCl in the starting buffer in 20 CV; Flow rate: 10 ml/min; Detection: UV 280 nm.; Fraction volume: 1600 µl. Fraction indicated by arrow contains the K5 type killer toxin corresponding to 120 mM NaCl.

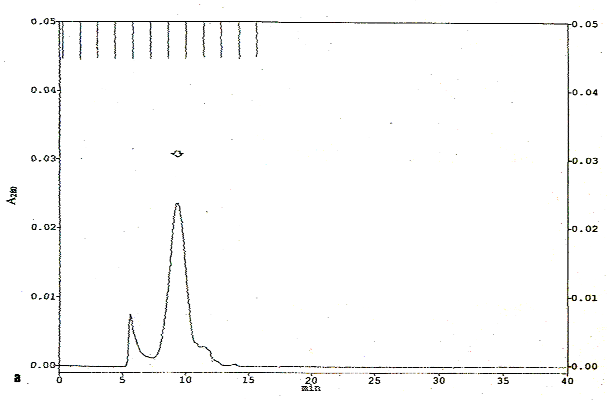


Figure 3.2 Elution Profile of the K5 Type Toxin on a TSK G2000SW Column Column size:7.5 mmD / 300 mmL; Sample: 40 µl; Elution buffer: 0.1M Na_2HPO4 pH 4.5 + 0.1 M Na_2SO4 ; flow rate 1 ml/min; detection 280 nm UV Fraction volume 1300 µl. Fraction containing killer protein is eluted at 8,5 ml and indicated by arrow.

Killer toxin containing fractions obtained from gel permeation chromatography were concentrated and buffer exchanged to Na_2HPO_4- citric acid buffer, pH 4.5.

3.3. Assessment of Protein Concentration

Protein concentration was adjusted to 32 µg /ml by Bradford assay using a standard curve drawn with bovine serum albumin (Figure 3.3.). The yield of the K5 type yeast killer protein from 1 L of the medium was 76.8 µg with a specific activity of 1041.7 U/mg.

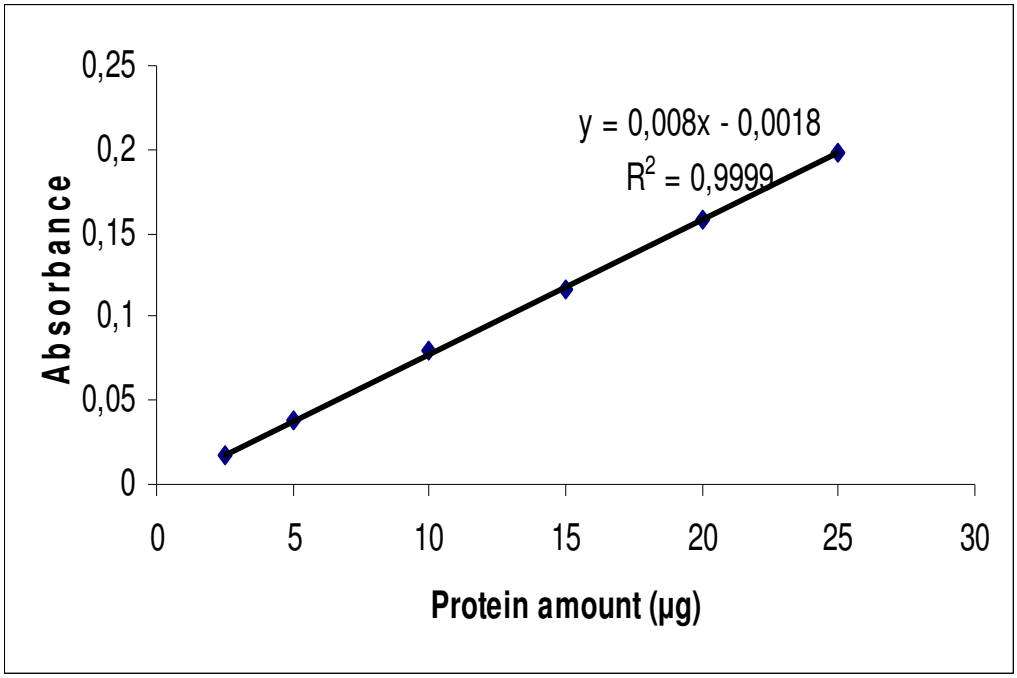


Figure 3.3. Standard Curve of Protein Amount versus Absorbance.
Bovine serum albumin solutions were used as standards. 100 µl of the purified K5 type yeast killer protein gave an UV absorbance of 0,0238 at 590 nm which corresponds to 3.2 µg

3.4. Determination of Killer Toxin Activity

Killing activity of the pure protein was tested with agar diffusion assay. Thirty µl of the protein was spotted onto YEPD pH 4.5 agar seeded with killer toxin sensitive *S. cerevisiae* cells. After 24 h incubation a clear growth inhibition zone of 13 mm which corresponds to 1 AU killer toxin was observed (Figure 3.4).

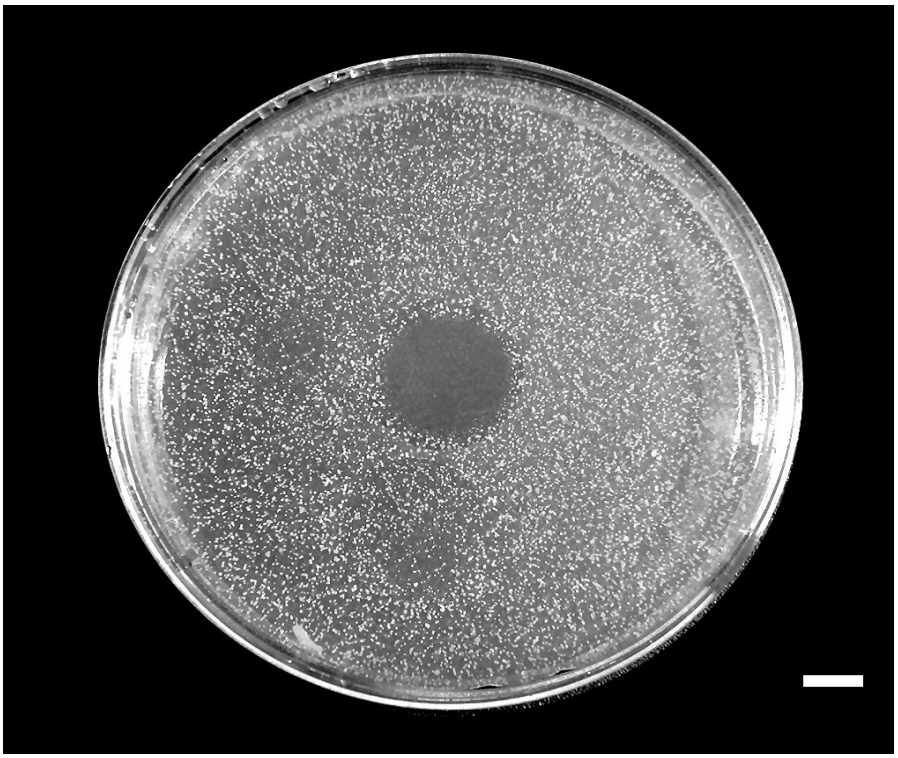


Figure 3.4. Killer Activity of the K5 Type Toxin Determined by Agar Diffusion Assay. Bar scale represents 5 mm.

3.5. SDS and Non-denatured SDS Polyacrylamide Gel Electrophoresis

Isolated killer protein (0.8µg) was electrophoresed on a 15% linear SDS-PAGE gel in a discontinuous buffer system under non-denaturing conditions to check its purity. Observation of single protein band on the silver stained gel indicates the absence of any contamination. Molecular weight of the K5 type yeast killer protein was determined with a 5%-20% linear gradient SDS-PAGE gel and was found to be 49 kDa (Figure 3.5.).

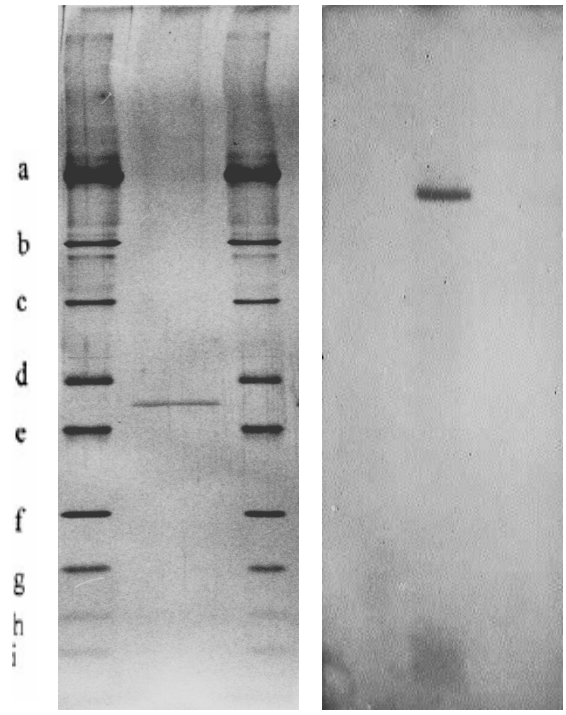


Figure 3.5. Gradient SDS-PAGE (**A**) and Non-denatured SDS-PAGE (**B**) Profiles of the K5 Type Yeast Killer Protein. Lanes 2-4 are K5 type yeast killer protein. Lanes 1-3 are molecular mass markers.(Da) a) α_2-macroglobulin (170,000), b) β-galactosidase (116,353), c) fructose-6-phosphate kinase (85,204), d) glutamate dehydrogenase (55,562), e) aldolase (39,212), f) triose phosphate isomerase (26,626), g) trypsin-inhibitor (20,100), h) lysozyme (14,307), i) aprotinin (6,500)

3.6. Antifungal Susceptibility Studies

Susceptibility of different pathogenic yeast isolates and dermatophytes to purified K5 type yeast killer toxin was tested in different concentrations ranging from 0.25 to 16µg/ml with a microtitre plate test. MICs were determined both visually (Figure 3.6.) and spectrophotometrically for the yeast strains whereas detection was only possible with visual inspection for the dermatophyte strains. MFCs of the K5 type yeast killer toxin for the yeast strains were tested by plating the content of all visually clear wells onto PDA plates (Figure 3.7.). The entire MIC data is presented as the average of 3 independent experiments.

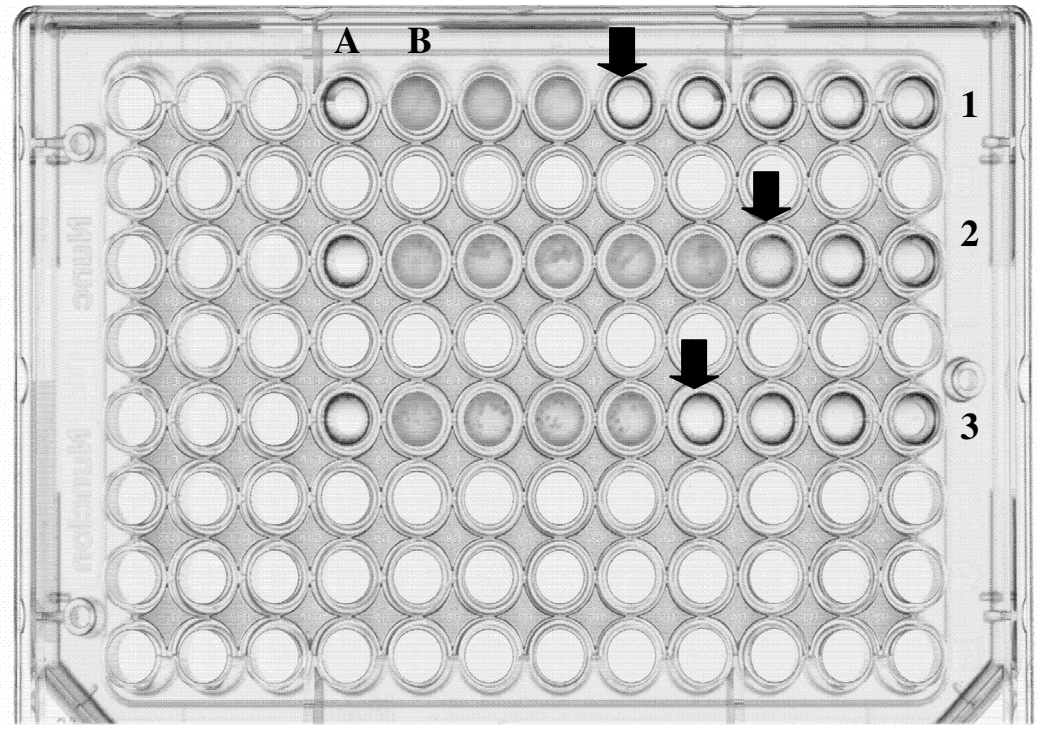
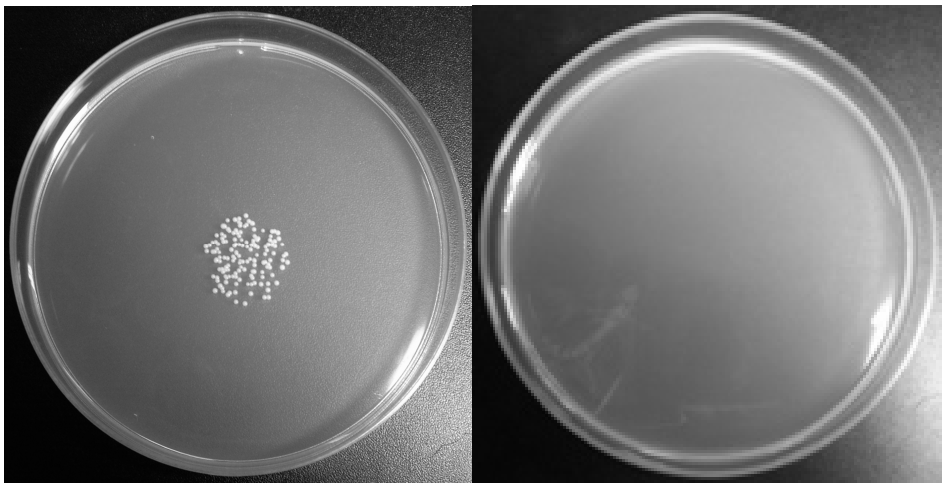
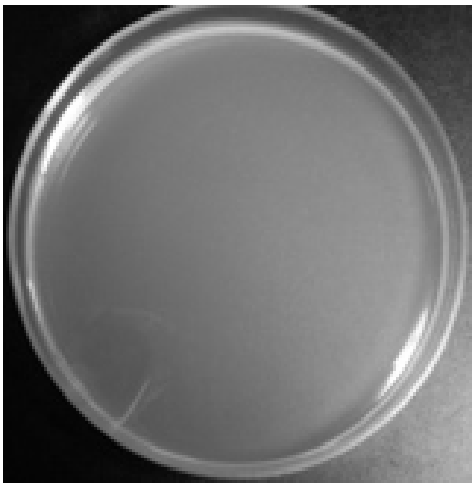


Figure 3.6. Antifungal Susceptibility Studies of the K5 Type Yeast Killer Toxin on a Microtitre plate. Arrows indicate the MIC_{100} (visually clear wells). 1) *Candida tropicalis* A15 $MIC_{100:}$ 1 µg/ml 2) *Candida albicans* ATCC 90028 MIC_{100}: 4 µg/ml 3) *Candida parapsilosis* ATCC 90018 MIC_{100}: 2 µg/ml A) Sterility control B) Growth control



4µg/ml 8µg/ml



16µg/ml

Figure 3.7 MFC Determination for *C.albicans* ATCC 36802 on PDA plates.

3.6.1. Susceptibility of Pathogenic Yeast Strains.

The results of susceptibility testing of the pathogenic yeast strains to the K5 type yeast killer protein ($MIC_{50,}$ MIC_{100} and MFC) are represented in Table 3.1. It showed a potent inhibitory activity against a variety of yeasts including *Candida albicans, Candida guilliermondi, Candida glabrata, Candida tropicalis, Candida krusei, Candida parapsilosis,* and *Saccharomyces cerevisiae.*

Table 3.1. Susceptibility ($MIC_{50,}$ MIC_{100} and MFC) of 26 Pathogenic Yeast Strains to the K5 Type Yeast Killer Protein.

STRAIN	**STRAIN NUMBER\ SOURCE**	**MIC_{50} µg/ml**	**MIC_{100} µg/ml**	**MFC µg/ml**
Candida albicans	ATCC MYA 2730	2	4	8
C. albicans	ATCC 10231	0.5	2	2
C. albicans	ATCC 26555	1	4	4
C. albicans	ATCC 36802	2	4	8
C. albicans	ATCC 90028	2	4	8
C. albicans	DSMZ 3454	1	2	2
C. albicans	A1	0.5	2	4
C. albicans	A2	2	4	4
C. albicans	A3	0.5	2	8
C. albicans	A4	1	4	4
C. albicans	A5	0.5	2	2
C. parapsilosis	ATCC 90018	1	2	8
C. parapsilosis	A6	0.5	1	4
C. krusei	ATCC 14053	4	8	>16
C. krusei	ATCC 6258	4	8	>16
C. krusei	A7	2	4	>16
C. krusei	A8	4	8	>16
C. guilliermondii	A9	1	2	4
C. guilliermondii	A10	2	4	4
C. guilliermondii	A11	1	4	8
C. guilliermondii	A12	0.5	1	2
C. guilliermondii	A13	1	2	4
C. pseudotropicalis	A14	0.25	1	1
C. tropicalis	A15	0.5	1	2
C. glabrata	ATCC 90030	0.25	0.5	1
S. cerevisiae	A16	0.25	0.5	1

3.6.2. Susceptibility of Dermatophyte Strains.

Susceptibility of 9 dermatophyte strains to the K5 type yeast killer protein was tested with a microtitre plate test. All of the strains including *Microsporum* and *Trichophyton* species were found to be susceptible to the toxin. MIC_{50} and MIC_{100} values are listed in Table 3.2. MIC_{50} range for the *Microsporum* species was 0,5-2 µg/ml and MIC_{100} range was 2-8 µg/ml whereas MIC_{50} range for *Trichophyton* species was 0,25-2 µg/ml and MIC_{100} range was 1-4 µg/ml

Table 3.2. Susceptibility ($MIC_{50,}$ MIC_{100}) of 9 Dermatophyte Strains to the K5 Type Yeast Killer Protein.

STRAIN	**STRAIN NUMBER\ SOURCE**	**MIC_{50} µg/ml**	**MIC_{100} µg/ml**
Microsporum audouinii	DSMZ 10649	1	2
M. canis	DSMZ 10708	2	8
M. gypseum	DSMZ 3824	0.5	2
Trichophyton rubrum	DSMZ 4167	1	4
T. mentagrophytes	DSMZ 4870	1	2
T. verrucosum	DSMZ 7380	0.25	1
T. interdigitale	DSMZ 12283	2	4
T. equinum	DSMZ 122284	1	2
T. tonsurans	DSMZ 122285	0.5	1

3.7. Cell Killing Kinetics of the K5 Type Yeast Killer Protein

Cell killing kinetics of the K5 type yeast killer toxin was studied on representative strains of the *Candida* genus. Selection of the strains was based on the sites of isolation. Results are given as time- kill curves in Figures 3.8.-3.21.

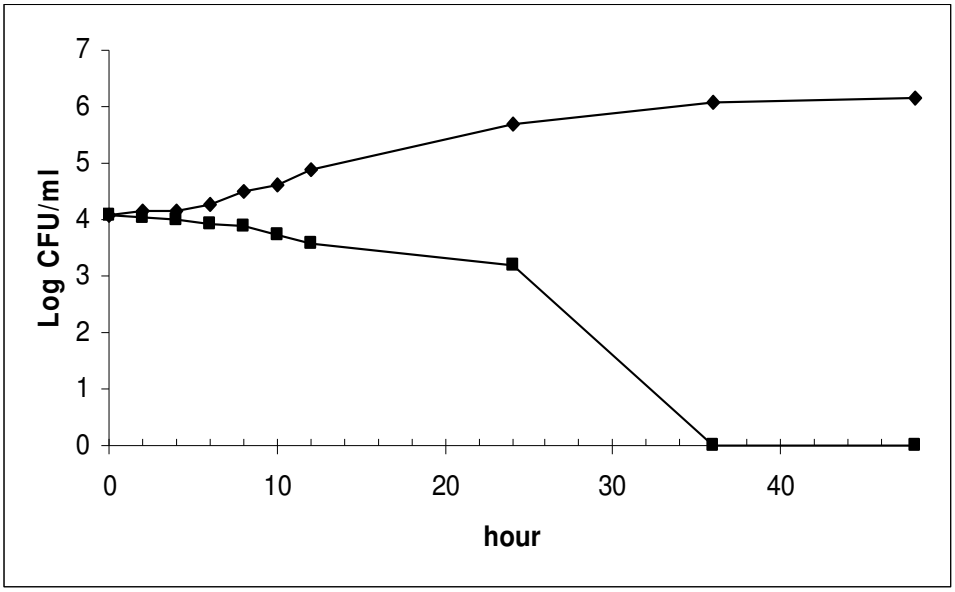


Figure 3.8. Time-kill Curve of *Candida albicans* (ATCC 26555)

■ With toxin ♦Without toxin

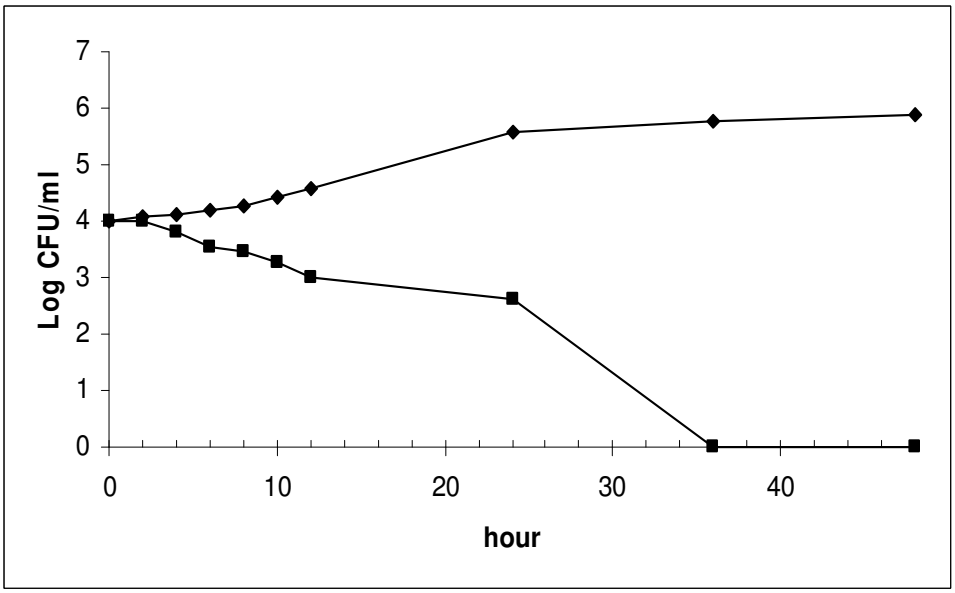


Figure 3.9. Time-kill Curve of *Candida albicans* (ATCC MYA2730)

■ With toxin ♦Without toxin

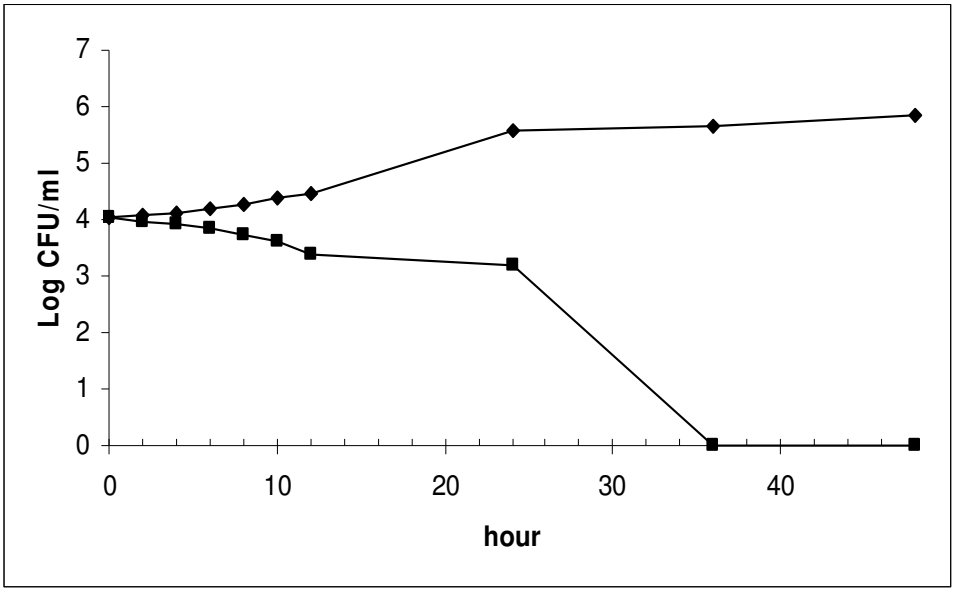


Figure 3.10. Time-kill Curve of *Candida albicans* (DSMZ 3454)

■ With toxin ♦Without toxin

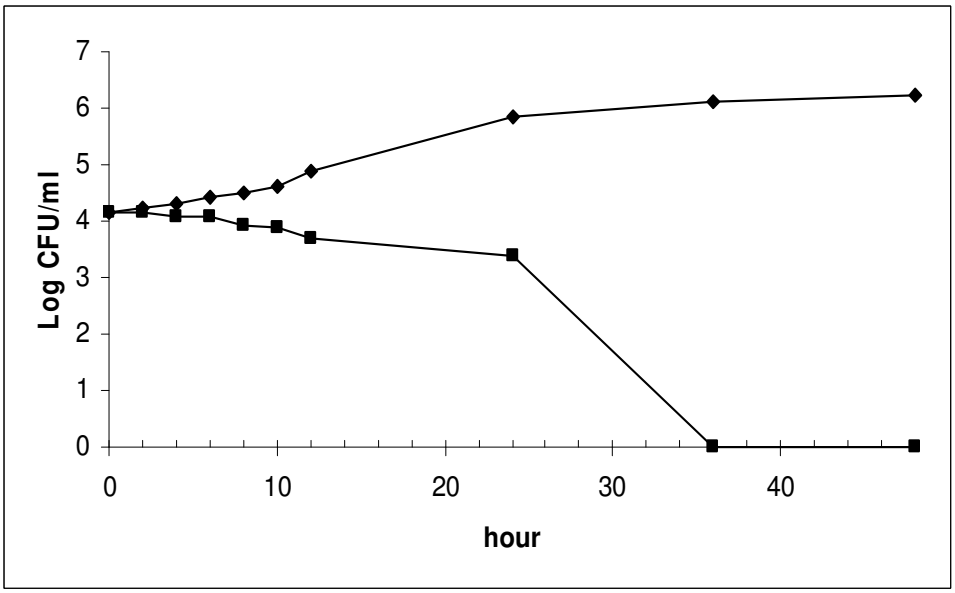


Figure 3.11. Time-kill Curve of *Candida albicans* (A1)

■ With toxin ♦Without toxin

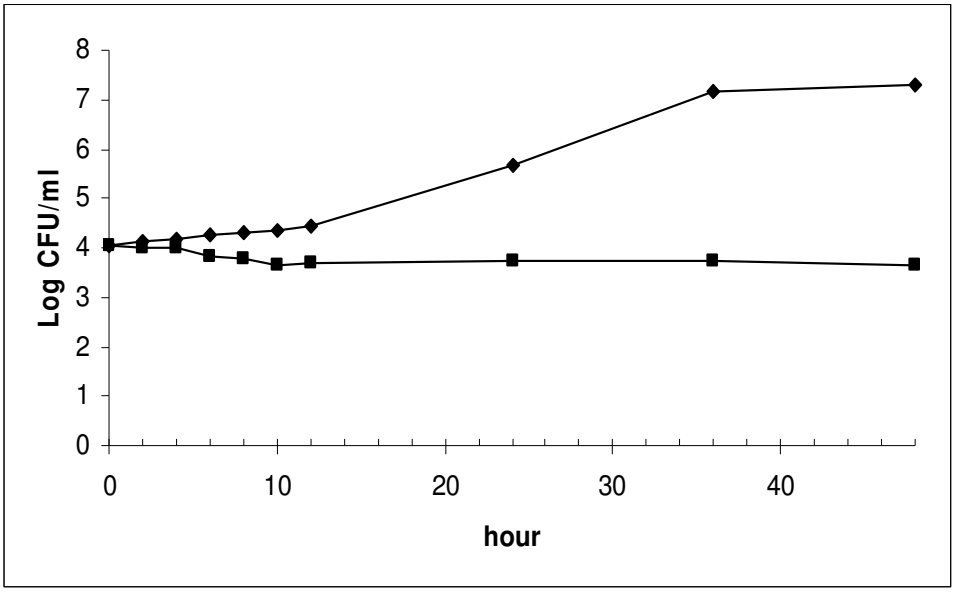


Figure 3.12. Time-kill Curve of *Candida krusei* (ATCC 14053)

■ With toxin ♦Without toxin

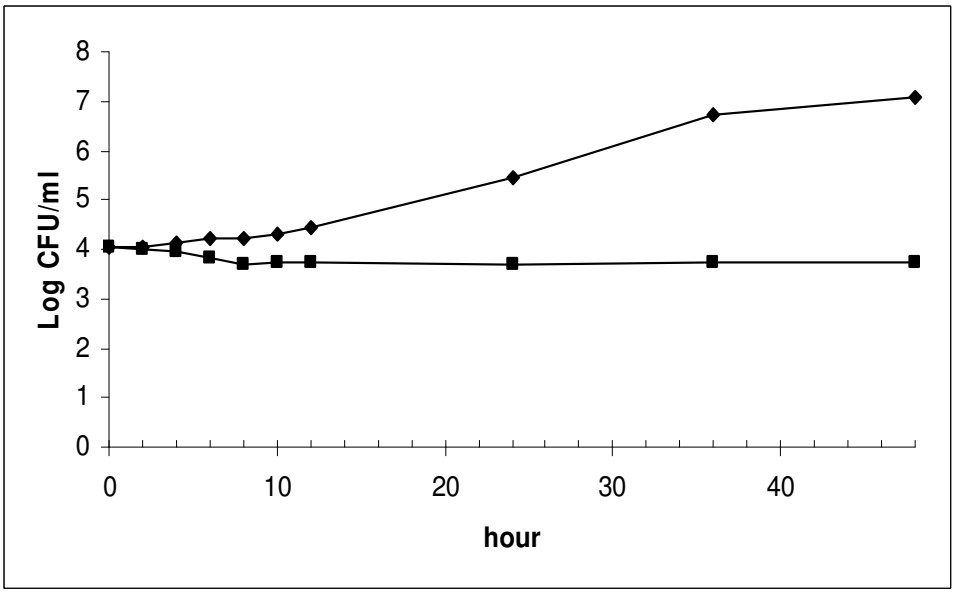


Figure 3.13. Time-kill Curve of *Candida krusei* (ATCC 6258)

■ With toxin ♦Without toxin

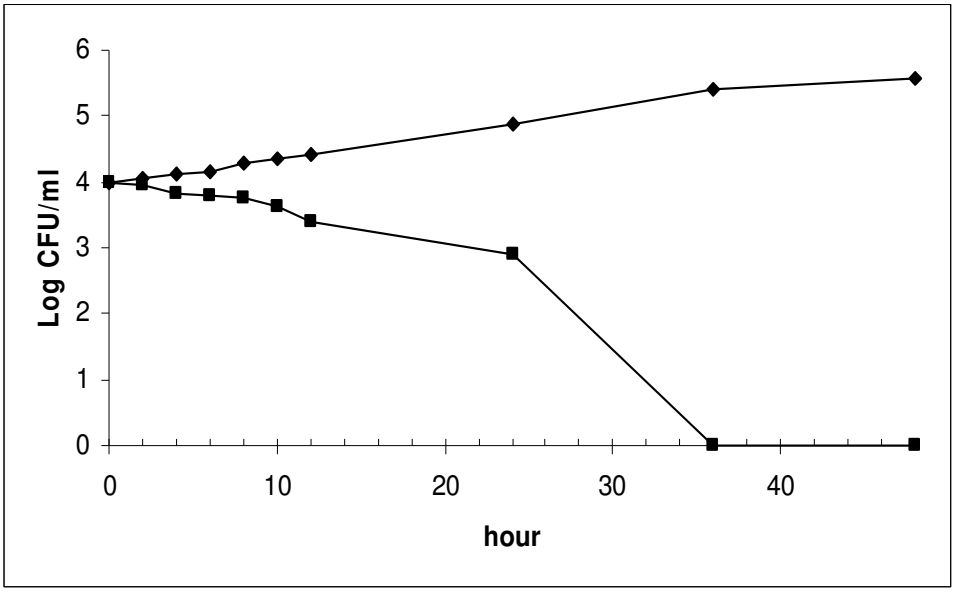


Figure 3.14. Time-kill Curve of *Candida guilliermondii* (A9)

■ With toxin ♦Without toxin

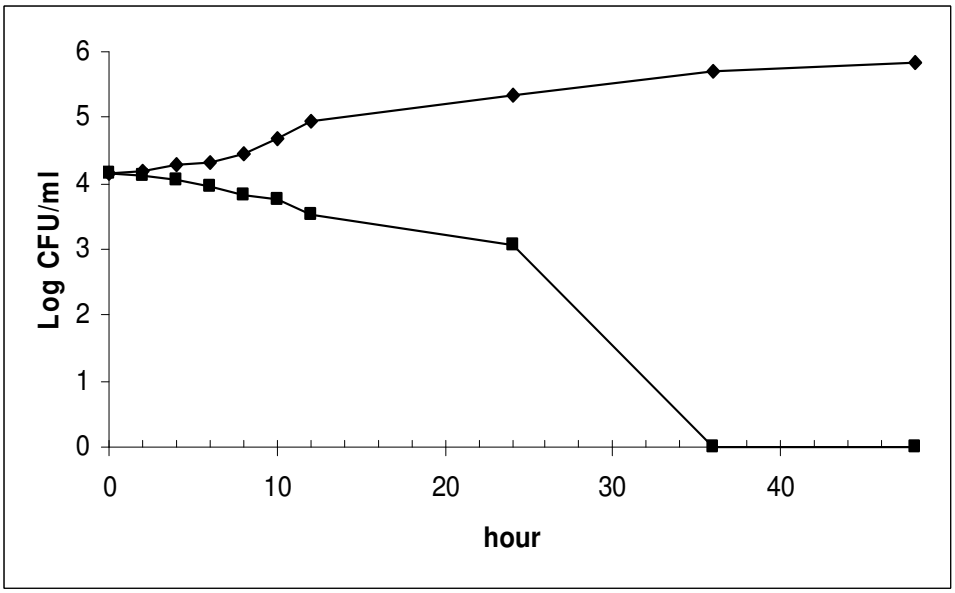


Figure 3.15. Time-kill Curve of *Candida guilliermondii* (A10)

■ With toxin ♦Without toxin

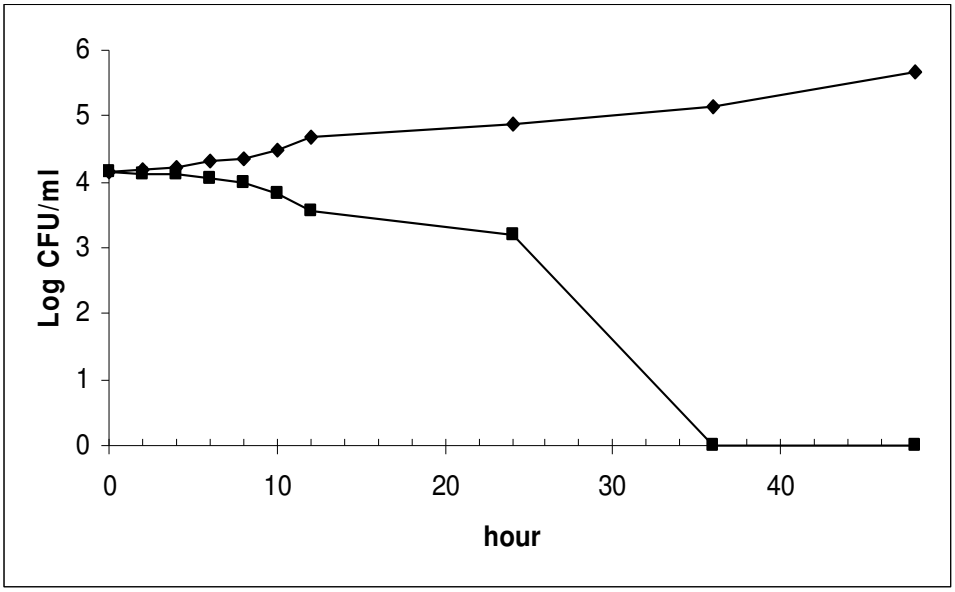


Figure 3.16. Time-kill Curve of *Candida guilliermondii* (A11)

■ With toxin ♦Without toxin

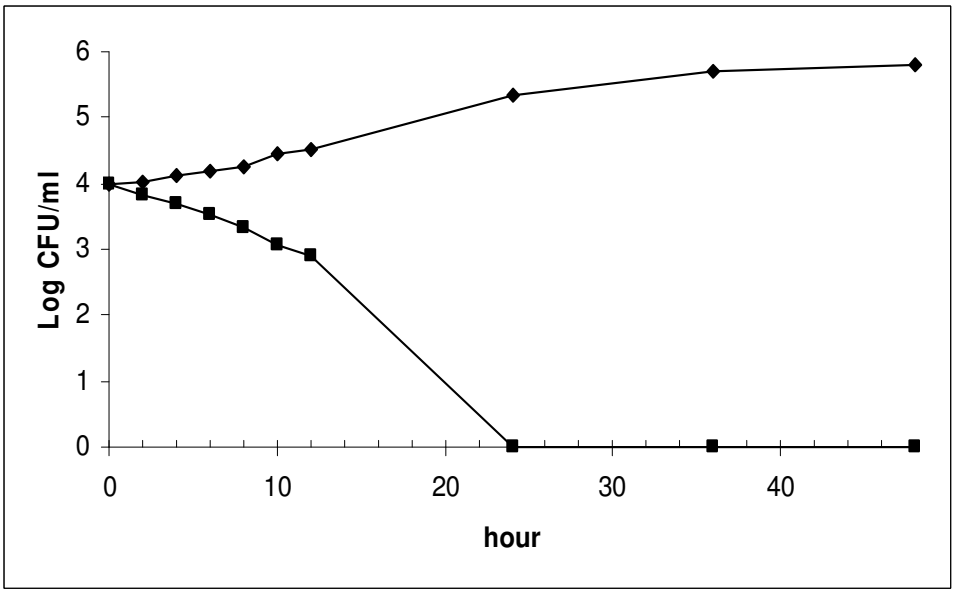


Figure 3.17. Time-kill Curve of *Candida pseudotropicalis* (A14)

■ With toxin ♦Without toxin

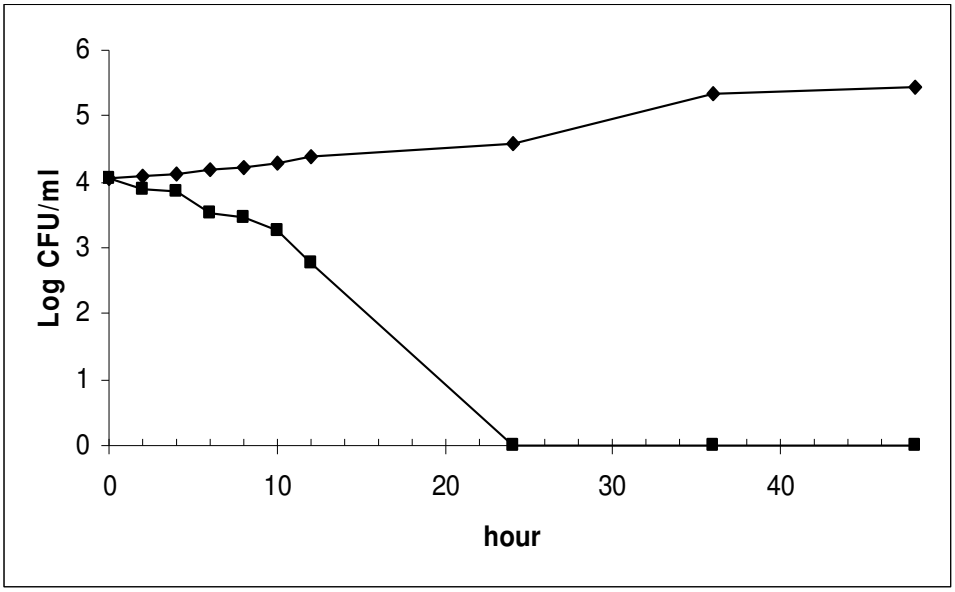


Figure 3.18. Time-kill Curve of *Candida tropicalis* (A15)

■ With toxin ♦Without toxin

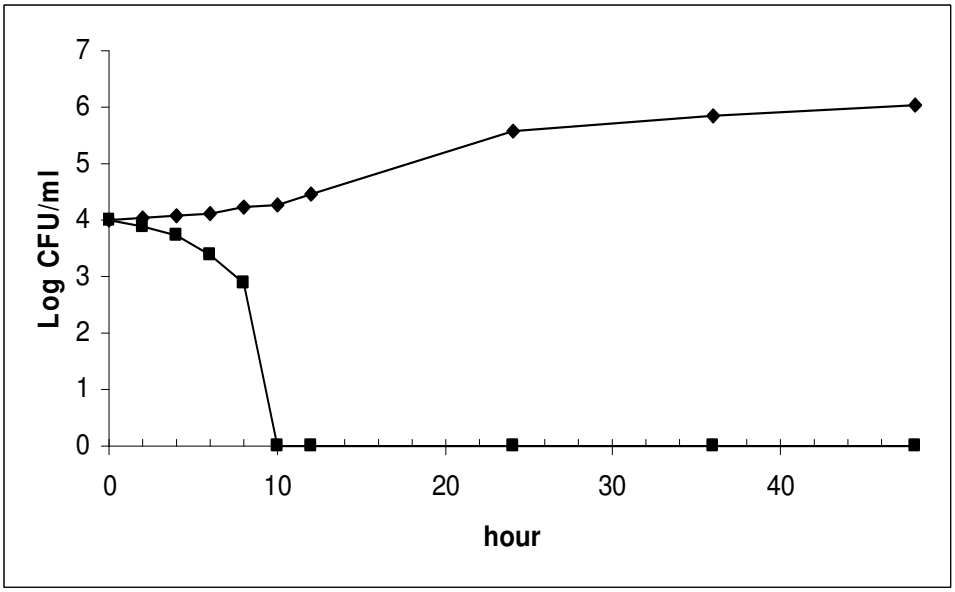


Figure 3.19. Time-kill Curve of *Candida glabrata* (ATCC 90030)

■ With toxin ♦Without toxin

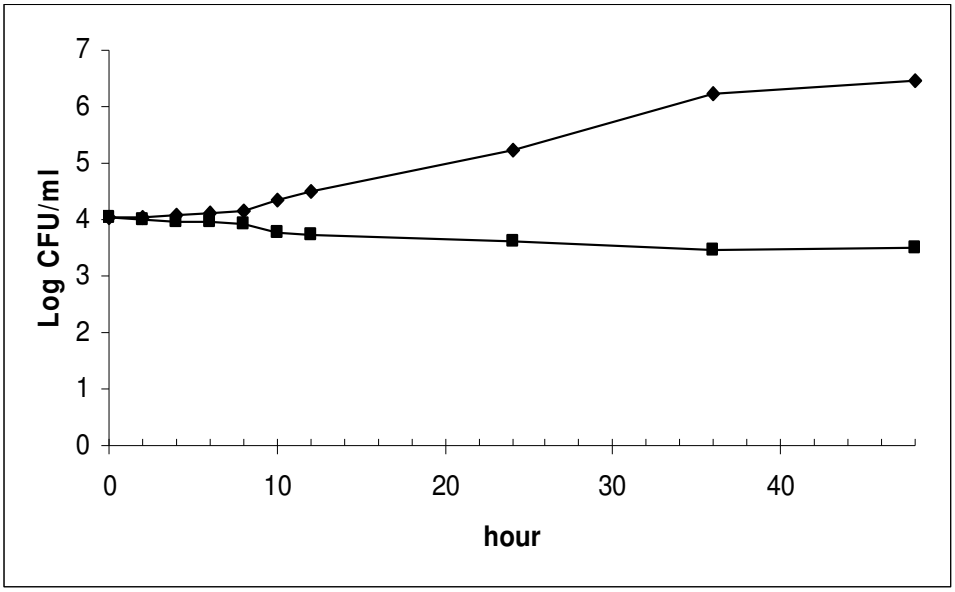


Figure 3.20. Time-kill Curve of *Candida parapsilosis* (ATCC 90018)

■ With toxin ♦Without toxin

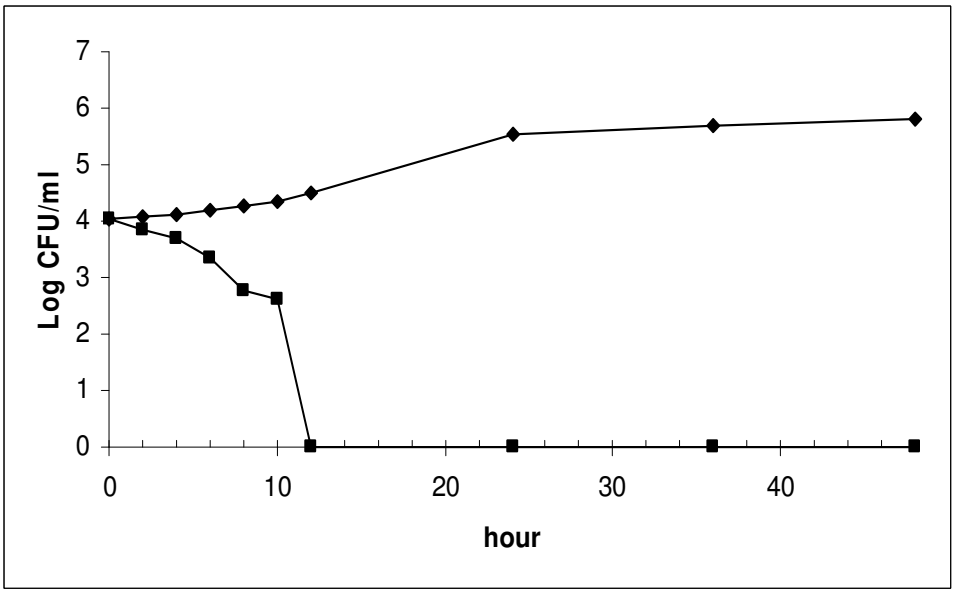


Figure 3.21. Time-kill Curve of *Saccharomyces cerevisiae* (A16)

■ With toxin ♦Without toxin

CHAPTER IV

DISCUSSION

Antifungal proteins, peptides and their synthetic derivatives have the potential for being used in the therapy of human fungal infections. Natural antifungal proteins are produced by a diverse group of organisms including bacteria, fungi, insects, vertebrates and invertebrates as well as plants. Yeast killer proteins are considered in this group and have been shown to have a broad spectrum of killing activity against various plant and human pathogens [44]. These toxic proteins inhibit the growth of various species and genera of yeast and fungi [9, 10, 11] as well as Gr (+) bacteria [12]. Thus researches on killer proteins are focused on the use of these agents as potential antifungals [44, 79]. Among the killer yeasts *Pichia anomala* NCYC 434 has been widely studied and was found to have an inhibitory affect on human and plant pathogenic fungi and K5 type yeast killer protein secreted by this strain was suggested as a potential antifungal agent [16-20]. The antifungal spectrum studies of the K5 type yeast killer toxin were based on the interaction of the killer protein producing strain *Pichia anomala* NCYC 434 with human fungal pathogens until this date. We have previously purified and characterized the K5 type yeast killer toxin in our laboratory [46] and now it is possible to determine the actual killing spectrum and cell killing patterns of this toxin on human pathogenic fungi.

Yeast infections have increased in humans for the past few years [61-63]. In immunosupressed patients these types of infections can be mortal. Particularly patients infected with HIV-1 are under the extreme risk of mycoses [98].

Therefore we have first determined the susceptibility of these organisms to the K5 type yeast killer protein by determination of MIC_{50}, MIC_{100} and MFC values of the toxin for pathogenic yeasts. Twenty five human pathogenic *Candida* species including standard strains and clinical isolates and 1 strain of *S. cerevisiae* were tested for their susceptibility to the toxin with a protein concentration ranging from 0.25 to 16 µg/ml. NCCLS recommendations of pH and temperature values of 7.0 and 35 ^{o}C were lowered to optimum values for the toxin which are pH 4.5 and 25 ^{o} C to ensure the stability of the toxin during testing. K5 type yeast killer protein were found to be affective against all of the tested 26 yeast strains since one of the major cell wall components of the yeasts is the β-1,3-glucan residues [42]. Susceptibility of the tested strains, that belongs to the different species, to the K5 type yeast killer protein varied due to different cell wall compositions and β-1,3-glucan content. Also minor differences were observed in different strains of the same species. These differences is probably due to the minor modifications of the β-1,3-glucan content of the cell wall that is related with the source of the *Candida* species.

K5 type yeast killer protein was tested against 11 *Candida albicans* isolates, the most common fungal pathogen, and also the organism responsible for the majority of localized fungal infections in humans [99]. MIC_{50}, MIC_{100} and MFC ranges were 0.5-2, 2 -4 and 2 -8 µg/ml respectively for these isolates.

Microorganisms gain resistance to conventional antimycotics especially in long term usages and these resistances spread rapidly among other microorganisms which are pathogenic to men and cause problems in the therapy of infections [80-82]. Fluconazole has been widely used since 1988 for the *Candida albicans* infections which resulted in the increase of the fluconazole resistant strains. Also it would appear likely that resistance to any azole used extensively in a similar setting could develop [100]. Fluconazole, itraconazole and ketoconazole MIC_{100}s for *Candida albicans* strains with reduced susceptibility to azole derivatives were found to be ≥ 100, 25, ≥25 respectively on a study conducted on 56 clinical isolates [101]. Our study included 1

fluconazole and 1 azole resistant strain of *C. albicans.* Toxin MIC_{50}, MIC_{100} and MFC values for these strains were 0.5, 2, 4 µg/ml for the fluconazole resistant and 2, 4, 4 µg/ml for the azole resistant strains of *C. albicans* respectively. These results indicate that K5 type yeast killer protein can be a new alternative in the treatment of azole and fluconazole resistant *C.albicans* strains.

We also included 3 C.albicans isolates from patients with vaginal candidiasis in our study. Vaginal candidiasis is a mucosal infection caused by *Candida* species and *C.albicans* is the most common etiological agent [102]. It is one of the most common infections in women [103]. An estimated 75% of all females experience at least one episode of the disease during their lifetime [102]. All the tested *C. albicans* strains isolated from vaginal candidiasis were found to be susceptible to the K5 type yeast killer protein in above mentioned MIC ranges.

K5 type yeast killer protein was also tested on several *C. albicans* strains isolated from human blood to determine the antifungal spectrum of the toxin and it was found to be effective on all the tested strains in above mentioned MIC ranges.

Results of the antifungal susceptibility studies against different *C. albicans* strains indicates that K5 type yeast killer protein is affective on all of the tested strains regardless the site of isolation.

Four *C. krusei* strains including both clinical and standard isolated were tested for their susceptibility to K5 type yeast killer protein. Although *Candida krusei* was reported as an infrequent isolate the widespread use of fluconazole to suppress fungal infections in these patients have contributed to a significant increase in *C. krusei* infection, particularly because of the high incidence of resistance of the yeast to this drug. Studies in both humans and animals have demonstrated the failure of fluconazole therapy against *C. krusei* due to increasing resistance of the organism to this azole [104]. Thus it has emerged as

a notable pathogen [105]. K5 type yeast killer toxin showed an inhibitory activity against all of the *C. krusei* strains within a MIC_{100} range of 4-8 µg/ml which indicates that the activity of the toxin is dose dependent whereas fluconazole MIC_{100} is higher than 64µg/ml for this species. Relatively higher MIC and MFC values of the K5 type yeast killer toxin for *C. krusei* isolates compared to other *Candida* spp. is probably due to the pseudohyphal growth of the strain with elongated and branched blastoconidia. Although MFCs of the toxin were higher than 16 µg/ml our findings revealed that K5 type yeast killer toxin can be used in fungal infections caused by *C. krusei*. This toxin can also be used at other *Candida* species infections without the risk of *C. krusei* invasion as a secondary infection since this pathogen is not resistant to K5 type yeast killer protein.

C. glabrata infections which have a high mortality rate are second in frequency after *C. albicans.* These infections are difficult to treat since resistance to many azole derivatives occurs during therapy [106]. We have included a standard strain of *C. glabrata* in our study and the results showed that it is susceptible to the K5 type yeast killer toxin at MIC_{100} of 2 µg/ml and MFC of 4 µg/ml whereas fluconazole MIC_{100} were found to be 32 µg/ml in a study conducted on 31 clinical isolates [107].

C. guilliermondii and *C. parapsilosis* are the other emerging pathogens that can infect various sites in human body were found to be susceptible to the K5 type yeast killer protein within the $MIC_{50,}$ MIC_{100} and MFC ranges of 0.5-2, 1-4, 2-8 µg/ml respectively.

Clinical isolates of *C. tropicalis* and *C. pseudotropicalis* isolated from human abscess and cutoneus candidiasis respectively were found susceptible to the K5 type yeast killer toxin. $MIC_{50,}$ MIC_{100} and MFCs were found to be 0.5, 1, 2 µg/ml for *C. Tropicalis* and 0.25, 1, 1 µg/ml for *C. pseudotropicalis* respectively. The use of the toxin against these types of cutoneus infections can be easily achieved since they do not require systemic therapy.

One strain of *S. cerevisiae* which is an opportunistic pathogen causing vaginal candidiasis [71] was also included in this study and found to be susceptible to the K5 type yeast killer toxin at both MIC_{100} and MFC of 2 µg/ml.

Therapy of serious *Candida* infections has been difficult because of the limited number of available antifungals [100]. Emergence of resistance to antifungal drugs does not appear to be a problem during their short-term use but is a major problem for long term therapies. So development of new antifungal agents especially with high selectivity on pathogenic yeast cells gained importance. K5 type yeast killer protein was proved to have a wide antifungal spectrum on pathogenic *Candida* species and can be proposed as a novel antifungal agent for alternative treatment of candidiasis. Especially for the mucosal fungal infections such as vulvovaginal candidiasis caused by *C. albicans* or *S. cerevisiae* this antifungal toxin can be readily used within topical formulations. Systemic use of this protein against fungal pathogens that colonise in the blood stream would be possible with formulations based on modern therapeutic systems such as nanospheres.

One of the most frequently observed skin diseases is the superficial mycoses [108]. Dermatophytes especially *Trichophyton* and *Microsporum* species are responsible for the most of the superficial infections [66]. Although the symptoms of these infections are mild and not life threatening, they can act as reservoir of organisms which can spread to other areas of the body or other individuals [66]. Thus we have tested the susceptibility of dermatophyte strains including *Trichophyton* and *Microsporum* species to the K5 type yeast killer toxin. All of the tested dermathophyte strains were found susceptible to the toxin since β-1,3-glucanases are known to inhibit germ tube elongation along with some morphological changes such as leakage of cytoplasm and cell swelling [109].

Tinea pedis (infection of feet) is one of the most prevalent superficial mycoses caused by *Trichophyton mentagrophytes* or *T. interdigitale* [110]. Also individuals with tinea pedis are susceptible to secondary bacterial infections [66].

Standard strains of *Trichophyton mentagrophytes* and *Trichophyton interdigitale* were tested against their susceptibility to the K5 type yeast killer toxin. Both of the strains were found to be susceptible to the toxin at MIC_{100} of 2 and 4 µg/ml respectively. Other strains of the genus including *Trichophyton rubrum, T. verrucosum, T. equinum* and *T. tonsurans* which are isolated from human skin infection were found to be susceptible to the K5 type yeast killer toxin in the MIC_{100} range of 1-4 µg/ml. Among these species *T. rubrum* is known as the primary causative agent of *tinea capitis* (infection of scalp) which is characterized with hair loss [66].

Microsporum species which are also responsible for tinea infections were also covered in this part of the study. Three standard isolates of this genus *M. audouinii, M. gypseum,* and *M. canis* were susceptible to the toxin at MIC_{100} of 2 µg/ml , 2 µg/ml , 8 µg/ml respectively.

Fluconazole, ketoconazole and griseofulvin MIC_{100} mean values for were found for the following species in a study conducted on 508 dermathophyte isolates as follows [111]. *T. rubrum* 8, 1, 4 µg/ml; *T. verrucosum* 64, 16, 8 µg/ml; *T. equinum* 64, 6.35, 8 µg/ml and *T. tonsurans* 64, 8, 4 µg/ml; *M. canis* 16, 1, 1 µg/ml; *M. gypseum* 32, 2, 1 µg/ml respectively. Our results indicate that K5 type yeast killer protein is more affective than Fluconazole, ketoconazole and griseofulvin that are currently used in the therapy of such infections against most of the dermathophyte species.

Dermatophytes mostly invade the superficial keratinized tissue. Thus they are treated with topical antifungal agents. K5 type yeast killer protein was found to be affective on all the selected test strains, its potential to be used as a topical antifungal agent for superficial fungal infections was proved in this study. K5 type yeast killer protein can be readily formulated as lyophilized powder, topical solutions or cream for the treatment of superficial infections caused by dermathophytes.

Cell killing kinetics of the K5 type yeast killer protein was analyzed on different *Candida* isolates at 2 times strain specific MIC_{100}. Representative strains were selected due to their sites of isolation. Results were correlated with the antifungal susceptibility studies of the toxin. Complete cell killing activity was faster for the strains with relatively lower MIC_{100} values such as *C. glabrata* and *S. cerevisiae*. The cell killing kinetics graphs also indicates that K5 type yeast killer toxin is more affective on actively growing cells since the killing affect was faster in the time periods when the log phase was observed for the growth control. Cell killing activity was started with in the first 2 hours for all of the tested strains. Complete cell death was observed at the same time periods for the different isolates of the same species. Only growth inhibition was observed for the *C. krusei* and *C. parapsilosis* strains since their MFCs are higher than the tested protein concentration. Time periods of the complete cell death was ranged from 10 to 36 hours for the other strains.

Activity of the yeast killer proteins are depended on pH and temperature of the environment which limits their use in relevant fields. K5 type yeast killer toxin has a wide range pH (2.5-5.5) and temperature (4-37 ^{o}C) stability and looses only 50% of its activity at 100 ^{o}C [46]. These characteristics along with its high affinity to β-1,3- glucans are enormously increasing the application area of this protein and especially highlight the use of this toxin as a novel antifungal agent.

There are two major problems in the treatment of fungal infections with the currently used antifungal agents such as amphotericin B, fluconazole, ketoconazole, itraconazole. These agents are not selective to the fungal cells and cause severe side affects on the host cells and these side affects are mainly related with liver problems [77]. Another problem is the resistance development to the antifungals especially in long term usages. Therefore development studies on novel antifungal drugs are focused on selective agents which target the components of yeast and fungal cell walls. These agents would not harm the host cell because mammalian cells do not have these components [46, 72]. K5 type

yeast killer toxin exerts its lethal effect by hydrolyzing β-1,3- glucans of the cell wall of sensitive fungal cells and causes loss of cell wall rigidity which leads to cell death due to the osmotic pressure. [46]. β 1,3 glucan hydrolyzing activity of the K5 type killer protein on sensitive cells highlighted the potential use of this protein as a highly selective antimycotic agent. The use of the K5 type yeast killer protein as an antifungal agent will be feasible with appropriate formulation studies upon the antifungal spectrum determination of the toxin in this study.

CHAPTER V

CONCLUSION

1. K5 type yeast killer toxin was found to be affective against all of the tested 26 strains of the genus *Candida* within the MIC_{50}, MIC_{100} and MFC ranges of 0.25-4, 0.5-8, 1-16 µg/ml respectively except for *Candida krusei* isolates.

2. K5 type yeast killer toxin was found to be affective against all of the tested 9 strains of human pathogenic dermathophytes within the MIC_{50} and MIC_{100} ranges of 0.25-2, 1-8 µg/ml respectively.

3. K5 type killer toxin exerts its cytotoxic effect within 2 h and kills the entire population at time periods between 10-36 hours when tested at MFC.

4. Wide antifungal spectrum and selectivity of the toxin due to its strong exo-β-1,3-glucanase activity highlights the use of the K5 type yeast killer protein against human fungal infections.

5. K5 type yeast killer protein would be used against human fungal infections with appropriate formulations.

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APPENDIX A

CHEMICALS AND THEIR SUPPLIERS

Acetic Acid (Merck, Germany)

Aceton (Merck, Germany)

Acrylamide (Boehringer Mannheim, Germany)

Ammoniumpersulphate (Pharmacia Biotech, Sweden)

Bacto-agar (Difco, USA)

Bacto-peptone (Difco, USA)

Bis-acrylamide (Boehringer-Mannheim, Germany)

Bovine Serum Albumine Fraction V (Boehringer-Mannheim, Germany)

Bromophenol Blue (Sigma, USA)

Butanol (Merck, Germany)

β-mercaptoethanol (Sigma, USA)

Citric Acid (Merck, Germany)

Coomassie Brilliant Blue R-250 (ICN, USA)

Coomassie Brilliant Blue G-250 (ICN, USA)

Dithiothreitol (DTT) (Boehringer Mannheim, Germany)

D-Glucose (Merck, Germany)

Di-sodium Hydrogen Phosphate (Merck, Germany)

Di-potassium Hydrogen Phosphate (Merck, Germany)

Ethanol (Merck, Germany)

Formaldehyde (Riedel-de Haen, Germany)

Glutaraldehyde (Fluka, Switzerland)

Glycerol (Merck, Germany)

Hydrochloric Acid (Merck, Germany)

Malt extract (Difco, USA)

Methanol (Merck, Germany)

PDA (Merck, Germany)

Phosphoric acid (Merck, Germany)

Potassium Dihydrogen Phosphate (Merck, Germany)

RPMI 1640 (Sigma, USA)

Silver Nitrate (Merck, Germany)

Sodium Carbonate (Merck, Germany)

Sodium chloride (Merck, Germany)

Sodium Dodecyl Sulfate (Merck, Germany)

Sodium Hydroxide (Merck, Germany)

Sodium Sulfate (Merck, Germany)

TEMED (Pharmacia Biotech, Sweden)

Trichloroacetic Acid (Merck, Germany)

Tris (Merck, Germany)

Yeast extract (Difco, USA)

APPENDIX B

BUFFERS AND SOLUTIONS

Buffers / Solutions	Composition
1.SDS-PAGE Monomer Solution	30.8% T , 2.7% C_{bis}
4X Running Gel Buffer	1.5 M Tris-Cl , pH 8.8
4X Stacking Gel Buffer	0.5 M Tris-Cl , pH 6.8
SDS	10%
Initiator	10% Ammonium Persulfate
2X Treatment Buffer	0.125 M Tris-Cl , 4% SDS , 20% Glycerol , 10% β-mercaptoethanol , 0.020% Bromophenol blue , pH 6.8
Tank Buffer	0.025 M Tris , 0.192 M Glycine , 0.1% SDS , pH 8.3.
2.SILVER STAIN Destain Solution I	40% Methanol , 7% Acetic Acid
Destain Solution II	5% Methanol , 7% Acetic Acid
Cross-linking Solution	10% Glutaraldehyde
(DTT) Solution	5 µg/ml
Silver Nitrate Solution	0.1% w/v
Sodium Carbonate	3% w/v
Developing Solution	3% sodium carbonate, 0.019% formaldehyde

RPMI 1640 COMPONENTS	Conc. (mg/L)
INORGANIC SALTS:	
Calcium nitrate (Ca(NO3)2 4H2O)	100.00
Potassium chloride (KCl)	400.00
Magnesium sulfate (MgSO4)	48.84
Magnesium sulfate (MgSO4 7H20)	100.00
Sodium chloride (NaCl)	6000.00
Sodium Phosphate (Na2HPO4)	800.00
OTHER COMPONENTS:	
Glucose	2000.00
Glutathione Reduced	1.00
Phenol red	5.00
AMINO ACIDS:	
L-Arginine	200.00
L-Asparagine	50.00
L-Aspartic Acid	20.00
L-Cystine dihydrochloride	65.00
L-Glutamic Acid	20.00
L-Glutamine	300.00
Glycine	10.00
L-Histidine	15.00
L-Hydroxyproline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lysine hydrochloride	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Tyrosine disodium, dihydrate	29.00
L-Valine	20.00
VITAMINS:	
Biotin	0.2
D-Ca Pantothenate	0.25
Choline Chloride	3.00
Folic Acid	1.00
i-Inositol	35.00
Niacinamide	1.00
p-Aminobenzoic Acid (PABA)	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20