INVESTIGATION OF CYTOCIDAL EFFECT OF K5 TYPE YEAST KILLER PROTEIN ON SENSITIVE MICROBIAL CELLS

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ABDULLAH SERTKAYA

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Approval of the Graduate School of Natural and Applied Sciences

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Prof. Dr. Canan ÖZGEN

Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

_____

Prof. Dr. Semra KOCABIYIK

Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

_____

Assoc. Prof. Dr. Fatih İZGÜ

Supervisor

Examining committee Members

Prof. Dr. Haluk HAMAMCI	(METU, FDE)	_____
Assoc. Prof. Dr. Fatih İZGÜ	(METU, BIO)	_____
Prof. Dr. Semra KOCABIYIK	(METU, BIO)	_____
Assoc. Prof. Mehmet BAYSALLAR	(G.A.T.A)	_____
Assist. Prof. Dr. A.Elif Erson	(METU, BIO)	_____

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Abdullah SERTKAYA

ABSTRACT

INVESTIGATION OF CYTOCIDAL EFFECT OF K5 TYPE YEAST KILLER PROTEIN ON SENSITIVE MICROBIAL CELLS

SERTKAYA, Abdullah

MSc, Department of Biology
Supervisor: Assoc. Prof. Dr. Fatih İZGÜ

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Some yeasts secrete polypeptide toxins, which are lethal to other sensitive yeast cells, gram-positive pathogenic bacteria and pathogenic fungi. Therefore these are designated as killer toxins. Killer toxins are suggested as potent antimicrobial agents especially for the protection of fermentation process against contaminating yeasts, biological control of undesirable yeasts in the preservation of foods. Moreover they are promising antimicrobial agents in the medical field; due to immune system suppressing diseases like AIDS, there is an increase in the incidence of fungal diseases and current antimycotics have low selectivity and severe side effects.

In this study our aim was to explain the cytocidal effect and enzymatic properties of K5 type yeast killer protein, which is secreted by *Pichia anomala* NCYC 434 cells, and known to have a broad range of killing spectrum. Competitive inhibition of the toxin with cell wall polysaccharides showed that primary binding site of toxin is β-1,3-glucans of sensitive cells. Toxin showed exo-β-1,3-glucanase activity which causes loss of cell wall rigidity leading cell death. K_m and V_{max} were found to be 0,3 mg/ml and 372,3 µmol/min/mg for laminarin hydrolysis. The toxin exerted its cytocidal effect after 2 h contact with the target cells. Toxin production was found to be dependent on β-1,3-glucan content of the media. Toxin activity was completely inhibited by Hg^{+2} ,while several metal ions and DTT increased the activity to different extends. Our findings revealed the characteristics of K5 type killer toxin which will help for its possible uses in near future.

Key words: Antimycotic agent, K5 type yeast killer toxin, Exo-β-1,3-glucanase, Enzyme kinetics.

ÖZ

K5 TİPİ ÖLDÜRÜCÜ MAYA PROTEİNİNİN DUYARLI HÜCRELER ÜZERİNDEKİ SİTOSİDAL ETKİSİNİN ARAŞTIRILMASI

SERTKAYA,Abdullah

Yüksek Lisans, Biyoloji Bölümü
Tez Yöneticisi: Doç. Dr. Fatih İZGÜ

Eylül 2005, 87 sayfa

Bazı mayalar ortama öldürücü proteinler olarak adlandırılan polipeptid toksinler salarlar ve bu toksinler diğer duyarlı mayalar, gram-pozitif patojenik bakteriler ve patojenik mantarlar üzerinde öldürücü etkiye sahiptir. Öldürücü toksinler özellikle fermantasyon sürecinin ve gıdaların kontamine edici mayalara karşı korunmasında ve tıp alanında potansiyel antimikotik ajanlar olarak önerilmektedir. Tıpta halen kullanılmakta olan antimikotiklerin düşük seçicilikleri ve ciddi yan etkilerinden, AIDS gibi immün sistem baskılayıcı hastalıkların yaygınlaşması nedeni ile artan mantar hastalıklarından dolayı yeni potansiyel antimikotikler önem kazanmışlardır.

Bu çalışmada insan patojenik mantarları da dahil geniş bir öldürme spektrumuna sahip olan *Pichia anomala* NCYC 434 tarafından üretilen K5 tipi maya öldürücü proteininin sitosidal etki ve enzimsel özelliklerini açıklamayı hedefledik. Toksinin öldürücü aktivitesinin hücre duvar polisakkaritleri ile yapılan karşılaştırmalı ihibasyonu, toksinin birincil bağlanma bölgesinin β-1,3-glukan olduğunu göstermiştir. Toksin hücre duvar sağlamlığını bozarak nihai hücre ölümüne neden olan ekzo-β-1,3-glukanaz aktivitesi göstermektedir. K_m ve V_{max} laminarin hidrolizi için sırasıyla 0,3 mg/ml ve 372,3 µmol/dak/ mg olarak bulunmuştur. Toksin sitosidal aktivitesini duyarlı hücrelerle 2 saatlik inkübasyondan sonra göstermeye başlamaktadır. Toksin üretimi besiyerindeki β-1,3-glukan varlığına bağımlıdır. Toksinin aktivitesi Hg^{+2} tarafından tamamen yok edilirken, bazı metal iyonları ve DTT toksin aktivitesine çeşitli oranlarda artırıcı etkide bulunmaktadır. Bulgularımız K5 tipi maya öldürücü proteinin yakın gelecekte kullanımında yardımcı olacak karakteristik özelliklerini açıklamaktadır.

Anahtar kelimeler: Antimikotik ajan, K5 tipi maya öldürücü proteini, Ekzo-β-1,3-glukanaz, Enzim kinetiği

To My Family

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TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF SYMBOLS

bp	Base pair
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic Acid
HPLC	High Performance Liquid Chromatography
(HK)	Hexo-Kinase
K^+	Killer character
K^-	Non-killer character
kb	Kilo base
kDa	Kilo dalton
K_m	The Michaelis constant, is the substrate concentration required to reach half-maximal velocity ($V_{max}/2$).
L dsRNA	Large size double stranded RNA
MWCO	Molecular Weight Cut-Off
M dsRNA	Medium size double stranded RNA
NCYC	National Collection of Yeast Cultures
R^+	Killer toxin immunity
R^-	Non-killer toxin immunity
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
V_{max}	The maximal velocity at saturation

CHAPTER I

INTRODUCTION

Yeasts are unicellular true fungi and generally in spherical or oval shape. The size of cells is usually 5-10 µm in diameter and they are facultative anaerobes. Yeast cells do not form filaments or a mycelium; the cells remain as single cells. These cells divide by budding; *Saccharomyces* and *Pichia* are known as "budding yeasts" due to the fact replication occurs by multilateral budding.

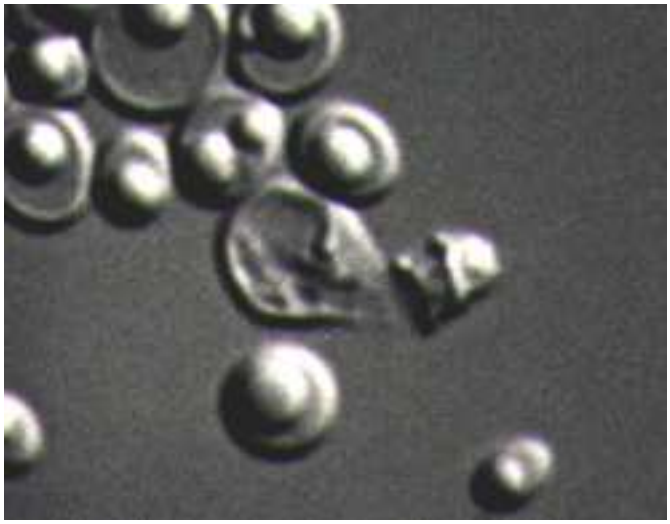


Figure 1.1. *Pichia anomala* cells [1].

Pichia anomala is a fungi belonging in the phylum; *Ascomycetes*, class; *Hemiascomycetes*, order; *Saccharomycetales*, family; *Saccharomycetaceae*, genera; *Pichia* (Hansenula), species; *anomala* [2]. *P. anomala* is present in many types of environments and have been isolated from fruit and plant material, cereal grain, maize silage, and from high sugar food products. *P. anomala* is frequently isolated from wine and has been reported to be a non-*Saccharomyces* wine yeast [2].

Yeast is thought to be the first domesticated microorganism because it has been used in brewing since Sumerian and Babylonian civilizations (6000-2000 BC) in parallel*, S. cerevisiae* strains have been used with grape cultivation in Georgia and for dough leavening in Egypt, therefore, yeast cells has a long history in human's life. As a consequence of this association of yeast with human life after the invention of microscope yeast has been one of the most important research subjects. Louis Pasteur showed the association of yeast metabolism with fermentation in 1857. Then the term "enzyme" was first used for this organism in 1877. Production of glycerol (1915), genetic map by Lindegren and discovery of mating type system (1949), first commercial pharmaceutical products from recombinant yeast such as Hepatitis B vaccine (1990-1994) and completion of the yeast genome project were some of the other important milestones of yeast history [2].

Yeasts are most widely used microorganisms in industry. They are cultured for the cells themselves, for cell components and for the end products that they produce during the alcoholic fermentation. Yeast cells are also used as sources of food, vitamins and growth factors [2]. The most well-known and commercially important yeasts are the related species and strains of *Saccharomyces cerevisiae*. These organisms have long been utilized to ferment the sugars from rice, wheat, barley, and corn to produce alcoholic beverages and in the baking industry to expand, or raise, dough. *Saccharomyces cerevisiae* is commonly used as baker's yeast and for some types of fermentation. Yeast is often taken as a vitamin supplement because it is

fifteen percent protein and is a rich source of B vitamins, niacin, and folic acid. To sum up, yeast cells are used in industry for bread making, for food supplement, for animal feeds, for alcohol and glycerol production, for enzyme production such as invertase, galactosidase, for production of vitamins including vitamin B and D, for production of beer, whiskey, wine, brandy, vodka, rum also yeast are used extensively in environmental technologies such as bioremediation, waste utilization, crop protection, biosorption of metals [3, 4].

Yeasts are not only used for conventional production processes but has also been used in new biotechnological purposes to produce heterologous proteins and other molecules mainly for pharmaceutical use, *S. cerevisiae* is the most attractive host organism for high level expression of proteins which have been used for pharmaceutical purposes. In recent years, several *Pichia* species have been found to be suitable for the expression of heterologous proteins. The pharmaceutical products produced in yeast are given in table 1.1. [5].

In contrast to antibacterial antibiotics, bacteriophages and bacteriocins, that were described at the beginning of the last century, a similar antibiotic phenomenon in yeast was demonstrated much later. In 1963, Bevan and Makower discovered the killer phenomenon in a *Saccharomyces cerevisiae* strain which was isolated as a brewery contaminant [6]. The killer phenotype they described is based on the secretion of a low molecular mass protein or glycoprotein toxin (the so-called killer toxin) which kills sensitive cells of the same or related yeast genera without direct cell to cell contact. Also killer toxins of certain yeast strains have potential growth inhibitory activity on gram positive pathogenic bacteria and plant pathogenic fungi [7, 8]. Besides toxin-secreting killer strains, a significant number of nonkiller yeasts can be isolated that have lost their ability to produce various killer toxins but nevertheless retain immunity.

Table 1.1 Pharmaceutical Products Produced in Yeast.

Prokaryotic products	Tetanus Toxin Fragment C; Streptokinase
Surface Antigens of viruses	Hepatitis B; HIV; Foot and Mouse Disease; Influenza; Polio; Epstein-Barr ;Oncogenic Retroviruses, Malaria Antigen
Animal Products	Hirudin; Porcine Interferon; Interleukin; Trypsin Inhibitor;
Human hormones	Insulin ; Parathyroid hormone; Growth hormone ;Chrionic Gonadotropin
Human Growth Factors	Insulin like Growth factor; Nerve Growth Factor; Epidermal Growth Factor; Tissue Factor; Granulocyte-Macrophage Colony stimulating factor; Tumor Necrosis Factor
Human Blood Proteins	Hemoglobin; Factors VIII and XIII ; Alpha-1- Trypsin; Antithrombin III Serum Albumin
Various Human Enzymes	Cystic Fibrosis Transmembrane Conductance regulator; Estrogen Receptor
Interferons	Interferon - α ; Interferon - β1

The killer phenotype, i.e. toxin production and functional immunity, is very frequent among yeasts and can be found both in natural yeast isolates and in laboratory yeast strain collections. In particular, for yeast strains living in their natural habitat, it has been shown that toxin production can confer a marked advantage in the competition with sensitive yeast strains when the available nutrients are limited [9, 10]. After the initial discovery of the killer phenomenon in *S. cerevisiae*, it soon became evident that killer strains are not restricted to the genus *Saccharomyces* but can also be found among many other yeast genera. Up to now, toxin-producing killer yeasts have been identified in *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Hansenula, Kluyveromyces, Pichia, Ustilago, Torulopsis, Williopsis*

and *Zygosaccharomyces*, indicating that the killer phenomenon is indeed widespread among yeasts [9-12].

Young and Yagiu (1978) described ten distinct killer classes (K1-K10) on the basis of the killing and immunity reactions between them, their response to killer-curing treatments, and analysis of dsRNA content and structural relationships between the toxins including three in *Saccharomyces spp.* strains (K1 , K2 , K3). K11 type was introduced by Wickner in 1975 to the Young and Yagiu classification [13]. The K1 phenotype is widely distributed among 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. The K3 group consists of strains derived from single wine yeast and has a killing activity on K1 and K2 type killers. The nomenclature used for killer phenotypes is summarized in Table 1.2. [14].

Table 1.2. Killer Phenotypes of the Genus *S. cerevisiae* [15]

Phenotype	Properties
$K_1^+ R^+_1$	Strains producing killer protein, immune to it and sensitive to types K2 and K3.
$K_2^+ R_2^+$	Strains producing killer protein, immune to it and sensitive to types K1 and K3.
$K_3^+ R_3^+$	Strains producing killer protein, immune to it and sensitive to types K1 and K2.
$K^- R^+$	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" phenotype; strains producing killer protein and exhibiting a decreased immunity to type K1.
$K^- R^-$	Sensitive strains not producing killer protein.

1.1. Molecular Biology of Yeast Killer Systems

Sacchromyces cerevisiae contains a haploid set of 16 well-characterized chromosomes, ranging in size from 200 to 2,200 kb. The total sequence of chromosomal DNA, constituting 12,052 kb, was released in April, 1996 [16]. In contrast to the genomes of multicellular organisms, the yeast genome is highly compact, with genes representing 72% of the total sequence. In addition, chromosomes contain mobile DNA elements, retrotransposons, which vary in number and position in different strains of *S. cerevisiae*, with most laboratory strains having approximately 30 retrotransposons. Other nucleic acid entities, presented in Figure 1.2, also can be considered as part of the yeast genome. The 2-µm circle plasmids, present in most strains of *S. cerevisiae*, apparently function solely for their own replication [16-19].

Strains of the yeast species harbor one or more nonhomologous species of double-stranded RNA (dsRNA), called L-A , L-BC, T, W and M(1-4). All of them show non-Mendelian inheritance and some of them are known to be families of natural variants such as totiviridae [20,21].

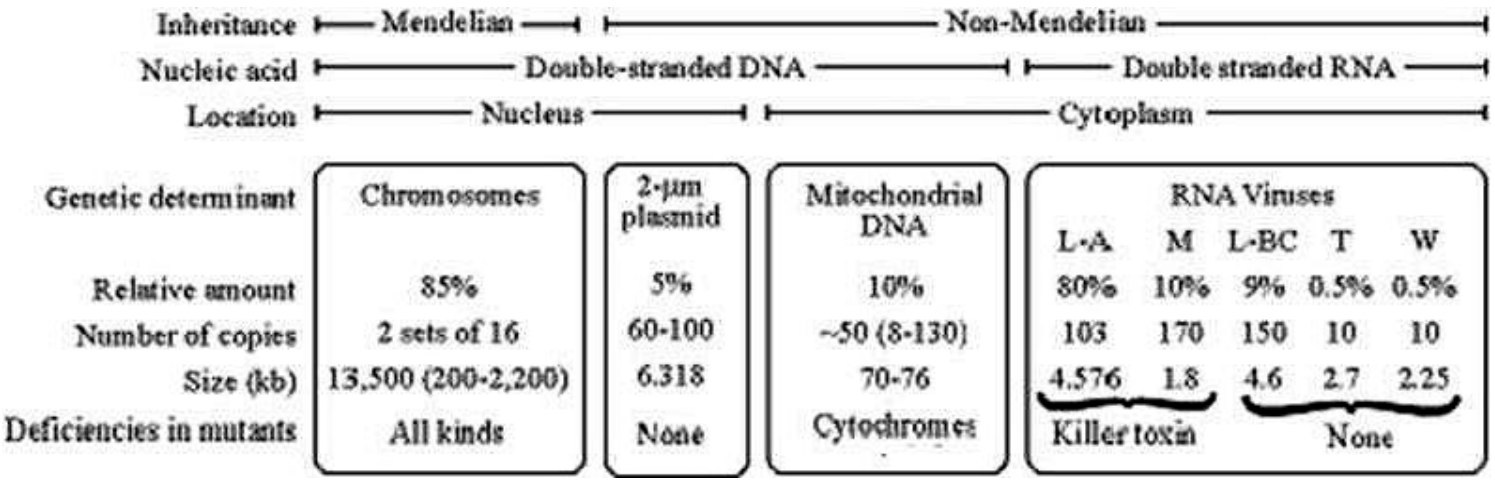


Figure 1.2. The Genome of a Diploid Cell of *S. cerevisiae*[17].

The genetic basis for killer phenotype expression can be quite variable; in the few cases where killer determinants have clearly been identified, they are either cytoplasmically inherited encapsulated dsRNA viruses, linear dsDNA plasmids or nuclear genes [22]. Table 1.3 shows genetic basis of some killer toxin producer yeast species.

Table 1.3.Genetic Basis for Killer Phenotype Expression in Yeast [21, 23, 24]

Yeast	*Genetic Basis*	*Toxin Gene*
Saccharomyces cerevisiae	dsRNA virus	M1- , M2- , M28
Hansenula uvarum	dsRNA virus	M-dsRNA
Zygosaccharomyces bailii	dsRNA virus	M-dsRNA
Ustilago maydis	dsRNA virus	M-dsRNA
Kluyveromyces lactis	linear dsDNA plasmid	pGKl1
Kluyveromyces fragilis	Chromosomal	Not identified
Pichia acaice	linear dsDNA plasmid	pPac1
Pichia inositovora	linear dsDNA plasmid	pPin1
Pichia kluyveri	Chromosomal	Not identified
Pichia farinosa	Chromosomal	SMK 1
Pichia anomala	Chromosomal	Not identified
Williopsis mrakii	Chromosomal	HMK
Williopsis saturnus	Chromosomal	Not identified
Schwanniomyces occidentalis	Chromosomal	Not identified
Debaryomyces hansenii	Chromosomal	Not identified
Candida glabrata	Chromosomal	Not identified

1.1.1. dsRNA Encoded Yeast Killer Systems

In *S. cerevisiae*, the killer phenomenon is based on the presence of cytoplasmically inherited double-stranded RNA (dsRNA) viruses. Persistent infection of yeast cells with these viruses is symptomless, and in contrast to certain fungal viruses that are associated with adverse phenotypic effects (like La France disease in *Agaricus bisporus* or plaque formation in *Penicillium*), yeast dsRNA viruses have no (so far recognized) reported adverse effects for host cell. In addition, yeast dsRNA viruses are considered non-infectious since no naturally occurring extra cellular route of transmission has been identified. They were therefore designated cryptic viruses or 'virus like particles' (VLPs) [19,21].

In contrast to the horizontal transfer of most pathogenic plant and animal RNA viruses, yeast dsRNA viruses only spread vertically during mating and heterokaryon formation *in vivo* and it has been suggested that this kind of virus transmission most likely represents a special adaptation since the frequent occurrence of mating and hyphal fusion in yeast and higher fungi makes an extra cellular route of virus transmission dispensable [25, 26]. L-A, L-BC and M are found in intracellular virus like particles, while these particles are not infectious, they can be introduced into spheroplasts by polyethylene glycol-induced fusion [19, 21, 27].

M dsRNA encodes the killer toxin and immunity of host cells. There are different M dsRNAs with different size M1 dsRNA (1.9 kB) , M2 dsRNA (1.7 kB) , M3 dsRNA (1.5 kB) correspond to individual types of strains ; i.e., K1 , K2 , K3 respectively. M dsRNAs present in high copy number in the cell about 10-100 [9, 12].

Another dsRNA (4,7kb) designated L dsRNA which is three-time larger than M is present in a high copy number (100 to 1000) in the cells. It is commonly closed

in virus like particles in almost all isolated strains of *S. cerevisiae* that usually do not contain M dsRNA and thus sensitive to the killer protein. The maintenance, encapsidation and replication of M type dsRNA is mainly dependent on L type dsRNA. L type dsRNA exists in several types. L type dsRNA contains two different types of RNA molecules called L-A, which codes for major capsid protein that are used for encapsidation of both the M and L types of dsRNA molecules, and L-BC having no homology between L-A type molecule. The second type, i.e. L-BC codes for a smaller capsid protein in the K1 type killer strains but several killer strains do not contain L-BC type dsRNA molecule indeed the direct function of both L-A and L-BC types in yeast killer phenomena has not been understood clearly [12, 19, 28]. In a series of *S. cerevisiae* strains other dsRNA types are also present which are designated as T, W and XL but their function and their relation with killer phenomenon is not known but several studies showed that they can be M dsRNAs with several deletions [12, 14].

1.1.1. a. Transcription and Replication of dsRNAs

Parental strands of L-A remain together during replication. It is suggested from the studies that (+) strands are made from dsRNA genomes and (-) strands are made on a (+) strand template to form L-A dsRNA. L-A dsRNA replication is asynchronous; i.e., (+) strands and (-) strands are not made at the same time [12].

It is proposed that the structure of the L-A-encoded capsid is designed to hold one L-A dsRNA molecule. The same capsid coded by L-A holds both itself and M dsRNA molecule. When M (+) strands are encapsidated in this coat and converted to dsRNA, the head is not full, because M (1.8 kb) is less than half size of L-A (4.6 kb). Thus, new M (+) strand transcripts often remain inside the viral particles, where they are converted to a second M dsRNA molecule. The mechanism of transcription

appears to be conservative with extrusion of the newly synthesized (+) when the capsid capacity is exceeded only then transcripts of the M dsRNA, which is responsible for the killer phenotype, are extruded to make new particles and translation of the killer protein. This mechanism is called “headful replication”. Headful replication implies that the L-A capsid size and structure are determined by the capsid protein structure. The replication cycles of L-A and M dsRNA are shown in Figure1.4. [14, 22, 29].

1.1.1. b. Genes Controlling the dsRNA Replication

The copy number of dsRNA viruses and their ability to replicate are controlled by both chromosomal and non chromosomal genes.

There are several gene families affecting the killer phenotype of killer cell. *MAK* genes, such as *MAK8* that codes for a ribosomal protein and *MAK1* codes for DNA topoisomerase, are needed for the K1 type killer phenotype and comprise at least 32 genes necessary to maintain M dsRNA .Likewise *clo* genes are related with the loss of L-A and L-BC type dsRNA virus like particles. Some yeast strains have superkiller character, which means more killing ability, and it is related with *SKI* gene family. Mutants carrying *ski2, ski3, ski4, ski6,ski7* recessive alleles produce more killer toxin and increased copy number of L-A and M dsRNA particles [19].

KEX genes consist of two genes and are needed to process toxin precursor. If a mutation occurs in the *KEX* genes the killing phenotype is lost but cells remain immune to toxin. *SEC* gene is also important as it is the gene for general protein secretion [19].

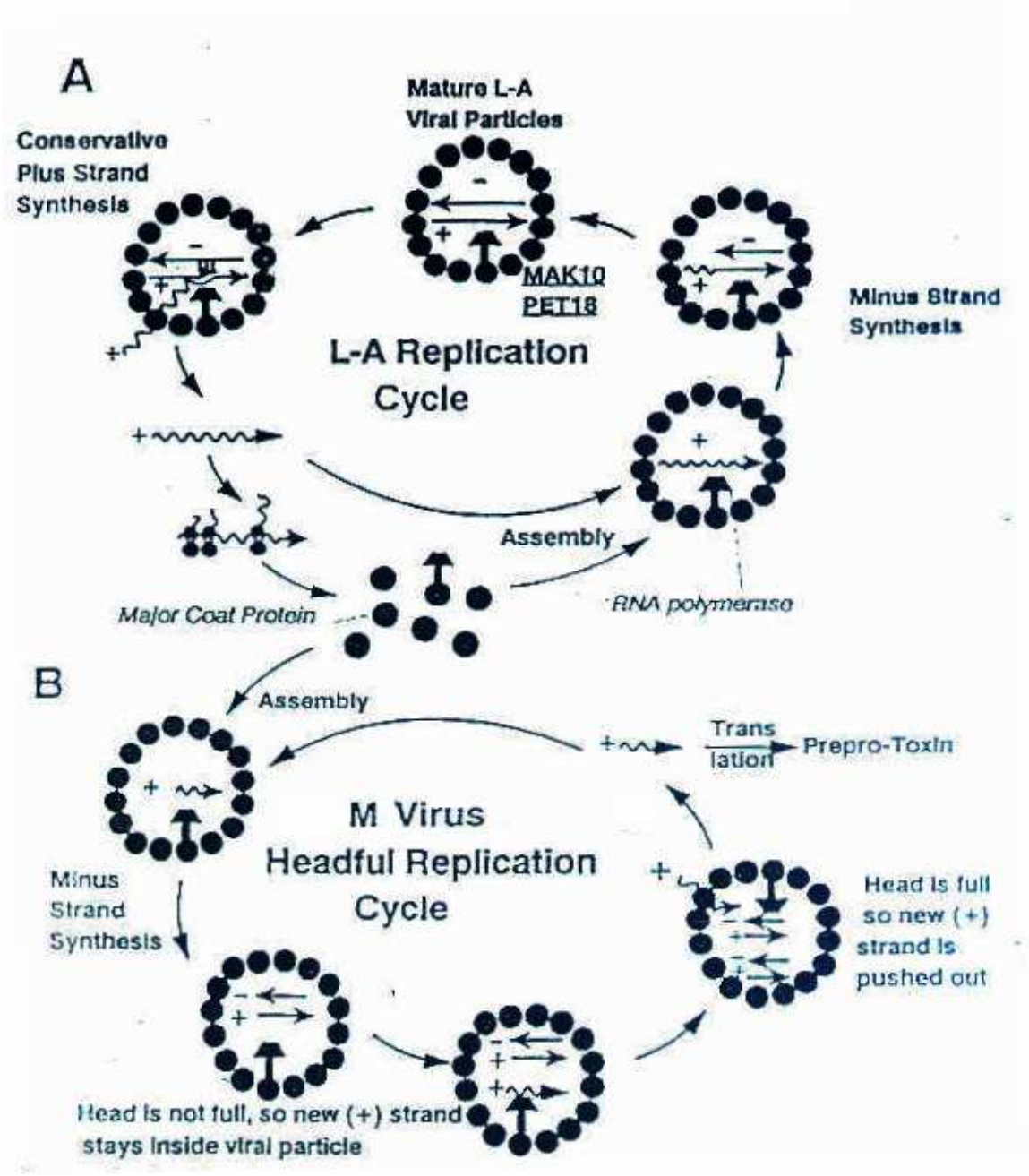


Figure 1.3.Replication Cycles of L-A and M dsRNAs [17].

KRE genes are needed for the toxin action sensitive cells, and code for the components of β-1,6-glucan synthase, in addition to this one other *KRE* gene affects the binding of the toxin to the cell wall [19].

Genes located on the dsRNA particle itself are also determinants of killer phenotype and these genes are given in Table 1.4.

Table 1.4 Cytoplasmic Genes Affecting The Killer Phenotype [19].

Cytoplasmic gene / mutant form	*Function of the gene*
[HOK]	*Helper of killer* This non-mendelian gene supplies the function needed by M for replication in a wild-type strain
[EXL]	*Excluder of M_2 dsRNA*; This non-mendelian trait provides the replication of certain forms of L-A, acts by lowering the copy number of the viral particle in the M_2 strain
[NEX]	M_2 Non excludable by [EXL], but does not prevent exclusion of M_2 by strains carrying M_1. [NEX] is located on a type of L-A found in K1 killer strain
[B]	A cytoplasmic gene which allows M to disperse with certain of the MAK gene products and elevates the copy number of M making the strain a superkiller
[KIL-d]	A mutant form of K1 killer strain in which haploid strains unstably maintain and incompletely express M but diploid strains stably maintain and normally express M
[KIL-n]	A mutant form of M dsRNA in which no active toxin is produced but cells are immune to toxin action

1.1.1. c. Structure, Processing and Secretion of Killer Toxins

The best-studied and best-known killer toxin, K1 (19 kDa), is secreted as a molecule consisting of two distinct disulfide bonded unglycosylated subunits, termed α (9.5 kD) and β (9.0 kD) derived from a 42-kDa glycosylated precursor molecule (protoxin). The α and β domains flank a segment called γ, which is not part of the mature toxin and assumed to be the immunity determinant. Protoxin is glycosylated at several sites within the γ segment [12, 30].

The primary translation product of M1 dsRNA is a 316-amino-acid, 35-kDa polypeptide (preprotoxin, M1p) consisting of a 44-amino-acid N-terminal leader sequence called d, which includes a 26-residue signal peptide, followed by the 103-residue α domain (positions 45 to 147) and the 83-residue β domain (positions 234 to 316) of toxin subunits, which are separated by a central γ peptide (positions 148 to 233) carrying all three potential N-glycosylation sites [12,14, 19].

Once synthesized, the preprotoxin enters the endoplasmic reticulum, directed by the leader sequence or some part of it, and the signal peptide is removed by a peptidase that probably cleaves at ValAla26 to produce protoxin. The remaining 27- to 44-amino-acid segment is presumably removed in the Golgi apparatus by an unidentified protease present in yeasts, which cleaves at ProArg44. In the endoplasmic reticulum, the γ domain is N-glycosylated and presumably folds into a form competent for translocation to the Golgi and for further processing, by a mechanism involving at least three proteolytic cleavages [12,19,31]. An endopeptidase encoded by the yeast *KEX2* gene (kex2p) is responsible for the cleavages which follow pairs of dibasic residues (ArgArg149, LysArg188, and LysArg233) and involve the termini of the a domain and the amino terminus of the β domain to generate both subunits. These cleavages presumably occur at different rates or with different efficiencies in the same vesicle in a late Golgi compartment, probably to minimize the production of lethal

fragments or to maximize the production of fragments involved in the expression of immunity [32, 33].

The α subunit is further processed by a serine carboxypeptidase, encoded by the yeast *KEX1* gene (kex1p), which removes dibasic C-terminal residues. Finally, the mature toxin is secreted outside the cell via the established yeast secretory pathway as a dimeric molecule in which the subunits are covalently linked by disulfide bonds that probably link the three cysteine residues in α and β [34]. Structure and processing of the preprotoxin is shown in Figure 1.4.

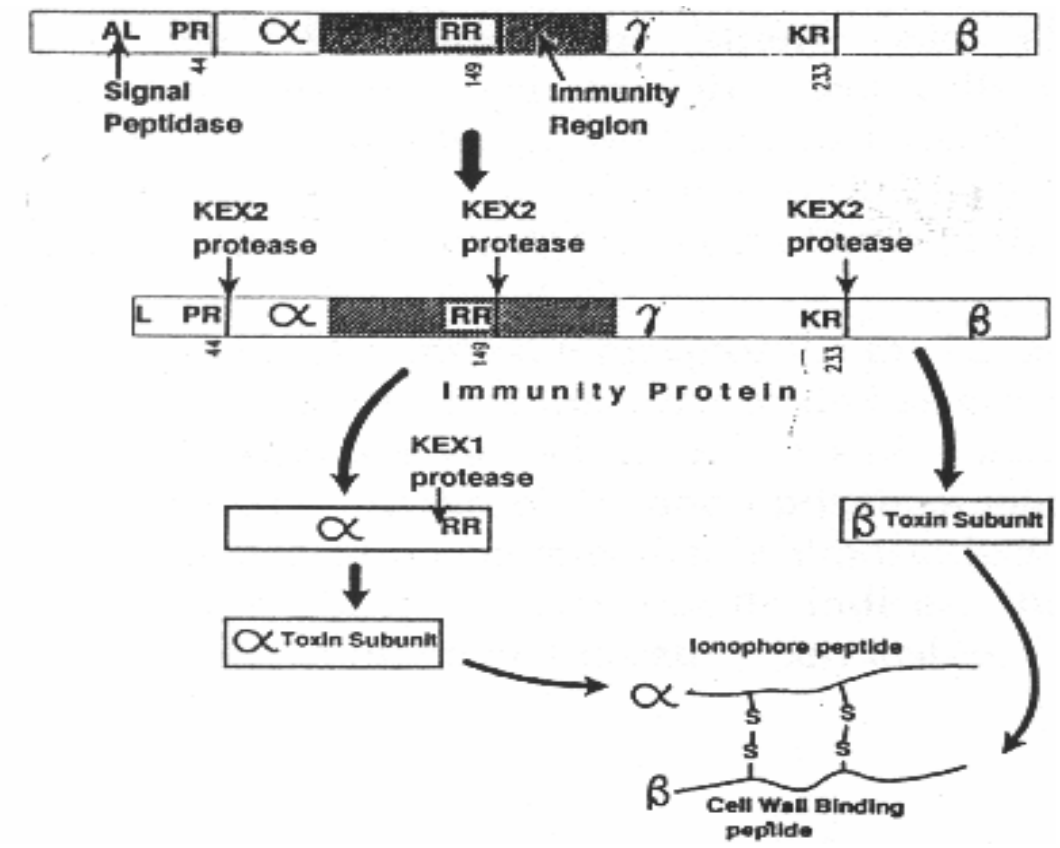


Figure 1.4.Structure and Processing of the Preprotoxin Encoded by M1 dsRNA [22].

The study of the K1 toxin maturation process has been extremely fruitful and has led to an understanding of most of the mechanisms involved in the processing and secretion of proteins, such as peptide hormones, in higher eukaryotes and to knowledge of the role of the kex proteases or their homologs in all eukaryotes [22].

Although the precise molecular basis for toxin immunity is still unknown, it has been speculated that it might be conferred by the toxin precursor itself. Due to fusion of the transport vesicles with the plasma membrane it is then transported out of the cell together with the immunity sequence which remains bound to the outer side of the membrane, probably to a specific receptor [12, 36]. This might act as competitive inhibitor of the mature toxin by saturating or causing elimination of a so far unidentified plasma membrane receptor that normally mediates toxicity. It was also shown that the unprocessed toxin precursor is sufficient to confer immunity [22].

1.1.2. Chromosomally Encoded and Plasmid Based Killer Systems

KHR(killer of heat resistant) and KHS(killer of heat susceptible) killer toxins have been described in *S. cerevisiae*. The mature KHR and KHS toxins are single proteins, with molecular masses of about 20 and 75 kDa, encoded on the left arm of chromosome IX and on the right arm of chromosome V, respectively. Since the mature toxins have molecular masses lower than those of their precursors, some protein processing is thought to occur during maturation. The *KHR* and *KHS* genes consist of 888 and 2,124 bp, respectively, with no homology to other killer genes [37, 38, 39].

The genetic basis for killer character in *Williopsis, Pichia, Candida, Debaryomyces, Cryptococcus, Torulopsis* and *Bullera* species is thought to be chromosomally encoded due to the absence of evidence that they are encoded by

dsRNA viruses or other cytoplasmic genetic elements such as linear plasmids that are common for several yeast species [40].

A novel type of killer toxin produced by the halotolerant yeast *P. farinosa* has been recently described. This toxin, termed SMKT (salt-mediated killer toxin), is a heterodimer (14.214 kDa), whose subunits (α, 6.6 kDa; β, 7.9kDa) are tightly linked under acidic conditions. It shows its maximum killer activity in the presence of 2 M NaCl. Although there is no sequence similarity to other toxins, the 222-aminoacid P. *farinosa* preprotoxin resembles the mode of action of *S. cerevisiae* K1 toxin [41, 42]. In addition to this the *P. kluyveri* killer toxin, a 19-kDa acidic glycoprotein, is associated with chromosomal genes and resembles the K1 type killer protein action in overall structure, hydrophobicity profile, and processing [43].

Williopsis mrakii produces two different killer toxins designated as HMK and K-500. HMK protein unlike other killer proteins is a basic unglycosylated polypeptide (10.7kDA), consisting of 88 aminoacids 10 of which are cysteines, shows high thermostability (100°C for 10 min) and pH stability between pH 2 and 11 [44, 45].

Recently, *Cryptococcus humicola* strains secreting small, acidic, thermostable, methanol-soluble, chromosomally encoded toxins, specified as microcins, have been isolated, suggesting that the killer phenomenon may be due to the excretion of two types of killer toxins: mycocins and microcins [46]. In addition, chromosomally encoded killer systems have been identified in almost all of the genera of yeasts comprising opportunistic pathogens, such as species of *Candida*, *Cryptococcus*, and *Torulopsis*, even though it is unlikely that killer toxins are important as virulence factors [47].

Kluyveromyces thermotolerans secretes a chromosomally encoded killer toxin which is different from every reported type of other killer factors. The killer

activity of the toxin depends on the concentration of NaCl and its killer spectrum also depends on the presence of NaCl [48].

Linear DNA plasmids have been identified for various yeast genera such as *Debaryomyces*, *Wingea*, *Kluyveromyces* and *Saccharomyces*. Killer strains of the dairy yeast *Kluyveromyces lactis* always contain 50 to 100 copies of each of two cytoplasmically inherited linear plasmids designated pGKL1 (k1) and pGKL2 (k2), which are 8,874 and 13,447 bp in size, respectively. *K. Lactis* toxin and the linear plasmids which are associated with the killer system are quite distinct from any other known yeast killer systems; in particular those which are encoded by double stranded RNA plasmid systems. k1 and k2 show an extremely high A+T content and have proteins covalently linked at their 5' termini and are almost certainly located cytoplasmically [49, 50].

Kluyveromyces lactis killer strains secrete a heterotrimeric toxin that inhibits the growth of a wide range of susceptible yeasts in the genera *Candida, Kluyveromyces, Saccharomyces, Torulopsis,* and *Zygosaccharomyces*, as well as nonkiller strains of *K. lactis.* The toxin consists of three subunits: a polypeptide with a single asparagine-linked oligosaccharide unit, designated α (99 kDa), and two smaller unglycosylated components, β (30 kDa) and γ (27.5 kDa), encoded by k1 [51, 52].

The plasmid k1 carries four open reading frames (ORFs) with gene functions involved in plasmid replication and expression of killer and immunity phenotypes. ORF1 codes for the plasmid-specific DNA polymerase, ORF2 and ORF4 encode the precursors of the subunits of the killer toxin (α, β, γ) and ORF3 is essential for toxin immunity. The plasmid k2 (13.5 kb), carrying eleven ORFs, provides essential maintenance functions presumably involved in transcription and replication of both plasmids. pGKL1 requires pGKL2 for its replication.k2 encodes plasmid-specific DNA and RNA polymerases, terminal proteins, capping

enzyme, helicase and single-stranded binding protein (SSB). Genetic organization of pGKL1 and pGKL2 is shown in Figure1.5. [53, 54].

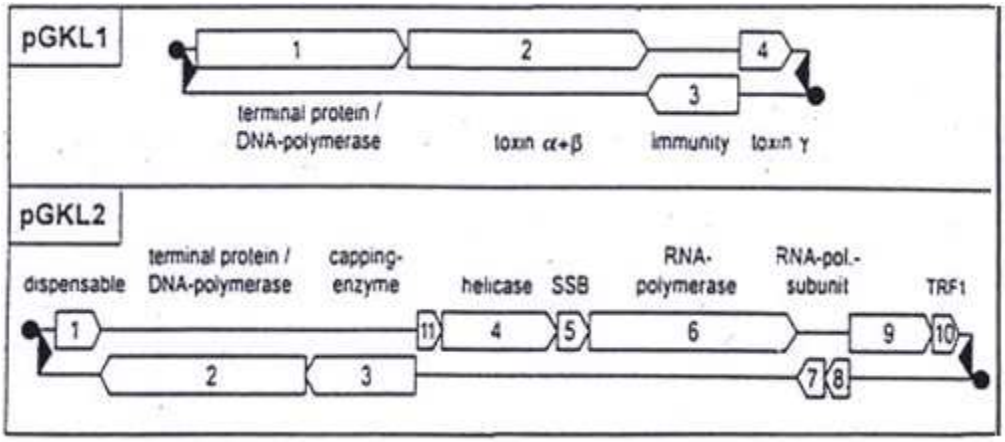


Figure 1.5. Schematic Representation of K. *lactis* Linear Plasmids [52].

Pichia acaciae killer strains have been shown to possess two linear plasmids, designated p*Pac*1-1 (13.6 kbp) and p*Pac*1-2 (6.8 kbp). These plasmids are quite similar in both function and structural organization to those found in *K. lactis*. p*Pac*1-2 was found to hybridize to k1 ORF2 and is thought to be involved in toxin production. Despite important similarities to *K.lactis* killer toxin, significant functional differences exist. *P.acaciae* toxin seems to be composed of three subunits (110, 39, and 38 kDa) with an associated chitinase activity. The presence of three linear dsDNA plasmids, of approximately 18, 13, and 10 kbp, has been reported in a killer toxin producing strain of *P. inositovora*. Only two of them (p*Pin*1-1 and p*Pin*1-3) seem to be associated with the killer phenotype, while the loss of p*Pin*1-2 has no effect on toxin production or susceptibility [47].

1.2. Toxicity of the Killer Proteins

Even though the killer toxins posses different modes of action, they do have one thing in common: all the secreted mature toxins can exert killer activity on susceptible cells by different mechanisms that require a specific initial binding to a cell wall receptor. After the adsorption of toxin actual killing processes which vary with the toxin type occurs.

1.2.1. Binding of Killer Toxin to the Target Cell: The Yeast Cell Wall

The mode of action of the K1 killer toxin has been the most extensively investigated killer protein in terms of toxicity properties. The first step of the killing action is strongly pH dependent with an optimum at pH 4.6 and is a low-affinity, high-velocity adsorption (1 min) of the killer toxin to the cell wall receptors. The constituents of the glucan fraction of the cell wall, mainly β-1, 6-D-glucan, have been identified as primary receptors for the toxin, and their assembly seems to require a number of yeast *KRE* (killer resistance) genes. Both subunits of the mature toxin seem to be necessary for receptor binding, with the hydrophilic β subunit being primarily responsible for the binding and the α subunit acting in a multifunctional way, with different overlapping regions of the polypeptide involved in killer activity, immunity, and binding [47].

The wall of a yeast cell is a remarkably thick (100 to 200 nm) and rigid envelope, which contains some 20 to 25% of the dry mass of the cell. Cell walls determine the shape of fungal cells and are essential for their integrity. Schematic structure of fungal cell wall and cell membrane is shown in Figure 1.7. The yeast periplasm is a thin (35-45 A), cell wall associated region external to the plasma membrane and internal to the cell wall. It mainly contains secreted proteins (mannoproteins) that are unable to permeate the cell wall, but fulfill essential functions in hydrolyzing substrates that do not cross the plasma membrane:

invertase converts sucrose into glucose and fructose; acid phosphatase catalyzes the liberation of free phosphate from organic compounds [4].

The wall consists of about 85 % - 90 % polysaccharide and 10 % - 15 % protein. The main components of the yeast cell wall are β-1,3-glucans (50%) that also contains some (1-6)-β-linked branches (5%) and mannoprotein, most of which is carbohydrate [66]. (1-6)-β-D-glucan, also containing some (1-3)-β-linked branches (14%), is a relatively minor constituent (15%), and chitin (0.6 to 9%) is present at an even lower level and the latter is concentrated in the bud-scar region. However the proportions of these different components vary according to the species [55]. Major relationships among cell wall components are represented in Figure 1.6.

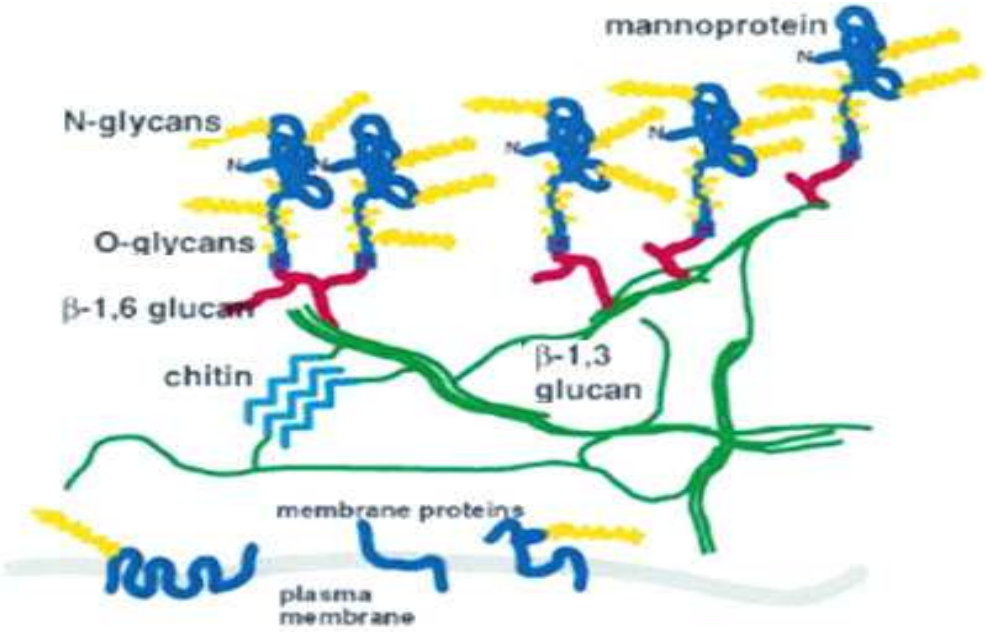


Figure 1.6. Relationships Among Components of *S.cerevisiae* Cell Wall [4].

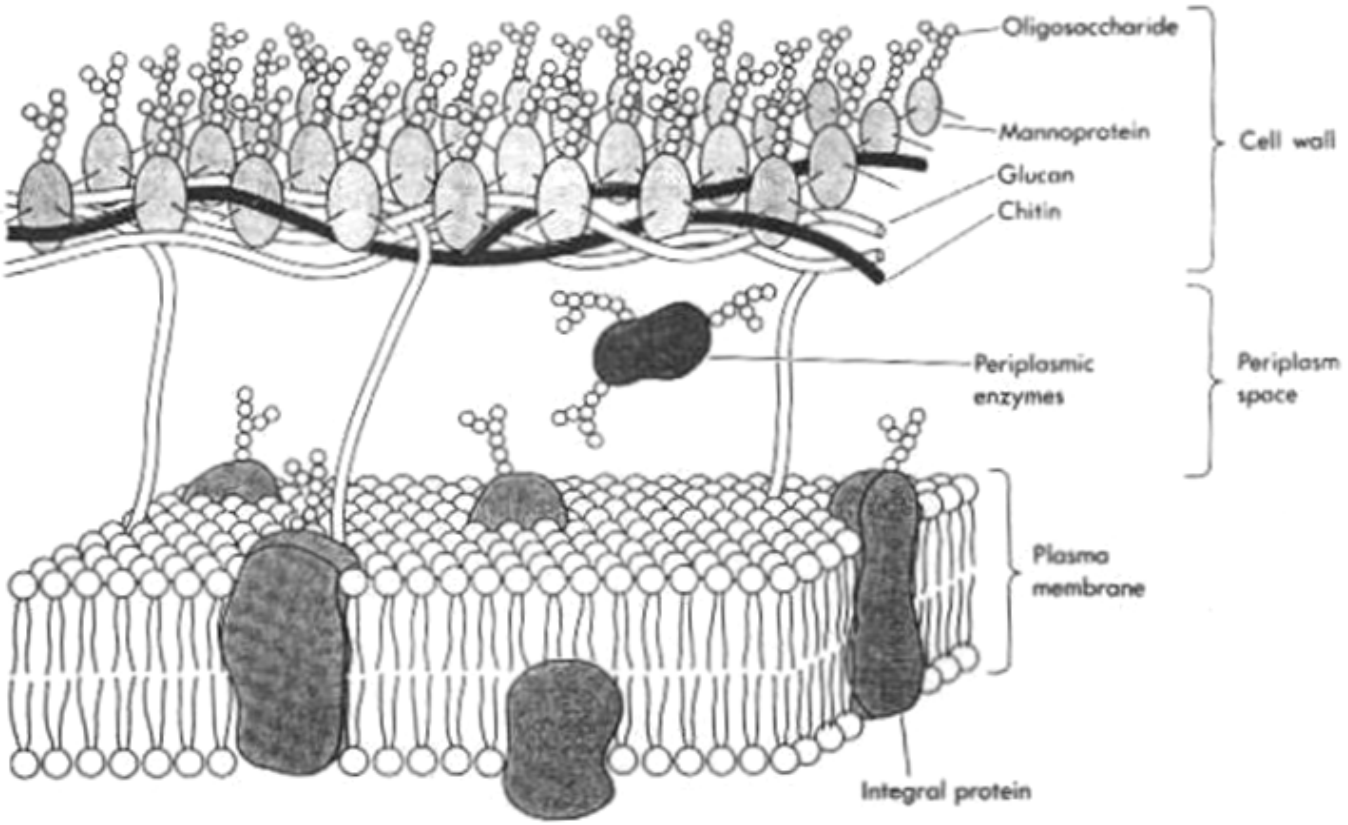


Figure 1.7. Schematic Representation of a Fungal Cell Wall [4]

There is an increasing commercial interest in the polysaccharides of yeast walls. One interest has focused on their applications in food processing as thickening agents, fat substitutes and as sources of dietary fibre [56]. Other attractions concern the ability of yeast wall glucans to stimulate the immune system and lower the serum cholesterol level, their antitumour activity and their potential use in cosmetics [57]. The mannoproteins exhibit biosurfactant properties [58]. To date, mainly the polysaccharides of *S. cerevisiae* have been considered in the context of these applications, possibly because we have detailed information about the wall composition of this species and this species is readily available as industrial yeast.

The role of cell surface polysaccharides as receptors for proteins in many cell events and mechanism of their action is an area of increasing interest. As well as acting as receptors for bacteria, viruses, and toxins, surface polysaccharides may be involved in cell interactions, such as cell associations, distribution, and turnover [57].

Various primary receptors for other killer toxins have been reported. β-1,3-D-Glucans and β-1,6-D-glucans probably act as cell wall receptors of *Hansenula mrakii.* The β-1,6-D-glucans are primary receptors for *S. cerevisiae* K1 and K2 killer toxins and *Hanseniaspora uvarum* killer toxin. Mannoproteins are receptors for KT28 of *S. cerevisiae* and *Zygosaccharomyces bailii* killer toxin, and chitin is a receptor for *Kluyveromyces lactis* killer toxin. Thus, any of the principal components of the cell wall could be the primary receptor for a killer toxin. Recently β-1,6-D-glucans are also found to be primary receptor also for *Pichia membranifaciens* and *Debaryomyces hansenii* killer toxins [47, 59].

1.2.2. Mode of Action of the Killer Toxins

The second step in the toxicity process of K1 type killer protein is a high-affinity, low-velocity, energy dependent interaction of the toxin with a probable plasma membrane receptor that leads to the actual lethal effect.

After binding to the yeast cell wall, K1 toxin is transferred to the cytoplasmic membrane and acts by forming voltage-independent cation transmembrane channels, which cause ion leakage and subsequent cell death. Two strongly hydrophobic regions near the C terminus of the α subunit have an α-helical structure separated by a short, highly hydrophilic segment and may act as a membrane- spanning domain responsible for channel formation [12, 17, 47].

K2 has virtually identical toxin activity to that of K1, despite a different structure, but K28 seems to act on the cell cycle in a different way. The K28 toxin binds primarily to the α-1,3-linked mannose residues of a 185-kDa cell wall mannoprotein, causing cell cycle arrest, apparently in the G2 phase, and leading to non-separation of mother and daughter cells, with the nuclear DNA confined to the mother cell. It is not clear if this early reversible inhibition of DNA synthesis is a primary or secondary effect of the toxin [47].

Killer toxin of *Kluyveromyces* lactis binds to the cell wall chitin as receptor; it is likely that a part of toxin or the entire molecule is translocated across the membrane into the cytoplasm. Then toxin which is a heterodimer causes a G_1 arrest and eventually loss of viability in S. cerevisiae cells, while permitting continued macromolecular biosynthesis. The killing activity mainly resides on its γ subunit while both α and β subunits are considered to be necessary for binding and/or uptake of the γ subunit [60, 61].

The killer toxins secreted by various strains of the yeast genus *Hansenula* strongly inhibit *de novo* β-1-3-D-glucan biosynthesis in yeast. It was shown that *Hansenula mrakii* toxin causes pore formation by inhibiting the β-1-3-D-glucan synthesis occurring at a budding site which results in leakage of cell material and eventual cell death [22 , 47].

The toxin from *Pichia kluyveri* causes ion-channel formation. 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 [41]. There is some evidence that K-500 toxin can act in a manner similar to *S. cerevisiae* K1 and *P. kluyveri* toxins by producing channels [47].

1.3. Application of Yeast Killer Systems

Killer yeasts and their toxins may have several applications. They have been used as model systems to study the mechanisms of regulation in eukaryotic polypeptide processing, secretion and toxin interaction with sensitive cells. Furthermore, the killer system in yeast provides useful models for the study of the control and expression of eukaryotic viruses. In addition, killer systems could have biotechnological applications both in fermentation industries and in medicine. In recombinant DNA technology, killer plasmids from *S. cerevisiae* and *K. lactis* have the potential to serve as cloning vectors for the effective secretion of expressed polypeptides [51].

1.3.1. Fermentation

Soon after the observation that toxin secreting killer yeasts can be the causal agent of stuck and / or protracted fermentations by antagonizing toxin-

sensitive yeasts and thus negatively affecting the quality of product, many efforts were undertaken to use natural or 'constructed' killer strains as starter culture in beer and wine fermentations. Immune industrial starter strains are constructed by using the methods of protoplast fusion or cytoduction. By means of genetic manipulation techniques starter culture can exhibit a significantly broader killing activity and are thus capable of outcompeting potentially contaminating yeasts like *Candida, Hanseniaspora, Kloeckera* and *Pichia* in mixed culture, this is especially important in the fermentation processes using mixed yeast strains [7, 37, 52].

1.3.2. Food Preservation

Growth of moulds in food and animal feed leads to reduced nutritional values and production of allergenic spores and hazardous mycotoxins. The production and storage of foods is frequently compromised by the growth of certain yeasts such as *S. ludwigii* and *Kloeckera apiculata* which produces low concentrations of ethanol and undesirable products of fermentation. Traditionally, fungicides have been used to deal with these problems, but factors such as consumer health and environmental concerns, resistance problems and a more strict legislation have made alternatives necessary. During recent decades, biological control of moulds has evolved as a possibility [50, 66].

A variety of microbial antagonists were reported to control several different pathogens on various fruits. Among these antagonists there were many yeasts and yeastlike organisms e.g. *Debaryomyces hansenii*, for control of post-harvest citrus rot and several species of *Cryptococcus* for control of post-harvest rot on apples and pears. The killer yeasts such as *Candida oleophila, Pichia anomala* have been demonstrated to be used as antagonist against most of the postharvest pathogens in the preservation of foods [67].

1.3.3. Heterologous Protein Expression

When expression of eukaryotic proteins is desired, bacterial systems often turn out to be ineffective hosts because of their limited capacity to perform multi-step post-translational modifications such as protein N-glycosylation, phosphorylation and acetylation. Therefore, unicellular eukaryotes such as the yeasts *S. cerevisiae, Pichia pastoris, Yarrowia lipolytica, Hansenula polymorpha, K.lactis* and *S.pombe* have become attractive hosts for the expression of heterologous proteins. During the past decade, an increasing number of medically and pharmaceutically interesting secretory proteins (such as mouse K-amylase, human Antithrombin III or placental alkaline phosphatase) have been expressed as extracellular proteins by using homologous secretion signals either derived from yeast invertase, acid phosphatase, pheromone P-factor, or from the plasmid-driven killer toxin of *K. lactis*. More recently it has also been shown that the secretion and processing signal derived from the *S. cerevisiae* ScV-M28 killer virus is fully functional in fission yeast *(S. pombe)* and can be used to target foreign proteins for secretion into the extracellular medium. Moreover, heterologous protein secretion driven by the K28 preprotoxin processing and secretion signal has been demonstrated as highly efficient for the green fluorescent protein (GFP), making fission yeast an attractive host for the processing and secretion of foreign proteins. Therefore, preprotoxin-based vectors might be an attractive means for efficient processing and high level secretion of heterologous proteins in yeast [22, 47].

1.3.4. Medicine: Antifungal and Antimicrobial Properties of Killer Toxins

Antifungal proteins, peptides and their synthetic derivatives possess the potential for being used in the treatment of human fungal infections which dramatically increased during the last two decades, particularly in immuno-compromised patients. Antimycotics such as flucanozole, ketoconazole, and

amphoterecin B, most of which are known to have severe side effects and disadvantages like causing resistance among target microorganism, are currently used antifungals in the treatment of fungal diseases[82,83]. In the search for novel and more selective antifungals, 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. Therefore antifungal research is currently focusing on the possible use of yeast killer toxins as novel antifungals [22,47].

The killer toxins from *Williopsis subsuffciens, Hanseniaspora uvarum, W. beijerinckii, W. mrakii* and *Pichia anomala* have anti-*Candida* activity. Some killer yeast strains have potential growth inhibitory activity on gram-positive pathogenic bacteria such as *Streptococcus pyrogenes, Bacillus subtilis, Sarcina lutea* and *Staphylococcus aureus* [7]. The 10.721-kDa toxin, produced by *W. mrakii*, was found to inhibit selectively β-glucan synthesis in the cell wall of susceptible yeasts by a mechanism similar to that of antifungal antibiotics such as aculeacin A, echinocandin B, and papulocandin B [47].

However, most yeast killer proteins exhibit their cytotoxic activity only within a narrow pH range and at temperatures between 20º C and 30º C, therefore, such yeast toxins are not suitable for oral and / or intravenous administration, but topical applications in the treatment of superficial lesions might well be possible [22]. Therefore killer toxins having antimicrobial activity at physiological temperature and pH are of interest.

Killer toxins produced by *Hansenula, Pichia, Kluyveromyces* strains are demonstrated to have antifungal activity against pathogenic fungi that threat human, animal and plant hosts [8, 68, 69]. Especially *Pichia anomala* killer toxin has been investigated and shown to have antagonistic effect against a broad range of microorganisms including taxonomically unrelated pathogenic microorganisms such as *P. carinii*, *Candida albicans* and *Mycobacterium tuberculosis*. It is

thought that *Pichia* toxins which shows relative high stability in comparison to toxins of other killer yeasts anti-microbial activity could be exploited in a therapeutic strategy [9, 14, 40, 70, 71].

According to the Young and Yagiu classification (1978), the K5 type yeast killer protein produced by *Pichia anomala* NCYC 434, was recently isolated and characterized [72]. The protein which is glycosylated has a molecular mass of 49 kDa with pI value of 3.7. K5 type killer protein is highly stable and active in the pH values between 3.0 and 5.5 and temperatures up to 37ºC. Its internal amino acid sequences show 100 % homology with exo-β-1-3 glucanase of *Pichia anomala* strain K [72].

β-1,3-Glucan is the major component of the most of fungi including pathogens that cause severe diseases. β-1,3-Glucanases hydrolyze this fiber causing the loss of cell wall rigidity due to the importance of the fiber in the cell envelope structure. Thus β-1,3-glucanases have been suggested as biocontrol agents in food preservation and fermentation industry. Moreover these enzymes are of great importance in fermentation and pharmaceutical technology where their use has been increasing as β-glucan modifiers [73]. In recent years β-1,3-glucanases have been increasingly used for the production of different formulations of β-(1-3)-glucan drugs and supplements which are used as immune-system inducer. In animal feed production, plant and animal chymus production it is used as viscosity reducer. In wine and beer industry β-1-3 glucanases are also used for taste improvement and facilitation of filtration [74].

Although there are studies investigating the nature of killer systems, information about this subject is limited with some killer systems especially that belong to *Saccharomyces* and *Kluyveromyces* of which genetic elements responsible for killer character responsible for the expression of killer protein is dsRNA and 2μ DNA plasmid.

High pH and thermo stability which are appropriate for both medical and industrial biocontrol purposes, broad spectrum of antimicrobial activity and proposed β-1,3-glucanase activity of the K5 type yeast killer toxin, suggest its possible applications in food biocontrol, fermentation process and as an antimycotic agent in medicine in near future.

In this study in order to highlight its importance, to supply required knowledge for its possible uses and to give more information about a chromosomally encoded killer toxin's nature, we aimed to investigate the cytocidal effect and enzymatic properties of K5 type yeast killer protein. We evaluated effect of media contents on its production. We found primary binding site of the toxin on sensitive target cells by competitive inhibition of toxin with different cell wall polysaccharides. We determined its cell-killing kinetic and its mode of action. We examined its classical Michaelis-Menten enzyme kinetics profile and evaluated the effect of several substances on its activity.

CHAPTER II

MATERIALS AND METHODS

2. 1. MATERIALS

2.1.1. Yeast Strains

Yeast strains were purchased from the National Collection of Yeast Cultures, Norwich, U.K. *Pichia anomala* (NCYC 434, K5) was used as the source of the K5 type killer toxin. *Saccharomyces cerevisiae* (NCYC 1006) was used as the killer toxin sensitive strain.

2.1.2. Culture Media

For routine growth, YEPD medium consisting of 1 % Bacto-yeast extract, 2 % Bacto-peptone and 2 % dextrose together with 2 % Bacto-agar at pH 5.5 was used. YEPD broth medium supplemented 5 % (v/v) glycerol buffered with citrate phosphate to pH 4.5 was used for killer toxin production. Killer activity assay was performed by using YEPD medium together with 2 % Bacto-agar buffered with citrate phosphate to pH 4.5. For polysaccharide binding, cell killing kinetic and measurement of effect of carbon source on killer protein production assays cells are grown on either YNBG (minimal synthetic medium) or Sobouraud medium. YNBG consists of 1 % yeast nitrogen base without aminoacids and 2% dextrose.

Sobouraud medium contains 1 % Bacto-peptone and 2 % dextrose. Malt extract broth was consisted of 1.7 % malt extract and 0.3 % bactopeptone. Basal medium which is used for the determination of effect of carbon sources on killer toxin production was consisted of 0.1% bactopeptone, 0.03 % urea, 0.2 % KH_2PO_4, 1.4 % $(NH_4)_2SO_4$, 0.03 % $MgSO_4.7H_2O$, 0.03 % glucose and supplemented with one of the carbon sources to be tested.

2.1.3. Chemicals

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

2.1.4. Buffers

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

2.2. METHODS

2.2.1. Sterilizations

The glassware was sterilized on dry-cycle at 200^o C for two hours. The media for stock cultures and for routine growth of the yeast cells were sterilized at 15 lb/sq inc. for 20 minutes on liquid cycle. The buffers used for the HPLC analysis was filtered through 0.45µm cellulose acetate filter discs (Sartorius, AG, Germany) using filter device (Sartorius, AG, Germany) before sterilization on liquid cycle.

2.2.2. Maintenance of the Yeast Cultures

The glass ampules that contain freeze-dried yeast cultures were opened in sterile conditions and about 0.5ml of YEPD broth were added to the dried material by using sterile pasteur pipette. After the freeze dried culture was dissolved completely, it was plated on YEPD agar at pH 5.5 and incubated at 25º C until the colonies are formed (1-5 days) [75].

2.2.3. Assessment of Killer Toxin Activity (Agar Diffusion Assay)

Toxin activity was tested according to agar diffusion method of Brown et al. [76]. Samples of 50µl were spotted onto YEPD plate (pH4.5 at 22º C) which was seeded with toxin sensitive strain, *Saccharomyces cerevisiae* (NCYC 1006). The killer activity was measured by the occurrence of clear zone of growth inhibition of the seeded killer toxin sensitive strain after 48 hours incubation. Killer toxin which gave a clear zone of 10mm in diameter was defined as 1 arbitrary unit (AU).

2.2.3 Killer Toxin Production

Killer toxin production, concentration and purification steps were made as described previously [72]. Pichia anomala NCYC 434 was grown to stationary phase in 1L of YEPD medium (containing 5 % glycerol) buffered to pH 4.5 with 0.1M citrate-phosphate buffer at 20^{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 5000 rpm for 10 min. at 4^{o} C and supernatant was filtered through a 0.45µm and 0.2µm cellulose acetate membranes (Sartorius, AG, Germany), respectively.

2.2.5. Concentration of the Killer Toxin

Cell free filtrate was ultrafiltrated through 30.000 MWCO centrifugal filter device (Vivaspin VS2021, Sartorius ,AG, Germany) at 4200 x rpm for 30-60 min. at 4°C (MR23i, Jouan, France). The concentrated cell free filtrates were then subjected to buffer exchange with 30mM N-methylpiperazine-HCl by using 5000 MWCO centrifugal filter device (Vivaspin VS2021, Sartorius). Buffer exchange step was performed three times at 4200 rpm for 15 min. at 4°C (MR23i, Jouan, France). Before injection onto the HPLC column, the samples were ultrafiltrated by 0.25µm syringe filter (Sartorius, AG, Germany) [72].

2.2.6. Purification of the Killer Toxin

The purification steps were done on a fully automated HPLC system (BioCAD 700E Perfusion Chromatography Workstation, Perseptive Biosystems Inc., USA) which included an automatic fraction collector (SF-2120 Super Fraction Collector, Advantec MFS, Japan). The buffers for the HPLC system were degassed with Helium gas before they were used. Detections were done with UV absorbance at 280 nm at an ambient temperature of 20 °C [72].

2.2.7. a. Anion Exchange Chromotography

Concentrated protein sample obtained from buffer exchange step was applied to an anion-exchange column (POROS HQ/M 4.6 mmD / 100mmL, Perseptive Biosystems,USA). The column was washed to twenty column volumes (CV) with 30mM N-methylpiperazine-HCl (pH 4.8). 750µL of sample (71 mg / ml) injected to the column and elution was done with a linear gradient of 0 to 500mM NaCl in the same buffer in 20 CV at a flow rate of 10 ml / min. The

fraction that corresponds to 120mM NaCl concentration was pooled. This pooled eluate containing active K5 type killer protein was then buffer exchanged to 100mM Na_2HPO_{4-} citric acid buffer, pH 4.5 by using 5000 MWCO centrifugal filter device (Vivaspin VS2021, Sartorius).

2.2.7. b. Gel-Permeation Chromatography

The sample obtained from the anion exchange chromatography step then subjected to a gel permeation chromatography column TSK G2000 SW (7,5 mmD/300mmL TosoHaas, Japan). The column was equilibrated with the eluent 100mM Na_2HPO_{4-} citric acid buffer , pH 4.5 ,containing 0.1M Na_2SO_4 , at a flow rate of 1 ml/min. The equilibration was performed until the baseline was stable [70]. 90µl of sample was injected. Elution was performed with the same buffer and the same flow rate. Then eluted fractions were tested for killer activity by applying 50µl on YEPD agar plates (pH 4.5) seeded with $1x10^5$ cells of the killer toxin sensitive strain *S. cerevisiae* NCYC 1006. After two days of incubation at 22^oC, killer inhibition zone was detected. Active eluate, which is composed of the fraction at 8.5 ml, pooled and was buffer exchanged with same buffer that contains 20 % glycerol (v /v) using 5000 MWCO centrifugal filter device.

2.2.8. Denaturing Gradient SDS-Polyacrylamide Gel Electrophoresis

The active fraction obtained from gel permeation chromatography was subjected to 5 %-20 % linear gradient SDS-PAGE gel in a discontinuous buffer system under denaturing conditions [77]. Gel electrophoresis was performed by using dual vertical slab gel electrophoresis unit SE600 (Hoefer, USA). 5 %-20 % linear gradient gel was prepared by using a gradient maker (Hoefer, USA). Gel was poured in a way that cross-linking was high at the bottom of the gels and it

gradually decreased towards the top. Separating and stacking gel compositions are given in Table 2.1 and Table 2.2 respectively [77].

After pouring the gel into the glass plate sandwiches, it was overlayed with 100µl water saturated n-butanol and left for one hour for polymerization. After the polymerization completed, n-butanol was poured off and the surface of the gel was rinsed with stacking gel buffer. Then, the stacking gel solution was poured onto the separating gel and a 1.5 mm thick 15 wells comb was inserted into the sandwich.

In denaturing SDS-PAGE method, treatment buffer contained 10 % β-mercaptoethanol, 0.125M Tris-Cl, 4 %SDS, 20 %Glycerol, 0.02 % bromophenol blue to 10ml dH_2O, pH 6.8. Samples and molecular weight markers were combined with equal volume of treatment buffer and were heated at 100^o C for 4 minutes. After the completion of polymerization of gel, sample and molecular weight markers were loaded onto the gel. Electrophoresis was performed at 15^o C using circulating cooling water bath (Heto Holten, Denmark) with power supply settings of 30mA (Power supply PP4000 ,Biometra, Germany) until the dye front reached the bottom. The gels were visualized by silver staining [78].

Molecular weight markers used were α_2-macroglobulin (170.000 kDa) , β-galactosidase (116.353 kDa) , fructose-6-phosphate kinase (85.204 kDa) , glutamate dehydrogenase (55.562 kDa) , aldolase (39.212 kDa) , triose phosphate isomerase (26.626 kDa) , trypsin-inhibitor (20.100 kDa) , (Roche Diagnostics).

Table 2.1. Separating Gel Mixtures.

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

Table 2.2. 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 ml
10% Ammonium persulfate*	50 µl
TEMED*	5 µl

*Ammonium Persulfate and TEMED were added after deaeration.

2.2.9. Protein Detection in Gels: Silver Staining

Silver staining was performed as previously described [93]. The gel was placed in destain I for 1 hour with gentle shaking. Destain I was replaced with

destain II and the gel was shaked slowly for 30 minutes. Destain II was discarded and cross-linking solution was added and again shaked slowly for 30 minutes. Cross-linking solution was poured off and 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 solution with slow shaking for 30 minutes. DTT (dithiothreitol) solution was removed and silver nitrate solution was added and again gel was shaken slowly for 30 minutes. The gel was washed with distilled water for two or three times and 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.

2.2.10. Determination of protein concentration

Protein concentration was estimated by the method of Bradford. [79] Bradford assay reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in the solution of 50 ml 95 % ethanol and 100 ml 85 % (w/v) phosphoric acid. Volume of reagent was completed to 1 L with distilled water and filtered through Whatman #1 paper just before use.

A series of concentration of bovine serum albumin including 10 to 100 µg / ml solutions were prepared as standard. A series of sample were prepared and marked as test tubes by diluting different amount of stock solution in 100mM Na_2HPO_4- citric acid buffer and total volume of the test series were also completed to 1ml. For each sample and standard tube 5 ml of Bradford reagent was added and incubated for 5 minute. After incubation absorbances were measured at 590nm by using UV visible spectrophotometer (model 1208, Shimadzu, Japan). A standard curve of absorbance versus µg protein was drawn and protein concentration was calculated.

2.2.11 Binding of Killer Toxin to Different Cell Wall Polysaccharides

Laminarin with β-(1-3) main glucosidic linkage type ; pustulan with β-(1-6) main glucosidic linkage type; β-Glucan with both β-(1-3) and β-(1-6) glucosidic linkage type; chitin with β-(1-4) main glucosidic linkage type; pullulan with α-(1-4) and α-(1-6) main linkage types; mannan with α-(1-6), α-(1-2) , and α-(1-3) mannosidic linkages were tested in terms of their binding abilities towards the killer toxin.

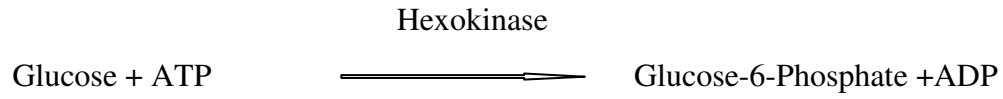
Saccharomyces cerevisiae cells which were grown on Sobouraud medium (1.10^5cells/ml) were mixed with the solutions of purified killer toxin (16µg/ml) and polysaccharide solution 9 mg/ml at a total volume of 300µL in 100mM Na_2HPO_4 –citric acid buffer. Incubations were made at 25º C at 120 rpm shaking for 24 hours. After the incubation the amounts of the killer activity remaining in the mixtures were assayed. Solutions were poured onto agar plates and plates were incubated for 48 hours at 30º C and colonies which formed after incubation were counted. As control, a cell solution which contain neither toxin nor polysaccharide were prepared. Another cell solution which was added the same amount of killer but not polysaccharides were also incubated in the same conditions for final comparison.

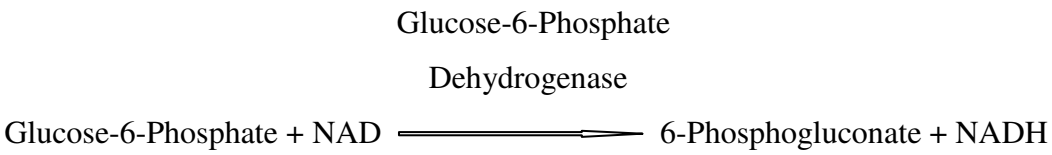
2.2.12 Exo-β-1,3-Glucanase activity assays

Exo-β-1,3-Glucanase activity of the K5 type yeast killer protein was analyzed by measuring the amount of glucose released from Laminarin as described by Bara et.al. [80]. Standard assays were done by adding 16 mg/ml pure K5 protein into 0.25 ml sodium acetate buffer containing 0.25 mg/ml Laminarin. Incubations were done at 25º C for 10-30 min. The reaction was stopped by boiling the tubes for 10 minute. Glucose formation in the samples was measured

by Glucose - Oxidase method which was done with Glucose (HK) Assay Kit (Sigma, USA) [81].

Glucose assay kit is used for the quantitative enzymatic detection of glucose in solutions. The kit ,which contains 1.5 mM NAD, 1.0 mM ATP, 1.0 unit/ml of hexokinase, and 1.0 unit/ml of glucose-6-phosphate dehydrogenase with sodium benzoate and potassium sorbate as preservatives in 20ml distilled water, utilizes two enzyme, hexokinase and glucose-6- phosphate dehydrogenase [82].





Glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Glucose-6-phosphate is then oxidized to 6-phosphogluconate in the presence of nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase. During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance is directly proportional to glucose concentration [82].

Table 2.3.Solutions Prepared for Glucose Oxidase Method.

Tube	Glucose Assay Reagent Volume	Sample Volume	Volume of Distilled water
Sample Blank	-	100µl	200µl
Reagent Blank	200µl	-	100µl
Test	200µl	100µl	-

The solutions given in Table 2.3 were pipetted to marked tubes and tubes were mixed and incubated for 15 min at 30ºC. After incubation tubes aseptically decanted into 96 well micro-titre plate. Absorbance of each tube were read at 340nm on an automatic micro-titre plate reader (Spectramax 190, Molecular Devices, USA) and using the following formula the glucose amounts in the solutions were calculated [82].

A Total Blank = A Sample Blank + A Reagent Blank

ΔA = A Test – A Total Blank

$$\text{mg glucose/ml} = \frac{(\Delta A)\,(TV)\,(\text{Glucose Molecular Weight})\,(F)}{(\varepsilon)(d)(SV)(\text{Conversion Factor for } \mu g \text{ to mg})}$$

$$\text{mg glucose/ml} = \frac{(\Delta A)\,(TV)\,(180.2)\,(F)}{(6.22)\,(1)\,(SV)\,(1000)}$$

$$\text{mg glucose/ml} = \frac{(\Delta A)\,(TV)\,(F)\,(0.029)}{(SV)}$$

A= absorbance measured at 340nm

TV = Total Assay Volume (ml)

SV = Sample Volume (ml)

Glucose MW = 180.2 g/mole or equivalently

180.2 µg/µmoles

F = Dilution Factor from Sample Preparation

ε = Millimolar Extinction Coefficient for NADH at 340 nm

Millimolar -1 cm –1 or equivalently (ml/µmoles) (1/cm)

d = Light path (cm) = 1 cm

1000 = Conversion Factor for µg to mg

2.2.13. Enzyme Kinetic Analysis

Initial velocities of the enzyme–catalyzed hydrolysis reaction were calculated by mixing equal amount of K5 type killer protein with varying concentrations of substrate, [S], Laminarin; between 0.16 to 17.2 mg/ml. Reactions were done in 0.25 ml 20 mM sodium acetate buffer, pH 4.5 for 30 minutes at 25ºC. After stopping the reaction by boiling for 10 minute, liberated reducing sugar, glucose amounts were calculated by Glucose-Oxidase method [81]. One unit of enzyme was defined as the amount of enzyme which liberates 1µ mol of reducing sugar equivalent to glucose per minute under standard assay conditions.

The kinetic parameters, Michaelis-Menten constants K_m and V_{max} were determined by application of the data which are initial substrate concentration and initial velocities measured for these substrate concentrations to non-linear-regression. Enzyme kinetic graphs and calculations made by using the enzyme kinetics module of Sigma Plot 9.0 software (Systat Software, Inc.,USA) on a personal computer.

2.2.14. Effects of Various Compounds on the Enzymatic Activity

Effects of chloride salts of several metals, DTT and EDTA on exo-β-(1-3) glucanase activity of K5 type killer protein determined in 20 mM sodium acetate buffer, pH 4.5, containing 2,56 mg/ml Laminarin at 25ºC in the presence of 2 mM concentration of compounds to be tested. Reactions were stopped after 30 minutes of incubation by boiling for 10 minutes. Then produced glucose amounts were determined by glucose-oxidase method [81]. Ca^{+2}, Ba^{+2}, Cd^{+2}, Cu^{+2}, Cr^{+2}, Mg^{+2}, Mn^{+2}, Li^{+}, Pb^{+2}, Fe^{+2} ,Ni^{+2}, Zn^{+2}, Hg^{+2}, Cs^{+} were tested by adding their water soluble salts.

2.2.15. Effect of Different Carbon Sources on Production of K5 Type Yeast Killer Protein

Killer producer, *Pichia anomala* NCYC 434 cells, was grown on YNBG medium (100 ml) for 16 hours at 30ºC. Preculture was precipitated by centrifugation at 4200 rpm and resolved in distilled water twice for the removal of medium itself. Then 1ml of this washed preculture were seeded by pipetting it into basal medium, pH 4.5, containing one of the carbon sources mentioned below. Incubations were made in 250ml erlenmayer flasks with total volume of 100 ml medium supplemented with glycerol, for 36 hours at 20º C at 120 rpm. Basal medium contains 0.1% bactopeptone, 0.03% urea, 0.2% KH_2PO_4, 1.4% $(NH_4)_2SO_4$, 0.03% $MgSO_4.7H_2O$, 0.03% glucose.

0,5% (v/v)Rye, 0,5% (v/v) barley, 0,5% (v/v) wheat bran, 0,5% (v/v) cellulose, 1% (v/v) glucose, 2% (v/v)glucose, 0,5% (v/v) β-glucan, 2% (v/v) mellebiose, 2% (v/v) melezitose, 2% (v/v) galactose were used as carbon sources in basal medium to be tested. Also Sobouraud broth, YEPD, malt extract broth and YEP broth were used for determination of their effects on killer protein production in the same conditions.

After incubation cells were removed by centrifugation at 4200rpm at 4ºC and filtered through 0.45 and 0.20 µm filters. These cell free culture filtrates were precipitated with acetone by incubating with %80 acetone at -20ºC for 3 hours. Then this acetone precipitated filtrate was recovered by centrifugation at 15000xg at 4ºC for 15 minutes. The supernatants were discarded and pellets were vacuum dried (Speedvac Evaporator RC10, Jouan, France) and resuspended in 100mM Na_2HPO_4 - citric acid buffer at pH 4.5 and 20µl of the samples were tested to evaluate killer protein production by exo-β-1,3 glucanase activity assay and agar diffusion assay.

2.2.16. Cell Killing Kinetics of K5 Yeast Killer Toxin

The time course of cell killing action of K5 type killer protein was determined by quantitative comparison of viable cell number of normal culture and toxin treated culture of *S. cerevisiae* strain. Cells ($1x10^5$cells/ml) were seeded into flask (250 ml) containing Sobouraud liquid medium, pH 4.5 and 5 ml of this culture pipetted into a tube and were mixed with killer toxin (16µg/ml). Another tube containing 5ml of culture but not toxin was also prepared for control. Incubation was done at 30 ° C at 120 rpm shaking for 24 hours. Every 30 minutes, 200µl of these cultures was aseptically taken and pelleted by centrifugation at 4200 rpm at 4º C and resuspended in 100mM citrate-phosphate buffer at pH 6.8 in order to inactivate any residual toxin. Then this cell solutions were poured and spread onto YEPD agar plates and plates incubated at 30º C for 2-4 days until colonies appeared and colonies were counted to determine the cell viability.

The toxin activity in hyper-osmotic medium was tested under the same experimental conditions as described above with the addition of 1 M sorbitol (Sigma) to the assay medium and resuspension buffer. Control tubes which lack toxin but contain 1M sorbitol were also prepared. After 7 h of incubation the resuspended cells were mixed with 5 ml of molten (45 °C) YEPD agar containing 1M sorbitol then poured onto the same agar plates. The plates were incubated for 2-4 days until the colonies appeared then colonies were counted.

CHAPTER III

RESULTS

3.1. Production and Purification of K5 Type Yeast Killer Protein

Toxin production by killer yeasts with a higher degree of activity depends on pH and temperature of the culture. The maximum production of K5 type yeast killer toxin is at pH 4.5 and 20-22ºC. Glycerol is added as toxin stabilizer [72]. Cell free liquid was concentrated by ultrafiltration and enriched for killer toxin by 7.2 fold. Then at pH 4.8 and at about concentration of 120mM NaCl on a POROS HQ/ M anion exchange column killer toxin was purified 280-fold. Killertoxin containing fraction obtained from anion exchange chromatography was then put through gel filtration chromatography using a TSK G 2000SW column and the fraction at 8.5 ml showed killer toxin activity [72].

Finally 400 fold purification was achieved [86]. The concentration of the purified protein was estimated as 16 µg/ml. Elution profiles of K5 type yeast killer protein on POROS HQ/ M column and TSK G2000SW column are shown in Figure 3.1 and 3.2 where fractions showing killer activity are indicated by arrows.

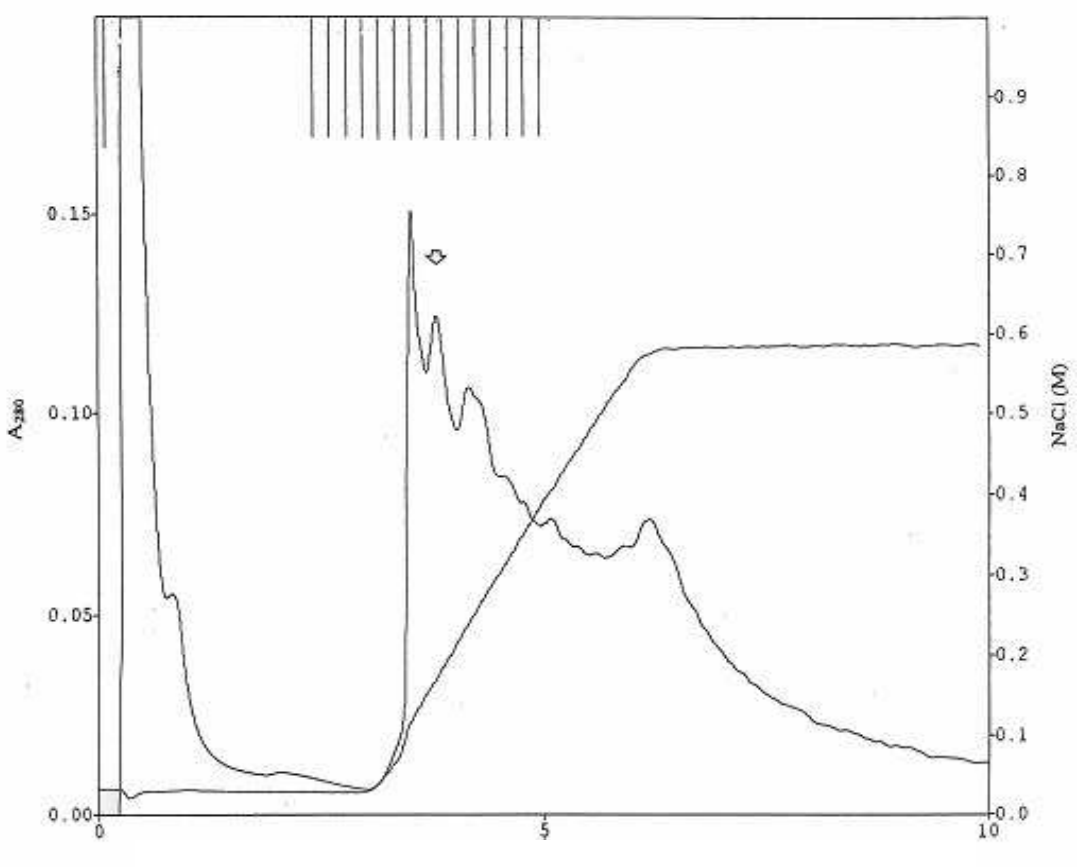


Figure 3.1. Elution Profile of K5 Type Yeast Killer

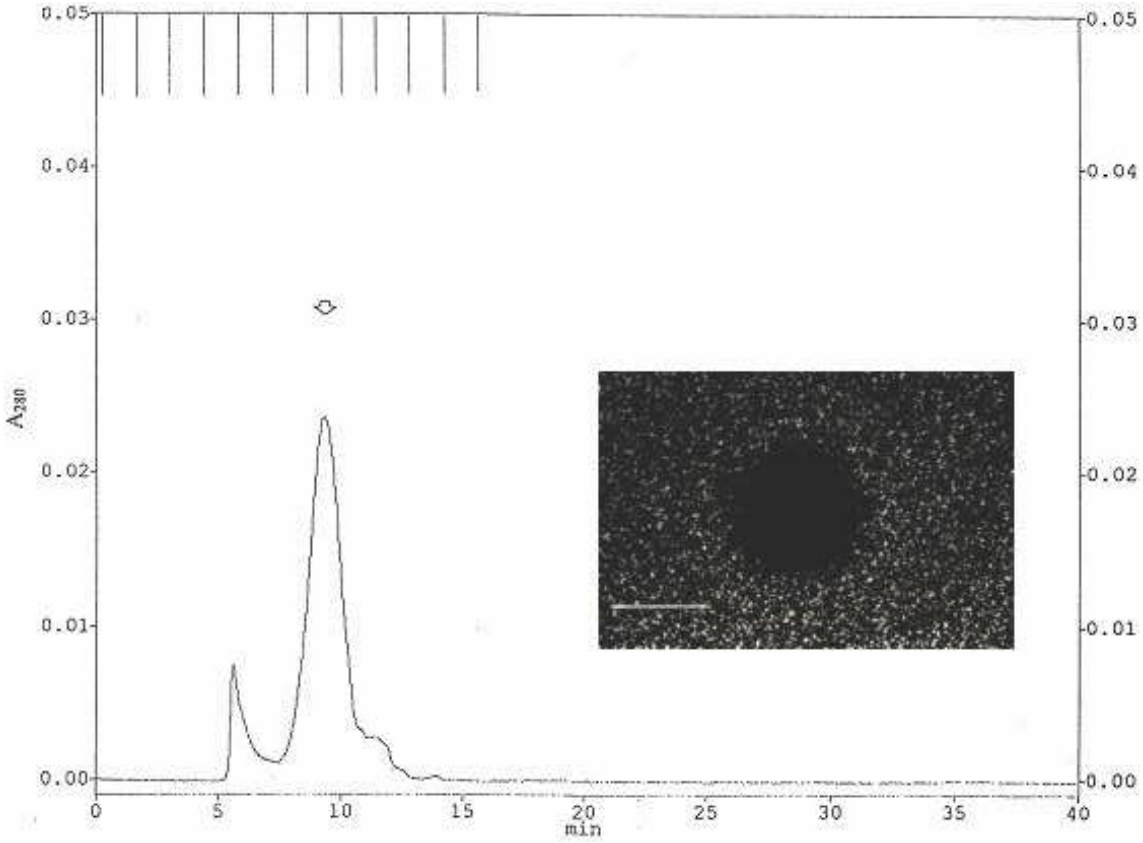


Figure 3.2. Elution Profile of K5 Toxin on a TSK G2000SW Column.
(50µl of the active fraction obtained from gel filtration chromatography gave clear zone of ~7 mm in agar diffusion assay which corresponds to 0.7 AU killer toxin (bar=5mm).)

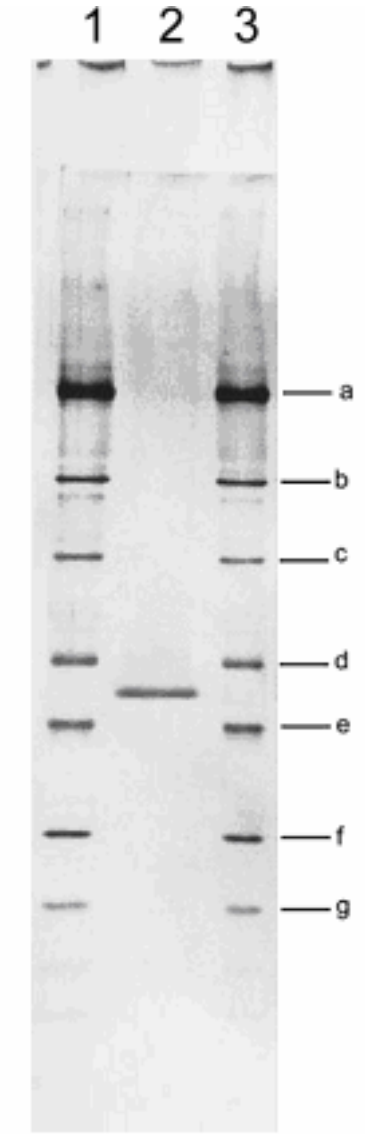


Figure 3.3. Silver Stained Denaturing Gradient SDS-Polyacrylamide Gel of K5 Type Yeast Killer Protein. Lane 2 is purified K5 type yeast killer protein and lane 1 and 3 are molecular weight markers ; a) α_2-macroglobulin (170.000 kDa), b) β-galactosidase (116.353 kDa), c) fructose-6-phosphate kinase (85.204 kDa) , d) glutamate dehydrogenase (55.562 kDa) , e) aldolase (39.212 kDa) , f) triose phosphate isomerase (26.626 kDa) , g) trypsin-inhibitor (20.100 kDa).

The killer toxin was homogeneous on a discontinuous gradient SDS-PAGE with a wide range of molecular mass marker, the toxin moved in a single band in front of glutamate dehydrogenase. The molecular mass of the toxin was 49,000 Da [72]. Discontinuous gradient SDS-PAGE profile of the K5 type yeast killer toxin is shown in Figure 3.3.

3.2. Binding of the Killer Toxin to Different Cell-Wall Polysaccharides

We have investigated the interaction of the K5 type yeast killer protein with cell wall components via competitive inhibition of the killing activity of the toxin by certain cell wall polysaccharides. The sensitive cell culture was grown on Sobouraud broth due the absence of β-Glucan and other possible cell wall components in this medium which would interact with killer toxin. The toxin sensitive cells ($1x10^5$cells/ml) seeded and incubated without killer toxin grew up to total of $1.2x10^7$ cells. When same culture was supplemented with 16 µg/ml purified killer toxin less then 50 cells could survive. Six independent series of same culture which contain killer toxin were prepared for the determination of effect of cell wall polysaccharides. These were mixed with each cell wall polysaccharides respectively. After 24 hour incubation at 24º C plated on to YEPD agar plates then colonies formed after 48 hours incubation were counted.

Among the tested cell wall polysaccharides pullulan and mannan had no reducing effect on the killing activity of the killer toxin. Both the control and sample tubes showed the same killing activity and less then 60 survivals remained on the agar plate. Other polysaccharides chitin and pustulan showed reducing effect on the killing ability of K5 type yeast killer protein but the inhibitory effect of these polysaccharides were weak in comparison to laminarin and β-Glucan which completely inhibited the killing action of killer toxin. Laminarin allowed the cells to reach up to 1.0×10^7 and β-Glucan inhibited the killer protein activity strongly and cells reached up to 1.1×10^7. These results showed that the K5 type

yeast killer protein interact with cell wall β-Glucan. Results of the binding experiment are given in the Table 3.1. Values represent the mean of three separate experiments.

Table 3.1. Competitive Inhibition of the Killer Toxin with Different cell Wall Polysaccharides

S.cerevisiae cells	POLYSACCHARIDE	Killer Toxin	Viable *S.cerevisiae cells* (x 10^2 cfu/ml)
$1x10^5$	**Control**(no polysaccharide)	No toxin	120000
$1x10^5$	**Control**(no polysaccharide)	16 µg/ml	0.48
$1x10^5$	**Pullulan** 9mg/ml	16 µg/ml	0.56
$1x10^5$	**Mannan** 9mg/ml	16 µg/ml	0.48
$1x10^5$	**Pustulan** 9mg/ml	16 µg/ml	27000
$1x10^5$	**Chitin** 9mg/ml	16 µg/ml	50000
$1x10^5$	**Laminarin** 9mg/ml	16 µg/ml	100000
$1x10^5$	**β-Glucan** 9mg/ml	16 µg/ml	110000

3.3. Exo-β-(1-3) Glucanase Activity Assays and Enzyme Kinetic Analysis

In order to define the enzymatic properties of the K5 type yeast killer toxin we evaluated β-glucanase activity of the toxin by analyzing toxin catalyzed laminarin hydrolysis reaction. Purified killer toxin (16 µg/ml) was incubated with Laminarin (0.25mg/ml) in Na_2HPO_4 – citric acid buffer for 30 minutes at 25º C. After the incubation, reaction was stopped by boiling for 10 minutes. The amount of reducing sugar, glucose, released from laminarin was calculated by glucose-oxidase method [81]. The purified killer toxin hydrolyzed laminarin and had a specific activity of 120 U/mg. According to these results K5 type yeast killer protein has hydrolytic activity on the cell wall β-(1-3) glucans.

The Michaelis-Menten constants K_m and V_{max} were determined by applying the initial substrate concentrations and initial laminarin hydrolysis reaction velocities, calculated by glucose–oxidase method, for these different substrate concentrations of, [S], laminarin.

Substrate concentrations were between 0.64 – 10.24 mg/ml and 30µL of purified killer toxin were added to the reaction mixtures. Total reaction volume was 0,25 ml and incubation time was 30 minutes. After incubation reaction was stopped by boiling, amount of glucose released from Laminarin determined by glucose-oxidase method. List of the data obtained from β-Glucanase assay and used for calculation of Michaelis-Menten constants, K_m and V_{max}, was shown in Table 3.2. All the data were the mean values of three separate experiments.

Table 3.2. List of data Used for The Calculation of Kinetic Parameters.

[Laminarin] ***mg/ml***	**Initial Velocity** ***µmol glucose/min/mg***
0.64	240.9230
1.28	312.9580
2.56	344.6350
5.12	348.6540
10.24	351.6020

Enzyme kinetic graphs and calculations made by using the equation below on enzyme kinetics module of Sigma Plot 9.0 software (Systat Software, Inc.,USA) on a personal computer. In this equation X represents the substrate concentration and expressed generally in µM or mM but here we expressed it as mg/ml due to the insufficient knowledge about the molecular mass of Laminarin. Y represents the initial enzyme velocity and expressed in units of product concentration produced per time and we expressed it by normalizing it to enzyme concentration so it was expressed as concentration of glucose released from Laminarin (µM) per time for per mg killer toxin.

Equation: Michaelis-Menten

$$Y=V_{max}*X/ (K_m+X)$$

The equation above describes an enzyme-catalyzed reaction obeying classical, or Michaelis-Menten, kinetics. V_{max} is the maximal velocity at saturation. K_m, or the Michaelis constant, is the substrate concentration required to

reach half-maximal velocity ($V_{max}/2$). Figure 3.4 displays the Michaelis-Menten plot of the β- glucanase reaction of K5 type yeast killer toxin.

We analyzed the data via non-linear regression calculated by Sigma Plot 9.0 software and they are given in Table 3.3, but for representation of data we have used Lineweaver-Burk plot which uses inverse of the Michaelis-Menten equation and produces less accurate K_m and V_{max} values calculated by linear regression. Lineweaver-Burk representation of data is given in the Figure 3.5.

Table 3.3. Michaelis–Menten Constants and Statistical Values

	Value	±Std. Error	95% Confidence Interval
V_{max}	372.2µmol/min/mg	10.81	337.86 to 406.66
K_m	0.3 mg/ml	0.05	0.1 to 0.49

Goodness of Fit	
Degrees of Freedom	3
R^2	0.99
Sum of Squares	525.95
Runs Test p Value	0.33

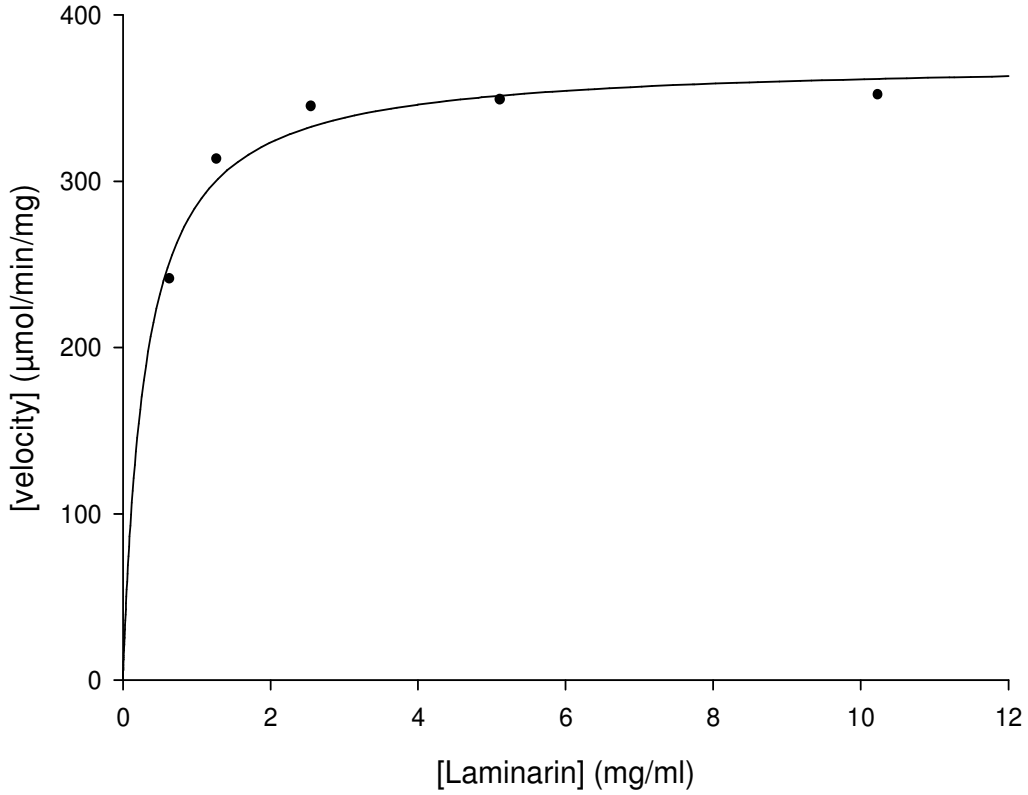


Figure 3.4.Michaelis-Menten Plot of The Exo-β-1,3-Glucanase Activity of K5 Type Killer Toxin.

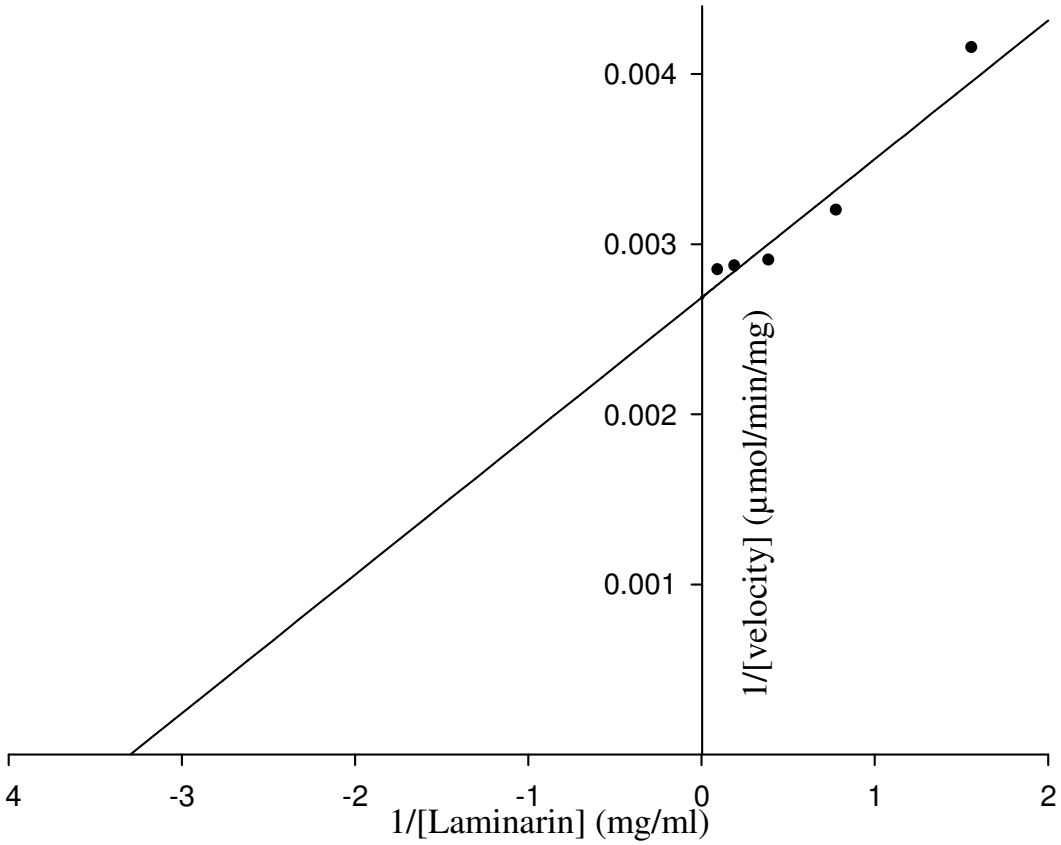


Figure 3.5. Lineweaver-Burk Plot of the Exo-β-1,3-Glucanase Activity of K5 Type Killer Toxin.

3.4. Effect of Different Carbon Sources on Production of K5 Type Yeast Killer Protein

β-Glucanase production in yeast is dependent on the carbon source available in media. Especially the presence of cell wall polysaccharides in culture medium strongly influences β-Glucanase production level of the cells [83, 84]. Therefore to further elucidate the nature of K5 type yeast killer protein, we have investigated the effect of different carbon sources on the toxin production by *P.anomala* cells.

The cells grew on all tested media consisted one of the carbon sources and basal medium. After grown of cells to stationary phase cells were removed and extracellular protein contents of the media were precipitated by acetone and recovered by centrifugation and vacuum-drying. Pellets were resuspended in Na2HPO4- citric acid buffer and β-Glucanase activities of each solution were determined via Glucose-Oxidase method.

The basal medium supplemented with sugars which does not contain any glucan source, such as galactose, % 1 glucose, % 2 glucose, % 2 mellebiose, % 2 melezitose did not induce production of killer toxin. There were not any killer toxin activity in cell free culture filtrates of these media in the agar diffusion assay and there were not any laminarin hydrolysis product in β-1,3-glucanase activity assay.

Carbon sources containing different rates and types of cell wall glucans showed different induction rate of killer toxin production which were parallel with their β-1,3-glucan content. β-Glucan and Barley showed relatively high killer toxin production than wheat, rye and cellulose.

The higher killer toxin production were displayed in the Malt extract broth and YEPD broth while Sobouraud broth did not induce any β-1,3-glucanase

production due to its deficiency of cell wall polysaccharides. The highest production achieved when the cells were grown in YEPD broth which is rich in β-1, 3-glucan which comes from its yeast extract content.

In order to examine the effect of glucose on the production of killer toxin also YEP broth was tested. In this medium killer protein production was quite low in compare to YEPD broth. This result suggests that glucose stimulates for the production of exo-β-1,3-glucanase when β-1,3-glucans are available in the media.

The results of killer activity determinations made by agar diffusion assay were parallel with the data obtained from exo-β-1,3-glucanase assay and they were both represented in Figure 3.6.

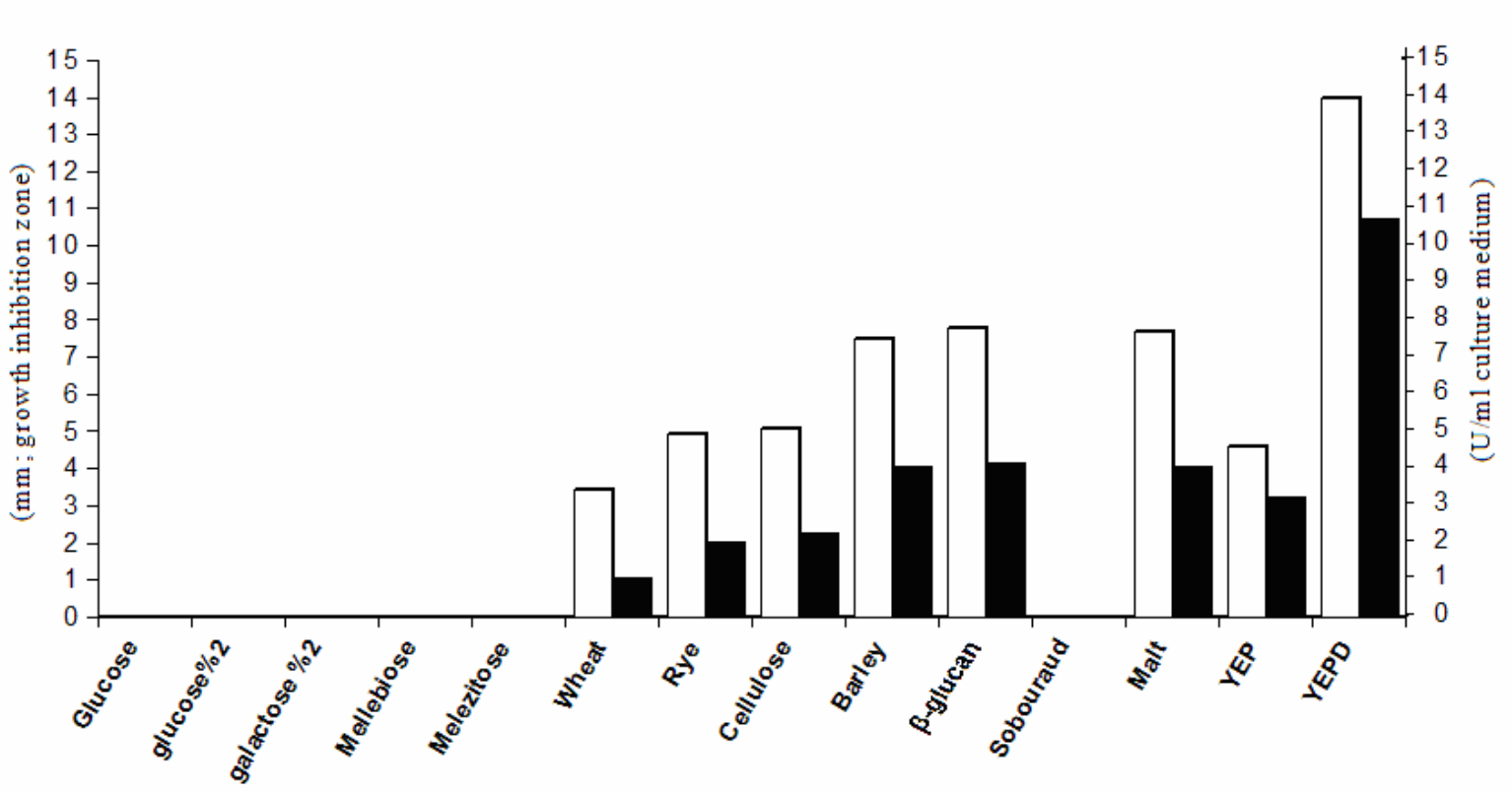


Figure3.6. Effect of Different Carbon Sources on Production of K5 Type Yeast Killer Protein

□ Determined by Agar Diffusion Assay Killer activity on sensitive S.cerevisiae cells (mm ; growth inhibition zone)

■ Determined by Exo-β-1,3-Glucanase Assay Exo-β-1,3-Glucanase Activity (U/ml culture medium)

3.5. Effects of Various Compounds on the Enzymatic Activity

Effect of several metal ions and other compounds such as EDTA, DTT were tested by comparing the velocity of test reactions containing one of the compounds tested with normal reaction.

Hg^{+2} completely inhibited the enzyme activity but all other metals tested increased the exo-β-1,3-glucanase activity of the K5 type yeast killer protein towards Laminarin. Rate of increase was variable in the range of approximately 14-63 %. The highest increase was in the reaction tube containing Pb^{+2} (62.6 %).

While EDTA did not have a measurable effect on the activity of killer toxin on Laminarin, DTT caused 15 % increase in the exo-β-1,3-glucanase activity of the killer toxin.

Reaction velocities of each of the reaction tube, containing additionally one of the chloride salts of metal ions or compounds listed in Table 3.4, were determined by glucose-oxidase method. All of the compound tested had 2mM concentration in the reaction volume.

Table 3.4. Effect of Various Compounds on β-Glucanase Activity of K5 Type Yeast Killer Toxin.

Compounds tested [2mM]	Reaction velocity (µmol [Glucose])	Relative Activity %
Ca^{+2}	0.5488	114.47
Ba^{+}	0.5901	123.08
Cd^{+2}	0.6418	133.87
Cu^{+2}	0.5847	121.96
Cr^{+2}	0.5703	118.94
Mg^{+2}	0.5716	119.22
Mn^{+2}	0.6051	126.21
Li^{+}	0.6094	127.11
Pb^{+2}	0.7796	162.60
Fe^{+2}	0.6308	131.58
Ni^{+2}	0.6062	126.30
Zn^{+2}	0.5625	117.20
Hg^{+2}	0	0
DTT	0.5531	115.7
EDTA	0.5727	100
Cs^{+}	0.5670	118.27
normal assay (Without any compound)	0.47945	100.00

3.6. Cell Killing Kinetic of the K5 Type Yeast Killer Protein

The sensitive *S. cerevisiae* cells (10^5cells/ml) grown on Sobouraud broth were treated with purified toxin (16 µg/ml) and viability of cells was determined every 30 minutes of the 24 hours incubation.

There were no significant viability change between toxin treated and normal cell cultures in the first 2 hours of incubation. According to the viable cell count data about 30% of the cells were killed after 2.5 hours incubation. Killing process continued increasingly by time and entire population died after 7 hours. Time dependent cell killing process of K5 type yeast killer protein is given in Figure 3.4. The data is presented as the average of three-independent experiments.

In contrast, the toxin showed no killer activity in the culture medium supplemented with 1 M sorbitol. There was no significant reduction in the cell viability of the sorbitol supplemented culture in compare to control culture which was supplemented with sorbitol in the absence of killer toxin. They both reached up to approximately $3.3x10^5$ cells.

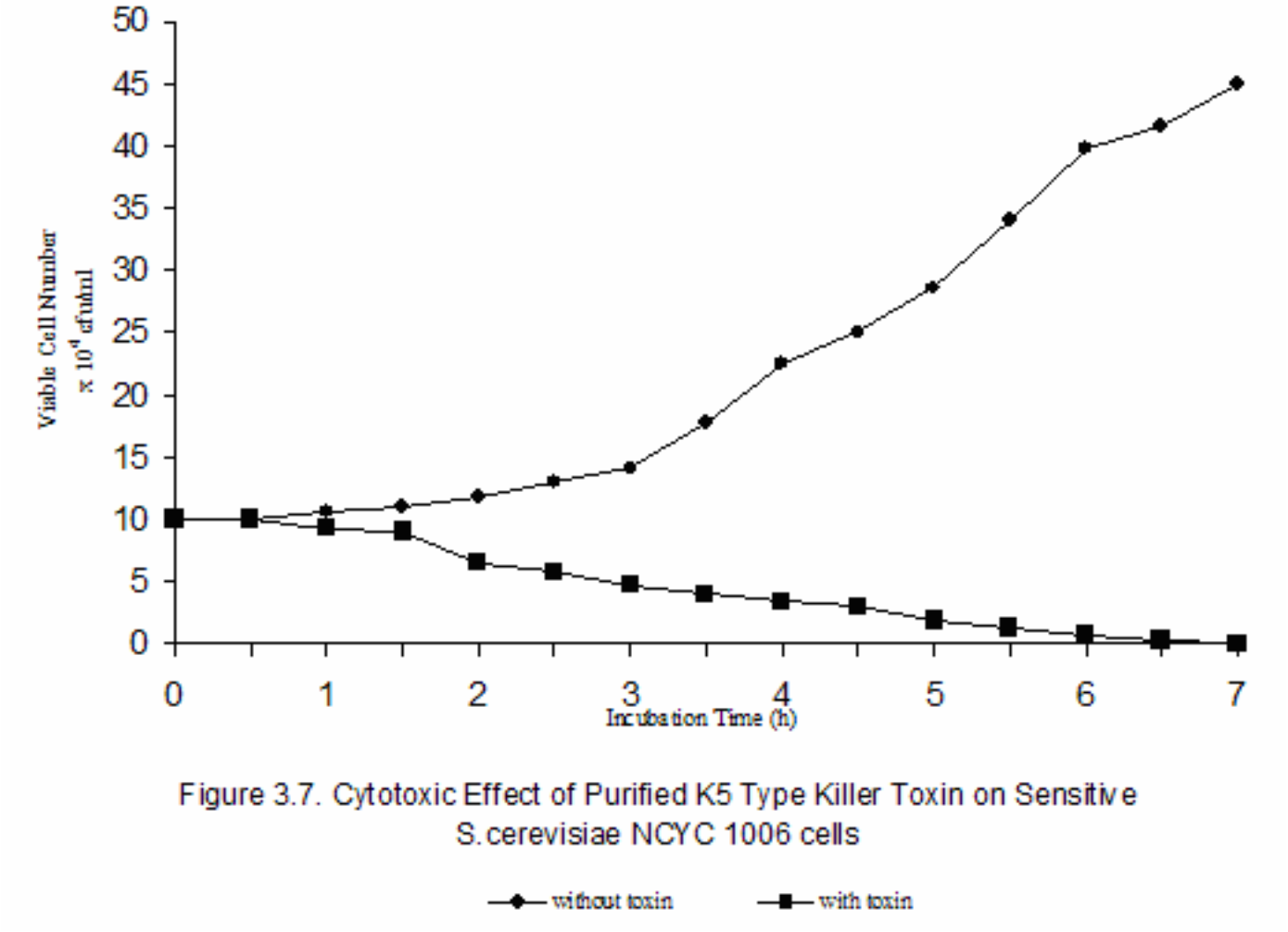


Figure 3.7. Cytotoxic Effect of Purified K5 Type Killer Toxin on Sensitive S.cerevisiae NCYC 1006 cells

CHAPTER IV

DISCUSSION

Since the first description by Bevan and Makower in *S. cerevisiae* different types of killer yeasts have been observed in several yeast genera and species. Killer yeasts secrete compounds in protein structure which are known as killer toxins to compete with other microorganisms in the medium [80, 85]. Some of these toxins have been found to be inhibitory to Gram (+) bacteria [7]. Having many application areas killer phenomenon of yeasts attracted many researchers. Although there is considerable amount of information about nature of killer toxins only a limited number of killer toxins were described with respect to cell wall binding site and mechanism of action. In this work our aim was to explain the nature of cytocidal effect and enzymatic properties of K5 type yeast killer protein which was previously isolated and purified in our laboratory [72].

Killer toxins generally have a drawback related with their temperature and pH dependency. Killer toxins generally stable at pH 4-5 and 20–25°C which affect

its killer activity. K5 type yeast killer toxin has been found to be highly stable at pH values between 3 and 5.5 and temperatures up to 37°C suggesting its possible use as an antimicrobial agent in medical technology, industrial and environmental biotechnology [72].

K1 and K2 type yeast killer protein binds to sensitive cells via cell wall β-1,6-D-glucans, K28 type yeast killer toxin uses mannoprotein layer of sensitive cell wall to penetrate and exert its killer activity, chitin is a receptor for *Kluyveromyces lactis* killer toxin. Recently β-1,6-D-glucans are also found to be primary receptor also for *Pichia membranifaciens* and *Debaryomyces hansenii killer* toxins. Receptor of killer toxin is an important parameter on activity of killer toxin and its antimicrobial spectrum [47, 72].

Therefore, we have started to investigate K5 type yeast killer protein by determination of its receptor on the cell wall of sensitive cells. In competition studies made with cell wall polysaccharides, laminarin, which is composed mainly of β-1,3-D-glucans and very few amount of β-1,6-D-glucans, most effectively adsorbed the toxin from solution and reduced the killer activity of solution allowing the cells to survive. β-Glucans which is composed of β-1,3-D-glucans and β-1,6-D-glucans were also effective in binding. Toxin bound pustulan which is composed mainly of β-1,6-D-glucans and β-1,3-D-glucans; and chitin composed mainly of β-1,4-D-glucans, less effectively. The binding of K5 type yeast killer toxin chitin and pustulan might be due to the slight affinity of β-1,3-glucanases toward chitin and pustulan which has been reported for other β-1,3-glucanases [86, 87].

The action of toxin on laminarin showed that K5 type killer protein exerts hydrolytic activity on the β-1,3-glucans in an exo like fashion because of the high amount of glucose release. All these results suggest that primary target of K5 type

yeast killer toxin is the cell wall β-1,3-glucans. Other killer yeasts characterized up to now are listed with their primary binding site on the sensitive cells in Table 4.1.

Table 4.1. Killer Toxins and Their Receptors on the Target Cell [24]

Killer toxin	Receptor
K1 Saccharomyces cerevisiae	β-1,6-D-Glucan
K2 Saccharomyces cerevisiae	β-1,6-D-Glucan
Kluyveromyces lactis	Chitin
Pichia acaciae	Chitin
HM-1 Williopsis mrakii	Cell wall β-1,6-D-Glucan
Pichia membranifaciens	β-1,6-D-Glucan
Debaryomyces hansenii	β-1,6-D-Glucan
Hanseniaspora uvarum	β-1,6-D-Glucan
KT28 Saccharomyces cerevisiae	Cell wall mannoprotein
Zygosaccharomyces bailii	Cell wall mannoprotein
*K5 Pichia anomala**	β-1,3-D-Glucan*

*from this study

Cell wall of fungal cells composed of about 85 % - 90 % polysaccharide and 10 % - 15 % protein. The main components of the yeast cell wall are β-1, 3-glucans (50 %) that also contains some 1,6-β linked branches (5 %) and mannoprotein, most of which is carbohydrate. β-1,6-D-glucan, also containing some 1,3-β linked branches (14 %), is a relatively minor constituent (15 %), and chitin (0.6 to 9 %) is present at an even lower level. In spite of differences among species, generally β-1,3-glucans are major component of the fungal cell wall and responsible for the rigidity of cell envelope [67].

Our results are consistent with the report that K5 type yeast killer protein had 100% homology with exo-β-(1-3)-glucanase of the *P.anomala* strain K [72]. In addition to this, its killing spectrum which includes *Candida albicans* with cell wall composition consisted of mainly β-1, 3-glucans confirms the exo- β-1, 3-glucanase activity of the K5 type yeast killer toxin [88].

When compared with purified and characterized exo-β-glucanases until today K5 type yeast killer toxin have a relatively low K_m value. K_m (0.25mg/ml) of the K5 type yeast killer toxin was lower than that of *Candida albicans* (3.9mg/ml), *Trichoderma harzianum* (2,1mg/ml) but slightly higher than those reported for *Trichoderma viride* (0.12 mg/ml), *Trichoderma asperellum* (0,87mg/ml) and *Trichoderma harzianum* TY (0.1 g/ml) [73, 80, 89, 90].

Having a strong exo- β-1, 3-glucanase activity indicates its possible use as cell wall degradation agent which is suggested as antimicrobial agents. Moreover its relatively high V_{max} and lower K_m values highlights its possible uses in industry that have an increasing demand of exo-β-glucanases for the enzymatic reactions which are used especially for modification of β-glucans. Recently these enzymes have been used for the production of different formulations of β-1,3-glucan drugs and supplements which are used as immune-system inducer. In animal feed production,

plant and animal chymus production it is used as viscosity reducer also in wine and beer industry exo-β-1-3- glucanases are used for taste improvement and facilitation of filtration [74].

K5 type killer toxin starts to kill sensitive target cells after 2 hours of incubation and kills entire population after 7 hours. According to these findings K5 type yeast killer toxin requires more time to kill entire population than *Zygosaccharomyces bailii* killer toxin, but it requires shorter time to kill the sensitive cells than K1 and KT 28 killer toxin of *S.cerevisiae* and *Kluyveromyces phaffi* KpKt toxins [47, 59].

The supplementation of 1 M sorbitol into the medium which is used for the treatment of cells with killer protein and agar plates used for the colony count showed no reduction in viability of sensitive cells. In other words hyper osmotic medium protects sensitive cells from killing action of K5 type killer toxin. This result indicates that K5 type yeast killer toxin disintegrates the cell wall components which causes death of sensitive cells under normal osmotic conditions. In other words this findings proves that exo-β-1, 3-glucanase activity of the toxin is responsible for the killing action.

Our findings showed that K5 type killer toxin has a different mode of action when compared to other yeast killer toxins, for instance after binding to the yeast cell wall β-1,6-glucans, K1 toxin is transferred to the cytoplasmic membrane and acts by forming voltage-independent cation transmembrane channels, which cause ion leakage and subsequent cell death. K2 killer toxin of *S.cerevisiae*, and killer toxin of *Pichia kluyveri* use same mode of action profile while the K28 toxin binds primarily to the α-1,3-linked mannose residues of a 185-kDa cell wall mannoprotein, causing cell cycle arrest, in the G2 phase, and leading to non-separation of mother and daughter cells, with the nuclear DNA confined to the mother cell [12, 14, 47]. Killer toxin of *Kluyveromyces lactis* causes a G_1 arrest and eventually loss of

viability in *S. cerevisiae* cells and *Hansenula mrakii* toxin causes pore formation by inhibiting the β-1-3-D-glucan synthesis occurring at a budding site which results in leakage of cell material and eventual cell death [22, 47, 91].

K5 type yeast killer toxin production by *P. anomala* cells was dependent on media composition especially carbon source available in the medium. According to our results K5 type toxin production level is induced in the presence of β-glucans. This increase in β-1,3-glucanase production may be for the utilization of β-1-3-D-glucan by hydrolyzing it to glucose. This finding is parallel with the reports for exo-β-glucanases of *Trichoderma harzianum* and *Trichoderma asperellum* [80, 92]. Furthermore when β-glucans are available in the medium, presence of glucose also stimulates the K5 type killer toxin production. It has been reported that production of exo-β-1,3-glucanase of *Candida oleophia* is also glucose dependent [93].

In the presence of metal ions K5 type killer toxin shows increased enzyme activity as reported for some other glucanases. Apart from Hg^{+2} all the metal ions increased β-1,3-glucanase activity of the K5 type yeast killer toxin to different rates in compare to control.

The complete inhibition by Hg^{+2} indicates that enzyme contains sulfhydryl groups that are important for exo-β-1,3-glucanase activity as reported for *Trichoderma harzianum* and *Trichoderma asperellum* [80, 90]. On the other hand, heavy metal ions stimulated enzyme to a certain extend but Pb^{+2} strongly increased the enzyme suggesting its use against plant pathogenic fungi in environments contaminated with heavy metal ions. In addition to our findings, there are similar reports about the increasing effect of these ions such as exo-β-1,3-glucanases of *T.harzianum* and *T.asperellum* and this findings may be helpful for the formulation of the toxin for possible future applications ,however, further studies may reveal exact reason of this effect [90, 92].

Slight increase in the enzymic activity was observed in the presence of DTT probably mainly because DTT protected sulfhydryl groups from oxidation and tabilized the enzyme [94]. There was no effect of EDTA which is a metal chelator suggesting that enzymic action is not dependent on a bivalent ion as cofactor [95].

The increasing demand on the new antifungals due to severe side effects and low selectivity of conventionally used antimycotics, the need of new and powerful biocontrol agents in food preservation and fermentation industry moreover exo-glucanase need of industry highlight the K5 type yeast killer toxin.

Its high activity in broad range of temperature up to 37ºC, increased activity in the presence of heavy metals, its relatively better enzymatic properties, broad range of antimicrobial activity due to its mode of action and receptor which is generally represented in a higher rate on the cell wall of pathogenic fungi and other pathogenic microorganisms but absent in higher eukaryotes, are some of the properties that make K5 type yeast killer protein potent antimicrobial agent. Further studies are needed for the determination of species specific minimum inhibition concentrations and cell killing kinetics on human pathogenic fungi, bacteria and plant pathogenic fungi which will open its use as an antimycotic agent.

CHAPTER V

CONCLUSION

1. Receptor of *P.anomala* killer toxin is found to be the cell wall β-1,3-glucans of the sensitive microbial cells.

2. K5 type yeast killer toxin exerts its lethal effect by hydrolyzing β-1,3-glucans of cell wall of sensitive microbial cells in an exo-like fashion and causes loss cell wall rigidity of sensitive microbial cells which leads to cell death due to the osmotic pressure.

3. Michaelis-Menten constants of K5 type killer toxin K_m and V_{max} were found to be 0, 3 mg/ml and 372, 3 µmol /min /mg by using Laminarin as substrate. These results suggest industrial use of K5 type yeast killer protein as an exo-β-1,3-glucanase.

4. K5 type killer toxin production is highly dependent on the composition culture medium especially carbon source. Presence of β-1,3-glucan induces production of the toxin. When β-1,3-glucans are available in the culture media glucose also stimulates the production of toxin.

5. The enzymatic activity of the K5 type yeast killer toxin depends on sulfhydryl groups. Toxin activity is not dependent on bivalent ions as cofactor but it is further stimulated by ions like Pb^{+2}, Cd^{+2}, Fe^{+2}.

6. K5 type killer toxin exerts its cytotoxic effect after 2 h contact with the sensitive *S.cerevisiae* cells and kills the entire population at the end of 7^{th} hour.

K5 type yeast killer protein, which have a strong exo-β-1,3-glucanase activity causing death of sensitive cells, may find possible uses in especially medical field due to its high selectivity given by its target ,β-1,3-glucan, which is common among lots of pathogens as major component responsible for cell wall rigidity, and lacked by higher eukaryotes.

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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)

Barium hydroxide(Sigma, USA)

Bis-acrylamide (Boehringer-Mannheim, Germany)

Bromophenol Blue (Sigma, USA

β-mercaptoethanol (Sigma, USA)

Cadmium iodure (Sigma, USA)

Calcium chlorure(Sigma, USA)

Cellulose (Sigma, USA)

Chitin from crab shells (Sigma, USA)

Citric Acid (Merck, Germany)

Coomassie Brilliant Blue R-250 (ICN, USA)

Cupper chlorure (Sigma, 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)

EDTA (Boehringer Mannheim, Germany)

Formaldehyde (Riedel-de Haen, Germany)

Ferrous chloride (Sigma, USA)

Glucose assay kit (Sigma, USA)

Glutaraldehyde (Fluka, Switzerland)

Glycerol (Merck, Germany)

Glycine (Merck, Germany)

Hydrochloric Acid (Merck, Germany)

Laminarin from *Laminaria digitata*(Sigma, USA)

Lead chlorure(Sigma, USA)

Malt extract (Difco, USA)

Mannan (Sigma, USA)

Mellebiose(Sigma, USA)

Melesitoze(Sigma, USA)

Methanol (Merck, Germany)

Nickelchoride

Potassium Dihydrogen Phosphate (Merck, Germany)

Pullulan (Sigma, USA)

Pustulan (Sigma, USA)

Silver Nitrate (Merck, Germany)

Sodium Carbonate (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)

Yeast nitrogen base without aminoacids(Merck, Germany)

Zincchloride (Merck, Germany)

APPENDIX B

BUFFERS AND SOLUTIONS

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