ISOLATION AND CHARACTERIZATION OF THE K4 TYPE YEAST KILLER TOXIN

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Killer yeasts secrete polypeptide toxins which kill sensitive cells of their own species and frequently those of other species and genera of yeasts. These protein compounds are designated as killer toxins. Also killer toxins of certain yeast strains have potential growth inhibitory activity on gram-positive pathogenic bacteria and plant pathogenic fungi. The yeasts are immune to their own killer protein. The killer phenomenon can be utilized for the protection of fermentation process against contaminating yeasts and for biological control of undesirable yeasts in the preservation of foods. The killer trait can also be used to produce large amount of foreign proteins in yeast. In the medical field , it is thought that their anti-microbial and anti-mycotic activity could be exploited in a therapeutic strategy.

Yeast killer toxins are classified into 11 types according to their killing spectra and immunity-specificities such as K1, K2, etc. Altough there is considerable amount of published information concerning the applications of yeast killer toxins , among the 11 types , only K1 , K2 and K6 have been characterized. In this study , it was aimed to purify and characterize the K4 type yeast killer toxin secreted by the *Hansenula anomala* NCYC 432. Gel permeation chromatography was performed to isolate the killer toxin by using a HPLC system. The toxin was shown to be a glycoprotein having a molecular mass of between 49.08 kDa and 47.25 kDa and isoelectric point of between 3.77 and 3.41.

Key words: Killer yeasts , K4 type yeast killer toxin , *Hansenula anomala* , Gel Permeation Chromatography

ÖZ

K4 TİPİ ÖLDÜRÜCÜ MAYA TOKSİNİNİN İZOLASYONU VE KARAKTERİZASYONU

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Öldürücü mayalar , kendi türlerinin hassas hücrelerini ve çoğunlukla başka tür ve cinslerden mayaları öldüren polipeptid toksinler salarlar. Bu proteinler öldürücü toksinler diye adlandırılır. Ayrıca bazı maya suşlarının salgıladığı öldürücü toksinler gram-pozitif patojenik bakterilerin ve patojenik bitki mantarlarının büyümelerini inhibe etmektedirler. Öldürücü mayalar ürettikleri bu toksinlere karşı bağışıklığa sahiptir. Öldürücü mayalar fermentasyon sürecinin ve gıdaların istenmeyen mayalara karşı korunmasında kullanılabilinir. Öldürücü karakter , yabancı proteinlerin mayada çok miktarda üretilmesinde de kullanılabilinir. Tıp alanında ise sahip oldukları bu anti-mikrobiyal ve anti-mikotik aktiviteler tedaviye yönelik olarak kullanılabilinir.

Öldürücü maya toksinleri öldürme spektrumu ve immunite özelliklerine göre 11 tip altında sınıflandırılmışlardır , örneğin K1 , K2 vb. Her ne kadar öldürücü maya toksinlerinin uygulamalarıyla ilgili çok sayıda yayımlanmış bilgi olsa da , 11 tip içinde şimdiye kadar yalnızca K1 , K2 ve K6 tipleri karakterize edilmiştir. Bu çalışmada *Hansenula anomala* NCYC 432 tarafından üretilen K4 tipi öldürücü maya toksininin izolasyonu ve karakterizasyonu amaçlanmıştır. Toksinin izolasyonu için HPLC sistemi ile jel geçirgenlik kromatografisi uygulanmıştır. Glikoprotein olduğu saptanan toksinin , moleküler ağırlığının 49.08 kDa ile 47.25 kDa ve izoelektrik noktasının 3.77 ile 3.41 arasında olduğu bulunmuştur.

Anahtar kelimeler: Öldürücü mayalar , K4 tipi öldürücü maya toksini , *Hansenula anomala* , Jel Geçirgenlik Kromatografisi.

To My Family

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AP	Alkaline Phosphatase
bp	Base pair
DIG	Digoxigenin
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol
HPLC	High Performance Liquid Chromatography
K^+	Killer character
K^-	Non-killer character
kb	Kilo base
kDa	Kilo dalton
[KIL-k1]	Normal K1 killer plasmid
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.

CHAPTER I

INTRODUCTION

Yeasts are unicellular fungi which are usually spherical or oval and cell division generally takes place by budding. Yeast do not form filaments or a mycelium and the population of yeast cells remains a collection of single cells. Some yeasts , such as *Saccharomyces cerevisiae* , also exhibit sexual reproduction by a process called mating , in which two haploid yeast cells (α and a type) fuse to form a diploid a/α cell. Within the fused cells , called zygote, spores form. For the most part , yeasts spread from place to place as ordinary vegetative cells rather than as spores and unlike most other organisms, have both a stable haploid and diploid state. Life cycle of a yeast *S. cerevisiae* is shown in Figure 1.1. [1, 2, 3].

Rapid growth , a budding pattern resulting in dispersed cells , the ease of mutant isolation , well-defined genetic system , and a highly versatile DNA transformation system are some properties which make yeast suitable for biological studies. Being nonpathogenic , yeast can be handled with few precautions [5].

Yeasts are the most important and the 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 other growth factors. Industrial uses of yeast is shown in Table 1.1. [1]

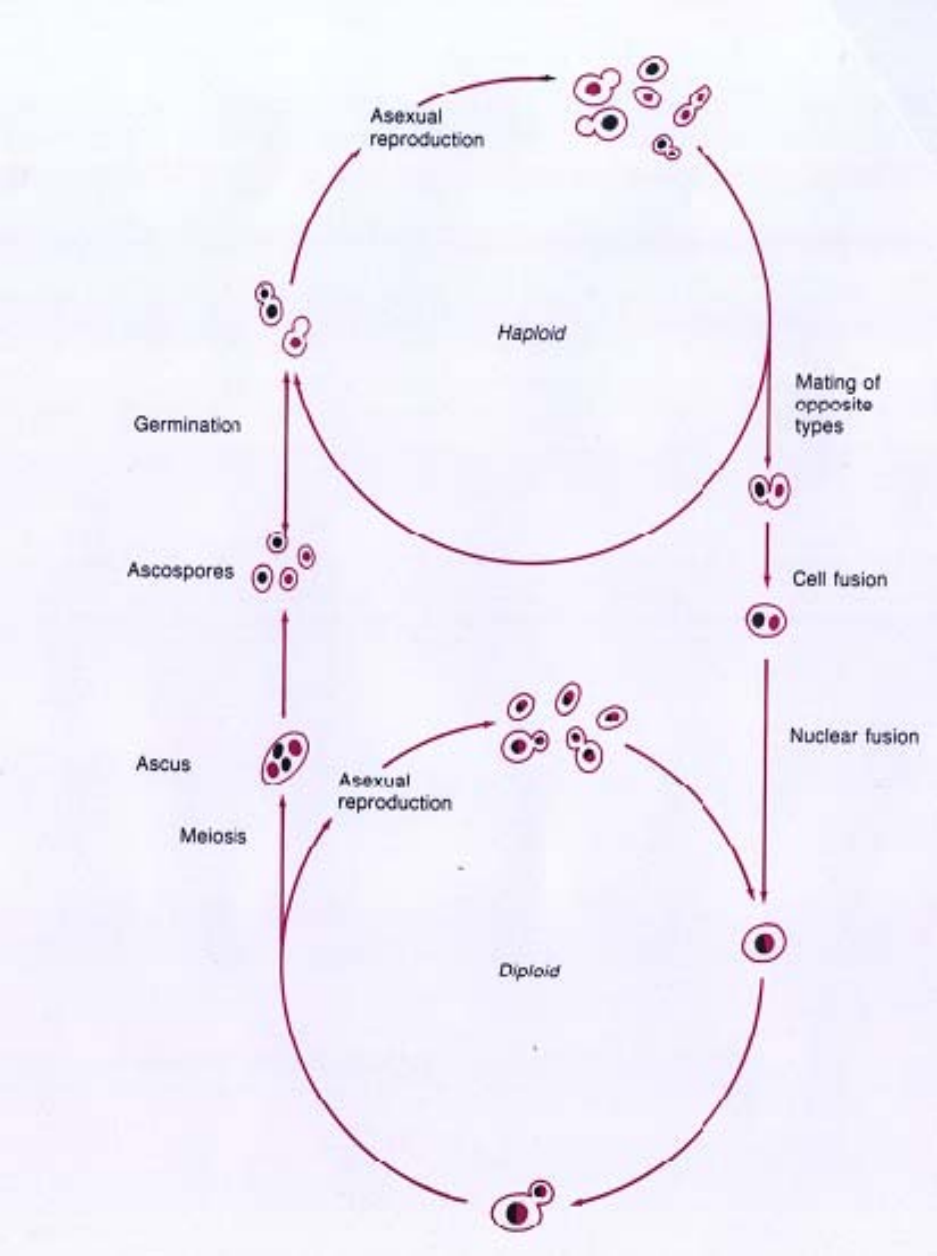


Figure 1.1. Life cycle of a typical yeast *S. cerevisiae* [5].

Table 1.1. Industrial uses of yeast [1]

Production of Yeast Cells
Baker's yeast , for bread making
Dried food yeast , for food supplements
Dried feed yeast , for animal feeds
Fermentation Products From Yeast
Ethanol for endustrial alchohol and as a gasoline extender
Glycerol
Yeast Products
Yeast extract for culture media ,
Enzymes for food industry ; invertase , galactosidase ,
B vitamins , vitamin D ,
Biochemicals for research ; ATP , NAD , RNA
Beverage Alchohol
Beer , wine , whiskey , brandy , vodka , rum

The yeasts currently used are descendants of the early *S. cerevisiae.* It is now possible to genetically change yeasts in the laboratory using genetic hybridization and cloning methods to produce strains of desirable qualities [4].

In many studies yeast has come to occupy the role of a model eukaryotic cell and many recent findings assume importance for cells of higher eukaryotes , including human. At the same time , yeast is a microbe of major economic and social significance. Not only does it provide the fermentations for most

breads and alcoholic beverages but increasingly it has been recruited to the "new biotechnology" to produce heterologous proteins and other molecules mainly for pharmaceutical use. Yeasts such as *S. cerevisiae* are attractive host organisms for high level production of heterologous proteins of commercial importance , because strains can easily be modified using molecular genetic techniques and their use in large scale industrial fermentations is well established. For example , the gene for human interferon and hormones such as insulin and somatostatin have been cloned and expressed in yeast [5 , 6].

The production of yeast killer toxins is a well-established phenomenon among many yeast genera and species [7]. The killer yeasts were first described in laboratory strains of *S. cerevisiae* by Makower and Bevan (1963) who demonstrated three phenotypes - killer , neutral and sensitive - with respect to the killer character [8, 9]. Killer yeasts , secrete polypeptide toxins which kill sensitive cells of their own species and frequently those of other species and genera of yeasts [10]. Also killer toxins of certain yeast strains have potential growth inhibitory activity on gram positive pathogenic bacteria [11] and plant pathogenic fungi [12].

These polypeptide toxins are commonly designated as killer factors , killer toxins or killer proteins. Terms such as zymocins or mycocins , anologous to bacteriocins have also been applied. The yeasts are immune to their own killer protein. This phenotype of toxin specific insensivity is termed immunity. To date , killer yeasts have been reported in strains of several yeast genera including *Saccharomyces, Candida ,Cryptococcus , Debaryomyces , Hansenula, Kluyveromyces, Ustilago, Williopsis, Hanseniaspora, Zygosaccharomyces* [13, 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 , analysis of dsRNA content and structural relationships

between the toxins including three in *Saccharomyces sp.* strains (K1 , K2 , K3) [14, 15, 16, 17]. K11 type was introduced by Wickner in 1975 to the Young and Yagiu classification [18]. The K1 phenotype is widely distributed among laboratory strains of *S. cerevisiae* and wild type strains of it , while K2 has been found almost exclusively among fermentation contaminants and are capable of killing K1 killers. The K3 group consist of strains derived from a single wine yeast and has a killing activity on K1 and K2 type killers. The nomenclature used for killer phenotypes is summurized in Table 1.2. [10].

Table 1.2. Killer phenotypes of the genus *S. cerevisiae* [10].

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" phenotpye ; strains producing killer protein and exhibiting a decreased immunity to tpye K1.
$K^- R^-$	Sensitive strains not producing killer protein.

The role and function of these toxins in natural yeast communities is uncertain. It is suggested that the killer phenomenon is a potential mechanism for

interference competition , where the production of toxic compounds prevents a competitor from gaining access to resources [19].

Several potential applications for the killer phenomenon have been suggested since it was determined and studied. In the fermentation and food industries the killer character can be used to combat wild , contaminating *Saccharomyces* strains during the production of beer , wine and bread. Killer yeasts have also been considered useful in biological control of undesirable yeasts in the preservation of foods. In recombinant DNA technology , killer plasmids of *S. cerevisiae* and *K. lactis* have the potential to be used as cloning vectors for the effective secretion of expressed foreign polypeptides. In the medical field , killer yeasts have been used in the biotyping of pathogenic yeasts , and killer toxins of *Hansenula anomala* and *Williopsis mrakii* have been proposed as antimycotic agents in the treatment of human and animal fungal infections. Yeast killer toxins have been used as model systems in fundamental research for studying the mechanisms of regulation of eukaryotic polypeptide processing , secretion and receptor binding [12, 20].

Killer toxins differ between species or strains , showing diverse characteristics in terms of gene that encodes them , the molecular size , the mature structure of the protein and mode of action of the toxin [21]. They cause membrane permeability changes in sensitive cells [22] , and in some cases inhibit DNA replication [23] or stop cell division at G1 phase [5].

Besides these differences among yeast killer toxins , they share a common characteristic in that they are pH and temperature dependent. They usualy active and stable at pH 4–5 and 20–25 °C and each toxin has an optimum pH and temperature at which it shows its killer character more intense [24].

1.1. Genetic Basis of Killer System

There are different genetic factor for each killer system (Table 1.3.) [25]. They are the dsRNA viruses that permanently resides in its host symbiotically , the linear cytoplasmic dsDNA plasmids and the chromosomal DNA [26 ,27].

Table 1.3. Genetic basis for killer phenotype expression in yeast [25].

Yeast	Genetic Basis	Toxin Gene
S. cerevisiae	dsRNA virus	M1- , M2- , M28
H. uvarum	dsRNA virus	M-dsRNA
Z. bailii	dsRNA virus	M-dsRNA
U. maydis	dsRNA virus	M-dsRNA
K. lactis	linear dsDNA plasmid	pGKl1
P. 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

1.1.1. dsRNA virus based yeast killer systems

In cooperation with nuclear genes , the killer phenomenon is brought about by virus like particles occuring in yeast cytoplasm. The similarity of these particles with viruses consist in the fact that their genome are usually dsRNA and encapsidated. The genetic determinants of the killer trait shows non-mendelian inheritance. The particles are non-infective but can be transfered by sexual hybridization, protoplast fusion or cytoduction [13, 28].

Strains of *S. cerevisiae* carry as many as five non-homologous species of dsRNA called L-A , L-BC , M , T , and W. L-A , L-BC and M are found in intracellular virus-like particles (VLPs). The genome of dsRNA viruses constitutes 0.1% of the total nucleic acid in *S. cerevisiae* [28]. Genome of diploid *S. cerevisiae* cell is shown in Figure 1.2. [4].

The nucleus of haploid *S. cerevisiae* cell contains approximately 14 000 kb of DNA subdivided into 16 linear chromosomal DNAs. In addition many strains carry 50 to 100 copies of 6.3 kb plasmid which is usually referred to as 2μm plasmid. The 75-80 kb of mitochondrial DNA is circular and each cell harbors 20-40 copies [29, 4].

	Mendelian		Non-Mendelian					
	Double-Stranded DNA			Double-Stranded RNA				
	Nuclear			RNA Molecules				
Characteristic	Chromosomes	2-μm plasmid	Mitochondrial	L-A	M	L-BC	T	W
Relative Amount (%)	85	5	10	80	10	9	0.5	0.5
Number of Copies	2 sets of 16	60-100	~50 (8-130)	1000	170	150	10	10
Size (kb)	14.000 (200-2200)	6.318	70-76	4.576	1.8	4.6	2.7	2.25
Deficiencies in Mutants	All Kinds	None	Cytochromes a, a_3, b	Killer Toxin		None		

Figure 1.2. Genome of diploid *Saccharomyces cerevisiae* cell [4].

M dsRNA encodes the killer toxin and immunity to it. There are different M dsRNAs with different size M_1 dsRNA (1.9 kB) , M_2 dsRNA (1.7 kB) , M_3 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 [14, 13]. M type dsRNAs are dependent for their maintenance and encapsidation on the capsid provided by L type dsRNAs. M viruses also requires the L virus encoded proteins for the replication of their killer toxin encoding genome.

Most *Saccharomyces* strains , whether killer or not , have a 4.5 kB linear dsRNA called L dsRNA. L dsRNA encodes proteins for the viral capsid and for the synthesis and encapsidation of ssRNA replication intermediates . L dsRNA is present in a higher copy number (100-1000) in the cell and commonly closed in virus-like particles in almost all isolated strains of *S. cerevisiae* that usually do not contain M dsRNA and are thus sensitive to the killer protein. L dsRNA comprises two unrelated families of molecules called L-A family and L-BC family. L-A encodes the major coat protein of the virus-like particles in which both itself and M dsRNA are encapsidated. L-BC dsRNAs have no apperent homology to L-A. It is not clear whether they have any functional relation to killer phenomena. Since some killer strains lack L-BC entirely. T and W have no homology with each other or with L-A , L-BC or M dsRNAs. They are present in very low copy number in the cells. No functional relation has yet been established with the killer systems [28 ,30].

Natural L-A dsRNAs variants carry various combinations of genes [HOK], [NEX] and [EXL] that in various ways influence the killer systems. [HOK] (helper of killer) gene supplies the helper function needed by M_1 or M_2 for replication in wilde type strains. It is located on certain forms of L-A ds RNAs namely L-A-HN , L-A-HE or L-A-H. [EXL] (excluder of M_2 dsRNA) is defined as the ability of certain L-A's to exclude M_2 from a strain not carrying [NEX]. [EXL] located on certain forms of L-A namely L-A-E , L-A-HE. [NEX] prevents [EXL] action. M_2 non-excludable by [EXL] , but does not prevent exclusion of M_2 by strains carrying M_1. [NEX] is located on L-A-HN the form of L-A found in

wild type K1 killer strains [31, 28].

There are some chromosomal genes effect the replication and expression of the cytoplasmic genetic elements of the killer system. They are shown in Table 1.4. [28]. [KEX] genes (killer expression) are needed for processing the toxin precursor. [MAK] genes (maintenance of killer) necessary to maintain M1 dsRNA. [SKI] genes (super killer) limit the viral propagation. Mutations on these genes result in enhanced toxin production [32, 28, 33].

Table 1.4. Chromosomal genes [28].

Chromosomal Genes	
MAK	Maintenance of [KIL-k_1] , MAK genes comprise at least 32 chromosomal genes necessary to maintain M_1 dsRNA.
Clo	A complex chromosomal defect resulting in loss of L-B or L-C.
SKI	Superkiller. Mutants carrying the recessive alleles of ski2 , ski3 , ski4 , ski6 ski7 or ski8 produce more killer toxin and have increased copy number of M_1 , M_2 , L-A and L-BC.
MKT	Maintenance of [KIL-k_2] in the presence of [NEX].
KEX	Killer Expression. Two chromosomal genes needed to process the toxin precursor. Mutants are K^-R^+.
REX	Resistance Expression. One chromosomal gene needed to express M_1 determined resistance to toxin.
KRE	Killer Resistant. Three chromosomal genes needed for normal toxin action on sensitive cells. KRE affects β(1,6)glucan , the normal toxin cell wall receptor.
SEC	Secretion. Chromosomal genes for general protein secretion.

1.1.1.a. Structure , processing and secretion of Killer Toxins

Killer toxin is composed of two subunits α (9.5 kD) and β (9.0 kD) , which originate from the amino-terminal and carboxy-terminal domains of the preprotoxin respectively. 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 [13, 32].

Preprotoxin (pptox) undergoes post-translational modifications via the endoplasmic reticulum , golgi apparatus and secretory vesicles resulting in the secretion of a mature α/β heterodimeric protein toxin. The N-terminal hydrophobic leading sequence is superimposed on the above 3 regions. This sequence with high affinity for endoplasmic reticulum permits penetration of the precursor (pptox) into the endoplasmic reticulum , where the region γ is glycosylated. Signal peptidase (SP) cleavage removes the toxin's N-terminal secretion signal (pre-region) in the endoplasmic reticulum. The secretory pathway of the *S. cerevisiae* killer toxin is shown in Figure 1.3.[13, 25].

A further modification proceeds in the golgi apparatus. During this stage the effect of sec7 and sec18 mutations can become manifested and products of genes KEX1 and KEX2 are also apparently involved. KEX1 and KEX2 genes were shown to encode proteases necessary for processing both killer toxin and α-factor precursor proteins. The combined action of these two proteases yields mature toxin from protoxin. Kex2p , the gene product of KEX2 , is an endopeptidase and cleaves the pro-region , removes the intramolecular γ sequence. Kex1p , the product of KEX1 , is a carboxypeptidase that removes the C terminal basic dipeptide exposed by Kex2p action. Figure 1.4. shows the preprotoxin processing in *S. cerevisiae* [13, 25].

Processing of the killer toxin also requires the products of SEC genes , responsible for general secretion. Mutations in these genes result in accumulation

of the glycosylated protoxin in the endoplasmic reticulum or secretory vesicles [34 ,28].

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. On the other hand , several lines of evidence suggest that the protoxin itself may be the immunity protein. The protoxin could , for example , bind to toxin receptors on the cell membrane much more tightly than toxin itself and would thus prevent the active toxin from binding and acting [13 ,35].

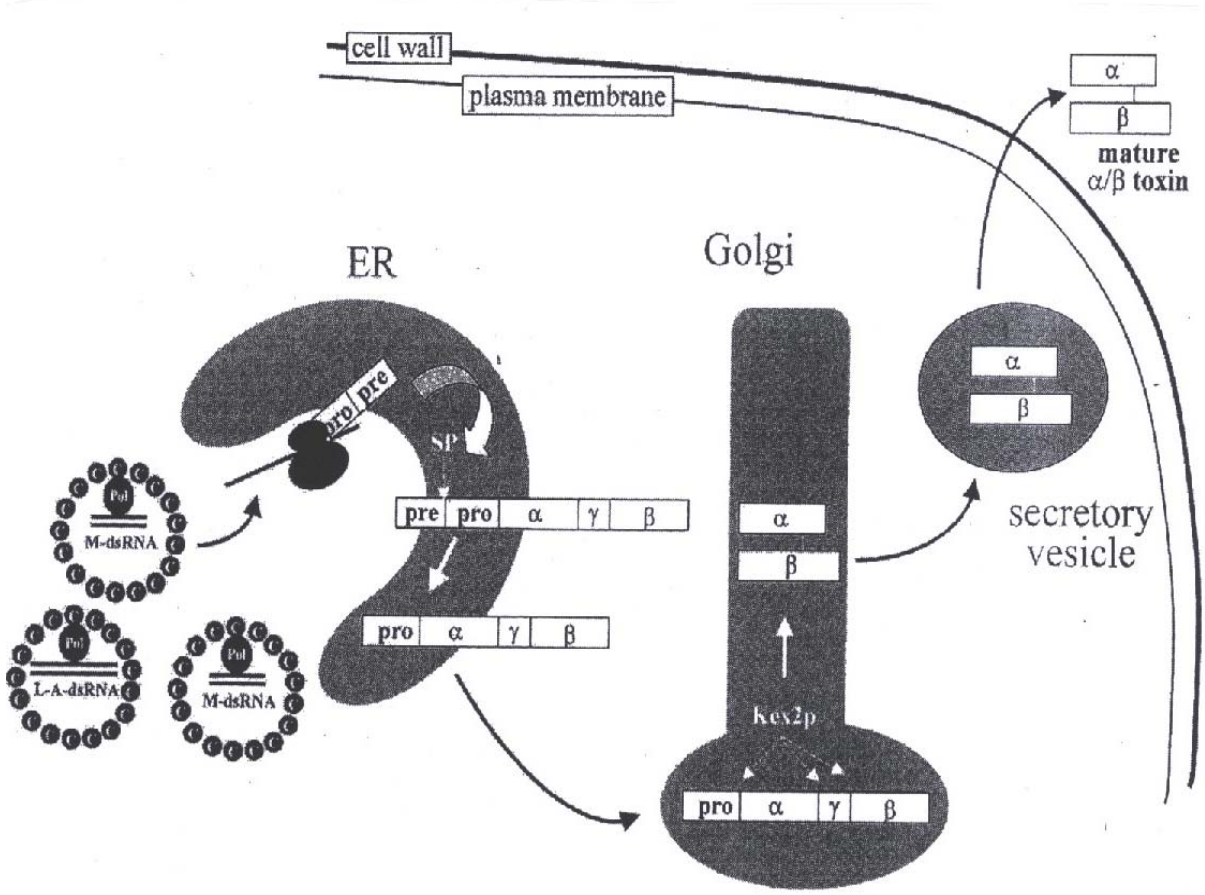


Figure 1.3. Secretory pathway of the *S. cerevisiae* killer toxin [25].

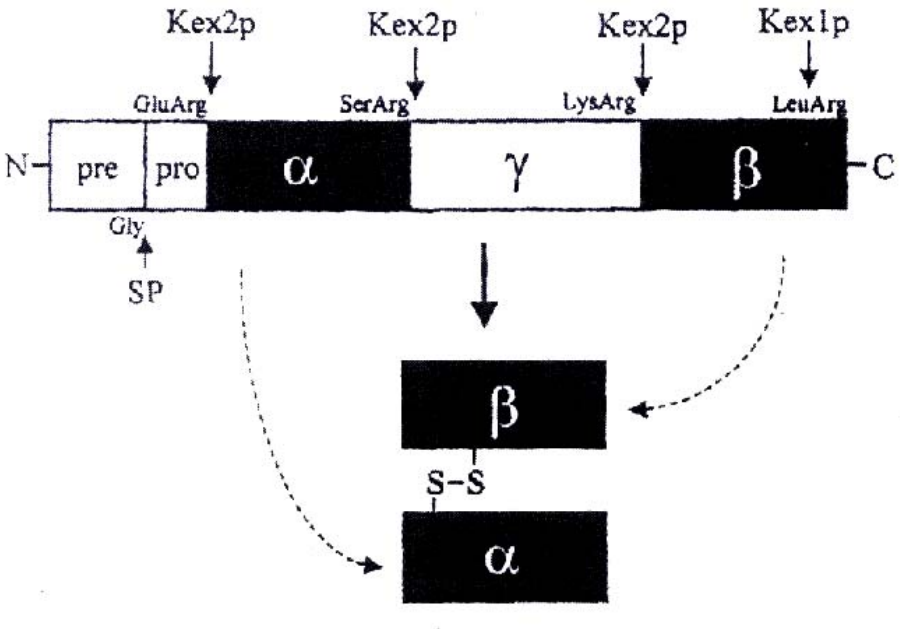


Figure 1.4. Preprotoxin processing in *S. cerevisiae* [25].

1.1.1.b. Transcription and Replication of dsRNAs

Synthesis of both (+) and (-) RNA strands occurs within the viral particles but at different points in the viral replication cycle [36]. Isolated intact VLPs containing both L and M dsRNAs also contain a transcriptase activity which produce ss transcripts having messenger activity for the known dsRNA gene products. The mechanism of transcription appears to be conservative with extrusion of the newly synthesized (+) strand. The replication cycle of L-A and M dsRNA is shown in Figure 1.5. [25].

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.(+) L-A ssRNA , rather than L-A dsRNA , is the form recognized by the packaging machinery for encapsidation into particles [35].

It is proposed that the structure of the L-A-encoded capsid is designed to hold one L-A dsRNA molecule. When M_1 (+) strands are encapsidated in this coat and converted to dsRNA , the head is not full , because M_1 (1.8 kb) is less than half size of L-A (4.6 kb). Thus , new M_1 (+) strand transcripts often remain inside the viral particles , where they are converted to a second M_1 dsRNA molecule. This mechanism is called “headful replication” [37].

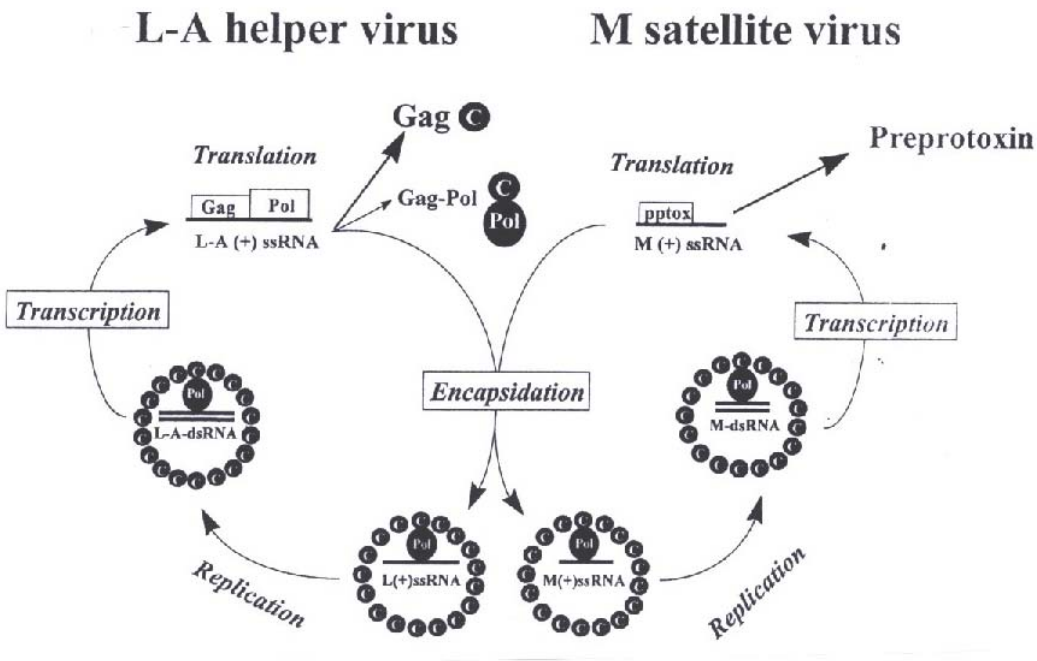


Figure 1.5. The replication cycle of L-A and M dsRNA [25].

1.1.2. Cytoplasmic Linear DNA Plasmid Based Killer Systems

Linear DNA plasmids have been identified for various yeast genera such as *Debaryomyces* , *Wingea* , *Kluyveromyces* and *Saccharomyces*. They are located in the cytoplasm and many of them confer a killer phenotype on their hosts. [38, 5].

The killer toxins differ from the toxin produced by dsRNA mediated killer yeast of *S. cerevisiae* in molecular weight , mechanism of killing action , and killing spectrum against various yeasts. In terms of gene function , the best-characterized of these yeast episomal systems is the cytoplasmically localized killer plasmid pair , pGKL1 (k1) and pGKL2 (k2) , of the dairy yeast *Kluyveromyces lactis*. k1 and k2 plasmids are extremely A+T rich , partially cross-hybridize to each other and carry terminal inverted repeats (TIRs) with covalently attached terminal proteins (TPs). They can be transferred to other species of yeast by cell fusion or transformation and in particular are stably maintained in *S. cerevisiae.* However the plasmids show curious incompatibilty with mitochondrial DNA in *S. cerevisiae* and are only stably maintained in ρ^0 (mitochondrial DNA-deficient) strains [38, 39 , 40].

The smaller plasmid k1 (8.9 kb) 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 specify subunits α , β , γ of the heterotrimeric killer toxin and ORF3 is essential for toxin immunity. The toxin causes an irreversible arrest of sensitive yeast cells in the unbudded (G1) phase of the cell cycle. The plasmid k2 (13.5 kb) , which carries 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)[38, 41, 42, 43]. Genetic organization of pGKL1 and pGKL2 is shown in Figure1.6. [41].

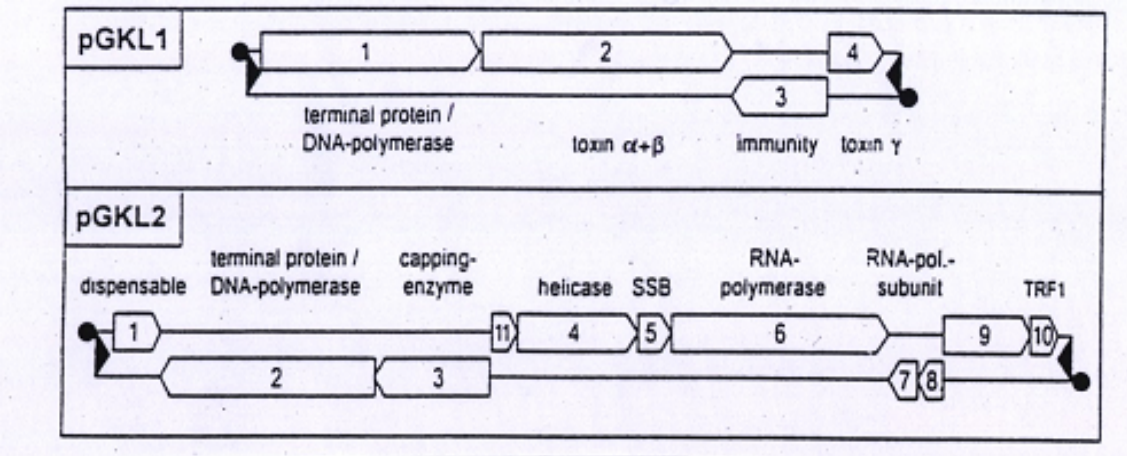


Figure 1.6. Shematic representation of *K. lactis* linear plasmids [41].

1.1.3. Chromosomally encoded yeast killer systems

There is no evidence as yet that killer character expressed by strains of *Williopsis , Pichia , Candida , Debaryomyces , Cryptococcus* and *Torulopsis* is dependent on plasmid-encoded systems. The genetic basis for killer character in these yeasts is , therefore , thought to be chromosomally inherited [44].

In a halotolerant yeast, *Pichia farinosa* , the killer gene (SMK1) is located on a chromosome and encodes a preprotoxin which is processed in a manner similar to that of the K1 toxin of *S.cerevisiae.* Expression of this toxin (SMKT , Salt Mediated Killer Toxin) is post-translationally controlled by NaCl [21, 45].

Kluyveromyces thermotolerans secretes 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 [46].

Goto et al. discovered two new killer activities in 1990. The killer genes were found to be encoded on chromosomal DNA of a strain of *S. cerevisiae*. One gene , designated KHR , is encoded on chromosome IX and another designated as KHS is on chromosome V [47].

The HMK gene , encoding a killer toxin of *Hansenula mrakii* , and the HSK gene encoding a killer toxin of *Hansenula saturnus* , were cloned. Killer protein encoded by these chromosomal genes have higher thermostability and wider pH-stability than other killer toxins [48].

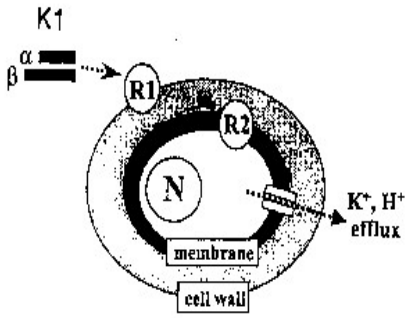
1.2. Mode of Action of the Killer Toxins

The mode of action of the killer toxin has been largely studied for the K1 and K2 killer system. Killing occurs by a two step mechanism ; binding to the cell wall and action at the cell membrane. It is assumed that after a certain lag period the killer protein is bound to a cell wall receptor 1,6-β-D-glucan. These receptors are found at large numbers on the cells of sensitive strains and strains of the killer phenotype. Binding to the cell wall receptor does not require energy supply.

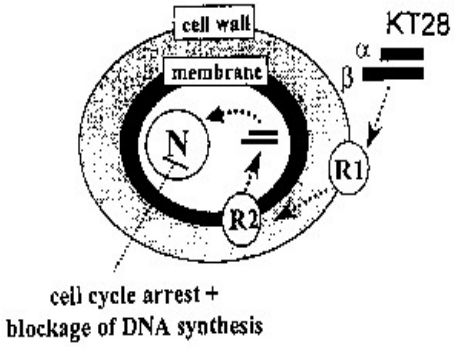
It is assumed that β-subunit is involved in binding to the cell wall receptor. α component might be responsible for the lethal effect on the membrane. The transfer and interaction with the membrane are energy-dependent processes.

It was demonstrated that damage of the cell by the killer protein caused by a changed permeabilty of the membrane and collapse of the proton gradient. This is followed by a pronounced decrease of pH in the cell, inhibition of metabolic process , release of potassium ions and ATP to the medium and eventual death of the cell. Mode of action of the K1 killer toxin secreted by *S. cerevisiae* is shown in Figure 1.7. a. [10, 13, 25, 49, 50].

Different from known killer toxins , the killer toxin from *S. cerevisiae* KT28 binds mannoproteins of the cell wall. Interaction with the membrane target causes rapid inhibition of the nuclear DNA synthesis. Cell viability is lost more slowly and toxin interrupts the cell division cycle at the S-phase (Figure 1.7. b.) [25, 51, 52].



(a) Mode of action of the K1 killer toxin



b) Mode of action of the *S. cerevisiae* KT28 killer toxin

Figure 1.7. Mode of action of the killer toxins secreted by *S. cerevisiae* [25].

Killer toxin of *Kluyveromyces lactis* causes an irreversible G_1 arrest and eventually loss of viability in *S. cerevisiae* cells , while permitting continued macromolecular biosynthesis. Native toxin is a heterotrimer but toxicity resides on its γ subunit.Both α and β subunits are considered to be necessary for binding and/or uptake of the γ subunit [41, 52, 53].

It was demonstrated that killer toxins secreted by varius strains of the yeast genus *Hansenula* strongly inhibit de novo β-1-3-D-glucan biosynthesis in yeast. For example , the toxin of *Hansenula mrakii* causes pore formation by inhibiting the β-1-3-D-glucan synthesis occuring at a budding site. This results in leakage of cell material and eventual cell death [25, 54 , 55, 56]

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 [49, 22].

1.3. Potential Uses of the Killer Yeast and Their Toxins

1.3.1. Fermentation Technology and Food Storage

Contamination with killer-toxin producing yeast species is a potential problem in fermantation process. The killer phenomenon can be utilized for the protection of fermentation process against contaminating yeasts by the introduction of the killer trait into the sensitive starter strains. Immune industrial strains is constructed by using the methods of protoplast fusion or cytoduction [11, 13, 57, 58, *59*].

In wine making , killer yeasts belonging to *S. cerevisiae* are currently used to initiate wine fermentation to improve the process of wine making and wine quality.

Stable double killer wine yeast strains have also been generated by gene replacement technology. They have a wider spectrum of killing and a potential competative advantage over other sensitive and killer strains of *S. cerevisiae* in wine fermentation [20 ,58].

At present killer yeasts appears to be promising biocontrol agents , providing alternatives to chemical fungicides in the postharvest storage of fruits and vegetables. 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. The biological control of these undesirable yeasts might be carried out by means of yeast cultures that produce yeast killer toxins [12, 16, 60].

1.3.2. Heterologous Protein Expression

The killer trait can be used to produce large amount of foreign proteins in yeast.Several heterologous genes,including genes coding biotechnologically important enzymes , has been expressed using killer plasmids as vectors.In this respect linear plasmids (pGKL1 and pGKL2) of *K. lactis* have some major advantages , when compared with circular plasmid such as their high copy number and extreme stability. Their cytoplasmic localization makes them independent of nuclear control in terms of replication and transcription [41, 61, 62].

The toxin cDNA clones has also been used to construct a secretion vector with the signal region of the toxin. This was applied to construct a yeast strain secreting the carboxymethylcellulase from *Cellomonas fimi* [35].

h-TPA (human tissue plasminogen activator) cDNA was fused with the leader sequence of *K. lactis* killer toxin in order to achieve high level expression of h-TPA in yeast [63].

Bacterial xylanase which is used in the paper manifacturing industry is expressed and secreted in *K. lactis* using a secretion signal derived from the pre- region of the *K. lactis* killer toxin [64, 65].

1.3.3. Medical Uses of the Killer Toxins

It was shown that some killer toxins have antifungal and antimicrobial effects. The killer toxins from *Williopsis subsuffciens* , *Hanseniaspora uvarum* , *W. beijerinckii* , *W. mrakii* and *Hansenula 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* [11, 66, 67].

Pichia anomala killer toxin (PKT) has been extensively investigated.It has been shown that this killer toxin active against a broad range of microorganisms including taxonomically unrelated pathogenic microorganisms such as , *P. carinii* , *Candida albicans* and *Mycobacterium tuberculosis*. It is thought that its anti-microbial activity could be exploited in a therapeutic staregy [7, 44, 68].

Yeast toxins are probably not suitable for oral and/or intravenous administration because most killer proteins exhibit their cytotoxic activity only within a narrow pH range and at temperatures between 20 ^{0}C and 30 ^{0}C. But topical applications in the treatment of superficial lesions might well be possible [25].

Although killer toxins have many potential uses in environmental and industrial biotechnology and in medical field , among the 11 types , only K1 , K2 and K6 have been characterized. In this study , we aimed to purify and characterize the K4 type yeast killer toxin secreted by *Hansenula anomala* NCYC 432.

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. *Hansenula anomala* (NCYC 432) was used as the source of the K4 type killer toxin. *Hansenula anomala* is an eukaryotic fungi belonging in the phylum; *Ascomycetes*, class; *Hemiascomycetes*, order; *Saccharomycetales*, family; *Saccharomycetaceae*, genera; *Pichia (Hansenula),* species; *anomala* . *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 containing 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.

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 were 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 25^{o}C until the colonies are formed (1-5 days) [4].

2.2.3. Determination of the Culture Conditions for Killer Toxin Production

The growth conditions for the toxin production was determined by the killer zone assay in a plate test as previously described [49, 69]. YEPD medium at different pH values were used and incubation was carried out at different temperatures. The cells of the killer sensitive *Saccharomyces cerevisiae* NCYC 1006 (1.10^5cells/ml) were resuspended in 15ml molten YEPD medium (45^{o}C). The suspensions were poured into sterile petri dishes and the plates were inoculated with a 50µl distilled water suspension of killer yeast cells just after the agar solidified. A zone of growth inhibition surrounding the killer yeast cell suspension indicates toxin production.

2.2.4. Killer Toxin Production

Hansenula anomala NCYC 432 was grown in 1L. of YEPD medium (containing 5% glycerol) buffered to pH 4.5 with 0.1M citrate-phosphate buffer at 22^{o}C for 36 hours at 120 rpm on a gyratory shaker (New Brunswick, USA). The cells were removed by centrifugation (KR 22i, Jouan, France) at 1,000 x g 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^{o}C (BR4i, Jouan, France). The concentrated cell free filtrates were then subjected to buffer exchange with 0.1M Na_2HPO_4 buffer at pH 4.5 containing 0.1M Na_2SO_4 by using the 5000 MWCO centrifugal filter device

(Vivaspin VS2021, Sartorius). Buffer exchange step was performed three times at 4200 x rpm for 15 min. at 4°C (BR4i, Jouan, France). The concentration of the sample was determined as 28mg/ml by measuring the absorbance at 280 nm using a UV-Visible Spectrophotometer 1208 (Shimadzu, Japan). Before injection onto the HPLC column the samples were ultrafiltrated by 0.25µm syringe filter (Sartorius, AG, Germany).

2.2.6. Isolation of the Killer Toxin

Isolation of the killer toxin by gel permeation chromatography was performed at 25°C on a high performance liquid chromatography (HPLC) system (BioCAD 700E Perfusion Chromatography Workstation, Perseptive Biosystems Inc.). The column used was gel permeation chromatography column TSK G2000 SW (7,5 mmD/300mmL TosoHaas, Japan). The buffers for the HPLC system were degassed with He gas before they were used.

The column was equilibrated with the eluent buffer 0.1M Na_2HPO_4 at 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 and detected at 280nm at an ambient temperature of 25ºC. Fractions were collected (850µl) automatically by fraction collector (Advantec SF-20 Super Fraction Collector, Japan) which was connected to BioCAD 700E Workstation. The collected peaks were concentrated seperately by ultrafiltration with 5000 MWCO ultrafilter device (Vivaspin VS2021, Sartorius Sartorius, AG). Fractions were tested for killer activity by applying 50µl of each on YEPD agar plates (pH 4.5) seeded with 10^5 cells of the killer toxin sensitive strain *S. cerevisiae* NCYC 1006. After two days of incubation at 22 °C , killer inhibition zone was detected.

The fraction with killer activity was collected for several runs , concentrated with 5000 MWCO ultrafilter device and 90µl of it was injected to gel permeation chromatography column TSK G2000 SW. Elution was performed with the same buffer and the same flow rate and detected at 280 nm at an ambient temperature of 25 ºC. The major peak was collected , concentrated and assayed for its killer activity.

2.2.7. Non-denaturing Gradient SDS-Polyacrylamide Gel Electrophoresis

The active fraction that was obtained from gel permeation chromatography was subjected to 5%-20% linear gradient SDS-PAGE gel in a discontinous buffer system under non-denaturing conditions [71]. 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 gradualy decreased towards the top. Seperating and stacking gel compositions are given in Table 2.1 and Table 2.2 respectively [71].

After pouring the gel into the glass plate sandwiches , it is 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 seperating gel and a 1.5 mm thick 15 wells comb was inserted into the sandwich.

In non-denaturing SDS-PAGE method , β-mercaptaethanol was not added to the treatment buffer (0.125M Tris-Cl , 4%SDS , 20%Glycerol , 0.02% bromophenol blue to 10ml dH_2O , pH 6.8). Sample was combined with equal volume of treatment buffer and were heated at 100 ^oC for 4 minutes. After the polymerization completed samples were loaded onto the gel. The gel was run at a constant current of 30mA (Power supply PP4000, Biometra, Germany) at

15 ^{o}C using circulating cooling water bath (Heto Holten, Denmark) until the dye front reached the bottom. The gel was visualized by silver staining [72].

Table 2.1. Seperating 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.

2.2.8. Denaturing Gradient SDS-Polyacrylamide Gel Electrophoresis

The isolated killer protein was subjected to 5%-20% linear gradient SDS-PAGE gel in a discontinous buffer system under denaturing conditions [71]. Denaturing SDS-PAGE method same as the non-denaturing SDS-PAGE method except the presence of β-mercaptaethanol in the treatment buffer. 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 polymerization completed , 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 either visualized by coomassie brilliant blue R-250 [73] or silver [72].

Molecular weight markers used for the molecular weight determination of the K4 killer protein 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) , lysozyme (14.307 kDa) and aprotinin (6.500 kDa) (Roche Diagnostics).

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

2.2.9. Peptide Mapping

In-gel digestion of the protein bands in the doublet excised from denaturing SDS-polyacrylamide gel was performed with endoproteinase Glu-C and tyrpsin proteases (Boehringer Mannheim Biochemica, Germany). Protein bands from denaturing SDS-polyacrylamide gel (12.5%, 0.75mm thick) were digested

without prior elution by placing gel slices containing these bands in the sample wells of a second SDS-polyacrylamide gel (15% , 1.5mm thick) and then overlaying each slice with protease. Digestion proceeded directly in the stacking gel during the subsequent electrophoresis [74]. The first gel , denaturing SDS-polyacrylamide gel (12.5% , 0.75 thick) in a discontinous buffer system , was prepared using a dual vertical slab gel electrophoresis unit (SE600 Hoefer, USA) as previously described [71]. Protein was loaded on to the gel after combine it with the equal volume of treatment buffer and heated at 100 ^{o}C for 4 minutes. The gel was run at a constant current of 20mA (Power supply PP4000, Biometra, Germany) at 15 ^{o}C using circulating cooling water bath (Heto Holten, Denmark). The first gel was stained and destained briefly by coomassie blue R-250 and destain 2 solution respectively. Then the gel was rinsed with cold water. The individual bands were then cut out with a razor blade , trimmed to fit the sample wells of the second 1.5mm thick gel and soaked in 10ml of a gel slice buffer (0.125 M Tris/HCl , 0.1%SDS , pH 6.8) for 30 minutes with occasional swirling.

The second denaturing SDS-polyacrylamide gel (15% , 1.5mm thick) was prepared which was cast with longer than usual stacking gel. The sample wells were filled with gel slice buffer and each gel slice was placed in. Spaces around the slices were filled by overlaying each slice with gel slice buffer containing 20% glycerol. Proteases , endoproteinase Glu-C and tyrpsin , were dissolved in a gel slice buffer containing 10% glycerol (0.1mg/ml) and overlayed seperately into each slot at a protease to substrate ratio of 1:20. Electrophoresis was performed in the normal manner (at a constant current of 30mA) with the exception that the current was turned off for 30 minutes when the dye front neared the bottom of the stacking gel. The gel was visualised by silver staining [72].

2.2.10. Native Polyacrylamide Gel Electrophoresis

Purified K4 type killer protein was electrophoresed on discontiuous polyacrylamide gel electrophoresis (1mm thick , 12.5%) under native conditions in slab gel. Different from the standart Laemmli SDS-PAGE protocol [71] , in native-PAGE , SDS and reducing agent were left out. Sample buffer contains no SDS or reducing agent (samples were not heated) and the gel and electrode solutions were prepared without SDS. Gel electrophoresis was performed by using SE250/SE260 Mighty Small slab gel unit (Hoefer, USA). The seperating and stacking gels were prepared according to Table 2.3.

The gel was run at a constant current of 20mA (Power supply PP4000, Biometra, Germany) at 15^{o}C using circulating cooling water bath (Heto Holten, Denmark) until the dye front reached the bottom. The gel was stained with coomassie brilliant blue R250 [73].

Table 2.3. Native-PAGE Seperating and stacking gel compositions

	Seperating Gel (12.5%T)	Stacking Gel (4%T)
Monomer Solution (30%T , 2.7%C)	4.2ml	0.44ml
4X Seperating Gel Buffer (1.5M Tris-Cl , pH:8.8)	2.5ml	-
4X Stacking Gel Buffer (0.5M Tris-Cl , pH:6.8)	-	0.83ml
ddH_2O	3.3ml	2.06ml
10% Ammonium persulfate*	50µl	16.7µl
TEMED*	3.3µl	1.7µl

*Ammonium Persulfate and TEMED were added after deaeration

2.2.11. Non-Denaturing SDS-Polyacrylamide Gel Electrophoresis

The purified K4 killer protein and the concentrated *H. anomala* culture media containing the active killer protein were electrophoresed on non-denaturing SDS-polyacrylamide gel (12.5%,1.5mm thick) in a discontinous buffer system [71]. Gel was prepared by using a dual vertical slab gel electrophoresis unit (SE600 Hoefer, USA). Seperating gel composition are given in Table 2.4. Stacking gel composition was the same as that of the Gradient SDS-PAGE (Table 2.2.)

Samples were combined with equal volume of treatment buffer containing no β-mercaptaethanol and were heated at 100^{o}C for 4 minutes prior to electrophoresis. After the polymerization was completed , samples were loaded onto the gel and the gel was run at a constant current of 30mA (Power supply PP4000, Biometra, Germany) at 15 ^{o}C using circulating cooling water bath (Heto Holten, Denmark) until the dye front reached the bottom. The gel was visualized by silver staining [72].

Table 2.4. Non-denaturing SDS-Polyacrylamide seperating gel (12.5%T) composition

Acrylamide-bisacrylamide (30:0.8)	12.5 ml
4X Seperating Gel Buffer (1.5M Tris-Cl , pH:8.8)	7.5 ml
10% SDS	0.3 ml
ddH_2O	9.6 ml
10% Ammonium persulfate*	150 µl
TEMED*	10 µl

*Ammonium Persulfate and TEMED were added after deaeration.

2.2.12. Isoelectric Focusing

The isoelectric point (pI) of the killer protein was determined by a high voltage vertical slab polyacrylamide gel electrofocusing system with a Hoefer SE600 electrophoresis unit as previously described by Giulian *et al.* (1984) [75, 76]. Focusing was carried out on pH 3-10 non-denaturing gradient 0.35mm thick gel containing 2.4% ampholytes and broad range pI markers (Ampholytes and pI markers were supplied from Pharmacia Biotech). The gel composition is given in Table 2.5.

Table 2.5. Isoelectric Focusing Gel Composition

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

*Ammonium Persulfate and TEMED were added after deaeration.

After the addition of ammonium persulfate and TEMED , the gel mixture was poured into the sandwich and the comb (0.35mm thick , 8 well) was inserted. The gel left for one hour for polymerization. After the polymerization completed the wells and upper chamber were filled with catholyte (0.02 M NaOH) and lower chamber of the electrophoresis unit was filled with anolyte (0.02 M acetic acid). The gel was prefocused at 10ºC for 10 minutes with maximum settings of 2500V, 300mA and 20W. Catholyte used was poured off after prefocusing. Upper chamber and sample wells were filled with new catholyte.

Then, K4 killer protein sample and pI markers were loaded on to the gel. Samples were combined with equal volume of sample buffer before loading (Table 2.6.). The focusing was performed at 10ºC using circulating cooling water bath (Heto Holten, Denmark) for 70 minutes at 2000V/h with maximum settings of 2500V , 300mA and 20W (Power supply PP4000; Biometra , Germany).

Table 2.6. Sample Buffer Composition for IEF

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

The gel was stained with coomassie blue R-250. In order to determine the isoelectric point of the K4 type killer protein with respect to the marker proteins , the gel was scanned and the data was processed with gel imaging and analysis system (Vilber Lourmat Gel Imaging and Analysis System, France).

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

2.2.13. Glyco-detection

Carbohydrate portion of the protein was investigated by an enzyme immunoassay method after its transfer to the nitrocellulose membrane by using the buffer system of Towbin *et al.* [77, 78, 79].

DIG-Glycan Detection Kit (Roche Diagnostics, Germany) was used according to the instructions of the manufacturer for glycodetection of immobilized proteins. Method involves the transfer of electrophoresed protein bands from a polyacrylamide gel onto a nitrocellulose membrane. The bound proteins are then available for analysis.

Denaturing SDS-polyacrylamide gel (12.5%,1.5 mm thick) was prepared using a dual vertical slab gel electrophoresis unit (SE600 Hoefer, USA) as previously described [71]. The purified K4 type killer toxin , transferrin and creatinase were loaded onto the gel. Transferrin and creatinase were used as a positive and negative control of glyco-detection , respectively. Sample and control proteins were combined with equal volume of treatment buffer and were heated at 100^{o}C for 4 minutes before loading. Electrophoresis was performed in the normal manner (at a constant current of 30mA ,Power supply PP4000, Biometra, Germany).

Stacking gel from SDS-PAGE gel was removed and discarded after the electrophoresis. The seperating gel was equilibrated in Towbin transfer buffer for 5 to 15 minutes. Nitrocellulose membrane was wet with distilled water and then it was soaked in transfer buffer for 2-5 minutes. Each component of the sandwich cassette were wet in the transfer buffer before assembly. The transfer sandwich was assembled in the proper order under buffer to minimize trapping air bubbles. After the tank was filled with towbin transfer buffer , the cassette was inserted in the proper orientation. Proteins were transferred at a constant current of 1.0 A for 1 hour at 10^{o}C using circulating cooling water bath (Heto Holten, Denmark). After the transfer , the gel was stained by coomassie blue R-250 to verify transfer.

Membrane was washed with dsH_2O for few seconds and was dried with whatman paper. From this point , all the incubations were performed by gentle agitation at room temperature except for color development.

Membrane was washed with PBS (Phosphate buffered saline) solution and then incubated in 10 mM sodium methaperiodate solution in order to oxidize the hydroxyl groups in sugars of glycoconjugates to aldehyde groups. Membrane was washed three times for 10 minutes each with PBS solution.

The membrane was then incubated in DIG-3-0-succinyl-e-aminocaproic acid hydrazid dissolved in sodium acetate buffer for one hour. DIG was covalently attached to aldehydes via a hydrazide group.

The membrane was washed three times for 10 minutes each with TBS solution. The membrane was then stained with Poncue S for 5 minutes and then rinsed with dsH_2O until the bands were visible. Proteins were marked with the help of a needle because Poncue S stain is disappeared during the incubation in the blocking solution last for 30 minutes.

The membrane was washed three times for 10 minutes each with TBS solution , then it was incubated in the Anti-Digoxigenin-AP solution for one hour and washed three times for 10 minutes each with TBS solution.

Lastly , the membrane was stained by immersing it in staining solution without shaking. Within the few minutes positive signals (black colour) was developed on the membrane in the marked positions corresponding to the killer protein and transferrin. In order to stop the reaction the membrane was rinsed with dsH_2O with several times and leaved for dry.

2.2.14. Protein Detection in Gels

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

2.2.14.1. Coomassie Blue Staining

Coomassie brilliant blue R-250 staining was performed as previously described by Wilson (1983) [73]. The gel was stained with staining solution for 2 hours with slow shaking. Then staining solution was replaced with destaining solution I for 30 min. to remove bulk of the excess stain and then the gel was transferred into the destaining solution II. Destaining solution II was changed twice a day until the backround was clear.

Isoelectric focusing gel was stained with different coomassie blue solution by using a method as previously described by Giulian *et al.*(1984) [75, 76]. IEF gel was first gently shaked in the fixative I solution for 5 to 10 minutes. Fixative I solution was replaced with fixative II solution and the gel was shaked slowly for 5 to 10 minutes. The gel was then washed with destaining solution for 5 to 10 minutes for two times and then the gel was stained with IEF staining solution for 1 hour. Destaining solution was changed until the backround was clear. Solutions were changed by the help of the peristaltic pump.

2.2.14.2. Silver Staining

Silver staining was performed as previously described [72]. 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 shaked 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.

CHAPTER III

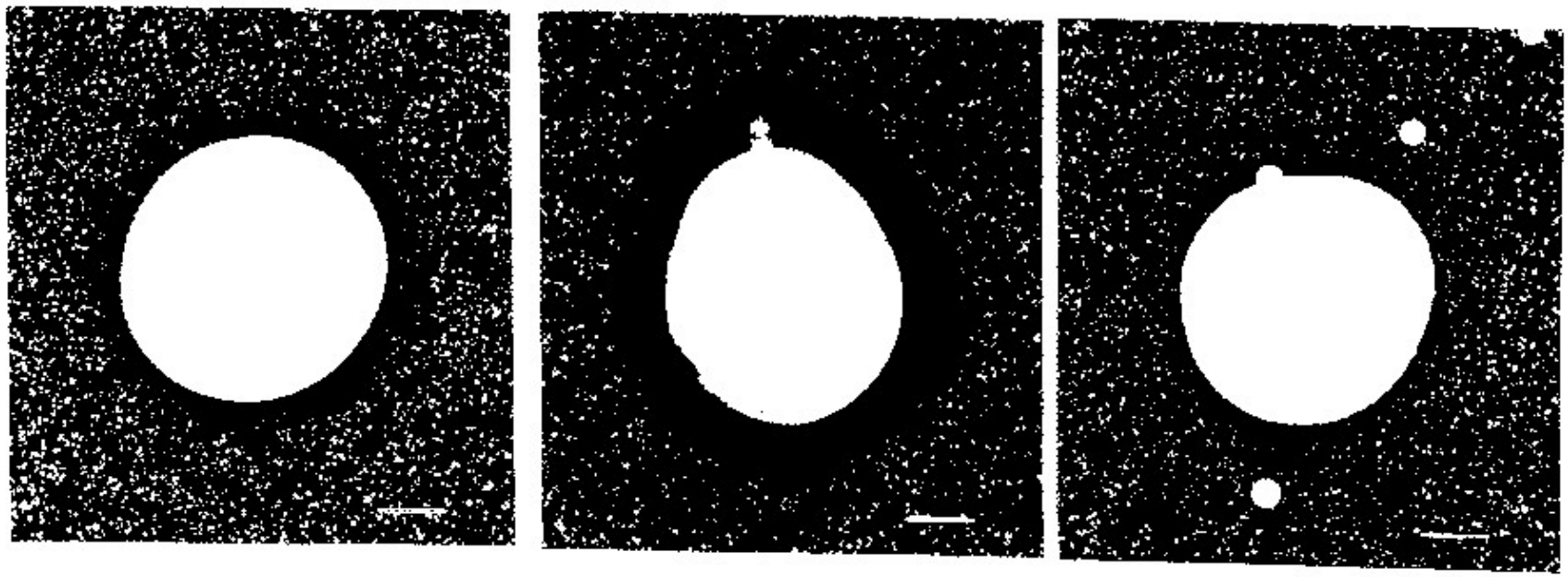
RESULTS

3.1. Determination of optimum culture conditions for killer toxin production

The production of yeast killer toxins was pH and temperature dependent [80, 81, 82]. The growth conditions for the toxin production was determined by the killer zone assay in a plate test [49,69]. *H. anomala* NCYC 432 was grown on YEPD plates seeded with cells of the toxin sensitive *S. cerevisiae* NCYC 1006 , at different pH values covering the range 3.0-6.0 in intervals of 0.5. Incubations were carried out at 16-35 ^{o}C in intervals of 2 ^{o}C. Toxin production was observed in a pH range from 3.0-6.0 and over a temperature range of 18-30 ^{o}C. The maximum production of toxin was at pH 4.5 and at 22 ^{o}C as revealed by the largest growth inhibition zone of the killer sensitive *S. cerevisiae* NCYC 1006 (Figure 3.1.).

3.2. Production and Concentration of Yeast Killer Toxin

For killer protein production , *H. anomala c*ells were grown in 1 liter YEPD medium (containing 5% glycerol) buffered to pH 4.5 with 0.1 M citrate-phosphate buffer at 22ºC for 36 hours at 120 rpm on a gyratory shaker. The cells were removed by centrifugation at 1000 x g for 10 minutes at 4 ºC and culture supernatant filtered through 0.4 μm and 0.25 μm cellulose acetate membranes respectively. The concentration of the protein mixture was 2,8 mg/ml.



pH: 3.5 pH: 4.5 pH: 6.0

Figure 3.1. Killer zone assay at pH 3.5 , pH 4.5 and pH 6.0 at 22 °C. Bar scale represents 4 mm.

Concentration of the killer protein in the culture supernatant was performed with 30.000 MWCO filter (Vivaspin VS2021, Sartorius) to a final concentration of 28 mg/ml. Thus the protein mixture was concentrated 10 folds. The killer activity of the protein in the concentrated cell free extract was determined by the killer zone assay in a plate test before it was used in the purification steps (Figure 3.2.).

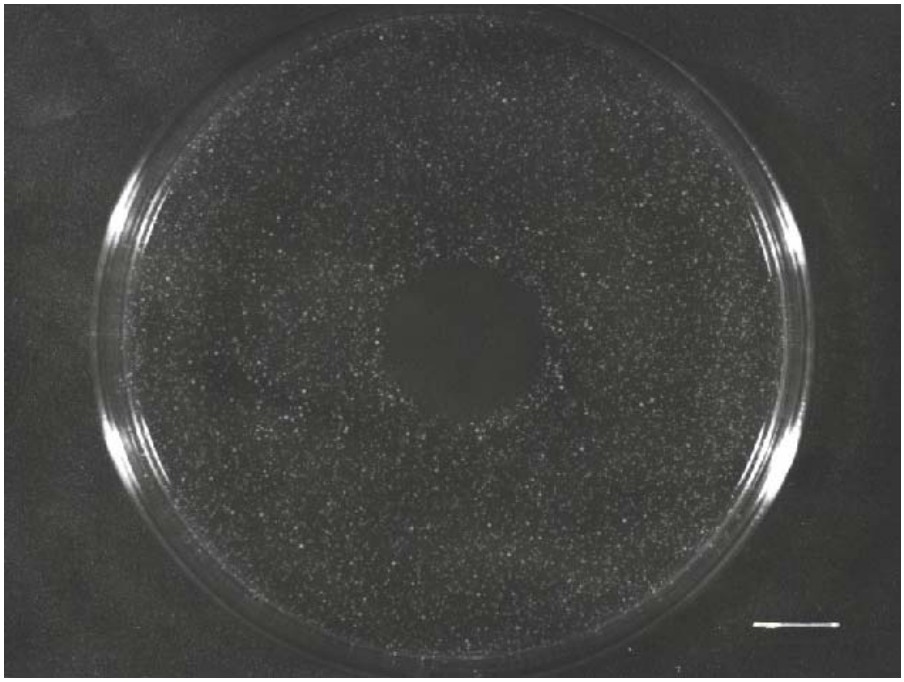


Figure 3.2. Growth inhibitory activity of the the killer toxin on sensitive *S.cerevisiae* NCYC 1006 in YEPD medium , at pH 4.5 , 22 ^{o}C. 25µl of concentrated protein mixture (28 mg/ml) was applied. Bar scale represents 11 mm.

3.3. Isolation of the Killer Toxin

Gel permeation chromatography was performed to isolate the killer toxin by using a HPLC system , BioCAD 700E Workstation. The column used was TSK G2000 SW (7,5 mmD/300mmL). Concentrated cell free extract was buffer exchanged to 0.1M Na_2HPO_4 at pH 4.5 containing 0.1M Na_2SO_4

with 5000 MWCO ultrafilter device. Buffer exchange step was performed three times at 4200 x rpm for 15 min. at 4°C. Concentrated and buffer exchanged protein mixture was then filtered through 0.25µm syringe filter before injected onto the column in order to prevent any particles to clog the HPLC system and the column. The column was equilibrated with the eluent buffer 0.1M Na_2HPO_4 at pH 4.5 containing 0.1M Na_2SO_4 at a flow rate of 1 ml/min. Then , 90µl of sample (28 mg/ml) was injected. Elution was performed with the same buffer and the same flow rate (Figure 3.3.).

The fractions were collected (850µl for each run) automatically by fraction collector and then they were concentrated seperately by ultrafiltration with 5000 MWCO ultrafilter device (Figure 3.4.). Concentrated fractions were then tested for killer activity by killer zone assay in a plate test. Fraction No:11 showed killer activity (Figure 3.5.). After several runs, the fraction with killer activity was collected , concentrated (6 mg/ml) and for its further purification , it was injected again to gel permeation chromatography column TSK G2000 SW (Figure 3.6.). The major peak was collected (Figure 3.7.) , concentrated (2 mg/ml) and assayed for killer activity to prove that it was the killer protein (Figure 3.8.). For further analysis , glycerol (15% v/v) was added to the killer protein solution and it was stored at –20 °C.

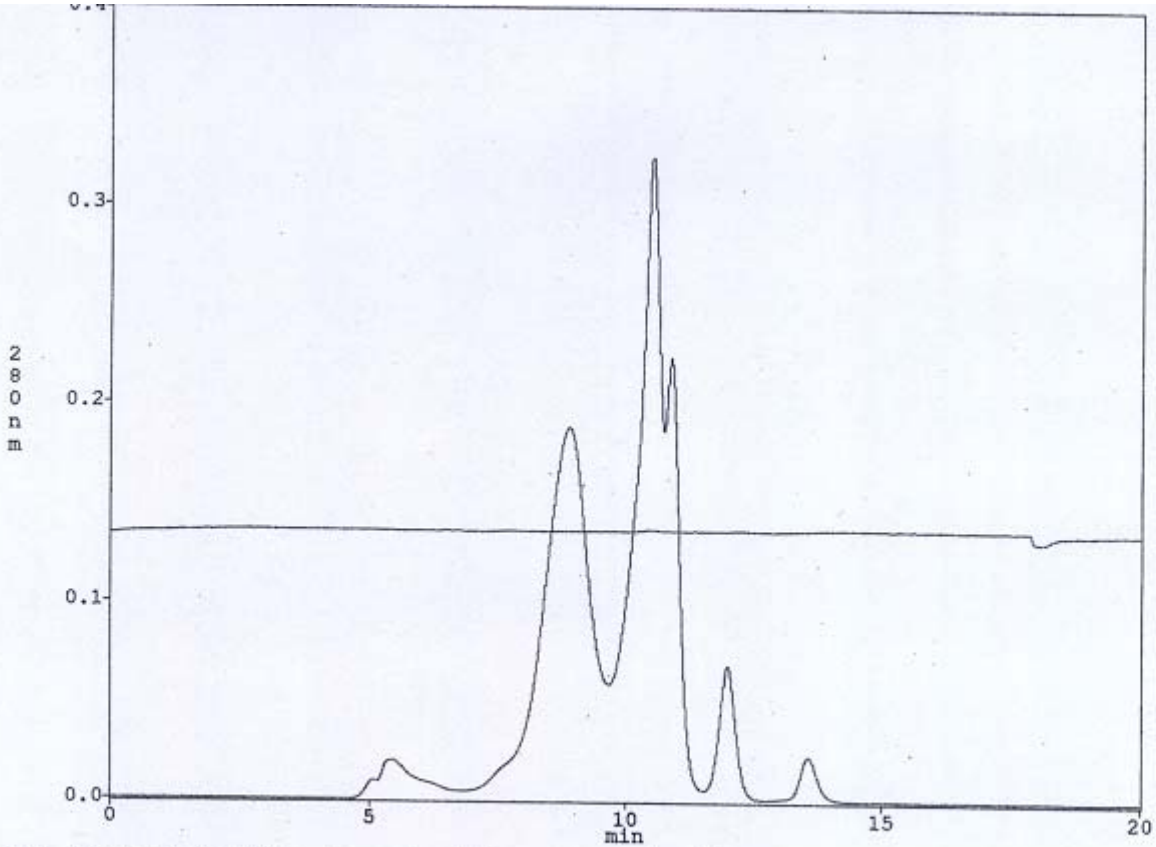


Figure 3.3. Gel Permeation Chromatography of the concentrated cell free extract containing active killer protein (28mg/ml protein mixture) ; sample , 90µl ; column TSK G2000 SW (7.5 mmD/300 mmL) ; buffer , 0.1M Na_2HPO_4 pH 4.5 + 0.1 M Na_2SO_4 ; flow rate 1ml/min ; detection , 280nm ; system , BioCAD 700E Perfusion Chromatography Workstation.

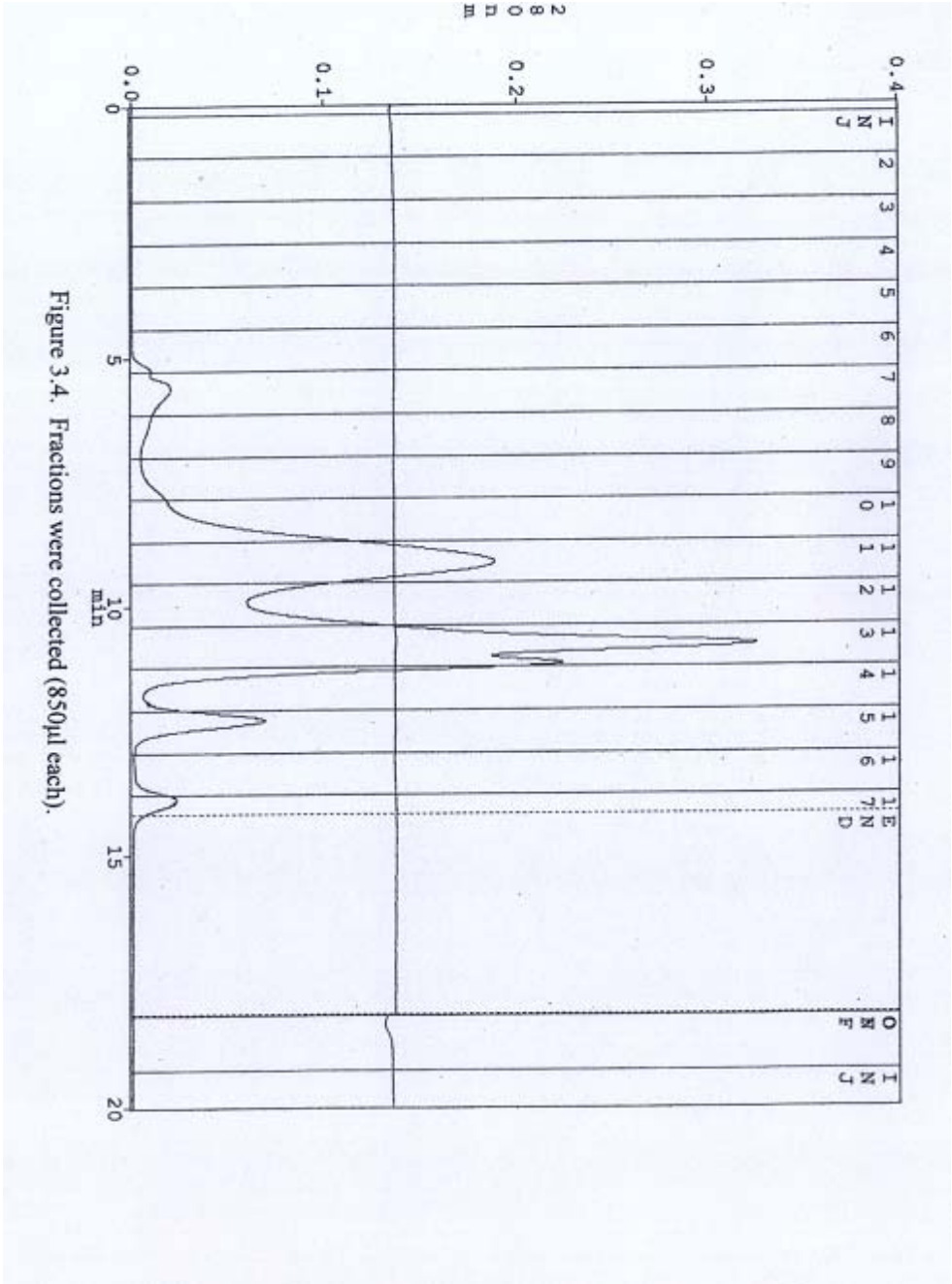


Figure 3.4. Fractions were collected (850µl each).

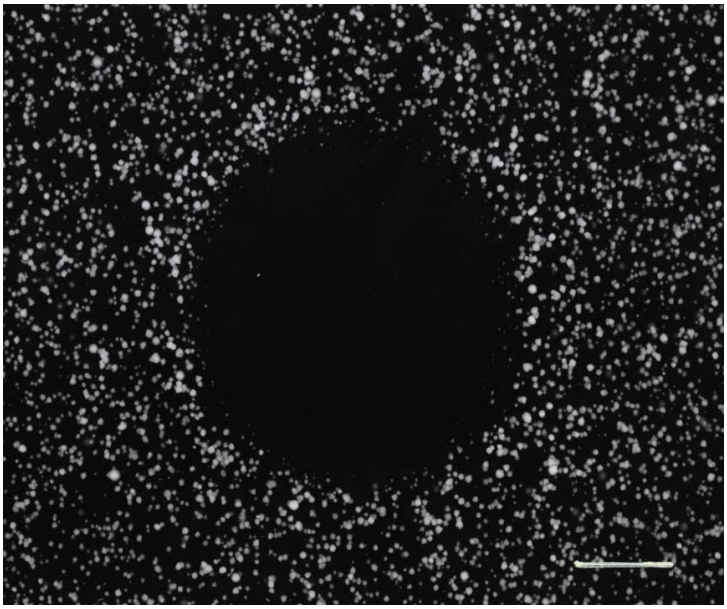


Figure 3.5. Fraction No:11 (10µl , 60µg) had the killer activity (~2.5 AU*). Bar scale represents 6 mm.

*One Arbitrary Unit is the amount of killer toxin which produces 8.5 mm diameter clear inhibition zone [47].

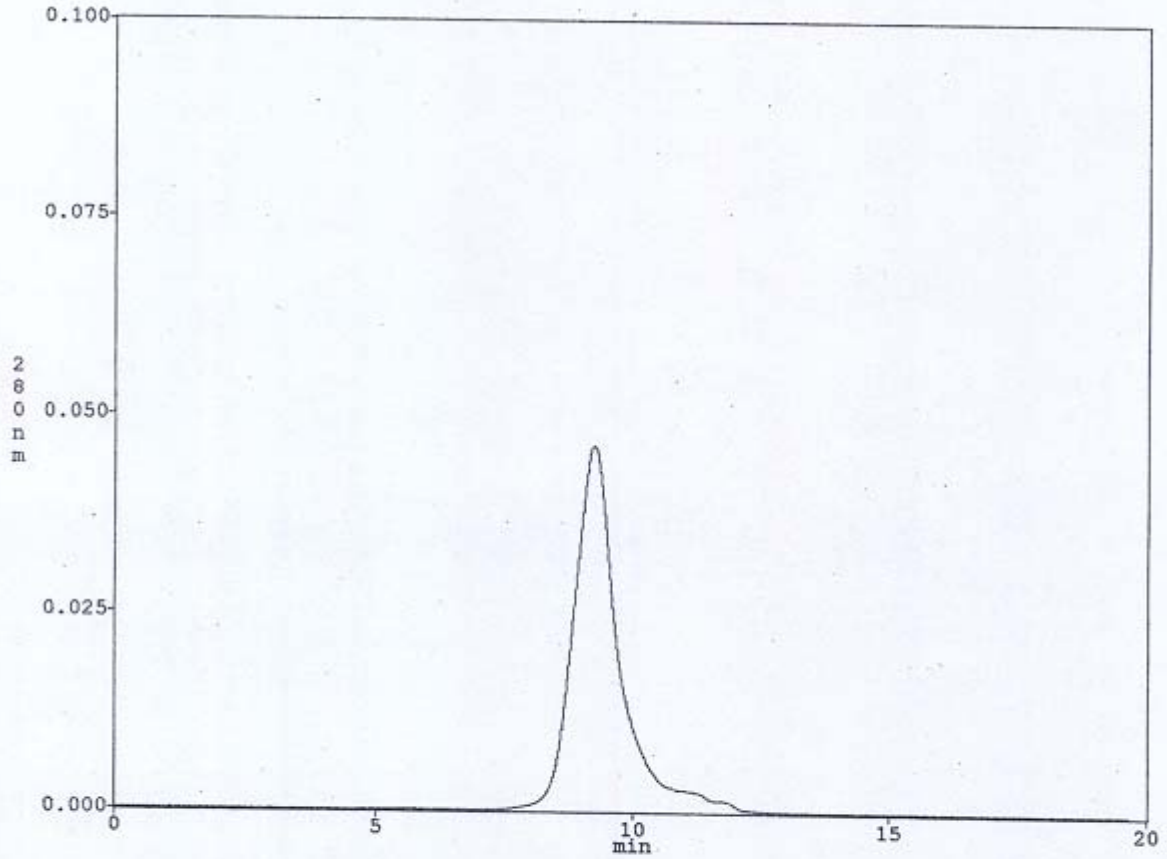


Figure 3.6. GPC separation of active fraction (No:11) obtained from first GPC. 90µl of sample (6mg/ml) was injected.

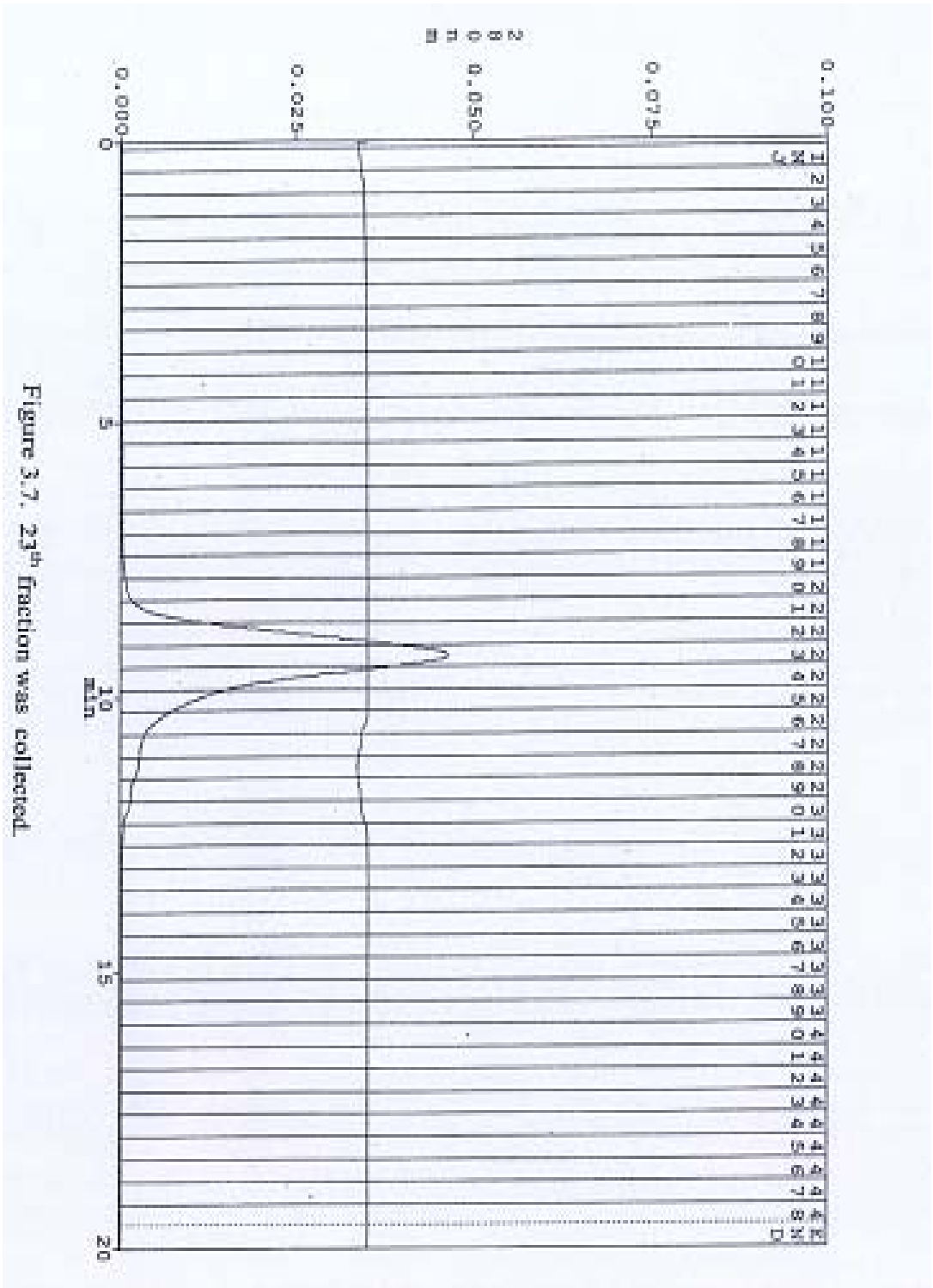


Figure 3.7. 23^{h} fraction was collected.

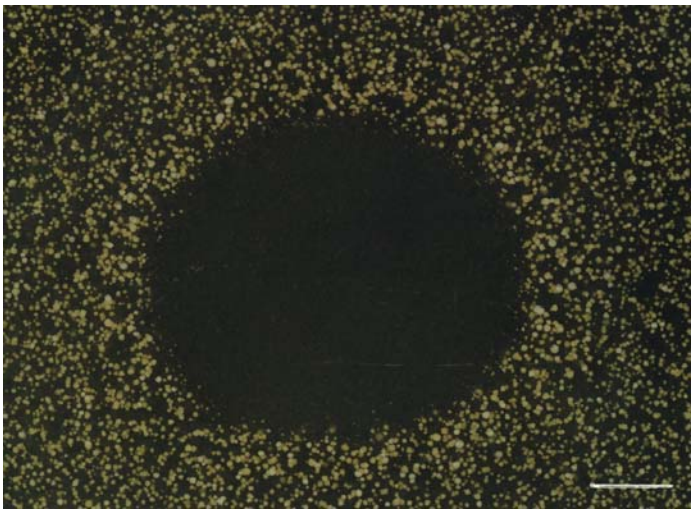


Figure 3.8. Killer activity (~1 AU) of the K4 type yeast killer toxin (10µl , 20µg) purified from GPC. Bar scale represents 2 mm.

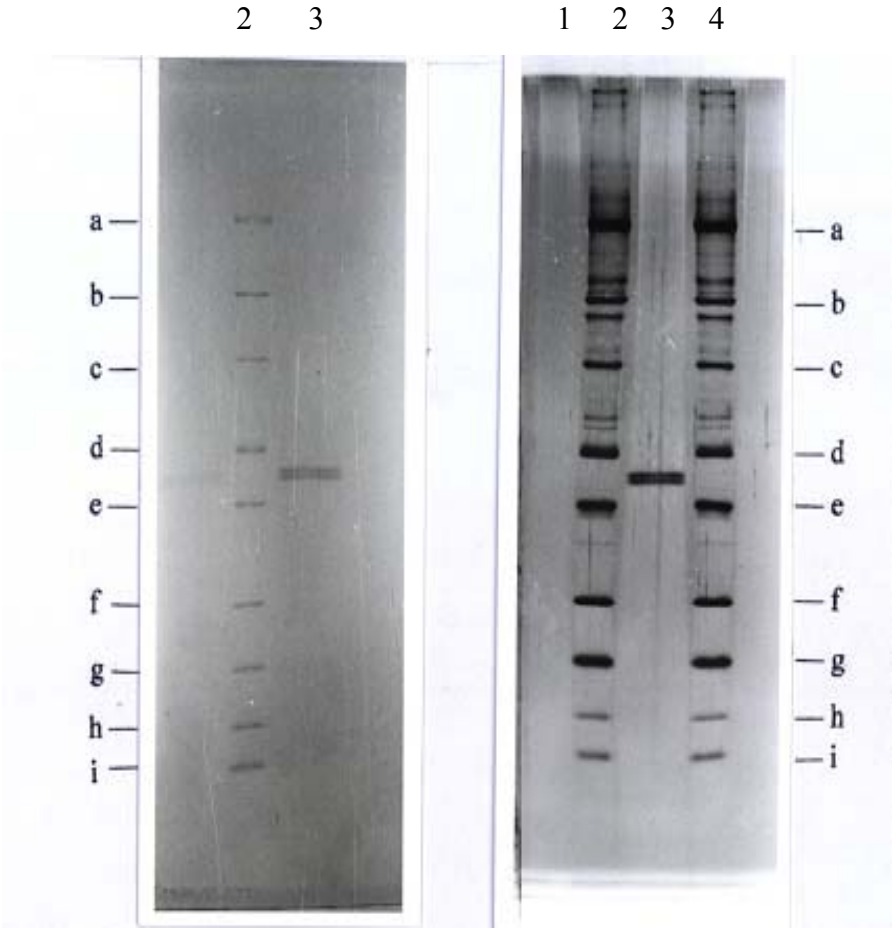
3.4. Non-Denaturing Gradient SDS-Polyacrylamide Gel Electrophoresis

Isolated killer protein (2µg) was electrophoresed on a 5%-20% linear gradient SDS-PAGE gel in a discontinous buffer system under non-denaturing conditions to check its purity. Observation of single protein band on the silver stained gel indicates the absence of any contamination (Figure 3.9.).

Figure 3.9. Non-Denaturing Gradient SDS-Polyacrylamide gel of the isolated K4 type yeast killer toxin.

3.5. Molecular Weight Determination

The purified killer protein (2µg) was electrophoresed on 5%-20% linear gradient SDS-PAGE gel in a discontinous buffer system under denaturing conditions. Molecular weight markers were used to determine its molecular weight. The protein migrated as doublet placed between glutamate dehydrogenase (55.562 kDa) and aldolase (39.212 kDa). (This will be discussed in discussion section). The molecular weight of the killer protein was between 49.089 kDa and 47.255 kDa. (Figure 3.10.)



A) B)

Figure 3.10. Denaturing Gradient SDS-Polyacrylamide Gel of K4 type yeast killer protein. A) Coomassie Blue and B) Silver Stained gels. Lane 2 and 4 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) , h) lysozyme (14.307 kDa) , i) aprotinin (6.500 kDa). Lane 3 is K4 type yeast killer toxin.

3.6. Peptide Mapping

In-gel digestion of the protein bands excised from denaturing SDS-polyacrylamide gel was performed with endoproteinase Glu-C and trypsin proteases. The second denaturing SDS-polyacrylamide gel in which digestion took place was visualised by silver staining. Observation of identical fragmentation patterns for both bands indicates isomeric forms of the isolated K4 type killer protein (Figure 3.11.).

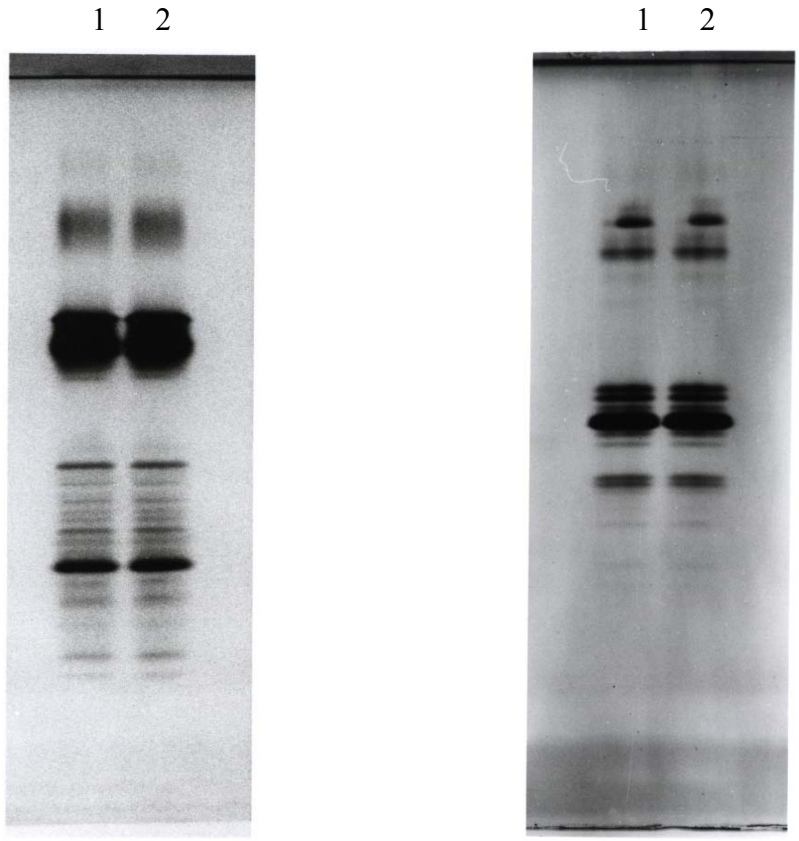


Figure 3.11. Peptide Maps of two bands in the doublet excised from denaturing SDS-polyacrylamide gel. Gel was visualised by silver staining. Endoproteinase Glu-C (a) and trypsin (b) proteases were used seperately for each band (at a protease to substrate ratio of 1:20). Lane 1 is upper band and lane 2 is lower band.

3.7. Native Polyacrylamide Gel Electrophoresis

The K4 type yeast killer toxin (2µg) was electrophoresed on 12.5% polyacrylamide gel in a discontinous buffer system under native conditions. The gel was stained with coomassie brilliant blue R250. Purified protein was seen as closely migrated two bands (Figure 3.12.).

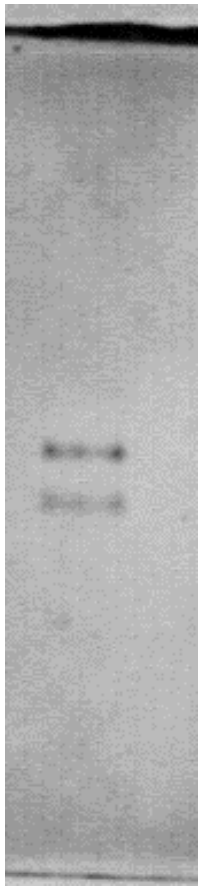


Figure 3.12. Native-PAGE profile of purified K4 type yeast killer toxin.

3.8. Non-Denaturing SDS-Polyacrylamide Gel Electrophoresis

The K4 type yeast killer toxin (2μg) and concentrated culture supernatant of *H. anomala* containing the active killer toxin was subjected to 12.5% non-denaturing SDS-PAGE in order to compare the protein profiles (Figure 3.13.).

Figure 3.13. Non-Denaturing SDS-PAGE profile of concentrated culture supernatant containing the killer protein (A) and the isolated K4 type killer toxin (B). Protein bands in the gel were visualized by silver staining.

3.9. Isoelectric Point Determination

The isoelectric point (pI) of the killer protein was determined by a high voltage vertical slab polyacrylamide gel electrofocusing in the pH range of 3-10 with broad range pI marker proteins. Protein appeared as closely migrated two bands in the gel with isoelectric points of 3.77 and 3.41 (indicated by arrows) (Figure 3.14.).

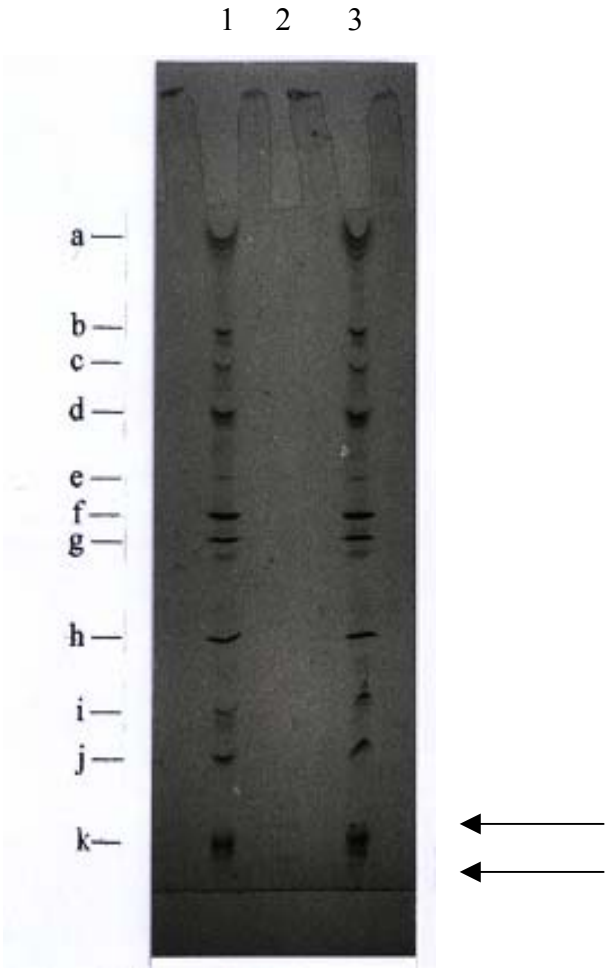


Figure 3.14. Polyacrylamide gel electrofocusing of K4 type yeast killer toxin in native state. Lane 1 and 3 are pI markers ; (a) trypsinogen 9.30; (b) lentil lectin-basic band 8.65; (c) lentil lectin-middle band 8.45; (d) lentil lectin-acidic band 8.15; (e) myoglobin-basic band 7.35; (f) myoglobin-acidic band 6.85; (g) human carbonic anhydrase 6.55; (h) bovine carbonic anhydrase 5.85; (i) β-lactoglobulin A 5.20; (j) soya bean trypsine inhibitor 4.55; (k) amyloglucosidase 3.50. Lane 2 is K4 type yeast killer toxin.

3.10. Glyco-detection

The purified K4 type yeast killer toxin was investigated for its carbohydrate portion by an enzyme immunoassay method using DIG-Glycan Detection Kit (Roche Diagnostics, Germany). Positive signals (black colour) on the membrane in the positions corresponding to the killer protein and the positive control protein (transferrin) indicates that the K4 type killer protein is a glycoprotein. Negative control protein (creatinase) gave no signal. It was also seen clearly that upper and lower bands of the doublet which was formed in the reducing SDS-PAGE gave positive signals seperately (Figure 3.15.).

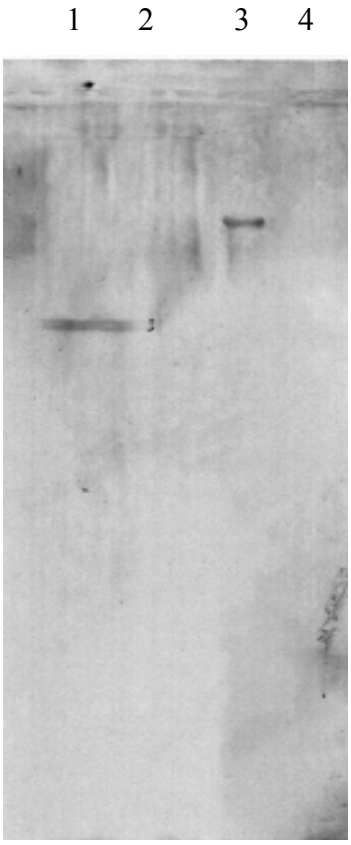


Figure 3.15. Glyco-detection of purified killer toxin. Positive signals on the nitrocellulose membrane indicate proteins having glycoconjugate. Lane 1, K4 type yeast killer toxin; Lane 3, Transferrin (glycoprotein) ; Lane 4, Creatinase (non-glycosylated protein).

CHAPTER IV

DISCUSSION

The killer phenomenon is widespread among various yeast genera and species. Killer yeasts secrete into the medium protein compounds known as killer toxins to eliminate competative strains and possess a resistance system against their own toxin[83, 84]. Also some of these toxins were inhibitory not only to other yeast species but also Gram (+) pathogenic bacteria [11] and plant pathogenic fungi [12]. Although there is considerable amount of published information concerning the applications of yeast killer toxins , only K1 , K2 , K6 have been characterized. In this study we aimed to isolate and characterize K4 type yeast killer protein secreted by the yeast *Hansenula anomala* NCYC 432.

Killer toxins share a common characteristic in that they are pH and temperature dependent. They usualy active and stable at pH 4–5 and 20–25°C and each toxin has an optimum pH and temperature at which it show its killer character more intense [19, 82, 85, 86, 87]. Thus , we first attempted to determine the optimum culture conditions of *H. anomala* for the production of K4 type killer toxin by killer zone assay test as previously described [49, 69]. *H. anomala* NCYC 432 was grown on YEPD medium at different pH values covering the range 3.0-6.0 in intervals of 0.5 and incubation was carried out at 16-35 ^{o}C in intervals of 2 ^{o}C. Toxin production was observed in a pH range from 3.0-6.0 and over a temperature range of 18-30 ^{o}C. The maximum production of toxin was at

pH 4.5 and at 22 ^{o}C as revealed by the largest growth inhibition zone of the killer sensitive *S. cerevisiae* NCYC 1006. Therefore , for the killer protein production , *H. anomala c*ells were grown in YEPD medium (containing 5% glycerol) buffered to pH 4.5 with 0.1 M citrate-phosphate buffer at 22ºC.

The culture supernatant was subjected to ultrafiltration with different cut-off values (10 , 30 , 50 , 100 kD) to determine the best cut-off value in which the killer toxin mostly retained in the supernatant while eliminate other proteins as much as possible. It was determined from the killer zone assay that the killer protein retained in the supernatant of 30kD filter and mostly filtered through 50kD filter. Thus , concentration of the killer protein in the culture supernatant was performed with 30kD centrifugal filter device.

Isolation of the K4 type killer toxin was performed by gel permeation chromatography (GPC). Gel permeation chromatography has been described by several other terms including , size exclusion , gel filtration , molecular sieve chromatography or even simply as gel chromatography. GPC seperates molecules on the basis of their size or more accurately their hydrodynamic volume. Gel permeation chromatography depends on the fact that within each particle of the stationary phase -that is the column packing material- there is a distribution of pore sizes. For small enough molecules , the pores are so large that the molecules can penetrate all of the internal volume of the particle resulting in delay in elution time. If the molecules are large enough , the pores are so small that the molecules are completely excluded from the internal volume , therefore exclusion of larger molecule is earlier than small molecule. Molecules in between will have access via diffusion to a portion of the internal volume but will be excluded by the smaller pores from the rest. Molecules are thus eluted in order of their dectreasing size [88, 89].

Different from other chromatographic modes , in gel permeation chromatography there should be no interaction between the sample and the surface of the stationary phase. The proper choice of pH and ionic strength is

essential to reduce the interaction between the column packing and sample during chromatographic method. As the ionic strenght of the mobile phase was increased , ionic interactions between the column stationary phase and the sample were reduced. On the other hand , with increasing ionic strength the hydrophobic interactions were increased between the column and sample. So , to minimize both ionic and hydrophobic interactions the ionic strength of the mobile phase was kept at 0.1M [90, 91, 92,93, 94].

The column used is also a very important factor that effects the seperation greatly. TSK gel columns were tried with different buffer compositions in order to obtain best resolution without loosing the activity of killer protein. The columns used were TSK G2000 SW , TSK G3000 SW and TSK G2000 SW_{XL}. These silica-based columns are bonded with hydrophilic compounds to reduce the adsorption of proteins. The resolution in the chromatogram obtained with TSK G2000 SW_{XL} column was poor. Although similar chromatograms were obtained with TSK G2000 SW and TSK G3000 SW , the resolution obtained with TSK G2000 SW column was better. The resolution difference between the chromatograms of these two columns was tought to be due to the seperation ranges of the columns. The seperation ranges of TSK G2000 SW and TSK G3000 SW were 5000-100.000 Da and 10.000-500.000 Da respectively [95, 96].

TSK G2000 SW column gave the best resolution with the eluent buffer of 0.1M Na_2HPO_4 buffer at pH 4.5 containing 0.1M Na_2SO_4. The column was equilibrated with this buffer at a flow rate of 1ml/min. Elution was performed with the same buffer and the same flow rate and detected at 280 nm at an ambient temperature of 25ºC. The concentrated cell free filtrates was buffer exchanged to eluent buffer with 5 kDa centrifugal filter device before it was applied to the column. Fractions were collected seperately by fraction collector and concentrated. Then, in order to determine the peak corresponding to killer protein , they were assayed for killer activity against toxin sensitive *S. cerevisiae* NCYC 1006 in a killer zone assay. It was observed that fraction No:11 had the killer activity and it was collected for several runs. Then , for its further purification again applied to

the gel permeation column. Major peak in the chromatogram had the killer activity and was collected for several runs for its subsequent analysis.

Although isolated killer protein was observed as a single band without any contamination in the silver stained non-reducing gradient SDS-PAGE gel , same protein was observed as doublet in the reducing gradient SDS-PAGE gel. (Figure 3.9. and 3.10.).

Several assumptions were considered regarding to doublet formation including possibility of killer protein having more than one subunit , incomplete reduction , variations in carbohydrate composition or isomerization [97].

The possibility of the killer protein having more than one subunit is highly unlikely , because if protein have subunits , they would have migrated faster in reducing SDS-PAGE gel. But migration patterns of protein observed in both reducing and non-reducing SDS-PAGE gel were identical. Additon of more reducing agent (β-mercaptoethanol) to the treatment buffer –before and after the treatment- or usage of another reducing agent (DTT) didn't change the result. Variations in carbohydrate composition is another possibility. But , both protein bands in the doublet was shown to be glycosylated and gave signal in the glyocodetection at the same intensity (Figure 3.14.).

It was thought that this doublet resulted from two isomeric forms of the K4 type toxin. In order to ensure , in-gel digestion of the protein bands in the doublet excised from the reducing SDS-PAGE gel was performed with endoproteinase Glu-C and trypsin proteases. The method , which is especially suitable for analysis of proteins which have been isolated from SDS gels , involves the partial digestion of proteins by proteases in the presence of SDS and analysis of the cleavage products by polyacrylamide gel electrophoresis. It is known that every protein in nature have unique peptide pattern for every proteolytic enzyme [74]. It was observed that peptide patterns so generated were identical for both protein bands.

So , it was concluded from this result that two bands in the doublet were the isomers of the killer toxin (Figure 3.11).

Observation of a closely migrated two bands in the isoelectric focusing and native-PAGE of the purified K4 type killer protein was due to the isomeric forms of the same protein.

Molecular mass of the purified K4 type killer protein (between 49.08 kDa and 47.25) was higher than K1 (20kDa) [49] , K2 (21kDa) [49] , K6 (42.3kDa) [15] type killer proteins. When compared with the others which have been defined but not yet classified it is larger than the toxins of *S. cerevisiae* (16kDa) [98] , *H. saturnus* (8.5kDa) [99] and *H. mrakii* (10.7kDa) [100] except that of *K. lactis* (180kDa) [101].

Isoelectric point of the K4 type killer protein (between 3.77 and 3.41) was lower when compared to previously characterized K1 (pI:4.3) [49] , K2 (pI:4.5) [102] and K6 (pI:5.9) [15] type killer proteins. It is an acidic protein as all the other known killer toxins with the exception of unclassified killer toxin of *H. mrakii* (pI:9.10) [100] and glycosylated. Some characteristics of the classified and unclassified killer toxins were given in Table 4.1.

K4 type killer protein has a wide killing spectrum and has a potential to be used in environmental and industrial biotechnology particularly in the medical field as a topical antimycotic agent. Purification and characterization of the K4 type yeast killer protein will be of help in large scale purification of this protein for industrial purposes.

Table 4.1. Comperison of the K4 type yeast killer toxin with other yeast killer toxins.

Sources: [a]This study , [b]Bussey *et al.* [49] , [c]Izgu *et al.* [15] , [d]Pfeifer P. *et al.* [102] , [e]Pfeifer P. And Radler F.[98] , [f]Ohta Y. *et al.* [99] , [g]Sugisaki Y. *et al.*[101] , [h]Yamamoto *et al.* [100].

	Classified Killer Toxins			Unclassified* Killer Toxins			
Property	K4 type *H.anomala*	K1 type *S. cerevisiae*	K2 type *S. cerevisiae*	K6 type *K. fragilis*	*S. cerevisiae*	*H. saturnus*	*K. lactis*	*H. mrakii*
Molecular weight (kDa)	[a]49 – 47.2	[b]20,65	[b]21	[c]42,313	[e]16	[f]8,5	[g]180	[h]10.7
Isoelectric Point (pI)	[a]3.7-3.4	[b]4,34	[d]4,5	[c]5,97	[e]4,5	[f]5,8	[g]4,8	[h]9.10

*Unclassified , indicates the killer toxins have not yet been classified according to the criteria of Young and Yagiu [14].

CHAPTER V

CONCLUSION

1. *H. anomala* NCYC 432 was found to be produce K4 type killer toxin in the pH range of 3.0-6.0 and over a temperature range of 18-30 ^{o}C. The maximum production of toxin was at pH 4.5 and at 22 ^{o}C.

2. Due to its isomeric form , the purified toxin migrated as doublet in the reducing SDS-PAGE gel and closely migrated two bands in the IEF and Native-PAGE gels.

3. It is a glycoprotein having molecular mass of between 49.08 kDa and 47.25 kDa.

4. Isoelectric point of the toxin was between 3.77 and 3.41.

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APPENDIX A

CHEMICALS AND THEIR SUPPLIERS

Acetic Acid (Merck, Germany)

Aceton (Merck, Germany)

Acrylamide (Boehringer Mannheim, Germany)

Ammoniumpersulphate (Pharmacia Biotech, Sweden)

Bacto-agar (Difco, USA)

Bacto-peptone (Difco, USA)

Bis-acrylamide (Boehringer-Mannheim, Germany)

Bromophenol Blue (Sigma, USA)

β-mercaptoethanol (Sigma, USA)

Citric Acid (Merck, Germany)

Coomassie Brilliant Blue R-250 (ICN, USA)

Dithiothreitol (DTT) (Boehringer Mannheim, Germany)

D-Glucose (Merck, Germany)

Di-sodium Hydrogen Phosphate (Merck, Germany)

Di-potassium Hydrogen Phosphate (Merck, Germany)

Ethanol (Merck, Germany)

Formaldehyde (Riedel-de Haen, Germany)

Glutaraldehyde (Fluka, Switzerland)

Glycerol (Merck, Germany)

Glycine (Merck, Germany)

Hydrochloric Acid (Merck, Germany)

Methanol (Merck, Germany)

Potassium Dihydrogen Phosphate (Merck, Germany)

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)

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.
Coomassie Blue Stain	0.025% Coomassie Brilliant Blue R-250, 40% Methanol , 7% Acetic Acid.
Destain Solution I	40% Methanol , 7% Acetic Acid
Destain Solution II	5% Methanol , 7% Acetic Acid

Silver Stain

Destain Solution I	40% Methanol , 7% Acetic Acid
Destain Solution II	5% Methanol , 7% Acetic Acid
Cross-linking Solution	10% Glutaraldehyde
Dithiothreitol(DTT) Solution	5 µg/ml
Silver Nitrate Solution	0.1% w/v
Sodium Carbonate	3% w/v
Developing Solution	3% sodium carbonate , 0.019% formaldehyde.

2. Isoelectric Focusing

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

3. Western Blotting and Glycodetection

Towbin Transfer Buffer	25mM Tris , 192mM Glycine , 20% MeOH , 0.1% SDS
PBS (Phosphate buffered saline)	50mM Potassium Phosphate , 150mM NaCl , pH:6.5.
Sodium Acetate Buffer	100mM Sodium Acetate , pH 5.5

Sodium metaperiodate	10mM Sodium metaperiodate in Sodium Acetate Buffer.
DIG Solution	6µl DIG in 30 ml Sodium acetate buffer
TBS (Tris Buffered Saline)	0.05M Tris-HCL , 0.15M NaCl pH 7.5.
Poncue S Solution	0.2%(w/v) Poncue S in 3% (w/v) Acetic Acid.
Blocking Solution	10 ml Blocking reagent + 90 ml TBS
Anti-DIG Solution	40µl Anti-DIG in 40 ml TBS
Staining Solution	1 ml NBT/X-phosphate solution in 50 ml Tris buffer (pH 9.5).