DETERMINATION OF ANTIMICROBIAL SPECTRUM OF K9 TYPE YEAST KILLER TOXIN AND ITS CELL KILLING ACTIVITY

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ABSTRACT

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Some yeast strains secrete extracellular polypeptide toxins known to have potential growth inhibitory activity on other sensitive yeast genera but are immune to their own toxins. These yeast strains are termed as killer yeasts and their toxins are designated as killer proteins or killer toxins. Killer phenotypes are classified into 11 typical types (K1-K11). The toxic actions of yeast killer proteins on sensitive cells show differences and one of the most important toxic actions involves the selective functional damage by hydrolyzing major cell wall components. Because mammalian cells lack a cell wall, novel highly selective antifungals tend to be harmless to people by targeting important cell wall components specific to fungi. We have previously characterized the K9 type yeast killer protein isolated from *Hansenula mrakii*. This protein is stable at pH and temperature values appropriate for its medical usage.

Antifungal activity of this protein was tested against 23 human pathogenic yeast and 9 dermathophyte strains. Pathogenic yeast strains found to be susceptible and both the MIC and MFC values ranged from 0.25 to 8 µg/ml except *C. parapsilosis* and *C guilliermondii* isolates. 9 dermatophyte strains were not susceptible to this protein and MICs were >64 µg/ml. According to the cell killing analysis toxin activity starts within the first 4 hours and complete cell death was observed for the 4, 8 and 16 times the MIC concentrations at 24 hour. The results obtained from this study might make the potential use of this protein possible as a selective antimycotic agent.

Key words: K9 type yeast killer toxin, antimycotic agent, human pathogenic fungi, MIC, MFC

ÖZ

K9 TİPİ ÖLDÜRÜCÜ MAYA PROTEİNİNİN ANTİMİKROBİYAL ETKİSİ VE HÜCRE ÖLDÜRME AKTİVİTESİ

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Bazı maya suşları diğer duyarlı maya cinslerinin potansiyel büyümelerini inhibe edici aktivitesi olduğu bilinen ekstraselüler polipeptid toksinler salgılarlar ve bu maya suşları kendi toksinlerine immündürler. Bu tip mantarlara öldürücü mantarlar ve salgıladıkları toksinlere öldürücü proteinler veya öldürücü toksinler adı verilmiştir. Öldürücü fenotipler 11 değişik tip altında sınıflandırılmışlardır (K1-K11). Bu maya öldürücü proteinlerinin hassas hücreler üzerindeki etki mekanizmaları farklılıklar gösterir ve bunların en önemlilerinden birisi de hücre duvarındaki bileşenleri hidrolize ederek seçici fonksiyonel hasar vermektir. Mantarlara özgü önemli hücre duvarı bileşenlerini hedefleyen yeni ve seçiciliği yüksek antifungallar, memeli hücrelerinin hücre duvarı olmadığı için insanlara zararsızdırlar. Laboratuarımızda yaptığımız çalışmalarda *Hansenula mrakii* suşundan izole edilen

K9 tipi öldürücü toksin tıp alanında kullanım için uygun olan sıcaklık ve pH şartlarında aktivitesini korumaktadır.

K9 tipi öldürücü maya proteininin antifungal aktivitesi CLSI metoduna göre 23 insan patojenik maya ve 9 dermatofit suşu üzerinde denenmiştir. Test edilen bütün 23 insan patojenik maya suşlarının bu proteine duyarlı olduğu ve toksinin MİK ve MFK'larının, *C. parapsilosis* ve *C. guilliermondii* izolatları haricinde, 0.25-8 µg/ml arasında olduğu saptanmıştır. Test edilen 9 dermatofit suşu üzerinde ise etkisinin olmadığı ve MİK değerlerinin 64µg/ml' den fazla olduğu bulunmuştur. *C. krusei* üzerinde yapılan hücre öldürme analizi toksin aktivitesinin ilk 4 saat içinde başladığını ve hücre ölümünün gerçekleştiği zamanın ise 4, 8 ve 16 x MİK konsantrasyonları için 24 saat olduğunu ortaya koymuştur. Yaptığımız bu çalışmalardan elde edilen sonuçlar bu proteinin seçici bir antimikotik ajan olarak kullanımını mümkün kılabilecektir.

Anahtar kelimeler: K9 tipi maya öldürücü proteini, antimikotik ajan, insan patojen mantarları, MİK, MFK

To My Family

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ATCC	American Type Culture Collection
bp	Base pair
Linear dsDNA	Linear double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DSMZ	German National Resource Centre for Biological Material
DTT	Dithiothreitol
HPLC	High Performance Liquid Chromatography
CLSI	Clinical and Laboratory Standards Institute
kb	Kilo base
kDa	Kilo dalton
L dsRNA	Large size double stranded RNA
MIC_{50}	Minimum concentration required to inhibit the growth of 50% of the isolates
MIC_{100}	Minimum concentration required to inhibit the growth of all the isolates
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

The group of microorganisms known as "yeasts" are simple unicellular true fungi and are considered as universal eukaryote model. Cells appear oval or spherical in shape and typically between 5-10 µm in diameter. Budding or binary fission is the most common mode of vegetative growth in yeasts and multilateral budding is a typical reproductive characteristic of ascomycetous yeasts, including *Saccharomyces cerevisiae*. Budding is an asymmetric process in which a daughter is initiated as an out growth from the mother cell, followed by nuclear division, cell-wall formation, and finally cell separation (Figure1.1.) [1,2].

Though vegetative growth is the major way of yeast reproduction, sexual reproduction is an alternative when nutrient supplies fall short. Although sexual reproduction is much less common than asexual reproduction, it introduces the possibility of variation into a population. To achieve sexual reproduction it is necessary to have two mating type haploid nuclei (α and a type) and they must fuse to form a diploid a/α cell, called zygote. That event is followed by the meiosis which produces four haploid sexual spores (a and α) called ascospores [3]. Life cycle of a yeast *S. cerevisiae* is shown in Figure 1.2. [4].

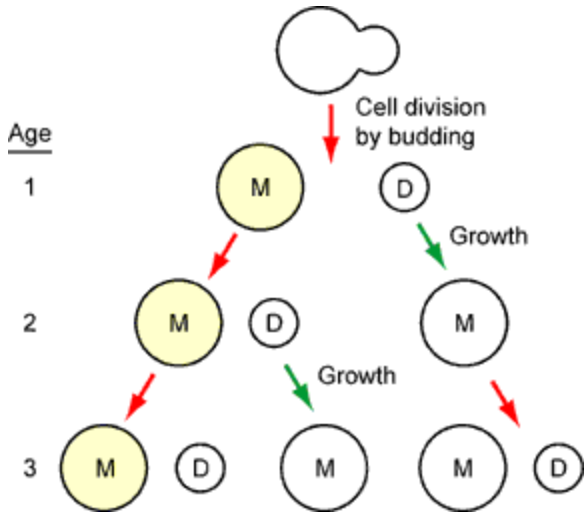


Figure 1.1. Budding Yeast Cell Division. Mother cell (M) gives rise to daughter (D) cells in an asymmetric process [2].

Among all eukaryotic model organisms, the yeast *Saccharomyces cerevisiae* is clearly the most ideal and the best understood universal model of a eukaryotic microorganism for biological studies [3]. Yeast has only a slightly greater genetic complexity than bacteria, and they share many of the technical advantages that permitted rapid progress in the molecular genetics of prokaryotes and their viruses. Some of the properties that make yeast particularly suitable for biological studies include rapid growth, a budding pattern resulting in dispersed cells, the ease of replica plating and mutant isolation, a well-defined genetic system, and the most important a highly versatile DNA transformation system. Being nonpathogenic, yeast can be handled with little precautions. Large quantities of normal bakers' or brewers' yeast are commercially available and can provide a cheap source for biochemical studies [5, 6].

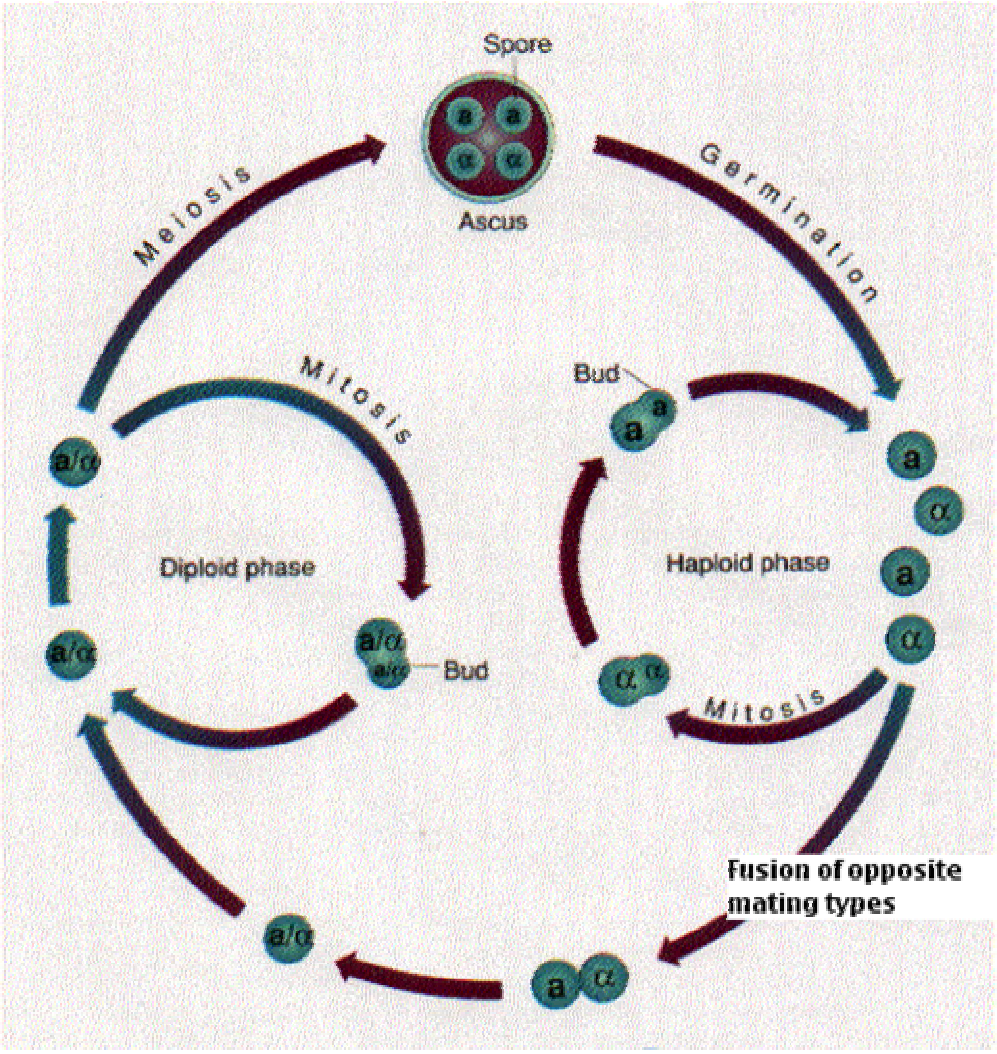


Figure 1.2. Life Cycle of a Typical Yeast *S. cerevisiae* [4].

Table 1.1. Yeast History [7].

Chronology	Milestones
6000-2000 BC	Brewing (Sumeria, Babylonia)
1680	Yeast under the microscope (van Leeuwenhoek)
1835	Alcoholic fermentation associated with yeast
1837	Name (S.cerevisiae) created for yeast observed in malt
1839	Sugar as a food source for yeast growth
1857	Fermentation correlated with metabolism (Pasteur)
1876	'Etudes sur la levure de bière' (Pasteur)
1877	Term "enzyme" (in yeast) introduced (Kühne)
1880	Single yeast cells and pure strains for brewing (Hansen)
1883	Alcohol and CO2 by cell-free extracts (Buchner)
1915	Production of glycerol
1920	Yeast physiology reviewed
1949	First genetic map (Lindegren); mating type system
1930-1960	Yeasts' taxonomy by Kluyver
1966	First tRNA structures from yeast
1978	First transformation of yeast (Hinnen, Hicks & Fink)
1990-1994	First commercial pharmaceutical products from recombinant yeast (Hepatitis B vaccine)
1990-1996	Completion of the yeast genome project

Table1.2. Industrial Uses of Yeasts and Yeast Products [8].

Production of yeast cells	Baker's yeast for bread making Dried food yeast for food supplements Dried feed yeast for animal feeds
Yeast products	Yeast extract for culture media B Vitamins, vitamin D Enzymes for food industry, invertase, galactosidase Biochemicals for research, ATP, NAD^+, RNA
Fermentation products from yeast	Ethanol for industrial alcohol Glycerol
Beverage alcohol	Beer Wine
Distilled beverages	Whiskey Brandy Vodka Rum

Yeasts can be considered man's oldest industrial microorganism. The unique properties of the yeast, *S.cerevisiae*, among some 700 yeast species and its enormous hidden potential which has been exploited for many thousands of years, made it a preferred organism also for research. Moreover, *S. cerevisiae* and other yeasts yielded a vast majority of industrial and medical applications beneficial to human life. The desired products of yeasts are either cells themselves that are cultured, the cell components or the end products that are produced during alcoholic fermentation [8].

Large-scale fermentation by yeasts is responsible for the production of ethanol for industrial purposes, but yeast is better known for its role in the manufacture of alcoholic beverages including beer, wine, and liquors by fermenting sugars from rice, wheat, barley, and corn. Besides these, industrial usage of yeast cells includes bread making, food supplements, animal feed, and glycerol production, enzyme production such as invertase, galactosidase, vitamin production including vitamin B and D. Yeasts are also used extensively in environmental technologies such as bioremediation, waste utilization, crop protection, biosorption of metals [9].

Moreover *S. cerevisiae* and other yeast protein expression systems are used for the production of many industrially relevant enzymes and are widely used by the research community to produce therapeutics that cannot be actively expressed in *Escherichia coli* or require glycosylation for proper folding and biological activity [10]. For example, the gene for human interferon and hormones such as parathyroid hormone and somatostatin have been cloned and expressed in yeast [11,12].

Antifungal proteins are produced by many organisms including bacteria, fungi, insects, vertebrates and invertebrates as well as plants. In 1963, Bevan and Makower discovered the killer phenomenon [K+] in laboratory strains of *S. cerevisiae* which were isolated as a brewery contaminant [13]. Some strains of *Saccharomyces* and other genera under certain conditions extrude into the

medium some polypeptide toxins known as killer toxins, killer proteins or killer factors. These extracellular polypeptide toxins are known to have potential growth inhibitory activity on other sensitive yeast genera but are immune to their own toxins. This phenotype of toxin specific insensivity is termed as self-immunity [14]. Yeast strains which secrete these polypeptide proteins are termed as killer yeasts. Also killer toxins of certain yeast strains have potential growth inhibitory effects on gram positive pathogenic bacteria including *Staphylococcus aureus* [15] and plant pathogenic fungi [16]. The role and function of these toxins in natural yeast communities is uncertain. It is suggested that the killer phenomenon is a potential mechanism for interference competition, where the production of toxic compounds prevents a competitor from gaining access to resources [17, 18]. When mycogenic yeasts are present in natural communities, a single killer strain usually predominates. Kinetic studies have shown that a killer strain can predominate in a mixed culture with a sensitive yeast strain [19].

Killer toxins have certain properties in common; they are proteinaceous substances, in general have low pH optima and are usually inactivated at elevated temperature [17]. Besides these common characteristics, the killer toxins differ between species or strains, showing diverse characteristics in terms of structural genes, molecular size, mature structure and immunity. The mechanisms of recognizing and killing sensitive cells differ for each toxin [20].

The production of cytotoxic proteins (killer toxins) is a widespread phenomenon among a great variety of yeast genera and species including *Saccharomyces, Candida, Cryptococcus, Debaryomyces, Hansenula, Kluyveromyces, Ustilago, Williopsis, Hanseniaspora* and *Zygosaccharomyces* [21-24]. These killer yeast species of different genera that have been isolated differ in their toxic compounds, in pH optima, temperature stability and sensitivity to proteolytic enzymes [17]. Studies on the nature of the killer factor produced by *Saccharomyces* have shown that this protein has a highly specific action spectrum and is dependent on specific pH, temperature and aeration conditions [25].

Young and Yagiu (1978) recognized 10 distinct groups (K1-K10) with respect to cross-killing and immunity interactions among killer strains of the genera [17]. This biochemical distinction was shown by analysis of the effects of proteolytic enzymes, temperature and pH on killer activity and by gel chromatography of crude preparations of killer factors. These killer strains which include the three strains of *Saccharomyces* genus (K1, K2, K3) are shown in Table 1.3. with their proteins. K11 type yeast killer protein isolated from *Torulopsis glabrata* was introduced by Wickner in 1975 to the Young and Yagiu classification [26].

Table 1.3. Killer Yeasts and Their Protein Toxins [17].

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
Torulopsis glabrata	K11

*The K1 phenotype belongs to the laboratory strains of *S. cerevisiae* and wild type strains of the species, while K2 has been found almost exclusively among fermentation contaminants and is capable of killing K1 killers.

Table 1.4. Nomenclature Used for Killer Phenotypes [14].

Phenotype	Properties
$K_1^+ R^+_1$	Strains producing killer protein, immune to it and sensitive to types K2 and K3.
$K^- R^+$	Neutral phenotype; strains do not produce killer protein and immune to it.
$K_1^{++} R_1^+$	"Super killer" phenotype; strains producing more active or more stable K1 killer protein.
$K_1^+ R_1^W$	"Suicidal" phenotpye; strains producing killer protein and exhibiting a decreased immunity to tpye K1.
$K^- R^-$	Sensitive strains not producing killer protein.

1.1. Genetic Basis of Killer System

Yeast is a simple single-cell eukaryotic organism that shares many characteristics with higher eukaryotic cells [9]. Therefore the complete sequence of yeast genome has proved to be extremely useful as a reference towards the sequences of human and other higher eukaryotic genes. The ease with which yeast can be manipulated genetically, coupled with its obvious industrial uses, has made this a favored system for studying gene regulatory events, and much progress has been made since the advent of recombinant DNA technology and the development of a transformation system for yeast in the late 1970s. The genomes of yeasts have been the subject of intense investigations during the last three decades. Even the largest yeast chromosomes are an order of magnitude smaller than human chromosomes and therefore provide simple models for investigating both chromosome structure and function.

In April 1996 the genome sequence of *S.cerevisiae* was finished and published together with extensive bioinformatic evaluations and speculative evolutionary implications. This unique collaboration of over 600 scientists from 100 laboratories in Europe, USA, Japan and Canada revealed the first complete set of DNA information from a eukaryotic organism [27]. The most widely investigated yeast, *Saccharomyces cerevisae*, contains 16 haploid chromosomes ranging in size from 200 to 2,200 kb. Pulsed- field gel electrophoresis has been used to separate chromosomes of *Saccharomyces cerevisae* by size [28]. Each chromosome contains multiple origins of replication, two telomeres and a centromere, and is in the form of chromatin in the nucleus. The total sequence of chromosomal DNA constitutes approximately 14 000 kb. Approximately 30% of the genes already have been characterized experimentally. Of the remaining 70% with unknown function, approximately one half either contain a motif of a characterized class of proteins or correspond to genes encoding proteins that are structurally related to functionally characterized gene products from yeast or from other organisms [29,30].

In addition; the 2µm circle plasmids, present in most strains of *Saccharomyces cerevisiae* apparently function solely for their own replication. It was first discovered by electron microscopy, and was named for its contour length and closed circular conformation as 2µm circles [31].

The killer character is genetically complex phenomenon since it depends on both on cytoplasmic factors and several chromosomal genes. Many types of killer toxins have been reported and their genomes were mapped either on cytoplasmically inherited encapsulated double-stranded RNA viruses, linear double-stranded DNA plasmids or on chromosomal genes [32-34].

1.1.1. ds RNA Virus Based Yeast Killer Systems

In cooperation with nuclear genes the killer phenomenon is brought about by dsRNA molecules. The dsRNA molecules are closed in virus-like particles (VLPs) thus termed as *Saccharomyces* virus (ScV) [14,35,36]. These yeast dsRNA viruses are considered as non-infectious because there is not an identified route of natural transmission. They are vertically transmitted and transmission occurs only by cytoplasmic mixing during budding, mating or by cell fusion and thus behaves as cytoplasmically genetic element [37]. The occurrence of a virus like particle in the cytoplasm and thus the secretion of a virally coded protein toxin, gives the cell its killer character. The strains lacking the virus are sensitive to the toxins produced by the harboring strains which are immune to their own killer factors [36].

There are five families of double stranded RNA in strains of *Saccharomyces cerevisiae* designated as L-A, L-BC, T, W and M, three of which (L-A, L-BC and M) are packaged separately by a common major capsid protein into virus like particle of the mycovirus. All show non-Mendelian inheritance [38].

Production of the killer toxin and immunity to the homologous protein are encoded by MdsRNAs. The variance of M called M1, M2, M3, etc. encodes different extracellular toxins and immunity to the respective killer protein. M1dsRNA, M2dsRNA and M3dsRNA encode toxin proteins for the K1, K2 and K3 killer systems respectively [38]. The replication of the MdsRNA is dependent on a larger dsRNA called L-dsRNA which is comprised of two unrelated families of molecules called L-A and L-BC family [39,40]. L-A encodes the major coat protein of the VLPs in which both LdsRNA and MdsRNA are encapsulated. It is not clear whether L-BC dsRNAs have any functional relation to killer phenomena since some killer strains lack L-BC entirely [40-42].

1.1.1. a. Synthesis, Processing and Secretion of the Killer Toxins

Among the virally encoded killer toxins of *S. cerevisiae* K1 and K28 are the most studied proteins in terms of their production and secretion. Although both toxins differ significantly in their amino acid composition and their molecular mode of action, they are homologous with respect to their synthesis, processing and secretion [36]. Most killer toxins have been shown to be produced from preprotoxins (pptox) through enzymatic processing. K1 and K28 toxins are both encoded by ds RNA viruses and information for the synthesis of the killer protein is stored in MdsRNA. Preprotoxin is secreted as a molecule consisting of two distinct disulfide bonded subunits, termed α (9.5 kD) and β (9.0 kD). The α and β domains flank a segment called γ , which is not a part of the mature toxin and assumed to be the immunity determinant [43-45].

The protein precursor enters the yeast endoplasmic reticulum via a highly hydrophobic N-terminal secretion signal. Than, undergoes post-translational modifications within the endoplasmic reticulum, and the golgi complex until it is finally secreted as mature α/β heterodimeric protein toxin [36].

Two gene loci KEX1 and KEX2 were identified when the mutants of K1 and K28 killers that failed to secrete the active toxin but remain immune to the toxin are studied [44]. The gene product of KEX2, Kex2p, is like an endoprotease which preferentially cleaves the proregion and removes the intramolecular γ sequence, while Kex1p is a serine carboxypeptidase that removes the C-terminal basic dipeptide of the α subunit exposed by Kex2p action [44,46].

Kex2p/Kex1p mediated preprotoxin processing removes the N-glycosylated γ sequence, trims the carboxy terminus of α and finally results in the secretion of 21 kDa α/β heterodimeric protein toxin whose α and β subunits are covalently linked through a single disulfide bond [47].

Secretion pathway of K28 is shown in Figure 1.3. and processing of K1 type yeast killer toxin is shown in Figure 1.4.

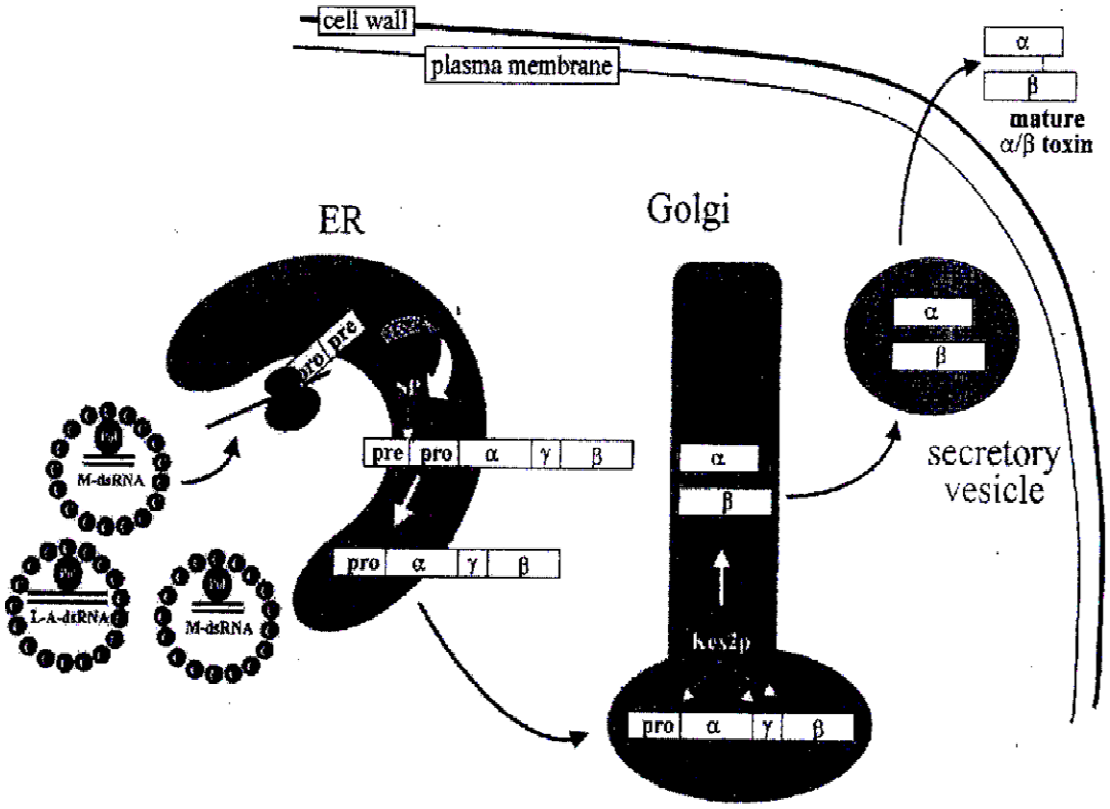


Figure 1.3. Secretory Pathway of Killer Toxin K28 in *S. cerevisiae* [36].

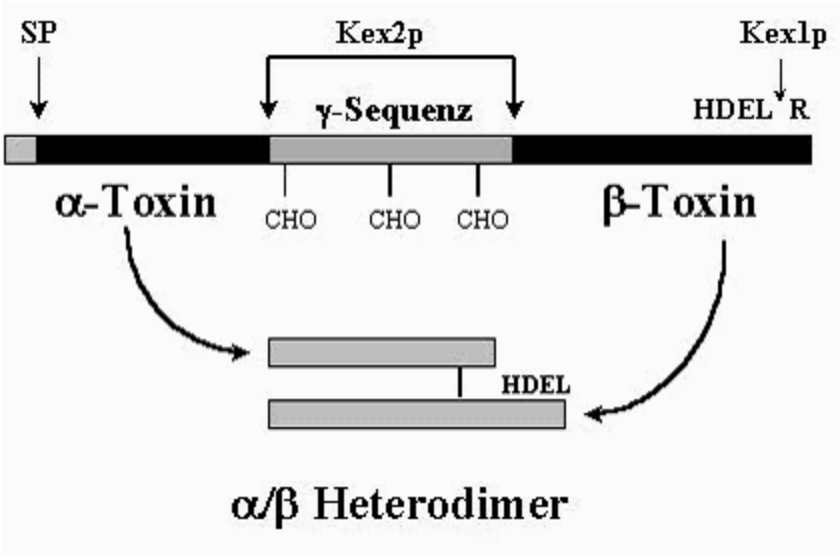


Figure 1.4. Structure and Processing of the K1 Type Yeast Killer Toxin [21].

1.1.2. Linear ds DNA Plasmid Based Killer Systems

Cytoplasmically localized linear yeast elements have been isolated from numerious genera such as *Debaryomyces, Wingea, Kluyveromyces* and *Saccharomyces*. The killer toxins differ from the toxin produced by dsRNA mediated killer yeast of *S. cerevisiae* in molecular weight, mechanism of killing action and killing spectrum against various yeasts [35].

Kluyveromyces lactis killer system showed that production of the toxin was dependent on the presence of two linear dsDNA plasmids which have been termed k1 (Pgkl1) and k2(Pgkl2) respectively. The smaller plasmid Pgkl1 encodes the killer and resistance characteristics, where as the larger plasmid Pgkl2 is likely to be necessary for replication and maintenance of the linear plasmids [33,34,48,49].

1.1.3. Chromosomally Encoded Yeast Killer Systems

There is no evidence available to indicate that killer character expressed by strains of *Williopsis, Pichia, Candida, Debaryomyces* and *Torulopsis* are associated with ds RNA viruses or ds DNA plasmids. Since there is no evidence for other systems, the killer character in these yeasts is assumed to be chromosomally inherited.

Two new killer activities were discovered in 1990 by Goto et al. which were weaker than known killer strains' activities. Their genes were found to be encoded on chromosomal DNA of *S. cerevisiae*. One gene designated KHR was encoded on chromosome IX and another one designated as KHS on chromosome V [36,50].

The HMK gene, encoding a killer toxin of *Hansenula mrakii* and the HSK gene encoding a killer toxin of *Hansenula saturnus* have been purified by Yamamoto et al. and Ohta et al., respectively. The toxins of these two genes show higher thermostability and wider pH stability than other killer toxins [51,52].

The genetic basis for the expression of killer proteins in different killer yeast strains is given in Table 1.5.

Table 1.5. Genetic Basis of Killer Phenotype Expression in Yeast [34].

Killer Yeasts	Genetic Basis
Saccharomyces cerevisiae	ds RNA virus
Hansenula uvarum	ds RNA virus
Zygosaccharomyces bailii	ds RNA virus

Ustilago maydis	ds RNA virus
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.Mode of Action of the Yeast Killer Proteins

The biological action of the killer toxins is considered to consist of two step receptor-mediated process. The first step involves a fast and energy-independent binding to a toxin receptor within the cell wall of a sensitive target cell. The second energy dependent step involves the toxin translocation to the cytoplasmic membrane and interaction with a secondary membrane receptor that leads to lethal effects [53-56].

β-Glucan is one of the major components of the yeast cell wall and is divided into two categories; (1-3)-β-D-linked glucan and (1-6)-β-D-linked glucan. Both types of β-Glucan, chitin and mannoproteins of the cell wall have been proposed as primary receptors of killer toxins [53-58].

The β-1,6-D-glucans are primary receptors for *Hanseniaspora uvarum*, *S. cerevisiae* K1 and K2 killer toxins, *Debaryomyces hanseni* and *Pichia membranifaciens* killer toxins. β-1,3-D-glucans are involved as primary receptors of *Hansenula mrakii*. Mannoproteins are receptors for *Zygosaccharomyces bailii* killer toxin and KT28 of *S. cerevisiae*; and chitin has been described as cell wall receptor for *Kluyveromyces lactis* and *Pichia acaciae*

killer toxins. Thus, any of the principal components of the cell wall could be the primary receptor for a killer toxin [54,55,59-61].

Binding of K1 killer toxin to β-1,6-D-glucan of the cell wall is the initial step for the action of this toxin. K1 toxin is known to cause ion channel formation which leads to produce uncontrolled leakage of potassium, protons, ATP and other small molecules from target cells and destroying the pH gradient of membrane leading to cell death [62].

The killer toxin of K28 of *S. cerevisiae* is different from known killer toxins, although resembles the K2 type. The receptors of the K28 are the mannoproteins of the cell wall. K28 causes cell cycle arrest, apparently in the G2 phase, and leading to non-separation of mother and daughter cells. K28 toxin irreversibly blocks DNA synthesis [61,63].

Another mode of action of killer toxin is seen in *Kluyveromyces lactis* in which the toxin acts by arresting the sensitive cells at G1 phase of the cell cycle and leads to loss of viability. The interaction of the killer toxin with the cell wall chitin is the first step in toxin action and then the toxin is internalized into the cell. Finally, the toxin causes G1 arrest and cells do not divide although they complete their cell cycles [64].

The toxin from *Pichia kluyveri* causes ion-channel formation that leads to sudden K^+ and ATP leakage. These channels are relatively non-selective for common physiological cations and anions. The toxin-induced channels would cause a 'leak pathway' for major ions such as K^+ and H^+ and dissipate the normal ionic gradients across the plasma membrane. Finally, these toxin induced channels result in sudden cell death [65].

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 [66].

Another toxin that interacts with the β-1,3-glucans of the cell wall of fungal cells is the HM1 toxin of *Hansenula mrakii.* HM1 has been shown to inhibit the synthesis of β-1,3-glucans in vivo and in vitro resulting in pore formation, leakage of material and eventual cell death during budding or responding to mating factor. Two genes of *S. cerevisiae* that confer sensitivity to HM1 are KNR4 and RHK1 which are involved in synthesis of β-1, 3-glucan. Disruption of KNR4 resulted in HM1 resistance and in a reduced level of β-1, 3-glucan synthase. In contrast over expression of RHK1 conferred HM1 resistance and increased the β-1,3-glucan content in the cell wall fraction . The designation RHK1 indicates that mutants are resistant to the HM-1 killer toxin from the yeast *Hansenula mrakii*, possibly due to defective glycosylation of the toxin receptor [67-71].

1.3. Toxin immunity

The killer cell is effectively protected against its own toxin. Although the precise molecular basis for toxin immunity is still unknown, there are studies showing that the unprocessed toxin precursor is sufficient to confer immunity in K1 and K28 strains [72].

A model was proposed in 1986 in which it was speculated that α, either alone or as part of a larger immunity determinant, would interact with the receptor during secretion, rendering it inaccessible or unrecognizable to exogenous α. Then it was shown that secretion of α fused to just an N-terminal fragment of γ component of the toxin was sufficient to confer immunity for the host cells [73].

On the other hand, for the K28 toxin a very different mechanism should be postulated because of its entering back into the host cell after secretion. The toxin re-enters the secretory pathway of a killer cell and reaches the cytosol, just as in a sensitive target cell. However the host cell is not killed but rather protected against the toxin effect. So, K28 immunity should work either within the yeast cytosol or eventually within the nucleus [36].

1.4. Applications of yeast killer phenomenon

Several potential applications for the killer phenomenon have been suggested since it was determined and studied. In fermentation industries, the killer character can be used to combat wild, contaminating *Saccharomyces* strains [74]. In the food industry, killer yeasts have been proposed to control spoilage yeasts in the preservation of food [75]. In the medical field, killer yeasts have been used in the biotyping of pathogenic yeasts [76], and the killer toxins of *Pichia anomala* [77,78], and *Hansenula mrakii* [67,79] have been proposed as antimycotic agents. Also they are used in fundamental research for studying protein processing and secretion, and recombinant DNA technology.

1.4.1. Food and fermentation industry

The biological control of the undesirable yeasts in the production and storage of various foods can be achieved by means of yeast cultures that produce yeast killer toxins. In wine making, killer yeasts belonging to *S. cerevisiae* are currently used to initiate wine fermentation as starter strains to improve the process of wine making and wine quality [74,79,80]. However, the main limit of the killer toxin of *S. cerevisiae* wine yeast (K2 type) resides in its narrow antiyeast spectrum which, being restricted to sensitive *Saccharomyces* strains, does not affect wild yeasts, such as those belonging to the genera *Hanseniaspora/Kloeckera, Pichia*, and *Saccharomycodes*. Stable double killer wine yeast strains have been generated by gene replacement technology. They

exhibit a significantly broader killing activity and are thus capable of competing potentially contaminating yeasts like *Candida, Hanseniaspora, Kloeckera* and *Pichia* in mixed culture [81].

Several ecological studies have clearly demonstrated that apiculate yeasts (*Hanseniaspora/Kloeckera*) predominate on grape surfaces and in freshly pressed juice. The control of the growth of apiculate yeasts in a nonsterile environment such as grape must is generally carried out by sulfur dioxide. However, several institutions, such as the World Health Organization and the European Economic Community, have highlighted the need to reduce the use of this antimicrobial agent in food products because of its toxicity. In this context, the use of a killer toxin as a control agent for apiculate yeasts in the prefermentative stage and during the fermentation of grape must be encouraged in order to reduce or eliminate the use of SO_2 [82,83]. For example; *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* [84].

1.4.2. Transgenic plants for expression of killer toxin

Plants are also exposed to a large number of pathogenic fungi; therefore, transgenic plants, which are capable of producing polypeptides that are toxic to disease causing pathogens, are being constructed for the improvement of pathogen resistance in crop plants [36].

The killer toxin which is naturally produced and secreted by virus infected strains of the fungal pathogen *U. maydis* has been shown to be an attractive and unique model for the introducing of the fungal resistance into tobacco plants [85-87]. *Ustilago* species are known as crop pathogens, including pathogens of maize, wheat, oats, and barley. *Ustilago* killer toxins appear to have a mode of action similar to that of the *Saccharomyces cerevisiae* killer toxin Kl, which introduces new ion channels in cellular membranes [88]. The UmV toxins of *U. maydis* are particularly attractive as biological control agents because they

have no known effects on plant or animal cells, presumably because specific receptor proteins are required for toxin binding and activity [89]. These virally encoded yeast toxins in crop plants may be a novel strategy to engineer biological control of fungal pathogens.

1.4.3. Heterologous protein secretion

The production of a functional protein is intimately related to the cellular machinery of the organism producing the protein. *E. coli* has been the "factory" of choice for the expression of many proteins because its genome has been fully mapped and the organism is easy to handle; grows rapidly; requires an inexpensive, easy-to-prepare medium for growth; and secretes protein into the medium which facilitates recovery of the protein. However, *E. coli* is a prokaryote and lacks intracellular organelles, such as the endoplasmic reticulum and the golgi apparatus that are present in eukaryotes, which are responsible for modifications of the proteins being produced. Many eukaryotic proteins can be produced in *E. coli* but are produced in a nonfunctional, unfinished form, since glycosylation or post-translational modifications do not occur. Therefore, researchers have recently turned to eukaryotic yeast and mammalian expression systems for protein production. Some unicellular eukaryotes such as yeasts including *Saccharomyces cerevisiae, Pichia pastoris, Yaravia lipolytica, Hansenula polymorpha, Kluyveromyces lactis* and *Schizosaccharomyces pompe* have become attractive hosts for the expression of heterologous proteins [90,91].

Kluyveromyces lactis has recently become an attractive microbial host for the expression of foreign genes and protein secretion for several reasons, including (i) its food grade status, since *K. lactis* is present in various milk products it is accepted as "GRAS" (generally recognized as safe); (ii) its excellent fermentation characteristics; (iii) the existence of both episomal and integrative vectors; and (iv) its ability to secrete high-molecular-weight proteins [90,92,93].

Bacterial xylanase which is used in the paper manufacturing industry is expressed and secreted in *K. lactis* using a secretion signal derived from the pro region of the *K. lactis* killer toxin [94].

An increasing number of pharmaceutically important secretory proteins such as mouse α-amylase, human antithrombin III or placental alkaline phosphatase have been expressed as extracellular proteins by using homologous secretion signals derived from plasmid driven yeast killer toxin of *K. lactis* [36,95,96].

Since the proteins produced in *P. pastoris* are typically folded correctly and secreted into the medium, the fermentation of genetically engineered *P. pastoris* provides an excellent alternative to *E. coli* expression systems. A number of proteins have been produced using this system, including tetanus toxin fragment, human serum albumin and lysozyme [97-99].

1.4.4. Medical applications of killer toxins

Humans and other animals are exposed to fungi from the moment of birth. Fortunately, only 200 or so species are pathogenic to mammals, although many nonpathogenic fungi cause allergy symptoms [100]. During the last two decades, the incidence of human fungal infections, especially involving population of immunocompromised patients due to intensive chemotherapy, immunosuppressive drugs and HIV, has dramatically increased [101-102]. Human fungal infections in Europe and large parts of the world are uncommon in normally healthy persons, being confined to conditions such as candidiasis (thrush) and dermatophyte skin infections such as athlete's foot. However, in the immunocompromised host, a variety of normally mild or nonpathogenic fungi can cause potentially fatal infections. However, in patients with compromised immune systems, infections even by fungal organisms with low virulence can be

life threatening; for example, systemic fungal infections of leukemia patients account for 50% of fatalities [103-104].

It is ironic that many of these patients succumb to fungal infections for which there are few or no drugs available for treatment. Encouragingly, naturally occurring antifungal proteins and peptides, as well as synthetic derivatives, have the potential to be very interesting clinical leads.

Fungal infections or mycoses are classified depending on the degree of tissue involvement and mode of entry into the host. These are:

Superficial - localised to the skin, the hair, and the nails.

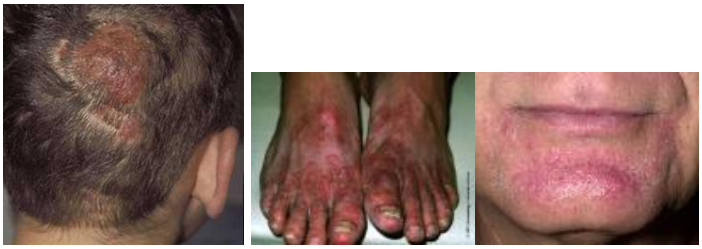
Subcutaneous - infection confined to the dermis, subcutaneous tissue or adjacent structures.

Systemic - deep infections of the internal organs.

1.4.4.a. Superficial Mycoses

In superficial mycoses, infection is localised to the skin, the hair, and the nails. An example is "ringworm" or "tinea", an infection of the skin by a dermatophyte. Ringworm refers to the characteristic central clearing that often occurs in dermatophyte infections of the skin. Dermatophyte members of the genera *Trycophyton, Microsporum* and *Epidermophyton* are responsible for the disease. Tinea can infect various sites of the body, including the scalp (tinea capitis), the beard (tinea barbae), the foot (tinea pedis: "athlete's foot"), the nails (tinea unguium) and the groin (tinea cruris-caused by *Epidermophyton floccosum*) [105-107]. Dermathophytes that are involved in the superficial infections and related diseases are given in Figure 1.5. [105].

Candida albicans is a yeast causing candidiasis or "thrush" in humans. As a superficial mycoses, candidiasis typically infects the mouth or vagina. *C. albicans* is part of the normal flora of the vagina and gastrointestinal tract and is termed a "commensal". However, during times of ill health or impaired immunity, the balance can alter and the organism multiplies to cause disease. Antibiotic treatment can also alter the normal bacterial flora allowing *C. albicans* to flourish [108].



A **B** **C**

Figure 1.5. Examples for Superficial Infections and Causative Agents.

A) Tinea capitis caused by *Trichophyton verrucosum, Trichophyton tonsurans* and *Microsporum audouinii*

B) Tinea pedis caused by *Trichophyton rubrum* and *Trichophyton mentagrophytes*

C) Tinea barbae caused by *Trichophyton verrucosum*

These infections are initially treated with topical antifungal agents such as azoles or allylamines since they infect the outer layer of the skin [109].

1.4.4.b. Subcutaneous Mycoses

These are infections confined to the dermis, subcutaneous tissue or adjacent structures. Infection may arise following the wounding of the skin and the introduction of vegetable matter. These mycoses are rare and confined mainly to tropical regions. They tend to be slow in onset and chronic in duration. An example is sporotrichosis caused by *Sporothrix schenckii* [110]. Infection usually follows an insect bite, thorn prick or scratch from a fish spine. Certain occupation groups appear to have increased risk from infection. These include florists, farm workers and others who handle hay and moss.

1.4.4.c. Systemic Mycoses

These are invasive infections of the internal organs with the organism gaining entry by the lungs, gastrointestinal tract or through intravenous lines. They may be caused by: (i) primary pathogenic fungi or (ii) by opportunistic fungi that are of marginal pathogenicity but can infect the immunocompromised host.

Primary Pathogenic Fungi: Infection occurs in previously healthy persons and arises through the respiratory route. Examples include histoplasmosis, blastomycosis, coccidiomycosis and paracoccidiodomycosis.

Opportunistic Fungi: In that of diseases, patients usually have some serious immune or metabolic defect, or have undergone surgery. The diseases include aspergillosis, systemic candidosis and cryptococcosis [105].

Aspergillosis: This is the name given to a number of different diseases caused by the mould *Aspergillus*. *A. fumigatus* is the most common species causing disease. The organism can infect the lungs, inner ear, sinuses and, rarely,

the eye of previously healthy persons. In the immunosuppressed host, *Aspergillus* can disseminate throughout the body [111].

Candidosis: In severely immunocompromised patients (e.g. those receiving chemotherapy) *C. albicans*, that is part of the normal human flora, can proliferate and disseminate throughout the body [112].

Cryptococcosis: This is a systemic infection caused by the yeast *Cryptococcus neoformans*. The most common manifestation is a subacute or chronic form of meningitis resulting from the inhalation of the organism. Pulmonary infection can also occur. The disease affects both healthy and immunosuppressed individuals [113].

Non-*Candida albicans* isolates are now emerging in immunocompromised or hospitalized patients. *Candida* species are the fourth most common pathogens isolated from blood cultures and the number of non-*Candida albicans* (NAC) with decreased susceptibility to anti-fungal agents is also increasing. These emerging pathogens include *Candida parapsilosis, Candida glabrata, Candida krusei, Candida guilliermondii* and *Candida keyfr* [114]. 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 [105].

The emergence and rapid dissemination of infectious agents resistant to antibiotic treatments have emphasized the need for alternative chemotherapies [115]. Among the new antimicrobial molecules under investigation, specific yeast killer toxins represent promising candidates because they display wide spectra of activity [116,117]. Especially toxins of the genus *Hansenula*, *Pichia*, and *Kluyveromyces*, because of their broad spectrum of killing activity, have been proposed as the basis for novel therapeutic strategies to treat human and animal insidious infectious diseases [16,79,118,119]. 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* [15].

The antimycotics which are currently used in the therapy of fungal infections can be grouped into three classes based on their site of action: azoles, which inhibit the synthesis of ergosterol (the main fungal sterol) such as Flucanazole and Ketaconazole; polyenes, which interact with fungal membrane sterols physicochemically such as Nystatin and amphotericin B; and antimetabolite fungal drugs such as 5-fluorocytosine, which inhibits both DNA and RNA synthesis [120]. The increased use of these antifungal agents in recent years has resulted in the development of resistance to these drugs and these resistances spread rapidly among other microorganisms which are pathogenic to man and cause problems in the therapy of those pathogens [121].

Yeast and fungal cell wall components represent attractive targets, since these structures are usually restricted to yeasts and higher fungi and do not occur in mammalian cells. Three basic constituents represent the major cell wall polysaccharides of the fungal cell: (i) branched polymers of glucose containing β-1,3 and β-1,6 linkages (β-glucans); (ii) unbranched polymers of *N*-acetyl-D-glucosamine (GlcNAc) containing β-1,4 bonds (chitin); and (iii) polymers of mannose (mannan) covalently associated with proteins (glyco[manno]proteins). The outer cell wall of dermatophytes contains glycopeptides that may evoke both immediate and delayed cutaneous hypersensitivity and they contain chitin and glucan as major cell wall polysaccharides [122]. Since the cell wall components was shown to act as the primary binding site and cell wall receptor for the different yeast killer toxins, antifungal research is currently focusing on the possible use of yeast killer toxins as novel antifungals [36,123].

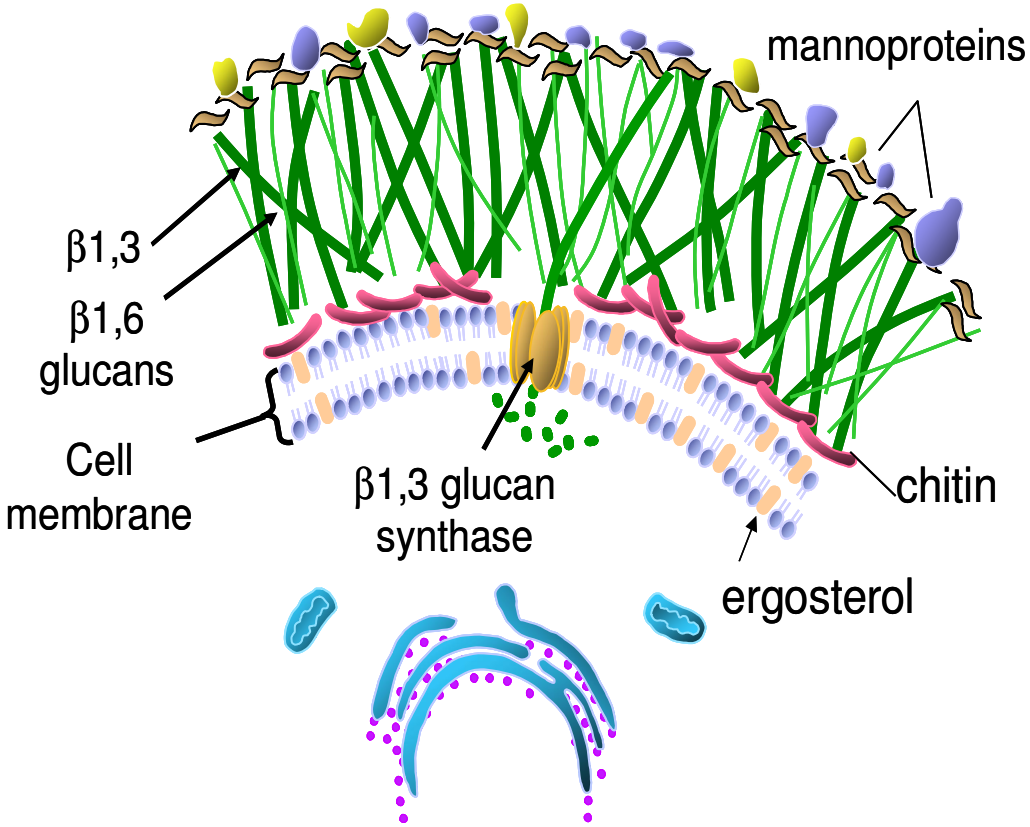


Figure 1.6. Yeast Cell Wall Structure [124].

1.5. K9 Type Yeast Killer Protein

K9 type yeast killer protein is produced by *Hansenula mrakii* (reclassified as *Williopsis mrakii*) [125] NCYC 500 according to the Young and Yagiu classification.

Figure 1.7. *Hansenula mrakii* Cells on YEPD Agar Medium.

Hansenula mrakii is a fungi belonging to the phylum; *Ascomycota*, class; *Saccharomycetes*, order; *Saccharomycetales*, family; *Saccharomycetaceae*, genera; *Hansenula* and species; *mrakii* [126].

Spore-forming ascomycetous yeasts of the genera *Pichia* and *Williopsis* displayed the broadest range of activity against sensitive strains of *Candida spp*. and *Saccharomyces cerevisiae*. *Williopsis mrakii* (NCYC 500) showed extensive anti-*Candida* activity against strains isolated from clinical specimens [79].

The proteinaceous killer toxin of *H. mrakii* have a wide-range intergeneric killing spectrum with relatively high stability against heat and it is unaffected over a wide range of pH changes, contrasting with most of the killer toxins. Consistent with the previous data on yeast killer toxins [15,46] it was shown that the production of the K9 type yeast killer toxin was pH and temperature dependent [127].

K9 type yeast killer protein was purified and characterized in terms of its biochemical properties for large scale production in our laboratory. It has a molecular weight of 46 kDa. This value is in the range of other known killer proteins' molecular masses. The isoelectric point was found 3.8 which showed that the K9 type killer protein is an acidic protein. This result was consistent with the data that with one exception all identified killer proteins are also acidic, as expected because of their acidic habitat that they live in. The optimum temperature and pH condition for the production of the K9 type killer protein was found to be around 18 ºC and pH 4.5. Mode of action of the K9 type yeast killer protein was also studied previously in our laboratory. The action of toxin on laminarin which is mainly composed of β-1,3- glucans showed that K9 type killer protein exerts hydrolytic activity on the β-1,3-glucans because of the presence of glucose release. 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 [127].

Cell killing activity of the K9 type yeast killer protein on sensitive cells highlighted the potential use of this protein as a highly selective antifungal agent since its hydrolytic 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 K9 type toxin at pH values between 3-5.5 and temperatures up to 37 ºC make its usage suitable in medical and industrial biotechnology.

This study deals with this potential antimycotic agent that may be exploited for medical application as an antifungal agent with an appropriate formulation. The purpose of this study is: (i) to determine the antifungal spectrum of the purified K9 type yeast killer toxin on the strains of the genus *Candida* and dermatophytes (ii) to examine the MIC_{50} (the MIC at which 50% of the strains tested were inhibited) and MIC_{100} (the MIC at which 100% of the strains tested were inhibited) values of the toxin (iii) to examine the value of the Minimum Fungicidal Concentration (MFC) endpoints as an alternative means of detecting the fungicidal activity of K9 type yeast killer toxin for different *Candida* species and dermathophytes and (iv) to evaluate the cell killing activity of the K9 type yeast killer toxin on *Candida krusei* ATCC 6258 isolate.

CHAPTER II

MATERIALS AND METHODS

2. 1. MATERIALS

2.1.1. Fungal Strains

The killer yeast strains used were *Hansenula mrakii* NCYC 500 from National Collection of Yeast Cultures (NCYC), UK. as the K9 type killer toxin producing strain and *Saccharomyces cerevisiae* NCYC 1006 as the killer toxin sensitive strain. A total of 9 dermatophyte (Table 2.1.) and 23 pathogenic yeast strains (Table 2.2.) were used as pathogenic fungal test isolates for antifungal susceptibility studies.

2.1.2. Culture Media

K9 type killer toxin producing strain *Hansenula mrakii* and killer toxin sensitive strain *Saccharomyces cerevisiae* were grown on yeast extract-peptone-dextrose (YEPD) agar plates at 25 °C and were maintained at 4 °C. The composition of YEPD buffered at pH 5.5 was as follows: 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose together with 2% Bacto-agar. *H.mrakii* cells were grown in YEPD broth medium supplemented 5 % (v/v) glycerol buffered with citrate phosphate to pH 4.5 for the K9 type killer toxin

production. For the killer activity assay, YEPD-agar medium together with 2 % Bacto-agar was buffered with citrate phosphate buffer and adjusted to pH 4.5.

All pathogenic fungal strains were freshly cultured on PDA (0,4 % potato extract, 2 % dextrose and 5 % agar) plates at 25°C for 48 to 72 h (or until they reached full growth) before the antifungal susceptibility studies. Synthetic RPMI 1640 medium with glutamine and without sodium bicarbonate dissolved in pH 4.5 50mM Na_2HPO_{4-} citric acid buffer was used for all in vitro susceptibility testing of pathogenic yeasts. Sabouraud broth buffered to pH 4.5 with 50mM Na_2HPO_{4-} citric acid buffer was used to investigate the activity of the cell killing.

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**	A3	Human blood
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, TÜRKİYE.

ATCC: American Type Culture Collection, USA

A: Our laboratory collection number

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

Before inoculation with the desired microorganisms, microbiological media and all materials coming into contact with it must be sterile. The glass cultureware were sterilized on dry-cycle at 240 ^{o}C for two hours. For the sterilization of the media for stock cultures and for routine growth of the yeast cells steam sterilization cycle at 121°C for 15 minutes was performed. The buffers used for the chromatographic purification steps were filtered through 0.45µm cellulose acetate filter discs (Sartorius, AG, Germany) using filter device (Sartorius, AG, Germany) before sterilization on liquid cycle. RPMI 1640 medium was filtered through 0.45µm and 0.22µm (Sartorius, AG, Germany) cellulose acetate filters respectively for sterilization.

2.2.2. Maintenance of the Fungal Cultures

Freeze-dried cultures of *Hansenula mrakii* and *Saccharomyces cerevisiae* in glass ampoules were opened aseptically and 0.5 ml of YEPD broth medium (pH 5.5) was added to dissolve the dried culture completely 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 [29].

Lyophilized cultures of dermatophyte strains are revived by breaking the ampule and placing adequate amount of sterile saline solution. Spores were scratched with the tip of a sterile pasteur pipette and 0.5 ml of the resuspended pellet was streaked onto YEPD pH 5.5 agar plates and incubated at 28 ºC. Dermatophyte strains were sub-cultivated to PDA plates to promote spore formation before 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.

All plate cultures were then stored at 4 ºC for a maximum of one month, after which subculturing onto a new plate was done.

2.2.3. K9 Type Killer Toxin Production

Hansenula mrakii NCYC 500 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 the cells were grown to stationary phase at 18 ^{o}C for 36 hours at 120 rpm on a gyratory shaker (Innova 4330, New Brunswick, USA). The cells were removed by centrifugation (KR 22i, Jouan, France) at 9,000 x g for 20 min. at 4 ^{o}C and supernatant was filtered through a 0.45µm and 0.2µm pore sized cellulose acetate membranes (Sartorius, AG, Germany), respectively [127].

2.2.4. Concentration of the Killer Toxin

Cell free filtrate was concentrated 50 fold by using 10 kDa MWCO centrifugal filter device (Vivaspin VS2001, Sartorius,AG, Germany) at 4200 x rpm for 30 min. at 4°C (BR4i, Jouan, France). The concentrated crude protein was buffer exchanged to 0.05M Na_2HPO_4 buffer at pH 4.5 containing 0.150M Na_2SO_4 by using the 10 kDa MWCO centrifugal filter device (Vivaspin VS2001, Sartorius). Buffer exchange step was performed three times at 4200 x rpm for 15 min. at 4°C to maintain YEPD free samples (BR4i, Jouan, France). Before injection onto the HPLC (High Performance Liquid Chromotography) column the samples were filtrated by 0.2µm syringe filter (Sartorius, AG, Germany) and checked for its killer activity by agar diffusion assay.

2.2.5. Determination of Killer Toxin Activity

Toxin activity at various stages of the study was tested according to Brown et al. [128] with an agar diffusion assay. Twenty five ml of molten YEPD agar (pH 4.5) was seeded with 1 ml of the killer toxin sensitive strain *S. cerevisiae* NCYC 1006 in sterile water at a density of 10^5 cells/ml and poured into petri dishes. Protein samples of 30 µl were spotted on YEPD agar plates. After two days of incubation at 25 °C, the killer activity was determined by measuring the clear zone of growth inhibition of the seeded killer toxin sensitive strain. Killer toxin which gave a clear zone of 10 mm in diameter was defined as 1 arbitrary unit (AU).

2.2.6. Purification of the Killer Toxin

The purification steps were done on a fully automated HPLC 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 .

The concentrated and buffer exchanged sample from the previous step was then subjected to gel permeation chromatography using a HiLoad 26/60 Superdex 75 prepgrade column. Superdex prepgrade is a modern preparative gel with a composite matrix of dextran and highly cross-linked agarose. Prior to injection of the sample, column was equilibrated with 50mM Na_2HPO_4- citric acid buffer, pH 4.5, containing 150mM Na_2SO_4 at a flow rate of 4 ml/min. The equilibration was performed until the baseline was stable. Five ml of sample was injected into the column and elution was done with the same buffer at a flow rate of 3.4 ml/min. Fractions were collected (10ml) automatically by fraction collector (Advantec SF-20 Super Fraction Collector, Japan) which was connected to BioCAD 700E Workstation. The collected peaks were concentrated seperately by ultrafiltration with 5000 MWCO ultrafilter device (Vivaspin VS2012, Sartorius Sartorius, AG) and checked for their killing activity. The fraction with killer activity was collected for several runs and then concentrated by using 5 kDa molecular cut-off ultrafilters (Vivaspin VS2012, Sartorius, AG, Germany) for further studies. 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.

The fractions containing killer protein that were collected for several runs subjected to the second gel permeation chromatography to get a single pick without any contamination. Five ml of killer toxin containing sample was injected into the same column and elution was done with the same buffer at a flow rate of 3.4 ml/min.

2.2.7. Assessment of Protein Concentration

Protein concentration was measured by using the protein-dye binding method of Bradford [129]. 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 50 mM Na_2HPO_{4-} citric acid buffer to construct a calibration curve ranging from 2.5 to 25 µg/ml.

Table 2.3. Volume of Solutions Used in Constructing BSA Standard Curve.

Solution	Buffer(ml)	BSA(µl)	BSA (µg)
1	795	5	2.5
2	790	10	5
3	780	20	10
4	770	30	15
5	760	40	20
6	750	50	25

These standard solutions were mixed with 200 µl filtered Bradford reagent. Then a blank sample was prepared by mixing 800 µl 50 mM Na_2HPO_{4-} citric acid buffer and 200 µl Bradford reagent. 10 µl protein sample was also diluted in 790µl of the same buffer and mixed with 200 µl Bradford reagent.

Then 100 µl from each standard solutions, protein sample and blank were pipetted into individual wells in a 96-well microplate (Nunclon 167008; Nunc, Denmark). Spectrophotometric measurements were taken after allowing the solutions to incubate at room temperature for ten minutes following the addition of dye reagent. The wavelength was set at 595 nm by using UV visible spectrophotometer (model 1208, Shimadzu, Japan). Absorbance values were recorded, and a calibration curve was determined for the protein concentration from the BSA standards, then the concentration of the protein in the sample was calculated.

2.2.8. Non-denaturing SDS-Polyacrylamide Gel Electrophoresis

Following gel permeation chromatography, the fraction containing killer toxin and the concentrated *H. mrakii* culture media containing the active killer protein were subjected to electrophoresis, according to the method described by Laemmli [130]. Gel electrophoresis was performed under non-denaturing conditions, using a 15 % and 0.75mm thick linear SDS polyacrylamide gel in a discontinuous buffer system to prove the purity of the toxin and also to compare the protein profiles by using a vertical slab gel electrophoresis unit SE 600 (Hoefer, USA).

After pouring the separating gel into the glass plate sandwiches, it was overlaid with 100µl water saturated n-butanol onto the top of the separating gel to avoid contact of the gel with air and left for polymerization for one hour. After the polymerization completed, n-butanol overlay was poured off and the surface of the gel was rinsed with water and stacking buffer respectively. Then, the stacking gel solution was poured onto the separating gel and a 0.75 mm thick 15 wells comb was inserted into the sandwich. Stacking gel was also left for polymerization for 1 hour. Separating and stacking gel components are given in Table 2.4. and Table 2.5.

In non-denaturing SDS-PAGE method, 2-β-mercaptoethanol as reducing agent was omitted in the treatment buffer (0.125M Tris-Cl, 4%SDS, 20%Glycerol, 0.02% bromophenol blue to 10ml ddH_2O, pH 6.8) preparation. Samples were combined with treatment buffer with a 1:1 (sample: treatment buffer) ratio and were heated at 100 ^oC for 4 minutes prior to electrophoresis. After the polymerization of stacking gel, samples were loaded onto the gel. The gel was run at a constant current of 20 mA (Power supply PP4000, Biometra, Germany) at 10 ^oC using a circulating cooling water bath (Heto Holten, Denmark) until the dye front reached the bottom. The gel was separated into two parts; one part containing the purified K9 type yeast killer protein to prove the purity of the toxin was visualized by silver staining [131] and the other part containing both the concentrated culture media and the purified killer toxin to compare the protein profiles was visualized by Coomassie brilliant blue R-250 [132].

Table 2.4. Separating Gel Mixtures (15%T).

Acrylamide-bisacrylamide (30:0.8)	15 ml
4X Separating Gel Buffer (1.5M Tris-Cl , pH:8.8)	7.5 ml
10% SDS	0.3 ml
ddH_2O	7.1 ml
10% Ammonium persulfate*	150µl
TEMED*	10µl

*Ammonium Persulfate and TEMED were added after deaeration.

Table 2.5. Stacking Gel Mixtures (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	6.0 ml
10% Ammonium persulfate*	50 µl
TEMED*	5 µl

*Ammonium Persulfate and TEMED were added after deaeration.

2.2.9. Denaturing SDS-Polyacrylamide Gel Electrophoresis

Molecular weight determination of the pure toxin was done under denaturing conditions, using a 15 % linear SDS polyacrylamide gel in a discontinuous buffer system [130] by using a vertical slab gel electrophoresis unit SE 600 (Hoefer, USA). Denaturing SDS-PAGE method was the same as the non-denaturing SDS-PAGE method except the presence of β-mercaptaethanol as reducing agent in the treatment buffer.

Sodium dodecyl sulfate (SDS), DiThioTreitol (DTT) or β-mercaptaethanol, and heat are responsible for the actual denaturation of the sample. SDS coats all proteins in proportion to their mass and ensures they all have a large negative charge. Therefore, during the electrophoresis proteins move through the gel towards the anode. The rate at which they move is inversely proportional to their molecular mass, larger proteins feel more resistance and hence taking longer to move. Heating the samples to at least 60 degrees shakes up the molecules, allowing SDS to bind in the hydrophobic regions and complete the denaturation. DTT's specific role in sample denaturation is to remove the last bit of tertiary and quaternary structure by reducing disulfide bonds.

The protein samples were mixed with equal volume of treatment buffer but the molecular weight (MW) markers were combined with treatment buffer with 1:4 ratio and were heated in boiling water for 4 minutes prior to electrophoresis. After the polymerization of the gel completed, MW markers (10µl) and the purified protein (20µl) were loaded into the stacking gel and run simultaneously. Electrophoresis was performed at 15 ^{o}C using circulating cooling water bath (Heto Holten, Denmark) with power supply settings of 20 mA (Power supply PP4000 ,Biometra, Germany) until the dye front reached the bottom. Subsequently, the proteins in the gel were either stained with Coomassie brilliant blue R-250 [132] or silver [131].

Molecular weight markers used for the molecular weight determination of the K9 type yeast killer protein were myosin (205.000 kDa), β-galactosidase (116.000 kDa), phosphorylase b (97.000 kDa), transferrin (80.000 kDa), albumin (66.000 kDa), glutamate dehydrogenase (55.000 kDa), ovalbumin (45.000 kDa), carbonic anhydrase (30.000 kDa), trypsin inhibitor (21.000 kDa), lysozyme (14.000 kDa) and aprotinin (6.500 kDa) (Amersham Biosciences).

For the molecular weight determination the SDS gel was scanned and the data was processed with gel imaging and analysis system (Vilber Lourmat Gel Imaging and Analysis System, France).

2.2.10. Isoelectric Focusing

The isoelectric point (pI) of the killer protein was determined by a high voltage vertical slab polyacrylamide gel electrofocusing system with a Hoefer SE600 electrophoresis unit as previously described [133, 134]. Focusing was carried out on pH 3-10 non-denaturing gradient 0.35mm thick gel containing 2.4% ampholytes and broad range pI markers (Ampholytes and pI markers were supplied from Amersham Biosciences). The gel composition is given in Table 2.6.

Table 2.6. Isoelectric Focusing Gel Composition.

Monomer Solution (30%T, 2.7%C)	2.2 ml
Glycerol	1.2 ml
ddH_2O	7.87 ml
Ampholytes (3 / 10)	0.72 ml
TEMED*	23 µl
10% Ammonium Persulfate*	50 µl

*Ammonium Persulfate and TEMED were added after deaeration.

After the addition of ammonium persulfate and TEMED, the gel mixture was poured into the sandwich and the comb (0.35 mm thick, 8 well) was inserted. The gel left for one hour for polymerization. After the polymerization completed the wells and upper chamber were filled with catholyte (0.02 M NaOH) and lower chamber of the electrophoresis unit was filled with anolyte (0.02 M acetic acid). The gel was prefocused at 10 ºC for 15 minutes with maximum settings of 2300 V, 300 mA and 20 W. Catholyte used was poured off after prefocusing. Upper chamber and sample wells were filled with fresh catholyte solution.

Then, K9 killer protein sample (15µl) was loaded onto the gel after the buffer exchange step with ddH_20 by using the 5000 MWCO microcentrifuge filter devise (Sartorius Centrisart-C4). Buffer exchange step was performed at 7500 rpm for 30 min. at 10 ºC (MR23i Jouan, France). Samples and pI markers were combined with equal volume of sample buffer before loading (Table 2.7.). The focusing was performed at 10 ºC using circulating cooling water bath (Heto Holten, Denmark) for 70 minutes with maximum settings of 2300 V, 300 mA and 20 W (Power supply PP4000; Biometra , Germany).

Table 2.7. Sample Buffer Composition for IEF.

ddH_20	395 µl
Glycerol	75 µl
Ampholytes (3 / 10)	30 µl

The pI markers used were trypsinogen (9.30), lentil lectin-basic band (8.65), lentil lectin-middle band (8.45), lentil lectin-acidic band (8.15), myoglobin-basic band (7.35), myoglobin-acidic band (6.85), human carbonic anhydrase (6.55), bovine carbonic anhydrase (5.85), β-lactoglobulin A (5.20), soya bean trypsine inhibitor (4.55), amyloglucosidase (3.50).

The gel was stained with coomassie blue R-250. In order to determine the isoelectric point of the K9 type killer protein with respect to the marker proteins, the gel was scanned and the data was processed with gel imaging and analysis system (Vilber Lourmat Gel Imaging and Analysis System, France).

2.2.11. Protein Detection in Gels

Protein bands in gels were visualised either by coomassie blue staining or silver staining.

2.2.11. a. Coomassie Blue Staining

Coomassie brilliant blue R-250 staining was performed as previously described by Wilson (1983) [132]. The gel was stained with staining solution for 2 hours with slow shaking. Then staining solution was replaced with destaining

solution I for 30 min. to remove bulk of the excess stain and then the gel was transferred into the destaining solution II. Destaining solution II was changed twice a day until the backround was clear.

2.2.11. b. Silver Staining

Silver staining was performed as previously described [131]. The gel was immersed in destain I for 30 minutes with gentle shaking. Destain I was replaced with destain II and the gel was shaken slowly. After 30 minutes Destain II was discarded and cross-linking solution was added and again shaken slowly for 30 minutes. Cross-linking solution was poured off and the gel was washed with several changes of distilled water over 2 hours or the gel was placed into 2 liters of water for overnight storage. The next morning, the gel was washed in fresh water for 30 minutes. The gel was incubated in DTT (dithiothreitol) solution with slow shaking for 30 minutes. DTT solution was removed and silver nitrate solution was added and again shaken slowly for 30 minutes. The gel was washed with distilled water for two or three times and washed with developing solution for once a time. Then fresh developing solution was added. Staining was occurred within 5-10 minutes. When the desired staining level was reached, development was stopped by replacing the development solution with the destain II.

2.2.12. Antifungal Susceptibility Testing

Killing activity of K9 type yeast killer protein was determined against 23 pathogenic yeast and 9 dermatophyte strains *in toto*. The susceptibility testing of *Candida* strains was performed according to Clinical and Laboratory Standards Institute (CLSI) -formerly NCCLS- M27-A2 (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts) methodology [135] and the susceptibility testing of dermatophyte strains was performed according to CLSI

M38-A (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi) [136] methodology; with minor modifications to provide the stability of the K9 type yeast killer protein during testing.

2.2.12. a. Susceptibility Testing of Pathogenic Yeast Strains

Minimum Inhibition Concentration (MIC) assays were performed in 96-well round-bottomed microtitre plates (Nunclon 167008; Nunc, Denmark) using a two-fold dilution series of K9 type yeast killer protein ranging from 64 to 0.250 µg/ml which were prepared in 50 mM Na_2HPO_4-citric acid buffer. A completely synthetic medium, RPMI 1640, was prepared at twice its final concentration (2x RPMI 1640) and buffered to pH 4.5 with the same buffer for susceptibility testing as the test medium. Yeast inoculum suspensions were obtained by taking five colonies (>1 mm diameter) from 24-h-old cultures grown on Potato dextrose agar plates. The colonies were suspended in 5 mL of sterile saline (0.85% NaCl). The inoculum suspensions were shaken for 15 s and the inoculum density was adjusted to the turbidity of a 0.5 McFarland (DEN-1 McFarland Densitometer, HVD, UK) Standard (equivalent to 1–5 x 10^6 cells/mL) with sterile saline. The suspensions were diluted 1:1000 with a 1:50 and 1:20 dilutions respectively in RPMI 1640 to obtain the twofold test inoculum (1-5 x 10^3cells/mL). Each microdilution well containing 100 µL of the corresponding two-fold protein dilution was inoculated with 100 µL of the cell suspension. This step diluted the medium (1x RPMI 1640) and protein concentrations (32 to 0.125µg/ml) and also inoculum densities (0.5-2.5 x 10^3 cells/ml) to the desired test concentrations. Growth and sterility controls were included for each isolate–protein combination. Growth control well was prepared by mixing 100 µl of cell suspension with 100 µl of Na_2HPO_4-citric acid buffer and sterility control well was prepared by mixing medium (RPMI 1640) with 100 µl of Na_2HPO_4-citric acid buffer**.** The microdilution trays were incubated at 25 ^oC and the wells were mixed with a multi-channel pipette at every 2 hours during 6 hours. MICs were determined visually after 48 h of incubation. The growth in each MIC well was compared

with the growth control well. MIC was defined as the lowest concentration of the K9 type yeast killer protein where wells were optically clear (without any visible growth/turbidity) or 95 % reduction in the growth which was determined by using an automatic microtitre plate reader at 590nm (Spectramax 190, Molecular Devices, USA). MIC_{50} (MIC at which 50% of the isolates were inhibited) and MIC_{100} (MIC at which 100% of the isolates were inhibited) values were also determined for all of the pathogenic yeast strains which were tested.

Minimum fungicidal concentrations (MFCs) were also determined for 22 *Candida* and 1 *S. cerevisiae* strains as described by Canton et al [137]. After 48 h of incubation 100 µl from each well that showed complete inhibition (optically clear) and from the growth control wells were subcultured on to PDA plates and incubated at 25 ^{o}C until growth was seen in the growth control subculture (usually 48 h). MFC was the lowest protein concentration that killed 99.9 % (with less than 2 colonies remaining) of the final inoculum.

2.2.12. b. Susceptibility Testing of Dermatophyte Strains

Minimum Inhibition Concentration (MIC) assays for 9 dermatophyte strains were also performed in 96-well microtitre plates by broth microdilution test. Two fold dilution series of the K9 type yeast killer protein was prepared in 50 mM Na_2HPO_4-citric acid buffer ranging from 128 to 0.250 µg/ml. Each dermatophyte isolate was subcultured onto PDA plates and incubated at 25°C for 7 days or until good conidiation was produced. A suspension of conidia in sterile saline was made by gently swabbing the colony surface with a sterile swab. The suspension was transferred into a sterile tube. After heavy particles were allowed to settle for 5 to 10 minutes, the upper homogenous suspension is transferred to a steril tube and vortexed (Heidolph, Germany) for 15 seconds. The resulting suspension was counted on a hemocytometer and was diluted in RPMI 1640 to the desired concentration of 4×10^5 to 50×10^5 spores/ml. Then, the suspensions were diluted 50-fold with 2 fold concentrated RPMI (pH 4.5) to obtain the

twofold test inoculum (0.8-10 x 10^4 spores/ml). Each well of microtiter plate contained 100 µl of two fold dilution series of protein ranging from 128 to 0.250 µg /ml and 100 µl of spore suspension. The final volume in each well was 200 µl and this resulted in the appropriate final concentrations of the medium (1x RPMI 1640), protein (64 to 0.125 µg/ml) and the spore suspension ($0.4x10^4$ to $5x10^4$ spores/ml) in the wells. Each row on the microtitre plate also included growth and sterility control wells. Dermatophyte plates were incubated at 25°C for 4 days to 6 days and during the incubation period the wells were mixed with a multi-channel pipette in every 2 hours for the first 4 hours. MICs of the dermatophyte isolates were determined visually at the end of the incubation.

2.2.13. Cell Killing Activity of the K9 Type Yeast Killer Toxin

K9 type yeast killer toxin was tested on the most crucial *Candida* spp., *C. krusei ATCC 6258*, for its cell killing activity. Analyses of cell killing activity were done according to İzgü et.al [138]. Before the test was performed, the isolate was subcultured and grown for 24 h at 25°C on SDA plates. The inoculum was adjusted to the density of a 0.5 McFarland turbidity standard which corresponds to 1 x 10^6 to 5 x 10^6 cells/ml. A hundred µl of the adjusted fungal suspension was then added to 9900 µl of 2 fold concentrated Sabouraud broth (pH 4.5). This resulted in a 1:100 dilution of the fungal suspension and yielded a starting inoculum of approximately 1 x 10^4 to 5 x 10^4 CFU/ml. The test solution tubes contained 250 µl fungal suspension and 250 µl appropriate concentrations of protein. The resulting protein concentrations in test solutions were equal to 1 (2µg/ml), 4(8µg/ml), 8 (16µg/ml) and 16 (32µg/ml) times the MIC for test isolate and the final cell suspension was equal to 5 x 10^3 to 25 x 10^3 cells/ml. Growth control tube included yeast suspension with toxin free buffer. Test solution tubes were incubated at 25°C without agitation for 48 hour. At predetermined time points (0, 2, 4, 6, 8, 10, 12, 24, 36, and 48 hours) 50 µl aliquots were aseptically removed from both the control tube (toxin free) and each tube with a test solution. Ten fold serial dilutions were performed on

samples in pH 6.8 citrate-phosphate buffer to deactivate the toxin. Hundred µl of the diluted sample was streaked on to SDA plates for colony count determination. All plates were incubated for 48 h at 25°C. When the colony counts were expected to be less than 1,000 CFU/ml, samples were diluted 2 fold in pH 6,8 citrate-phosphate buffer.

CHAPTER III

RESULTS

3.1. Production of the Killer Toxin

For killer protein production, *H. mrakii c*ells were grown in 1 liter YEPD medium (containing 5% glycerol) buffered to pH 4.5 with 0.1 M citrate-phosphate buffer and incubated at 18 ºC for 36 hours at 120 rpm on a gyratory shaker to provide the optimum culture conditions for the maximum killer toxin activity. *Hansenula mrakii* cells were removed from the culture liquid by centrifugation at 9000 x g for 20 minutes at 4 ºC and culture supernatant filtered through 0.45 µm and 0.25 µm cellulose acetate membranes respectively. The concentration of the protein mixture was 10 µg/ml (0.01mg/ml) [127].

3.2. Concentration of the K9 Type Yeast Killer Toxin

Concentration of the killer protein in the culture supernatant was performed with 10.000 MWCO filter (Vivaspin VS2001, Sartorius) to yield 50 fold concentrated protein equivalent to a final concentration of 500 µg/ml (0.5mg/ml). The killer activity of the protein in the concentrated cell free extract was determined by the killer zone assay in a plate test before it was used in the purification steps.

3.3. Determination of Killer Toxin Activity

Toxin activity before every stage of the study was tested with an agar diffusion assay. The killer activity of the protein in the concentrated cell free extract was determined by spotting 30 µl of the protein onto YEPD pH 4.5 agar plates seeded with killer toxin sensitive *S. cerevisiae* NCYC 1006 cells before it was used in the purification steps (Figure 3.1.).

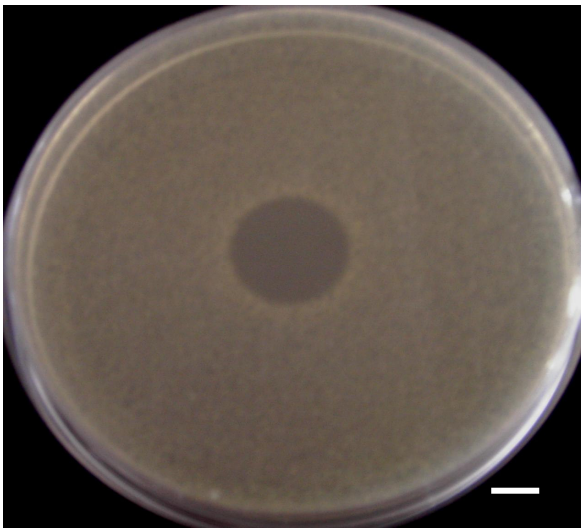


Figure 3.1. Growth Inhibitory Activity of the Killer Toxin on Sensitive *S.cerevisiae* NCYC 1006 in YEPD Medium, at pH 4.5, 25 ^{o}C. 30 µl of concentrated protein mixture (500 µg/ml) gave a clear growth inhibition zone of 17 mm which corresponds to 1.7 AU killer toxin. Bar scale represents 5 mm.

3.4. Purification of the Killer Toxin

Crude toxin obtained from ultrafiltration step was buffer exchanged with 50 mM Na_2HPO_4 at pH 4.5 containing 150 mM Na_2SO_4 to purify the killer toxin by using a HPLC system, BioCAD 700 E Workstation. K9 type yeast killer protein was purified by gel permeation chromatography using a HiLoad 26/60 Superdex 75 pg column for the optimal resolution (Figure 3.2). Each fraction were collected (10ml) by a fraction collector into sterile tubes and the collected fractions were concentrated separately by ultrafiltration with 5000 MWCO ultrafilter device (Vivaspin VS2012, Sartorius).

Concentrated fractions obtained from gel permeation chromotoghraphy were then tested for killer activity by killer zone assay in a plate test and after 24 h incubation a clear growth inhibition zone of 15 mm which corresponds to 1,5 AU killer toxin was observed in the Fraction No: 4 and Fraction No: 5 (Figure 3.2.).

After detecting the fraction with killer activity, it was pooled for several runs, concentrated and subjected to the second gel permeation chromatography. The pick profile observed in the second gel permeation chromatography indicates the absence of any contamination (Figure 3.3.). The antifungal susceptibility studies were done by the protein obtained from the first chromatography because in every chromatographic process protein loses its killing activiy.

350 fold concentrated protein with the final concentration of 400 µg/ml obtained from gel permeation chromatography gave a clear growth inhibition zone of 15 mm which corresponds to 1.5 AU killer toxin (Figure 3.4.)

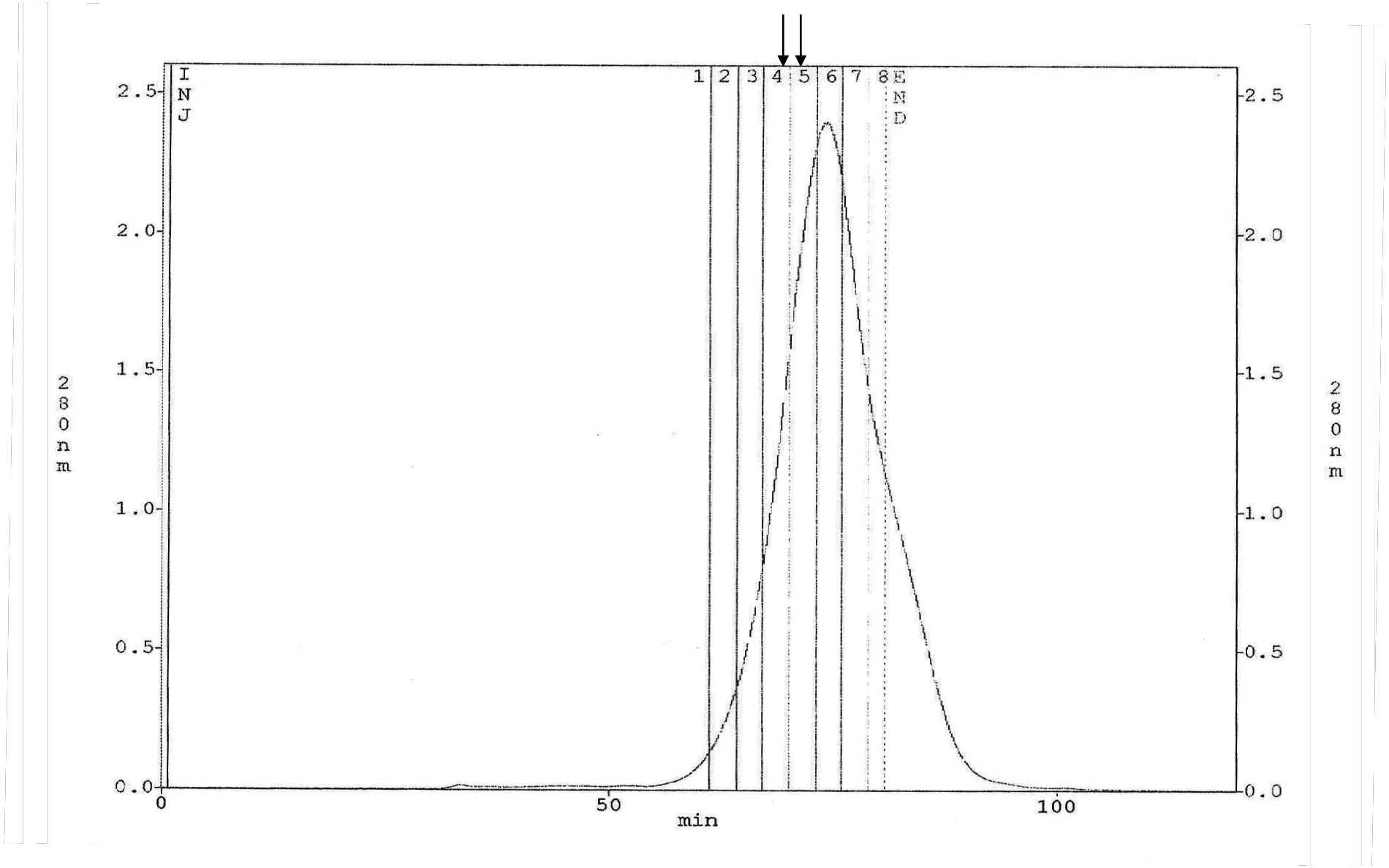


Figure 3.2. Elution Profile of the K9 Type Toxin on a HiLoad Superdex Column

Column size: 26 mmD / 600 mmL; Sample: 5000 µl; Elution buffer: 50mM Na_2HPO_4 at pH 4.5 + 150mM Na_2SO_4 ; Flow rate: 3.4 ml/min; Detection: 280 nm UV; Fraction volume: 10ml. Fractions containing killer protein are eluted at 225 ml and indicated by arrows.

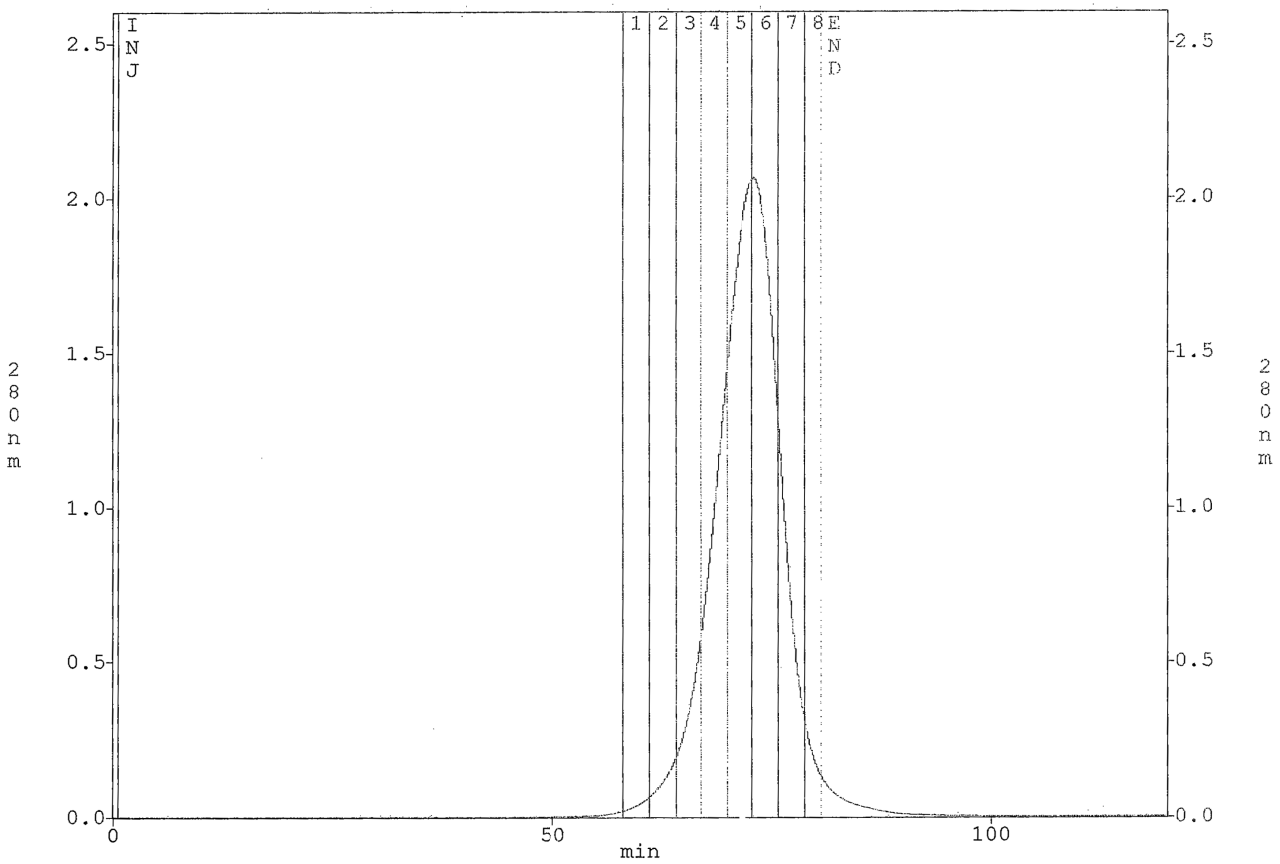


Figure 3.3. Second Gel Permeation Chromotography of the K9 Type Toxin on a HiLoad Superdex Column

Column size: 26 mmD / 600 mmL; Sample: 5000 µl; Elution buffer: 50mM Na_2HPO_4 at pH 4.5 + 150mM Na_2SO_4 ; Flow rate: 3.4 ml/min; Detection: 280 nm UV; Fraction volume: 10ml.

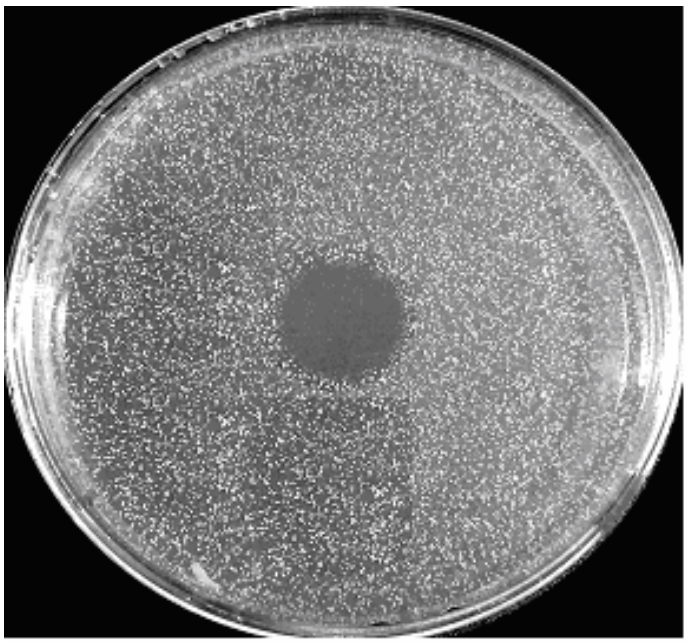


Figure 3.4. Killer Activity of the K9 Type Toxin Determined by Agar Diffusion Assay. 30µl of the active fraction obtained from gel permeation chromatography gave a clear growth inhibition zone of 15 mm which corresponds to 1.5 AU killer toxin (12µg). Bar scale represents 5 mm.

3.5. Assessment of Protein Concentration

The concentration of protein was measured by Bradford dye assay using a calibration curve drawn with bovine serum albumin and adjusted to desired concentration for further studies before each antifungal susceptibility testings (Figure 3.5.).

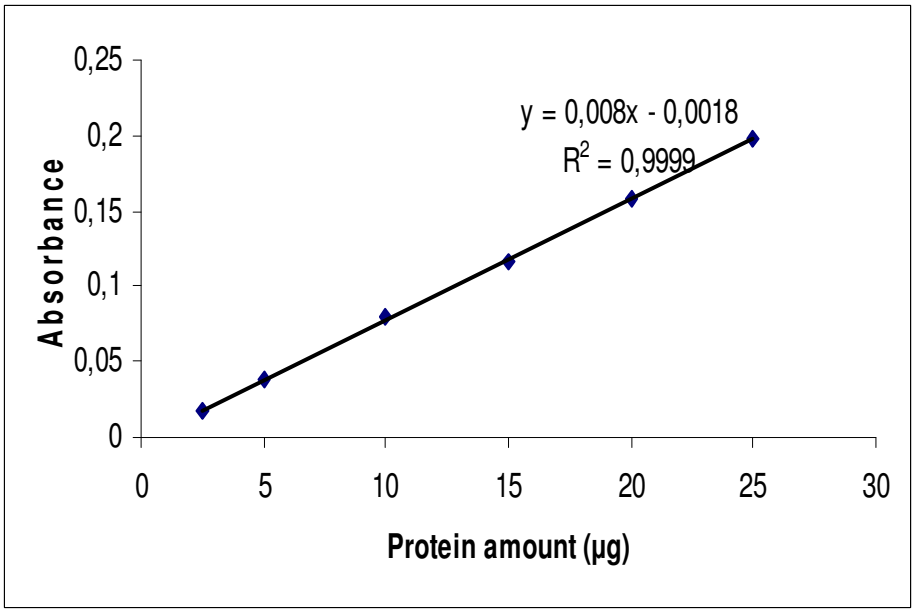


Figure 3.5. Standard Curve of Protein Amount versus Absorbance. Bovine serum albumin solutions were used as standards. 10 µl of the purified K9 type yeast killer protein gave an UV absorbance of 0,0302 at 590 nm which corresponds to 4 µg.

3.6. Non-Denaturing SDS-Polyacrylamide Gel Electrophoresis

Purified killer protein (2µg) and concentrated culture supernatant of *H. mrakii* containing the active killer toxin were electrophoresed on a 15% linear SDS-PAGE gel in a discontinous buffer system under non-denaturing conditions to check its purity and to compare the protein profiles. Observation of single protein band on the silver stained gel indicates the absence of any contamination (Figure 3.6.). Comparision of protein profiles is shown in Figure 3.7.

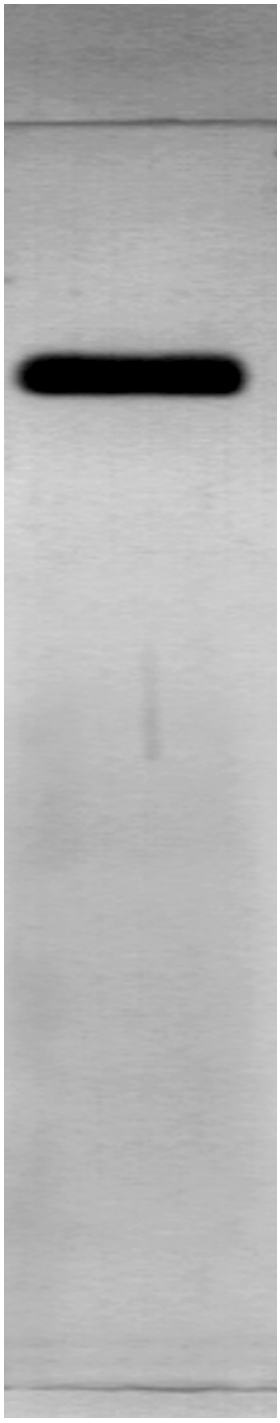


Figure 3.6. Non-Denaturing SDS-Polyacrylamide Gel of the Purified K9 Type Yeast Killer Toxin.

A B

Figure 3.7. Non-Denaturing SDS-PAGE. Profile of Concentrated Culture Supernatant Containing the Killer Protein (A) and the purified K9 type killer toxin (B).

3.7. Denaturing SDS-Polyacrylamide Gel Electrophoresis

The purified killer protein (2μg) was electrophoresed on 15% linear SDS-PAGE gel in a discontinous buffer system under denaturing conditions. Molecular weight markers were used to determine its molecular weight. For the molecular weight determination of the K9 type killer protein, the gel was scanned and the data were processed with Vilbert Lourmat Gel Imaging and Analysis System, France. The migrated protein placed between glutamate dehydrogenase (55.000 kDa) and ovalbumin (45.000 kDa). The molecular weight of the killer protein was found 46.504 kDa (Figure 3.8.).

3.8. Isoelectric Point Determination

The isoelectric point (pI) of the killer protein was determined by a high voltage vertical slab polyacrylamide gel electrofocusing in the pH range of 3-10 with broad range pI marker proteins. Protein appeared as a band in the gel with isoelectric point of 3.867 (indicated by arrow) (Figure 3.9.).

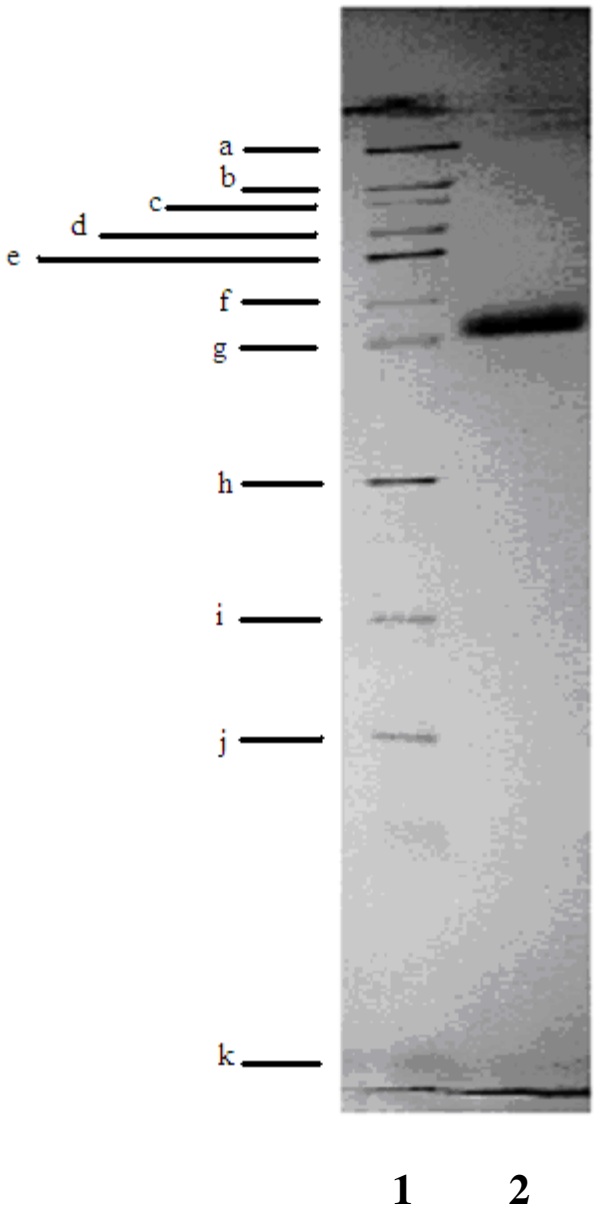


Figure 3.8. Denaturing SDS-Polyacrylamide Gel Electrophoresis of the K9 Type Yeast Killer Toxin. Lane 2 is K9 type yeast killer toxin. Lane 1 is the molecular weight markers; a) myosin (205.000 kDa), b) β-galactosidase (116.000 kDa), c) phosphorylase b (97.000 kDa), d) transferrin (80.000 kDa), e) albumin (66.000 kDa), f) glutamate dehydrogenase (55.000 kDa), g) ovalbumin (45.000 kDa), h) carbonic anhydrase (30.000 kDa), i) trypsin inhibitor (21.000 kDa), j) lysozyme (14.000 kDa) and k) aprotinin (6.500 kDa).

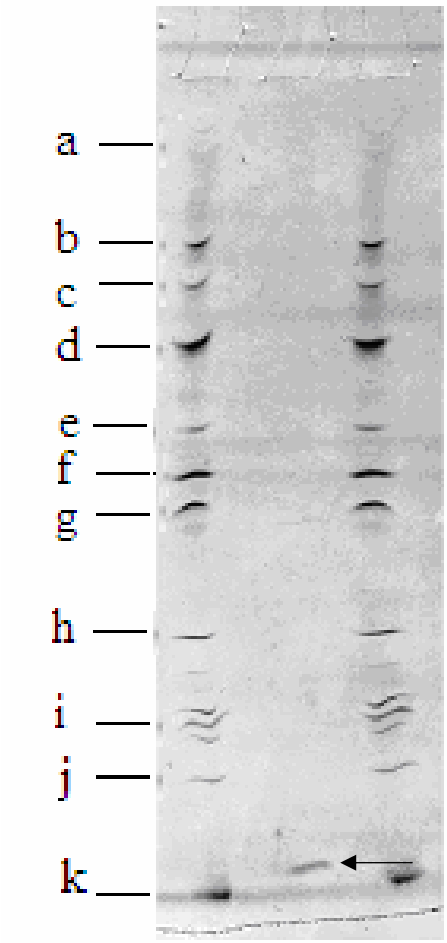


Figure 3.9. Polyacrylamide Gel Electrofocusing of K9 Type Yeast Killer Toxin in Native State. Lane 1 and 3 are pI markers ; (a) trypsinogen 9.30; (b) lentil lectin-basic band 8.65; (c) lentil lectin-middle band 8.45; (d) lentil lectin-acidic band 8.15; (e) myoglobin-basic band 7.35; (f) myoglobin-acidic band 6.85; (g) human carbonic anhydrase 6.55; (h) bovine carbonic anhydrase 5.85; (i) β-lactoglobulin A 5.20; (j) soya bean trypsine inhibitor 4.55; (k) amyloglucosidase 3.50. Lane 2 is K9 type yeast killer toxin.

3.9. Antifungal Susceptibility Studies

The antifungal susceptibilities of different pathogenic yeast isolates and dermatophytes to purified K9 type yeast killer toxin was tested in different protein concentrations ranging from 32 to 0.125 µg/ml and 64 to 0.125 µg/ml according to Clinical and Laboratory Standards Institute M27-A and M38-A methodology. MICs were determined both visually (Figure 3.10.) and spectrophotometrically for the yeast strains whereas detection was only possible with visual inspection for the dermatophyte strains. The entire MIC data is presented as the average of 3 independent experiments.

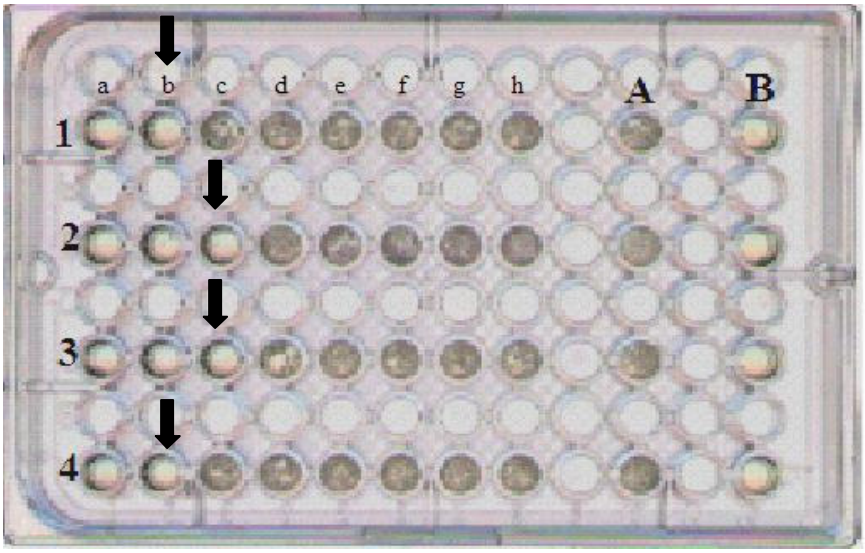
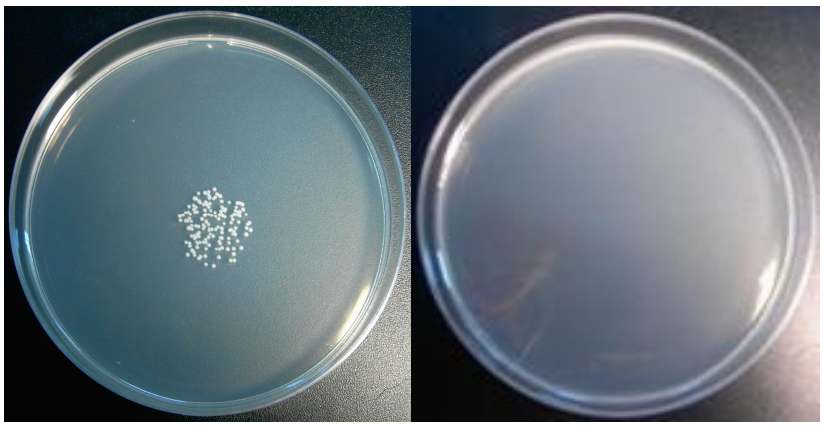
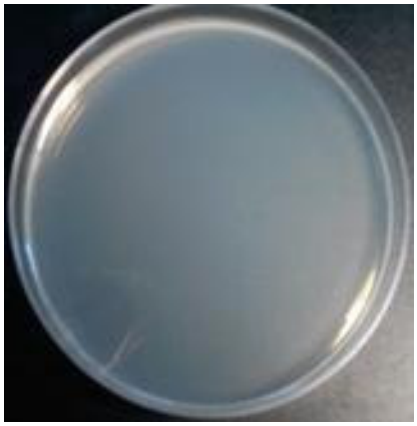


Figure 3.10. Antifungal Susceptibility Studies of the K9 Type Yeast Killer Toxin on a Microtitre Plate. Arrows indicate the MICs (visually clear wells). Protein concentrations; a) 16 µg/ml b) 8 µg/ml c) 4 µg/ml d) 2 µg/ml e) 1 µg/ml f) 0.5 µg/ml g) 0.25 µg/ml h) 0.125µg/ml. 1) *Candida albicans* A1 MIC: 8 µg/ml 2) *Candida albicans* A3 MIC: 4 µg/ml 3) *Candida guilliermondii* A9 MIC: 4 µg/ml 4) *Candida albicans* ATCC 10231 MIC: 8 µg/ml
A) Growth control B) Sterility control.

MFCs of the K9 type yeast killer toxin for the yeast strains were tested by plating the 100 µl of all visually clear wells onto PDA plates (Figure 3.11.).



4µg/ml 8µg/ml



16µg/ml

Figure 3.11. MFC Determination for *C.albicans* ATCC 90028 on PDA plates.

3.9.1. Susceptibility of Pathogenic Yeast Strains.

The results of susceptibility testing of the pathogenic yeast strains to the K9 type yeast killer protein (MIC and MFC) are represented in Table 3.1.

Table 3.1. MICs and MFCs of K9 Type Yeast Killer Protein for 23 Pathogenic Yeast Strains.

STRAIN	**STRAIN NUMBER\ SOURCE**	**MIC µg/ml**	**MFC µg/ml**
Candida albicans	ATCC MYA 2730	8	8
C. albicans	ATCC 10231	8	8
C. albicans	ATCC 26555	4	4
C. albicans	ATCC 36802	8	8
C. albicans	ATCC 90028	8	8
C. albicans	DSMZ 3454	8	8
C. albicans	A1	8	8
C. albicans	A3	4	4
C. parapsilosis	ATCC 90018	>32	>32
C. parapsilosis	A6	>32	>32
C. krusei	ATCC 14053	2	4
C. krusei	ATCC 6258	2	4
C. krusei	A7	2	8
C. krusei	A8	2	4
C. guilliermondii	A9	4	8
C. guilliermondii	A10	16	16
C. guilliermondii	A11	8	>32
C. guilliermondii	A12	16	16
C. guilliermondii	A13	>32	>32
C. pseudotropicalis	A14	2	2
C. tropicalis	A15	0.25	0.25
C. glabrata	ATCC 90030	0.25	0.25
S. cerevisiae	A16	0.5	0.5

The MIC_{50} (the MIC value able to inhibit 50% of the strains tested belongs to the same species) and MIC_{100} (the MIC value able to inhibit 100% of the strains tested belongs to the same species) results of the K9 type yeast killer protein against pathogenic yeast isolates are represented in Table 3.2.

Table 3.2. MIC_{50} and MIC_{100} Values of K9 Type Yeast Killer Protein against Pathogenic Yeast Isolates.

Species (no. of isolates)	µg/ml		
	MIC Range	MIC_{50}	MIC_{100}
Candida albicans (8)	4-8	4	8
Candida parapsilosis (2)			>32
Candida krusei (4)			2
Candida guilliermondii (5)	4- >32	16	>32
Candida pseudotropicalis (1)			2
Candida tropicalis (1)			0.25
Candida glabrata (1)			0.25
Saccharomyces cerevisiae (1)			0.5

3.9.2. Susceptibility of Dermatophyte Strains

Susceptibility of 9 dermatophyte strains to the K9 type yeast killer protein was tested with a broth microdilution testing according to CLSI M38-A methodology. All of the strains including *Microsporum* and *Trichophyton* species were found to be not susceptible to the toxin and the MICs for these isolates were >64 µg/ml.

Table 3.3. Susceptibility of 9 Dermatophyte Strains to the K9 Type Yeast Killer Protein.

Strains	**MIC µg/ml**
Microsporum audouinii.....	>64 µg/ml
M. canis.....	>64 µg/ml
M. gypseum.....	>64 µg/ml
Trichophyton rubrum.....	>64 µg/ml
T. mentagrophytes.....	>64 µg/ml
T. verrucosum.....	>64 µg/ml
T. interdigitale.....	>64 µg/ml
T. equinum.....	>64 µg/ml
T. tonsurans.....	>64 µg/ml

3.10. Cell Killing Activity of the K9 Type Yeast Killer Protein

Time-kill curve of the K9 type yeast killer toxin was determined for one of the most crucial *Candida* genus, *Candida krusei* (ATCC 6258) in 2 fold concentrated Sabouraud broth (pH 4.5) with concentrations of protein equal to 1, 4, 8 and 16 times the MIC to assess the correlation between MIC and fungicidal activity. The plot of the killing activity of the K9 type yeast killer toxin as the log10 CFU/milliliter versus time is presented as time- kill curve in Figure 3.12.

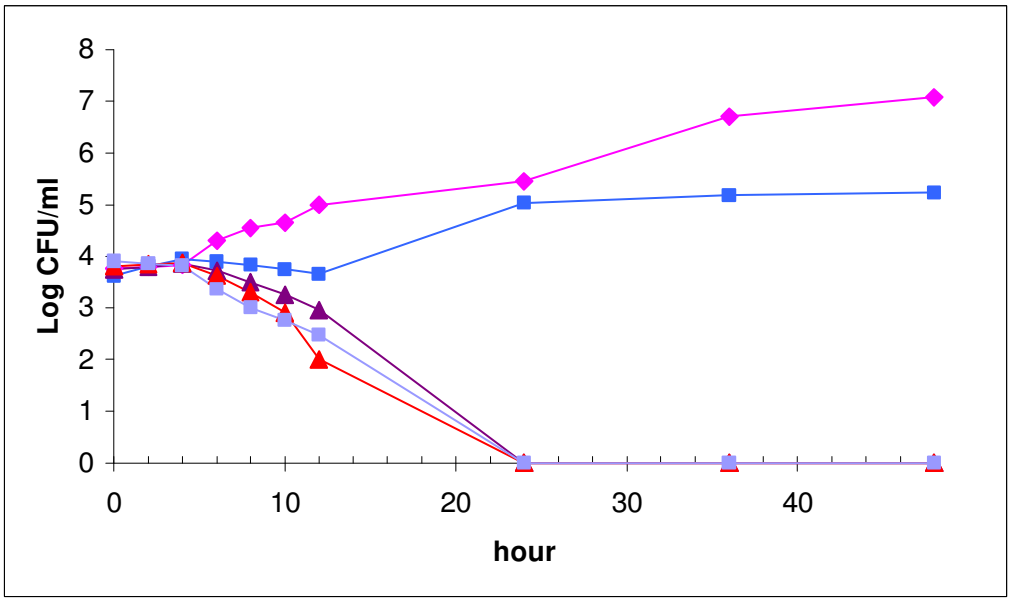


Figure 3.12.Time-kill Curve Plot for *Candida krusei* (ATCC 6258) at the following concentrations: the MIC: 2 µg/ml (■), 4 times the MIC: 8 µg/ml (▲), 8 times the MIC: 16 µg/ml (■) and 16 times the MIC: 32 µg/ml (▲). ♦, growth control.

CHAPTER IV

DISCUSSION

The need for safe and effective antifungal agents increases in parallel with the expanding number of immunocompromised patients at risk for invasive fungal infections. The emergence of fungal pathogens resistant to current therapies causes the dearth of antifungal agents. Currently available antifungal compounds act on targets also found in mammalian cells, which may result in toxicity or an adverse drug interaction. It is therefore imperative to find antifungal compounds that are not toxic to mammalian cells. The past decade has witnessed a dramatic growth in knowledge of natural peptides. Natural antifungal proteins are produced by a diverse group of organisms including bacteria, fungi, insects and mammals 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 [139]. These toxic proteins inhibit the growth of various species and genera of yeast and fungi [16,59] as well as Gr (+) bacteria [15]. Thus research on killer proteins are focused on the use of these agents as potential antifungals [67,139]. Among the killer yeasts *Hansenula mrakii* NCYC 500 has been widely studied and presented findings suggest that *H. mrakii* killer toxin has potential as a novel antimycotic agent [67-71]. K9 type yeast killer protein was previously purified and characterized in our laboratory [127] and now it is possible to determine the actual killing spectrum and cell killing patterns of this toxin on human pathogenic fungi.

Fungal infections are a major cause of morbidity and mortality in immunocompromised patients [102,105]. The *Candida* species are the most common cause of fungal infections [108]. *C. albicans* is the most frequently isolated yeast associated with human infections. There are now at least 17 species of *Candida* that have been shown to cause disease in humans including *Candida albicans, Candida guilliermondi, Candida glabrata, Candida tropicalis, Candida krusei* and *Candida parapsilosis* [140]. Therefore we have first determined the susceptibility of these organisms to the pure K9 type yeast killer protein by determining the MIC and MFC values of the toxin for pathogenic yeasts. MICs were determined by using two endpoints: (i) the MIC at which 50% of isolates are inhibited (MIC_{50}) and (ii) the MIC at which 100% of isolates are inhibited (MIC_{100}) compared to the growth in the control wells containing no toxin. Twenty two 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 32 to 0.125 µg/ml. In this study, the CLSI recommendations has been adapted for testing approximately 23 pathogenic yeast strains and 9 dermatophytes including the most common species. Modifications to the method included a decrease in the incubation temperature from 35°C to 25°C and in the pH value from 7.0 to 4.5 to provide the stability of the K9 type yeast killer protein during testing.

Susceptibility of the tested strains, which belongs to the different species, to the K9 type yeast killer protein varied due to different cell wall compositions and different polysaccharide content of the cell wall (β-glucans-chitin- mannan). K9 type yeast killer protein may not be the agent of choice for infections caused by *C. parapsilosis* and *C. guilliermondii* because they displayed higher MICs while remaining susceptible. *C. tropicalis, C. pseudotropicalis, C. glabrata* and *S. cerevisiae* had lower MICs considering *C. albicans* isolates since they were moderately susceptible to the K9 type yeast killer protein than the others.

K9 type yeast killer protein was tested against 8 *Candida albicans* isolates which is the most frequently isolated causative agent of candidal

infections in humans (more than 50%) and is generally accepted as the most pathogenic species of the genus *Candida* [141]. MIC and MFC ranges were the same, 4-8 µg/ml for 8 *C. albicans* isolates. MIC_{50} and MIC_{100} values were 4 and 8 µg/ml respectively.

Microorganisms can develop resistance to antifungal agents especially in long term usages by mutation of targets within the organisms or by transfer of resistance genes from other organisms by transformation, transduction or conjugation and these resistances spread rapidly among other microorganisms which are pathogenic to men and cause problems in the therapy of infections. The development of antifungal drug resistance in *Candida albicans* following prolonged exposure to azole antifungals is well established [142]. Fluconazole has been widely used since 1988 for the *Candida albicans* infections which resulted in the increase of the fluconazole resistant strains. Among the tested *C.albicans* isolates, our study included 1 fluconazole resistant strain. Toxin MIC and MFC values for that fluconazole resistant strain was 8 µg/ml. That result indicates that K9 type yeast killer protein may be a new alternative in the treatment of fluconazole resistant *C.albicans* strains.

In our study we also tested one *C.albicans* isolate from a patient with vaginal candidiasis. Vaginal candidiasis is a fungal infection of any of the *Candida* species, of which *C. albicans* is probably the most common. The majority of cases of vaginal candidiasis occurs in perfectly healthy women and responds well to conventional antifungal treatments for *Candida* vulvovaginitis [143]. An estimated 75% of all females experience at least one episode of the disease during their lifetime [144]. This tested *C. albicans* strain isolated from vaginal candidiasis was found to be susceptible to the K9 type yeast killer protein with a MIC value 8 µg/ml.

K9 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.

One of the most noteworthy aspects of this study is the good activity of K9 type yeast killer toxin against the four strains of *C. krusei* including both clinical and standard isolates tested. Although *Candida albicans* is the most common human yeast pathogen, other *Candida* species such as *C. krusei* are now recognized as emerging agents, especially in patients with human immunodeficiency virus (HIV) disease. *C. krusei* is inherently resistant to the widely used triazole antifungal fluconazole and poses therapeutic problems, especially in systemic candidiasis. It is thought that the wide use of the newer triazole drug, fluconazole, in HIV-infected individuals is contributing to this phenomenon. Studies in both humans and animals have now demonstrated prophylactic and therapeutic failure of fluconazole against *C. krusei* due to increasing resistance of the organism to this azole. The widespread use of the newer triazole, fluconazole, to suppress fungal infections in human immunodeficiency virus (HIV) -infected individuals has contributed to a significant increase in *C. krusei* infection [145,146]. K9 type yeast killer toxin showed an inhibitory activity against all of the *C. krusei* strains with a MIC value of 2 µg/ml whereas fluconazole MIC range is between 16 and 64 µg/ml for this species [135]. MFC range for these 4 *C. krusei* strains was between 2-8 µg/ml. The MFC values of the K9 type yeast killer protein was relatively higher than the MIC values for *C. krusei* isolates compared to other *Candida* spp. This may be due to the growth characteristics of the organism. Pseudohyphal growth of the strain with elongated and branched blastoconidia may play a role in the difference of the MFC values. Our findings demonstrated that K9 type yeast killer toxin may 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 K9 type yeast killer protein.

Until recently, *Candida glabrata* was considered a relatively nonpathogenic commensal fungal organism of human mucosal tissues. However, following the widespread and increased use of immunosuppressive therapy together with broad-spectrum antimycotic therapy, the frequency of mucosal and systemic infections caused by *C. glabrata* has increased significantly. In fact, depending on the site of infection, *C. glabrata* is often the second or third most common cause of candidiasis after *C. albicans.* A major obstacle in *C. glabrata* infections is their innate resistance to azole antimycotic therapy, which is very effective in treating infections caused by other *Candida* species, especially fluconazole. Consequently, *C. glabrata* infections have a high mortality rate in compromised, at-risk hospitalized patients [147,148]. We have included a standard strain of *C. glabrata* in our study and the results showed that it is susceptible to the K9 type yeast killer toxin at MIC and MFC of 0.25 µg/ml.

C. guilliermondii and *C. parapsilosis* which are the other emerging pathogens that can infect various sites in human body were found to be moderately susceptible to the K9 type yeast killer protein within the MIC and MFC ranges of 4->32 and 8- >32 µg/ml for *C. guilliermondii* respectively, >32 µg/ml in the means of both MIC and MFC values for *C. parapsilosis*. These two *Candida spp*. had the highest MIC and MFC values among the other *Candida spp*. This result may be due to the difference in cell wall compositions and may be the difference in polysaccharide content of the cell wall which is used as a target by the toxin.

Clinical isolates of *C. tropicalis* and *C. pseudotropicalis* isolated from human abscess and cutoneus candidiasis respectively were found susceptible to the K9 type yeast killer toxin. Both the MICs and MFCs were found to be 0.25 µg/ml for *C. tropicalis* and 2 µg/ml for *C. pseudotropicalis*.

Saccharomyces cerevisiae is a ubiquitous, ascomycetous yeast and is commonly considered to be nonpathogenic. However, in the past several years this organism has been noted to be a human pathogen, particularly in

immunocompromised patients. Vaginitis caused by *Saccharomyces* species has been reported only rarely [149]. One strain of *S. cerevisiae* which is an opportunistic pathogen causing vaginitis was also included in this study and found to be susceptible to the K9 type yeast killer toxin at both MIC and MFC of 0.5 µg/ml.

Opportunistic fungal infections resistant to antifungal agents have been increasingly documented in recent years and their frequency will likely continue to increase. This phenomenon appears due largely to the extensive use of antifungal agents to treat fungal infections that typically occur in severely immunocompromised and/or critically ill patients. The azoles have been widely used to treat a variety of both superficial and invasive fungal infections. Unfortunately, this broad usage led to both development of acquired resistance (especially among *C. albicans*) and to shifts in flora away from the *C. albicans* and towards the less susceptible non-albicans species [150]. 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. This study demonstrates that K9 type yeast killer protein may be a potent antifungal for alternative treatment of candidiasis, as evidenced by the low MICs obtained when it was tested against a variety of fungal species. Especially for the mucosal fungal infections such as vulvovaginal candidiasis caused by *C. albicans* or *S. cerevisiae,* this antifungal toxin may be readily used within topical formulations. Systemic use of this protein against fungal pathogens that colonise in the blood stream might be possible with formulations based on modern therapeutic systems such as nanospheres.

Dermatophytes are a group of morphologically and physiologically related molds that cause well-defined infections in vertebrates. The incidence of dermatophytoses has increased over recent years, particularly in immunocompromised patients. *Trichophyton*, *Microsporum*, and *Epidermophyton* are the causative agents of dermatophytosis and infect the hair,

skin, and nails [105]. In this study we also tested 9 dermatophyte strains including *Trichophyton* and *Microsporum* species for their susceptibilities to K9 type yeast killer protein. All of the MIC values for tested dermathophyte strains were found >64 µg/ml. Considering this it might be said that K9 type yeast killer toxin can not show its hydrolytic activity against these isolates. This may be due to the different cell wall composition structure of these isolates or due to the difference in the binding affinities of the K9 type yeast killer toxin to the major cell wall components of these isolates. In this study we observed that K9 type yeast killer toxin can not be used as a topical antifungal agent for superficial fungal infections caused by dermatophytes tested in this study.

Cell killing kinetic of the K9 type yeast killer protein was analyzed on fluconazole resistant *Candida krusei* ATCC 6258 at the concentrations of MIC, 4, 8 and 16 times of the strain specific MIC value. Partial inhibition of growth was noted at the concentration of MIC; however, the toxin showed fungicidal activity against *C. krusei* 6258 at concentrations four or more times the MIC. Thus, increasing the concentration of the toxin in solution, up to the maximal concentration tested (16x MIC), resulted in improved antifungal activity. K9 type yeast killer toxin showed its fungicidal activity (99.9% decrease in the log10 of the number of CFU/milliliter compared with starting inoculum) against *C. krusei* 6258 at ≥4× the MIC. Cell killing activity was started with in the first 4 hours and reached the fungicidal endpoint at 12 h. Complete cell death was observed for the 4, 8 and 16 times the MIC concentrations at 24 hour. This study also demonstrates that K9 type yeast killer toxin has dose-dependent effects against tested *Candida krusei* ATCC 6258 because with the increasing concentrations of the toxin the killing activity is more profound.

Drugs that are the currently used against fungal infections such as amphotericin B, fluconazole, ketoconazole or itraconazole are not selective to the fungal cells and cause severe side affects on the host cells. New antifungal drugs which target the components of yeast and fungal cell walls are underdevelopment for treatment of fungal infections. These drugs would not

harm the host cell since the mammalian cells lack a cell wall [151]. K9 type yeast killer toxin exerts its lethal effect by hydrolizing the 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. This hydrolytic activity on sensitive cells makes the K9 type yeast killer protein highly selective antimycotic agent especially against *Candida* species. The use of the K9 type yeast killer protein as an antifungal agent might be possible with appropriate formulation studies upon the antifungal spectrum determination of the toxin in this study.

CHAPTER V

CONCLUSION

1. K9 type yeast killer toxin was found to be affective against all of the tested 22 strains of the genus *Candida* within the MIC and MFC ranges of 0.25-8 µg/ml except for *Candida parapsilopsis* and *Candida guilliermondii* isolates.

2. K9 type yeast killer toxin was found effective against fluconazole resistant *Candida krusei* isolates with a MIC value of 2 µg/ml for all of the tested *C. krusei* isolates.

3. MIC values of the K9 type yeast killer toxin on the tested 9 strains of human pathogenic dermathophytes were determined as >64 µg/ml.

4. K9 type yeast killer toxin exerts its cytotoxic effect within 4 h and kills the entire population at 24 hour.

5. Wide antifungal spectrum and selectivity of the toxin due to its strong hydrolytic activity highlights the use of the K9 type yeast killer protein against human fungal infections.

6. K9 type yeast killer protein might 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)

Laminarin from *Laminaria digitata*(Sigma, 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
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.
Coomassie Blue Stain	0.025% Coomassie Brilliant Blue R-250, 40% Methanol , 7% Acetic Acid.
Destain Solution I	40% Methanol , 7% Acetic Acid

Destain Solution II	5% Methanol , 7% Acetic Acid

Silver Stain

Destain Solution I	40% Methanol , 7% Acetic Acid
Destain Solution II	5% Methanol , 7% Acetic Acid
Cross-linking Solution	10% Glutaraldehyde
Dithiothreitol(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.

Isoelectric Focusing

Monomer Solution	30.8% T , 2.7% C_{bis}
Anolyte	0.02 M Acetic Acid
Catholyte	0.02 M Sodium Hydroxide
Fixative I	20% Trichloroacetic Acid
Fixative II	40% Ethanol , 10% Acetic Acid , 0.25% SDS.
Stain	40% Ethanol , 10% Acetic Acid , 0.125% Coomassie Blue R-250.
Destaining Solution	40% Ethanol , 10% Acetic Acid

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