Dr. habil. Anna Teresa Salek
Study was carried at the Technical University of Munich, Germany
at the University of Regensburg, Germany
and at the INROPHARM, Veterinary-Pharmaceurical Products GmbH & Co. KG,
Passau, Germany, Director: Dr. med. vet. Ferdinand Rott

YEAST ANTIMICROBIAL PROTEINS

INTRODUCTION

1. Specific secretory system - glycosylated yeast killer protein

The killer phenomenon has been reported for strains of the genera *Saccharomyces, Kluyveromyces, Hansenula* (or *Pichia*), *Hanseniaspora, Candida, Torulopsis, Debaromyces, Cryptococcus* and *Ustilago* (1, 2, 3, 4, 5, 6, 7, 8). The above-mentioned yeasts produce toxins which act against sensitive strains of the same or closely related species as well as against unrelated microorganisms, including pathogenic yeasts.

The mechanisms of toxicity are various: 1). a pore-forming channels in the cell wall (i.e. enter the cytosol and attack essential constituents), 2). an inhibition of protein synthesis, or 3). an arrest of the G_1 phase of the cell cycle (6, 9, 10, 11, 12, 13).

Many of yeast killer toxins are glycoproteins. Most of them consist of membrane-binding subunits that interact with carbohydrates, such as 1,6-β-D-glucan (found e.g. on the cell wall of *Saccharomyces cerevisiae* (6, 10) or *Williopsis mrakii* (14) or with α-mannan (from the surface of *Candida albicans* (14)) while toxin α-subunit of *Kluyveromyces lactis* has a potential chitinase activity and may play an essential role in initial contact of the toxin with the cell surface (15). Moreover, the γ toxin subunit of *Kluyveromyces lactis* is transported inside the cell and modifies the cell cycle or the activity of critical cellular components (1).

Generally, the sugar-binding membrane anchor subunits of each killer toxin are very diverse in amino acid sequence and able to bind to various carbohydrate receptors, and can, therefore, kill various microorganism, including pathogenic yeast.

In the killer phenomenon of yeasts, various genetic determinants encode killer toxins (1, 2, 6, 10, 16). The best characterised yeast killer system (K_1 killer) of *Saccharomyces cerevisiae* is mediated by two linear dsRNA plasmids, M_1dsRNA and L_{1A}dsRNA, which reside in Virus Like Particles, VLPs. Such killer system produces in the secretory pathway (through the Golgi apparatus (17)) a glycosylated preprotoxin (43 kD) as the intracellular precursor of a secreted protoxin. This protoxin contains the leader peptide, the toxin (α and β-heterodimeric subunits of 9.0 and 9.5 kD, respectively) and the glycosylated γ peptide (which remains in the cell wall during secretion of the α-β heterodimer). The toxin of above-mentioned *Saccharomyces cerevisiae* is an unglycosylated protein (18 kD) and kills sensitive strains of the same or related species (6).

Further types of toxins (K_2, K_3, K_{28}) have also been found in the genus of *Saccharomyces*. These are distinguishable from K_1 killer, and from each other, by their killing and immunity specificities (3). Generally speaking, K_1 toxin is normally found in laboratory

strains (6, 10, 18), K_2 and K_{28} occur in wine yeast (19, 20) whilst the K_3 is produced by other strains of *Saccharomyces* sp. (10).

A number of killer yeast which belong to *Kluyveromyces, Hansenula/Pichia* and *Debaromyces* carry linear double-stranded DNA (dsDNA) plasmids. As far as examined, however, the linear dsDNA-plasmids from *Kluyveromyces lactis* and *Pichia acaciae* are responsible for the killer activity only (1, 12, 21, 22, 23, 24). *Kluyveromyces lactis* strains, harboring pGKL1 and pGKL2 plasmids, secrete a killer toxin (glycoprotein), consisting of three subunits, α (97 kD), β(31 kD) and γ (28 kD). This toxin has a very broad killer spectrum against yeasts of different genera and species (1). In *Pichia acaciae* two plasmids, p*Pac*1-1 and p*Pac*1-2 are responsible for killer protein production (23).

On the other hand, some killer toxins of *Pichia* spp., *Hansenula, Debaromyces hansenii* and recombinant strains of *Saccharomyces cerevisiae* are chromosomally coded (1, 25, 26, 27). These killer toxins are produced as single peptides or proteins, or glycoproteins.

Despite, our knowledge about above genera, i.e. toxins from some killer strains, including those *Williopsis* (or *Hansenula*) sp., have undefined genetic origins. For instance, *Williopsis mrakii* LKB 169 (13, 25) secreted two toxins (a protein and a single polypeptide with molecular weight 10.7 and 8.9 kD, respectively) which showed identical killer activities (disruption of the impermeability of the cell membrane leading to ATP leakage) and killed yeast which belong to various genera (28). Strain of *Williopsis mrakii* NCYC 500 secreted only one active acidic polypeptide with molecular weight 1.8-5.0 kD (29). In the above-mentioned strains, it has been difficult to regulate the killer protein synthesis due to lack of exactly knowledge of their genetic background.

Pichia/Hansenula/Williopsis form a particularly interesting category of killer yeast because their proteins/glycoproteins (e.g. from *Hansenula/Pichia anomala* (29) act against pathogenic strains of yeast (2, 23, 30, 31, 32, 33, 34, 35, 36), bacteria (37, 38, 39) and mycelial fungi (28, 40).

The therapeutic effect of killer protein from well genetically-characterized laboratory yeast strain of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* is presently unknown, because their proteins are weak killer to variety pathogenic microorganisms.

2.   Predicted biotherapeutic effect of killer toxins

It has been observed that an increasing number of pathogens are becoming resistant to antibiotics in current use. The need for novel, broad-spectrum antimicrobial agents is increasigly important in today´s medical field. Therefore, biotechnology is turning to the natural product to find new bio-therapeutic agents, active against pathogenic bacteria or yeast.

Natural products are the most consistently successful source of antimicrobial leads. They continue to provide greater structural diversity than standard combinatorial chemistry and so they offer major opportunities for finding novel low molecular weight lead peptide or protein that are active against a wide range of assay targets (41).

Recently, a prophylactic and therapeutic antimicrobial strategy, based on a specific physiological target, has become effective due to the use of killer yeast directed against their natural competition. On several occasions, differential susceptibility to the toxic effect of yeast killer protein has been proposed as a potentially useful bio-therapeutic agent for improvement of the human or animals health (29, 37, 42, 43, 44, 45, 46, 47) as well as for environmental control (28, 48).

The first exploitation of the yeast killer system seems to have been noted as an epidemiological marker for intraspecific differentiation of opportunistic microorganisms (4, 29, 31, 32, 33, 34, 38, 39, 40, 49, 50, 51). These results led to the evaluation of the potential therapeutic effect of selected killer toxins from yeast of *Williopsis mrakii* or *Pichia anomala*

and they were successfully applied to laboratory animals and used as anti-microbials against *Candida albicans, Candida glabrata, Nocardia asteroides, Malassezia pachydermatis, Pneumocystis carinii, Microsporum canis* (2, 28, 30, 37, 42, 43, 44, 45, 46, 47, 49).

Even though, the yeast killer toxins, mostly glycoproteins, are lethal to a wide spectrum of pathogenic microorganisms. They have a potential therapeutic effect and cannot be comprehensively utilised without a very specific investigations. Such investigations aim at characterising the genetic background of killer yeast and the biochemistry of their toxins as well as receptors (ligands) from pathogens which bind the killer glycoprotein (a mechanism of binding is similar to lectin action). Study of the mechanisms of antimicrobial actions are complicated and diverse.

The combination of transcriptome with proteome and metabolome research and the elucidation of structure-function relationships of biomolecules, as killer proteins, will eventually result in true understanding of whole-cell functioning.

A preliminary requirement would be knowledge about an influence of some genetic determinants on biosynthesis and secretion of killer proteins/glycoproteins. A previously study (52, 53), relative to the frequency of electrotransformation of dsRNA killer plasmids into the *Saccharomyces cerevisiae* protoplasts (strains $\underline{rho}^-/\underline{rho}^o$ and $\underline{rho}^+$), showed that their genotype (haploid, MATα, *pet*18, thermo-resistant, osmophilic) was responsible for the stable maintenance of VLPs (encapsulated dsRNA plasmids) inside of transformed cells, and also for a substantial yield of killer proteins. Studies on the stability of dsDNA killer plasmids (pGKL1 and pGKL2) from *Kluyveromyces lactis* IFO 1267, transformed into mitochondrial mutants strains of *Saccharomyces cerevisiae* have recently been carried out in laboratory of Gunge and co-workers (1).

## 3.	**Killer protein biochemistry**

A phylogenetic study on killer yeasts of genus *Hansenula* showed that yeast species *Williopsis mrakii* or *Pichia anomala* with saturn-shaped ascospores had a strong killer activity to ward various yeast species and will be useful for breeding in wine making and will have a wide application as „antibiotics" (4, 28, 29, 35, 40, 47, 54).

A study performed by Ashida (25) and Yamamoto (13) showed that *Williopsis mrakii* LKB 169 secrete into culture media two toxins (K-I and K-II). Toxin K-I is composed of 88 amino acids residues with a molecular size of 10.7 kD. The K-II toxin is a single polypeptide with molecular weight of about 8.9 kD. Both toxins were very stable against heat (boiling for 3 min at pH 4) and in the pH range of 4-11 at 25º C. They showed identical killer actions (disrupts the permeability of the cell membrane and ATP leakage) and killed yeasts which belong to various genera (e.g. *Hansenula, Pichia, Candida, Saccharomyces, Kluyveromyces, etc.*) (28). In contrast, Hodgson and co-workers (29) informed that strain of *Williopsis mrakii* NCYC 500 secreted only one active killer toxin, acidic polypeptide (possessing 125 amino acids), with a relative molecular weight between 1.8-5.0 kD and stable in the range of pH 2.4-4.0. The genetic basis of the *Williopsis mrakii* was partly identified (exactly for strain IFO 0895) by analysis of nucleotide sequences (55).

Hansenula anomala, halophilic strain, natural isolates (56) secreted two killer toxins, both glycoproteins: one (K-I) - with molecular weight 300 kD (53% protein, 47% carbohydrate) and the pI at pH 2.9 as well as second (K-II, as disulphide-bond formation) - with molecular weight 700 kD (49% protein, 51% carbohydrate) with the pI at pH 3.6. The K-I toxin was more stable at high temperature (35º C) than K-II. Both toxins were active between pH 2.5-5.0 (at 5º C) for 18 hours, but only in the presence of NaCl. The killer spectra of these toxins were different from those of killer toxins known as K_1-K_{11}.

Some strains of *Pichia anomala* species, WC65, showed killer activity to a variety of yeasts, including pathogenic *Candida albicans* (54). That effect came from glycoprotein with molecular weight of 85.3 kD, stable at pH 2-5, with the pI at pH 5.0.

In the light of above-mentioned study it was performed biochemical characteristic of other yeast proteins. Established tools in life-sciences research, such various chromatography and electrophoresis techniques, are now being more useful in protein biochemistry. Therefore, in the case of purification of killer proteins some of their methods were used.

METHODS

1.   Methods of separation and purification of killer proteins

1.1.   <u>Ion exchange chromatography (IEX): Fast Flow on Sepharose SP</u>

Some killer proteins were preliminary characterised by Salek [Report III, 1995/96; confidential material]. They are most stable at low pH and have an acidic (positive charge) isoelectric point, pI. For these applications in present study a strong cation exchange medium, such Sepharose SP (Fast Flow), was chosen. A purification was performed in follow way.

Concentration of crude killer toxin and dialysis

Killer toxin was recovered from the cell-free culture (initially 10 l) after 20-fold concentration by freeze-drying (till about 500 ml) and next dialysed for about 48 hours against water with 0.02% NaN_3 (MWCO 3500 D membrane). After dialysis, the more than 500 ml of concentrate was again concentrated by freeze-drying (till 200-fold).

Small amounts of concentrates were ultra-filtrated (and dialysed) by centrifugation ($7500^.$ g/2 h/4° C) in „Centricon"-3 concentrators, firm „Amicon", with a membrane of MWCO 3000 D.

Medium for IEX chromatography Fast Flow

SP Sepharose Fast Flow medium was used because all characterised killer proteins have pI in range of acidic pH, positive charge, what was previously reported [Salek, Report III, 1995/96; confidential material]. That strong cation exchanger is based on 45-165 μm agarose beads, pre-swollen and ready for packing of column. A higher degree of cross-linking (the bead size and bed volume does not change with changes in ionic strength or pH) is used to give medium greatly physical and chemical stability. SP Sepharose Fast Flow ion exchanger is highly substituted with strong ion exchange groups ($-O-CH_2-CHOH-CH_2-O-CH_2-CH_2$ - CH_2SO_3, which remain negatively charged and maintain consistently high capacities, 180-250 μmol protein per ml gel, over broad working pH range of 3-14) and has an exclusion limit of approximately 4×10^6. That conditions allowed the selection of a pH value and buffer, suit to the properties of the sample with killer proteins. For separation of killer proteins in dialysed concentrates (min. 200x) from yeast culture the pre-swollen SP Sepharose Fast Flow beads (in deionized water, 1:2) was used. The column 11A with 1cm bed volume has been prepared.

Buffer for equilibration, loading of killer protein sample and washing of column

0.01M citric (Na^+) buffer at pH 3.0 was used. Flow rate achievable with SP Sepharose Fast Flow medium was 9.3 ml/h. For equilibration of column with above-mentioned matrix used to 18 ml of buffer (during 2 hours) and the same volume of buffer was used to for loading (bonding) of sample and washing of ion exchanger.

Elution of killer protein in salt′s gradient

For elution of killer proteins from matrix, in gradient of salt (0-0.5M NaCl), 0.5M NaCl solution buffered by 0.01M citric (Na$^+$) buffer at pH 3.0 was used. Flow rate with SP Sepharose Fast Flow medium (1 ml of bed volume) has been regulated, giving 0.620 ml efflux pro 1 fraction (during 4 minutes). Total time of elution was 130 minutes, i.e. for using 20 ml of 0.5M NaCl, buffered by 0.01M citric (Na$^+$) buffer at pH 3.0.

Regeneration of matrix in the column 11A

For regeneration of SP Sepharose Fast Flow exchanger (in the column) after elution of sample in salts gradient (0-0.5M NaCl, buffered by 0.01M citric buffer at pH 3.0) used to 1M NaCl, buffered also by 0.01M citric (Na$^+$) buffer at pH 3.0. The time of regeneration was 130 minutes (for using 20 ml of buffered 1M NaCl).

Biological test for killer activity in fractions after IEX separation

All fractions, which showed peaks in loading, elution and regeneration phases were tested for killer activity, according to the previously written methods [Salek, Report III, 1995/96; confidential material], i.e. 1 arbitrary/lethal Unit of killer protein activity it is 10 mm^2 of clear zone surrounded by dead cells (a field w/o of well area). The clear zone became such effect of killer activity of toxin (in volume 20 μl) against sensitive strain (e.g. *Saccharomyces cerevisiae* S·6/1) in test medium.

Therefore, 1 Unit, 10 mm^2, it is $\varnothing$=1.37 mm of clear zone w/o life cells (5.37 mm of external diameter with dead cells minus 4 mm of wells diameter).

Moreover, killer protein with 1 arbitrary/lethal Unit of activity kills 7.71 · 10^3 cells of *Saccharomyces cerevisiae* S·6/1 or 7.71·10^4 cells of *Pseudomonas fluorescens* DSM 50106 (or eventually other bacteria strains). In practice the clear zone with a diameter less than 2 mm (i.e. 1.6 U) was not considered on account of non-precise measurements.

1 arbitrary Unit of killer activity = 5,7·10^3 killed sensitive yeast cells or 5,7·10^4 killed bacteria cells.

1 Unit of killer toxin corresponds to:
- 3.1 ng proteins (including killer) from *W. mrakii AS/15 ρ$^-$* on peptide-free medium,
- 2.2 ng proteins from *Pichia anomala* USCS 25F,
- 3.1 ng proteins from *Saccharomyces globosus* BKM y-438,
- 3.9 ng proteins from *Pichia subpelliculosa* NCYC 16,
- 3.9 ng proteins from *Hansenula anomala* NCYC 435,
- 3.7 ng proteins from *Hanseniaspora valbyensis* 13cs/6ρ$^-$.

The fractions with a strong killer activity were collected, dialysed (10 kD MWCO membrane, for 24 hours in water, at 4° C) and next concentrated (till 2000x) by freeze-drying method. These samples have been stored at -20° C for a long time. Above-mentioned preparations were necessary for SDS-PAGE electrophoresis to avoid problem of concentrated buffer salt and for visible purity of separated protein by IEX chromatography.

1.2   Ion exchange chromatography: Fast Performance Liquid Chromatography (FPLC)

For separation (purification) of killer proteins was used also a method of the ion exchange chromatography with column:
- Resource Mono S™, 1 ml (the firm „Pharmacia") or
- Resource Mono S™ HR 5/5 (also the firm „Pharmacia").

Resource S is a strong cation exchange columns based on beaded hydrophilic resin with one of the narrowest particle size distributions available. Mono S beads have a particle size of 10 μm. At a flow rate of 1.0 ml/min., a Mono S HR 5/5 column operates at back pressure of ca. 10 bar (1 MPa, 150 psi). The charged group on the gel is -CH$_2$-SO$_3^-$. Ionic capacity of the gel

is 0.13-0.18 mmoles/ml. Separations of substances with molecular weights up to 10^7 have been carried out successively. Mono S HR 5/5 columns can be used in aqueous solutions in the pH range 2-12. They are stable in alcohol/water solutions (C_1-C_4 alcohols).

In the case of killer protein separation (Column Resource Mono S$^®$) a manual running the programm B_1 (way 2) was used:

Time (min.)	%B	Vale (position)	Port (position)	Flow rate (ml/min.)
0	0	1.1	6.0	0.5
1	0 loading	1.2	6.1	0.5
10	0	1.1	6.1	0.5
40	100	1,1	6.1	0.5
45	100	1.1	6.0	0.5
50	0	1.1	6.6	0.5

Collector: 1 ml/2 minutes, i.e. 25 tubes with fractions.

Buffer systems and gradient separation
For an equilibration of Resource Mono S$^®$ column and for a separation of killer proteins the 10 mM citric (Na$^+$) buffer system, at pH 3.20, was used. Elution was running in gradient of 0 - 0.5 M NaCl (NaCl prepared in above buffer system, at pH 3.20).

For the purpose of the best purification, the separation process was also run in other systems, e.g.:
- 10 mM citric (Na$^+$) buffer at pH 4.50,
  gradient of salt 0 - 0.5 M NaCl in above buffer system,
  Resource Mono S$^®$ column
  killer toxin of 250 and 50x concentrated;
- 50 mM malonic acid (Na$^+$) buffer at pH 5.50
  gradient of salt 0 - 0.5 M NaCl in above buffer system
  Resource Mono Q column
  killer toxin 250 and 50x concentrated.

In all cases, 25 collected fractions were analysed for killer activity.

1.3.   Gel filtration chromatography (GF)

A column and matrix
Separation of killer proteins presented in concentrated (min. 200x) and dialysed samples of yeast cultures have been performed on the column of TSK G 2000 SW (firm "Pharmacia") with silica beads for separation of protein in the range of 0.5-60 kD.

Killer protein elution
Samples were eluted with equilibration buffer, i.e. by 50 mM citrate/phosphate at pH 4.7 and 4º C. The volume of fraction was 600 μl, collected during 12 minutes. Total time of separation usually has taken max. 17 hours.

Molecular weight standards
In this case as standards the cytochrom c (MW 12.5 kD), chymotrypsinogen A (25 kD), albumin fraction V (66 kD) and aldolase (158 kD) were used.
Biological test for killer activity in fractions after gel filtration chromatography

All fractions which showed peaks have been tested for killer activity and then active fractions were collected, dialysed (10 kD MWCO membrane, for 24 hours, in water at 4º C) and next concentrated by freeze-drying method (till 2000x). These samples were ready to SDS-PAGE electrophoresis, and storage at -20º C for a long time.

1.4   Molecular weight determination

Native PAGE (vertical set ups)
Native PAGE (7.5% polyacrylamide with gel-buffer at pH 8.8 and electrode-buffer at pH 8.3) was used for first estimation of the bands in samples obtained after IEX or gel filtration chromatography of concentrated cultures. For sample preparation it was used only simultaneous desalting and concentration in „Centricon-3" (firm "Amicon").

SDS-PAGE electrophoresis (10% T)
Method polyacrylamide gel electrophoresis in reducing conditions, i. e with sodium dodecyl sulphate (10% polyacrylamide gel matrix, suitable for protein in the range 20-100 kD) was used for determination of the approximate molecular weight of studied killer proteins. As marker have been used High-Range Marker, M_r 39.2-200 kD, firm Boehringer-Mannheim.
Below is presented the protocol of gels preparation (thickness 0.75 mm) for small system (vertical set up), i.e. Midget Electrophoresis Unit, LKB 2050.

Solution	Stacking gel	Separating gel
Acrylamide Gelling Solution (mix): - 30% Acrylamide, - 0.8% Bis-acrylamide	0.44 ml	5.00 ml
Stacking Gel Buffer: - 4x concentrated at pH 6.8	0.83 ml	-
Separating Gel-Buffer: - 4x concentrated at pH 8.8	-	3.75 ml
Deionized water	2.06 ml	6.10 ml
Well deaeration		
10% Ammonium persulphate solution	0-0.2 ml	0.15 ml
Total volume	3.35 ml	15.00 ml

Stacking gel buffer 4x conc. contained: 0.5M Tris-Cl at pH 6.8 with 0.4% SDS and 0.4% TEMED. Separating gel buffer 4x conc. contained: 1.5M Tris-Cl at pH 8.8 with 0.4% SDS and 0.4% TEMED. For sample preparation (min. 0.7 μg protein/ml) was used sample buffer 2x conc. which contained: 0.12M Tris-Cl at pH 6.8 with 4% SDS, 20% glycerol, 2mM EDTA and 0.02% bromophenol blue. Samples (15 μl/band) were diluted with above buffer (as

1:1) and heated at 100° C for 4 minutes, cooled and before loading a short time centrifuged (to remove any debris). For running a gel the tank buffer with final concentration: 0.025 M Tris, 0.19 M glycine and 0.1% SDS was prepared.

*IPG - Dalt (horizontal set ups) for 2-D electrophoresis with **immobilized pH gradient** in the first dimension (57).*

Immobiline dry strips (gradient pH 4-9 from Immobiline pK 3.6, 4.6, 6.2, 7.0, 8.5, 9.3; SDS polyacrylamide 12%T) were used for the first dimension (isoelectric focusing, IEF, for determination of pI purified proteins). Immobilized pH gradient gel strips were prepared by cutting dry IPG slab gels (cast on plastic backing: GelBond, 110 mm high) into 4 mm wide strips. Before IEF, the dry IPG gel strips were rehydrated to the original gel thickness of 0.5 mm in a mould filled with a solution containing the necessary additives (e.g. urea and detergent) for first-dimensional isoelectric focusing. The rehydrated IPG gel strips were then placed on a flat-bed cooling plate. Samples (prepared with soluble buffer, i.e. 9 M urea, 2% CHAPS, 1% DTT, 0.8% Ampholytes pH 3-10, 2 mM proteinase inhibitor) were applied into special frames, placed onto the gel surface. Samples (about 4000-6000x concentrated) of 10 μl for 2-D and 50 μl for electroblotting (selective detection of proteins) were used.

Running conditions for first-dimensional IEF with IPG gel strips were follow:
- Temperature: 20° C,
- Current max.: 5 mA per strip,
- Power max.: 3.0-5.0 W per strip,
- Voltage max.: 150 V for 30 min.
  - 300 V for 60 min.
  - 3500 V 17000 Vh, to the steady state.

After IEF, ready gel strips were <u>not</u> stained. If IPG gel strips were not used immediately, they were stored at -80° C (in plastic bag).

For the second-dimensional (2-D) electrophoresis (determination of M_r and the pI of killer proteins) the twice-equilibrated IPG gel strips (first in Tris-HCl buffer with 6 M urea, 30% glycerol, 2% SDS, 1% DTT and then in Tris-HCl buffer with bromophenol blue) were embedded onto horizontal homogeneous SDS-PAGE (12% T). Low-Molecular-Weigth Marker for the range 14 - 94 kD (α-lactoalbumin - 14.4 kD, Soybean Trypsin Inhibitor - 20.1 kD, Carbonic anhydrase - 30 kD, Ovoalbumin - 43 kD, Bovine Serum Albumin - 67 kD, Phosphorylase - 94 kD) from PharmaciaBiotech was used. Running conditions were follow:
- Temperature: 20° C,
- Current max.: 30 mA per strip,
- Power max.: 4 W per strip,
- Voltage max.: 200 V for 45-50 min.
  - 600 V for 2.5 h, to the steady state.

After 2-D electrophoresis gel strips were silver stained (30, 58).

Silver stain

For separation of protein at the very low level (e.g. 10 ng/band) the silver staining in follow way was used:

- Fixation I: 50% methanol, 12% TCA, 2% $CuCl_2$ 5 minutes
- Fixation II: 10% ethanol, 5% acetic acid   5 minutes
- Oxidation: 0.01% $KMnO_4$ 5 minutes
- Fixation III: Solution from Fix. II   1 minute
- Washing I: 10% ethanol   5 minutes

- Washing II: deionized water   5 minutes
- Silver stain: 0.1% $AgNO_3$ 5 minutes
- Development: 2% K_2CO_3, 0.01% formaldehyd till visible bands
- Washing III: Solution Fix. II   20 sec.

After staining the gels were washed in 10% ethanol and stored in plastic bag (at $4-8°C$).

<u>*Molecular Weight Standard: High Range Boeringer-Mannheim*</u>:

Myosin	200.00 kD
β-Galactosidase	116.25 kD
Phosphorylase B	97.40 kD
Bovine serum albumin	66.20 kD
Aldolase	39.20 kD

1.5 <u>Isoelectric point determination.</u>

<u>*Separation and isolation of killer protein : preparative scale of isoelectric focusing*</u>

The RF3 Recycling Free Flow Focusing Apparatus (Protein Technologies Inc., Rainin Instrument Co. Inc.) was used. This eliminated the need to elute protein from gels or chromatographic media, and minimised losses due to protein adsorption to solid supports.

The concentrated of cell-free culture after dialysis and the second times freeze-drying concentration (200x) contained 0.06-0.26 mg protein/ml (it depended on the type of killer strain). Therefore, 5.5-24.0 mg (volume of reaction chamber: 92.3 ml dialysed concentrate + 10.5 ml 87% glycerol + 2.5 ml Ampholyte such Servolyte at pH 3-7) was used per run.

After the above *preparative isoelectric focusing* all 30 fractions were tested to identify killer activity and analysed for protein content (absorbance, A_{280}). After dialysis, for removal ampholytes by native-IEF-PAGE, the pI of the proteins present in each active fractions was determined. In this case 10% of polyacrylamide with Ampholyte, such Servalyte („Serva") at pH 3-7, was used.

<u>*Analytical scale*</u>

Isoelectric focusing (IEF) on PAGE for identifying some critical impurities of separated killer proteins was performed on 10% polyacrylamide (thickness 0.2 mm) as follow:

Acrylamide 29.8%	1.72 ml
Bis-acrylamide	0.75 ml
Glycerol (87%)	2.30 ml
Deionized water	4.63 ml
Servalyte[*]	0.70 ml (0.2 ml pH 3-7 + 0.5 ml pH 4-5)
30% $(NH_4)_2S_2O_8$ (Starter)	50 μl

Next, after deaeration the acrylamide 2 h was polymerized. Running conditions for first-dimensional separation are:

Pre-focusing	30 minutes at 500 V and loading of samples
	(18 μl/well on the cathode or anode size),
Focusing	30 minutes at 500 V,
Focusing	60 minutes at 1000 V,
Focusing	120 minutes at 2000 V.

Gel after separation were silver stained, like in SDS-PAGE and final washed in solution of 5% glycerol and protected by cellophane. After air-drying they were storage in plastic bag.

1.6   Protein assay

Lowry method (chemical)

Protein in cell-free supernatant, as well as in other samples was determined using a classical Lowry method (22), adapted for plastic microtiter dishes [Technical University of Munich - internal method], modified for dilute solutions and for the presence of interfering substances (59).

For assay proteins in samples with more than 25μg proteins per 1ml they were precipitated by 3 M TCA (1 ml 10x concentrated cell-free culture supernatant + 0.2 ml of 3 M TCA) and after centrifugation (7500·g/10 min.) were re-dissolved in 60 μl deionized water. For spectrophotometric measurements a 40 μl of this solution was taken.

Samples with less than 25μg per ml of proteins were mixed with RNA (25μl soluble RNA at 5 mg/ml in 1 ml of sample) and after incubation (45 min. in bath-ice) as well as centrifugation (for 10 min. at the 27000 g, 20°C) pellets were dissolved in 0.1 N NaOH. Proteins were assaying according to Lowry (22), but with twice the concentration of sodium tartrate and copper sulphate. For spectrophotometric measurements a 40 μl of the above-mentioned solution was used.

Bovine Serum Albumin (BSA), at 1 mg/ml or 5 mg/ml was used as standard (the range of standard curve: 1-50 μg/240 μl of reaction solution).

Protein blotting for identification of glycoproteins in killer fractions with killer activity (57)

Immobiline strips (50 μl sample) after IFE were electroblotted on nitrocellulose membrane (Western-electroblotting). For specific identification of glycoproteins the lectin, i.e. Concanavalin A (Con A) was used. It is able to bind α-D-glucose and α-D-mannose. The glycoprotein-lectin complex was treated with horseradish peroxidase. Visualisation of glycoproteins used chromogen-substrate stain.

RESULTS

Development of killer proteins

Optimal production of killer proteins from 6 different yeast strains (*Williopsis mrakii* AS/15ρ⁻, *Pichia anomala* UCSC 25F, *Saccharomyces globosus* BKM y-438, *Pichia subpelliculosa* NCYC 16, *Hansenula anomala* NCYC 435 and *Hanseniaspora valbyensis* 13cs/6ρ⁻) were performed on media described previously [Salek, Report II 1994/95; confidential material]. They were developed after a number experimental cultures, tested for their killer activity against few different sensitive strains.

For biochemical characterisation of killer proteins, from examined strains, was used *peptide-free medium* (described also in Report II). That one was used for the purpose of escape a lot of non-killer polypeptides/proteins from medium. These proteins usually cause some difficulties during interpretation of results. Contents of total proteins in above-mentioned yeast killer cultures are shown in Tab.1.

In order to assay of proteins in supernatant with amount in very low level (>25μg/ml), especially in culture of killer yeast on *peptide-free medium*, it was necessary to use a modification of Lowry method (with RNA, see Methods). Biochemical examined strains had the content of protein on *peptide-free medium* in the range of 0.3-0.9 μg/ml. Therefore, for

further purification system (using ion exchange or gel filtration chromatography), were used dialysed concentrates, in about 200-fold concentration (i.e., 0.60-0.18 mg/ml).

When at first the isoelectric focusing separation (analytical scale, on 10% polyacrylamide gel) has been made, it was identified many bands with different isoelectric points (Fig. 1).

The isoelectric points for all killer proteins lay on anodic size, but number of proteins for some strains (e.g. for *Williopsis mrakii* AS/15ρ⁻, *Pichia anomala* UCSC 25F and *Pichia subpelliculosa* NCYC 16) was not clear, because often bands showed quite big and not sharp spots. It have been supposed that they presented glycoprotein images.

The next step of protein purification it was a preparative large-scale separation of proteins in solution based on differences of isoelectric points (pI) (for instance see Fig. 2).

Concentrated samples, after dialyse, were put to trial of separation on the gel filtration column (for protein in the range 0.5-60 kD).

Biological test applied for determination of killer activity in over 30 fractions, obtained after gel chromatography separation, showed that all analysed concentrated samples have killer activity in very big range of fractions, e.g. from no. 8 till fraction no. 25 (for instance see Fig. 3 a-b). This means that a molecular weight of all analysed proteins should be between 45 kD and 60 kD, but never less than 45 kD. For that - the elution time of separated killer proteins was compared with elution time of proteins standard, e.g. cytochrom c (12.5 kD), chymotrypsinogen A (25 kD) and egg albumin (60 kD).

Fractions with maximal killer activity (after biological test) were collected, concentrated/dialysed by using „Centricon-3" concentrator and tested for the pI as well as for number of proteins (bands on native PAGE). It was found that the molecular weights of isolated/purified killer proteins were estimated to be quite high, more than expect, when they were compared with elution of standards (cytochrom c, chymotrypsinogen A, egg albumin). Therefore, it has supposed that killer toxins (often glycoproteins, detected by the concanavalin A-peroxidase method after blotting to nitrocellulose) might have undergone subunit aggregations. That effect also has been previously reported by Kagiyama (56). Moreover, in many cases (e.g. *W. mrakii* AS/15ρ⁻, *P. anomala* USCS 25F) on PAGE-native it has been shown one-big-wide spot, which seems to come from proteins with different glycosylation level. When the isoelectric focusing separation on PAGE (analytical-scale) was made, it has identified a minimum 2 proteins bands with different the pI (see also a separation in Fig. 3b).

That problem was partly resolved by using IPG-Dalt method for 2-D electrophoresis because in this specific conditions, for sample preparation, have ensured some dissociation of toxin-carbohydrate complexes (Fig. 4).

After that more easy was to interpret results and to determinate a real killer proteins. Dissociate conditions for toxin-carbohydrate complex (w/o inactivation of its) in this separation method (sample soluble in buffer contained: urea, DTT, detergent) showed that killer-active fractions of some strains possessed agglomerated proteins with different origin (some of glycosylated), molecular weight and the pI. However, when killer toxin from *Williopsis mrakii* AS/15ρ⁻ was collected after gel filtration and subjected into IPG-SDS, obtained protein bands did not show any detectable carbohydrate moieties (data not shown).

In connection of above problems, during continued work on the purification of killer proteins, mainly the ion exchange chromatography as the best method for separation was used. Of course, the method of gel filtration was also has been performed, but only in order to comparison of both techniques by using SDS-PAGE and IEF.

In Figure 5 it is shown IEX chromatography separations of proteins from concentrated samples (20-60-times) of studied yeast cultures. Each fraction from individual separation was controlled for killer activity. It was found that the best separations (with single peaks) were performed in column Resource Mono S®, by using of 10 mM citric (Na⁺) buffer at pH 3.20, in gradient of salt 0 - 0.5 M NaCl (in that buffer). Used to concentration of killer toxin was 250x.

After elution in gradient of salt all fractions with highest killer activity (Nr. 9, 10 and 14, see Tab. 2) were collected (about 30 ml) and next dialysed in the bag of MWCO 3.5 kDa (24 hours at 4°C in redest. water).

After dialyses the solution from the bag (about 50 ml) was lyophilized. It was obtained 15,6 mg powder with 5.42 mg of killer proteins (according to Lowry method). That powder was dissolved in 5 ml MilliQ steril water (preparat - a pure killer protein) and finally were proved on purity using SDS-PAGE or IEF technique.

In the Fig. 6 a-b they are presented pictures as average result of SDS-PAGE and IEF-PA, which have drawn up on the ground of many ion exchange chromatography and gel filtration separations.

Therefore, for summation of biochemical characteristic of killer proteins belong to 6 different yeast strains the results only from ion exchange and gel filtration chromatography were used.

In the Table 2 are presented actual, biochemical characteristic of killer proteins, i. e. their molecular weight and isoelectric point, as results of experiments recently performed.

Characteristic of killer proteins from 6 strains of yeast
A. *Williopsis mrakii* AS/15ρ⁻.

Williopsis mrakii AS/15ρ⁻, the yeast with saturn-shaped ascospores, show quite strong killer activity to ward various yeast species [Salek, Report II, 1994/95; confidential report]. That strain, _rho⁻_ , was isolated, as a subclone AS/15ρ⁻, from polyclones of *Williopsis mrakii* NCYC 500 after mitochondrial mutation (mtDNA) by ethidium bromide (53). *Williopsis mrakii* mutant AS/15ρ⁻ possesses a better extracellular secretion of killer toxins, what was testify using sensitive strain of *Saccharomyces cerevisiae* S·6/1 in biological test.

For optimal production of killer protein (type K_9) should be use *YPG medium* (pH 5.0) and 3 days cultivation at 25°C with vigorous shaking at 1st day, gently shaking at 2nd day and w/o shaking at 3rd day.

Table 3 (or Fig. 6) presented these, purified killer toxins from above strain:
• polypeptide with molecular weight (M_r) about 14 kD and some (?) with less than 10 kD,
• the glycoprotein with a molecular weight 67 kD.

They have the pI at pH 4.87 and pH 5.1, respectively. Moreover, they were very stable against stomach digesting enzymes (pepsin, pancreatin and also bromelin), show killer activity in the range of pH 2.5-4.7 at 25° C, as well as the activity at temperature 37° C [Salek, Report I, 1992/93; confidential material].

B. *Pichia anomala* UCSC 25F.

Pichia anomala USCS 25F is also the yeast with saturn-shaped ascospores, secreted very active killer toxins against quite a big range of microorganisms, including pathogenes [Salek, Report II, 1994/95; confidential material].

For optimal production of killer protein should be use also, like for *Williopsis mrakii* AS/15ρ⁻, *YPG medium* (pH 5.0) and the same conditions for cultivation.

The purified killer toxins are (Table 3 or Fig. 6): a polypeptide with M_r less than 14 kD and glycoprotein with M_r 60 kD. Isoelectric points for these are at pH 5.23 and 5.50, respectively. These toxins have distinct killer activity in the range pH 3-5 and at the temperature until 35° C as well as did not lose all activity during operation of digesting enzymes.

C. *Saccharomyces globosus* BKM y-438.

The genetic origin of killer proteins from above strain (HO/HO, pet/pet and trp/trp mutant, kindly obtained from Nesterova, St. Petersburg University) is dsDNA plasmid with 14 kb. Such *trp⁻* mutant, strain BKM y-438, could not produced killer toxin on *peptide-free medium YNBglu2-medium* (w/o amino acids). That yeast needed small amount of yeast extract (*YNBYEglu2-medium)*. Optimal medium for production of its killer toxin was *YPG-medium* and a propagation according to Report II [Salek, Report II, 1994/95; confidential material].

Purified and separated killer proteins (type K_4) were active in the range pH 3.0-4.7 and at temperature 25-37° C [Salek, Report I, 1992/93; confidential material]. They were presented by:

• polypeptide with M_r about 14 kD and the pI at pH 5.0,

• other two glycoproteins with M_r = 62 kD each, but different the pI, e.g., at pH 4.7 and 5.43.

Saccharomyces globosus BKM y-438 yeast, like above strains of *Williopsis mrakii* and *Pichia anomala,* was resistant to digesting enzymes and showed evident the killing effect to quite big spectrum of microorganisms, including pathogenic fungi and bacteria.

D. *Pichia subpelliculosa* **NCYC 16.**

This strain showed optimal growing and production of killer toxins (type K_5) on *YPG-medium* and secreted three proteins:

• protein with M_r 20.2 kD and the pI at pH 4.86,

• protein with M_r 67 kD, the pI at pH 4.75 and

• glycoprotein with M_r 67 kD but with the pI at pH 5.34.

Above proteins possessed activity between pH 2-4.7 at 25° C against tester strain of *Pseudomonas fluorescens* DSM 50106 [Salek, Report I, 1992/93; confidential material]. Digested enzymes had no destructive influence on killer proteins.

Toxins from *Pichia subpelliculosa* NCYC 16 were especially active against bacteria, e.g. *Staphylococcus aureus, Micrococcus* sp., *Enterococcus avium, Streptococcus faecium, E.coli, Salmonella,* etc.

E. *Hansenula anomala* NCYC 435.

Type of toxin K_8. For optimal production of killer protein needed *YPG-medium*, and conditions according to described in Report II [Salek, Report II 1994/95; confidential material]. They were represented by:

• glycoprotein with M_r 51 kD and the pI at pH 5.22,

• protein with M_r 25 kD and the pI at pH 5.15.

Killer proteins from *Hansenula anomala* NCYC 435 were active between pH 3-5 and at temperature not high than 35° C. It was found that *Hansenula anomala* NCYC 435 is especially active against pathogenic bacteria, such *Micrococcus* sp., and *Escherichia coli* [Salek, Report II, 1994/95; confidential material].

F. *Hanseniaspora valbyensis* **13cs/6ρ⁻.**

This strain with very small dimension of cells (about 2 μm) showed much less amount of protein production in peptide-free medium than above-mentioned strains (about 0.3 μg/ml), therefore purification process was very difficult. Obtained toxins, type glycoprotein, were in the range 40-52 kD with the pI at pH 4.36.

Nevertheless, anti-microbial activity of these proteins showed big effect on the killing of pathogenic bacteria and fungi [Salek, Report II, 1994/95; confidential material] what probably was resulting from specific receptors and a presence of organic acids (at a quite high level), such lactic acid or acetic acid in tested samples (data not shown).

DISSCUSION

Selection of biochemical methods for purification of killer protein.

As might be expected, chromatographic separation of proteins should be complex and diverse. Both, target molecules and impurities, show considerable variation and complexity. The size and complexity of proteins means that changes in chromatographic conditions can

have a profound effect. However, in chromatography must be performed a balance of separation with the preservation of biological activity and protein stability.

It is known that adsorption chromatography depends upon interaction of different types between solute molecules and ligands immobilised on a chromatography matrix, such in ion exchange chromatography.

Ion exchange chromatography is among the most precise methods for the fractionation of biological substances and it is based on differences in charge characteristic (cationic/anionic) as well as is thus dependent on the pH of the system and the isoelectric points of proteins (pI, i. e. the value of pH when the total number of positive charges equals the total number of negative charges, resulting in a net charge of zero). When the buffer pH is above the pI of that, an anion exchanger should be used. When the system pH is below the pI of the protein, a cation exchanger should be used. Biomolecules (such proteins) with even small differences in surface charge characteristic can be separated by ion exchange chromatography. Very high resolution usually is obtained during gradient elution by optimising the ionic strength or pH of the gradient. The high capacity of commercial ion exchange media allows large volumes of dilute sample to be processed and then eluted in a concentrated form.

Ion exchange chromatography is widely used in the separation of proteins because the relatively mild binding and elution conditions allow high protein recovery with intact biological activity. All proteins have some ionic character, therefore, conditions used for ion exchange chromatography (aqueous, buffered salt solutions) are highly compatible with majority proteins. Most protein separation schemes involves one or more ion exchange steps (binding and elution of proteins).

The bonded phase of an ion exchange packing consists of functional groups that have either a positive charge (anion exchange), used to separate negatively charged target molecules (anions), or a negative charge (cation exchange), used to separate positively charged target molecules (cations). Anion or cation exchange functional groups are classified as either „strong" or „weak". Weak ion exchange groups are titratable, i.e. they gain or lose electrical charge as the pH of the mobile phase changes. Note that the terms „strong" or „weak" do not refer to the strength of the binding but only to the effect of pH on the charge of the functional groups. Because of the substantial energy involved in charge-charge interactions, the laws of physics dictate that the number of positive and negative charges in any given volume must be almost exactly equal.

Ion exchange binding occurs when the salt concentration or ionic strength of the mobile phase is reduced to the point that the ionic groups on the sample molecules begin to serve as the counter ions for the charged groups on the stationary phase. This causes the sample molecules to bind to the surface. Elution takes place when the ionic strength of the mobile phase is increased. As this happens, salt molecules displace the bound sample molecules with the same charge as the bonded phase (called co-ions) bind to the charge groups on the sample molecules.

The choice between anion and cation exchanger depends upon the charge characteristics and the effect of pH on stability and solubility of both the target protein molecule itself and the other molecules in the sample. The maximise binding strength, select an operating pH range that is either above or below the isoelectric point of the target, based on where the protein is most stable and soluble.

Gel filtration chromatography (syn. gel permeation chromatography) of killer protein were performed according to differences in their molecular weight and sizes as they passed through a column packed with a chromatographic medium which was gel. A gel was a heterogeneous phase system in which a continuous liquid phase, usually aqueous, was contained within the pores of a continuous solid phase, the gel matrix. In gel filtration, the pores of gel had a carefully controlled range of sizes, and the matrix was chosen for its chemical and physical stability.

For small enough killer protein molecules, the pores were so large that the molecules could penetrate all of the internal volume of the particles. If the killer proteins were large enough, the pores have been so small that the molecules were completely excluded from the internal volume.

Gel filtration chromatography was most often used as a final polishing method, since it is the only separation method available to remove aggregated protein species without any chemical or physical change that may cause more aggregates to form.

Gel filtration chromatography has limited usefulness as a high throughput technique. The separation mechanism has been required a slow flow rate, and in most cases, sample load had only 1-5% of the column bed volume to ensure good results.

Both, for identification and control of assessment purity, a single method, such as SDS-PAGE, was used. In other cases, it was needed an additional method, e.g. IEF, isoelectric focusing on PAGE, for identifying one or more critical impurities that later was removed from the target. In all cases, however, it was limited the screening assays to only the information needed to identify „good" results and where to move forward with the chromatographic development.

As in Results mentioned, that the molecular weight of characterised toxins/proteins was previously estimated from the elution profile of gel permeable chromatography. It was found that the values of molecular weight of isolated/purified killer proteins were to be quite high, more than expect. In some cases, after the isoelectric focusing separation on PAGE (analytical-scale), in most killer-active fractions were detected minimum 2 proteins bands with different the pI.

It was only possible to resolve such problem when IPG-Dalt method for 2-D electrophoresis was used. Dissociate conditions for toxin-carbohydrate complex in this separation method showed that killer-active fractions after gel filtration possessed agglomerated proteins with different origin (some of glycosylated), molecular weight and the isoelectric point.

In recapitulation of above it is important to say that generally in purification process of killer proteins the ion exchange chromatography has a big advantage of the gel filtration chromatography. Therefore, the ion exchange chromatography (with cation exchanger) as optimal method for separation and purification of killer protein in the laboratory and technical scale should be performed by good (quick) organisation way.

Biological activity of killer proteins.

The killing effect is mediated by diffusable toxins since sensitive cells are killed/inhibited when have been incubated in the presence of the cell-free supernatant of liquid cultures.

It was found that the area of the inhibition zone (arbitrary or lethal units) is directly proportional to the concentration/dilution of culture, but only in the range 5 – 6 Units/ml, respectively.

Moreover, the killing of sensitive cells (e.g. *Saccharomyces cerevisiae* S·6/1) was strongly depended upon physical parameters of the environment (i. e. buffered medium, where the toxin function and stability depended on folding stabilised by ionic bonds from mineral substance) and the growth state of the sensitive cells (considering the amount of synthesised β-1,6-D-glucan in the cell wall of yeast).

Generally, lethal/killer activity of each toxin/protein strongly depended on pH (usually less than 5) and a number of specific receptors on surface of cell wall in sensitive acceptor strains (e.g. in pathogenic fungi such *Candida albicans* or bacteria, as *Staphylococcus aureus*, etc.). It is important to know how many molecules of killer toxin are required to kill one definite cell of sensitive yeast or bacteria strain. Palfree and Bussey (18) have estimated such amount for *Saccharomyces cerevisiae* which is 10^4 molecules of toxin per cell of sensitive yeast. In our

study was detected that killer toxin of 1 arbitrary unit (3-4 ng killer protein) killed $5,7 \cdot 10^3$ yeast cells or $5,7 \cdot 10^4$ bacteria.

It was also found that the adsorption of killer toxin to the yeast cell wall (because a specific receptors) was irreversible, as dilution of the toxin treated cells by $5 \cdot 10^5$ /ml did not „rescue" the cells (quite fast reaction of permeabilisation). In the case of *Williopsis mrakii* AS/15ρ⁻, *Pichia anomala* UCSC 25F and *Saccharomyces globosus* BKM y-438 was observed much debris cells (on the test-medium for activity as a dark-blue front of the clear zone).

Furthermore, very important for killer activity was cell-excretion which for all examined toxins was detectable from the middle exponential phase and were maximal in stationary phase of growth (data not shown). Good excretion of heavily glycosylated proteins was not unusual in most determined killer strains (in this study). It was evident that the interplay between glycosylation, growth conditions and secretion of protein existed quite complex (see also (60)).

Since the discovery of killer toxins from yeast has been performed over 30 years and this phenomenon has been the subject a lot of papers - purification of toxins has proved difficulties (18, 19, 20).

What was reported previously by some authors (61), the toxin (protein) of killer *Saccharomyces cerevisiae* was absorbed by Sephadex and was thus not fractionated by gel filtration with Sephadex G-150. Because of a broad distribution of the killer toxin in many fractions after gel filtration on Sephadex 4B, assumed that the toxin consists of a multicompound complex, which can polimerizing at high concentrated protein (62).

As was reported, the instability of toxins represented a major problems in their purification. Ouchi et al. (63) informed that polyhydric alcohols (such as glycerol) stabilised killer activity, therefore in many cases of this study was used this type of preservation. Moreover, even was used glycerol, for an ultrafiltration technique through „Amicon" membrane PM10 or PM30, showed evident disruptions of killer proteins, especially for *Williopsis mrakii* AS/15ρ⁻, *Pichia anomala* USCS 25F and *Saccharomyces globosus* BKM y-438. Much more protective was freeze-drying technique or „Centricon-3" concentrator.

An instability of killer activity was also observed during purification of proteins by ion-exchange chromatography, using a cation exchange bach method with „Source^{TM} 30Q" (Pharmacia Biotech AB). Probably it was caused by conditions for a separation which were not optimal for activity examined toxins.

Anyway, the killing activity of purified proteins was stable only within a narrow pH range (i.e. at 3.5-5.5 for all studied toxins) and at temperature above 35° C quickly lost such action (to cell wall receptors).

The best killer toxins in the sense of:
- the lethal, specific activity to sensitive strains, especially to pathogenic microorganisms,
- the amount of produced protein (including killer) and their stability during a purification process, and
- demands for production of killer proteins -

were toxins from *Williopsis mrakii* AS/15ρ⁻, *Pichia anomala* USCS 25F and *Saccharomyces globosus* BKM y-438. Their killer phenomenon with lethal actions for many pathogens and as a model system for the fungal viruses may also prove to be a model for latent viral infection of higher eukaryotic organisms, because did not find toxicity effect of such proteins in mammalian cells (data not shown).

REFERENCES

(1)   Gunge N., (1995), Ed. Kück, The Mycota. Genetics and Biotechnology, 189-209. Springer-Verlag, Berlin, Heidelberg.

(2)   Magliani W., Conti S., Gerloni M., Bertolotti D., Polonelli L., (1997), Clinical Microbiology Reviews, 10, 369-400.

(3)   Philliskirk G., Young T.W., (1975), Antonie van Leeuwenhoek J. Microbiol. Serol., 41, 147-151.

(4)   Polonelli L., Morace G., (1986), J. Clinical Microbiology, 24, 866-869.

(5)   Stumm C., Hermans J.M., Middlebeek E.J., Croes A.F., De Vries G.J.M.L., (1977), Antonie van Leeuwenhoek J. Microbiol. Serol., 43, 125-128.

(6)   Tipper D.J., Bostian A., (1984), Microbiological Reviews, 48, 125-156.

(7)   Young, T.W., Yagin, M., (1978), Antonie van Leeuwenhoek 44, 59-77.

(8)   Young, T.W., (1987), Ed. Young T.W., The Yeast, vol.2, 2nd edition, Academic Press Inc., pp. 132-164, London.

(9)   Georgopapadakou N.H., (1998), Current Opinion in Microbiology, 1, 547-557.

(10)   Bussey H., C. Boone, A. Dmochowska, D. Greene, Zhu H., Lolle S.J., Vernet T., Dignard D., Thomas D.Y., (1988), Viruses of Fungi and Simple Eukaryotes, Ed. Koltin Y., Leibowitz M., 161-178, Marcel Dekker.

(11)   Piddock L.J.V., (1998), Current Opinion in Microbiology, 1, 502-508.

(12)   Stark M.J.R., Boyd A., Mileham A.J., Romanos M.A., (1990), Yeast, 6, 1-29.

(13)   Yamamoto T., Hiratani T., Hirata H., Imai M., Yamaguchi H., (1986), FEBS, 197, 50-54.

(14)   Kasahara S., Inoue S.B., Mio T., Yamada T., Nakajima T., Ichishima E., Furuichi Y., Yamada H., (1994), FEBS Letters, 348, 27-32.

(15)   Butler A.R., O´Donnel R.W., Martin V.J., Gooday G.W., Stark M.J.M., (1991), Eur. J.Biochem., 199, 483-488.

(16)   Stark M.J.R., Boyd A., (1986), EMBO J., 5, 1995- 2002.

(17)   Glick B.S., (2000), Current Opinion in Cell Biology, 12, 450-456.

(18)   Palfree R., Bussey H., (1979), Eur. J. Biochem., 93, 487-493.

(19)   Pfeiffer P., Radler F., (1982), J. Gen. Microbiol., 128, 2699-2706.

(20)   Pfeiffer P., Radler F., (1984), Arch. Microbiol., 137, 357-361.

(21)   Bolen P.L., Eastman E.M., Cihak P.L., Hayman G.T., (1994), Yeast, 10, 403-414.

(22)   Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J., (1951), J. Biol. Chem., 193, 265-275.

(23)   Worsham P.L., Bolen P.L., (1990), Current Genetics, 18, 77-80.

(24)   Yu-Sheng Cong, Yarrow D., Yu-Yang Li, Fukuhara, H., (1994), Microbiology, 140, 1327-1335.

(25)   Ashida S., Shimazaki T., Kitane K., S. Hara, (1983), Agric. Biol. Chem., 47, 2953-2955.

(26)   Gunge N., Fukuda K., Morikawa S., Murikami K., Takeda M., Miwa A., (1993), Curr. Genet., 23, 443-449.

(27)   Starmer W.T., Ganter Ph.F., Aberdeen V., (1987), Can. J. Microbiol., 33, 783-796.

(28)   Walker G.M., McLeod A.H., Hodgson V.J., (1995), FEMS Microbiol. Letters, 127, 213-222.

(29)   Hodgson V.J., Button D., Walker G.M., (1995), Microbiology, 141, 2003-2013.

(30)   Cailliez J.G., Seguy N., Denis C.M., Aliouat E.M., Mazars E., Polonelli L., Camus D.E., (1994), Journal of Medical & Veterinary Mycology, 34, 227-239.

(31)   Morace G., Archibusacci C., Sestito M., Polonelli L., (1983/84), Mycopathologia, 84, 81-85.

(32)   Polonelli L., Fanti F., Conti S., Campani L., Gerloni M., Castagnola M., Morace G., Chezzi C., (1990), J. Immunological Methods, 132, 205-209.

(32)   Polonelli L., Conti S., Gerloni M., Magliani W., Chezzi C., (1991), Critical Reviews in Microbiology, 18, 47-87.

(33)   Polonelli L., Morace G., Conti S., Gerloni M., Magliani W., Chezzi C., (1992),

Curr. Top. Med. Mycol., 4, 137-157.

(35) Sawant A.D., Abdelal A.T., Ahearn D.G., (1988), Appl. Environ. Microbiol., 54, 1099-1103.

(36) Sawant A.D., Abdelal A.T., Ahearn D.G., (1989), Antimicrobial Agents and Chemotherapy, 33, 48-52.

(37) Cailliez J.C., Seguy N., Aliouat E.M., Polonelli L., Camus D., Deicas E., (1994), Medical Hypothesis, 43, 167-171.

(38) Morace G., Dettori G., Sanguinetti M., Manzara S., Polonelli L., (1988), Eur. J. Epidemiol., 4, 99-103.

(39) Morace G., Manzara S., Dettori G., Fanti F., Conti S., Campani L., Polonelli L., and Chezzi C., (1989), Eur. J. Epidemiol., 5, 303-310.

(40) Polonelli L., Dettori G., Cattel C., Morace G., (1987), Eur. J. Epidemiol., 3, 236-242.

(41) Harvey A., (2000), Drug Discovery Today, 5(7), 457-463.

(42) Aliouat E.M., Caillez C.J., Sequy N., Deicas E., Polonelli L., Gerloni M., Conti S., Camus D., (1993), Serodiagn. Immunotherap. Infect. Disease, 5, 102-106.

(43) Pettoellomantovani M., Nocerino A., Polonelli L., Morace G., Conti S., Dimaritino L., Deritis G., Iafusco M., Guandalini S., (1995), Gastroenterology, 109, 1900-1906.

(44) Polonelli L., Lorenzini R., De Bernardis F., Gerloni M., Conti S., Morace G., Magliani W., Chezzi C., (1993), Scand. J. Immunol., 37, 105-110.

(45) Polonelli L., De Bernardis F., Conti S., Baccanera M., Gerloni M., Morace G., Magliani W., Chezzi C., Cassone A., (1994), J. of Immunology, 152, 3175-3182.

(46) Polonelli L., De Bernardis F., Conti S., Boccanera M., Magliani W., Gerloni M., Cantelli C., Cassone A., (1996), The Journal of Immunology, 156, 1880-1885.

(47) Seguy N., Cailliez J.C., Polonelli L., Dei-Cas E., Camus D., (1996), Parasitol. Res., 82, 114-116.

(48) Petersson S., Schnürer J., (1995), Appl. Environmental Microbiol., 61, 1027-1032.

(49) Polonelli L., Lorenzini L., Bernardis F., Morace G., (1986), Mycopathologia, 96, 103-107.

(50) Polonelli L., Manzara S., Conti S., Dettori G., Morace G., Chezzi C., (1989), Mycopathologia, 108, 211-215.

(50) Provost F., Polonelli L., Conti S., Fisicaro P., Gerloni M., Boiron P., (1995), J. Clinical Microbiology, 33, 8-10.

(51) Salek A., Schnettler R., Zimmermann U., (1990), FEMS Microbiology Letters, 70, 67-72.

(52) Salek A., Schnettler R., Zimmermann U., (1992), FEMS Microbiology Letters, 96, 103-110.

(54) Sawant A.D., Abdelal A.T., Ahearn D.G., (1989), Antimicrobial Agents and Chemotherapy, 33, 48-52.

(55) Kimura T., Kitamoto N., MatsuokaK., Nakamura K., Iimura Y., Kito T., (1993), Gene, 137, 265-270.

(56) Kagiyama S.T., Aiba T., Kadowaki K., Mogi K., (1988), Agric. Biol. Chem. 52, 1-7.

(57) Görg, A.,(1996), GDCh Fortbildungskurs 365/96, TUM-Weihenstephan.

(58) Blum, H., Beier, H., Gross, H.J., (1987), Electrophoresis, 8, 93-99.

(59) Polachek, I., Cabib, E., (1981), Anal. Biochem., 117, 311-314.

(60) Cailliez J.G., Cantelli C., Seguy N., Conti S., Gerloni M., Morace G., Polonelli L., (1994), Mycopathologia, 126, 173-177.

(61) Woods D.R., Bevan E.A., (1968), J. General. Microbiology, 51, 115-126.

(62) Bussey H., (1972), Nature New Biology, 235, 73-75.

(63) Ouchi K., Kawase N., Nakano S., Akiyama H., (1978), Agric. Biol. Chem., 42, 1-5.

Yeast antimicrobial proteins

S u m m a r y

Many yeasts secrete proteins which are toxic for pathogenic and non-pathogenic microorganisms. These toxins, mostly glycoproteins, consist of membrane-binding subunits which interact with carbohydrate (e.g. $1,6$-β-D-glucan or α-mannan) on the cell wall of sensitive strains. The killing effect is presented by membrane permeation, cell lysis or inhibition of the cell cycle.

It is also suggested that these killer glycoproteins, similar in structure to lectins, can mediate self-adhesion of the pathogenic microorganisms, so stimulating their excretion from the intestines of infected mammals. It is supposed that above interactions could be important for therapeutic application, especially to enteric diseases.

In order to fully understand the structural basis of the functions of killer glycoproteins, it is essential to characterize their glycosylation state and to determine the structure of all glycans attached to the proteins.

In this paper, a strategic approach to the purification of yeast protein from complex biological mixtures is presented. The approach is structured into seven subassignments, each of which is essential for the successful isolation of a pure and biological active yeast protein.

The subassignments are: 1) decide upon the use of the purified protein; 2) collect information about the chemical, physical and biological properties of the protein; 3) establish assays for the protein and its biological activity; 4) decide on the source of raw material; 5) develop an efficient extraction method; 6) learn and develop a purification method; 7) establish optimum conditions for storage of the purified protein.

Key words:

Yeast killer protein/glycoprotein/toxin, secretory system, therapeutic effect.

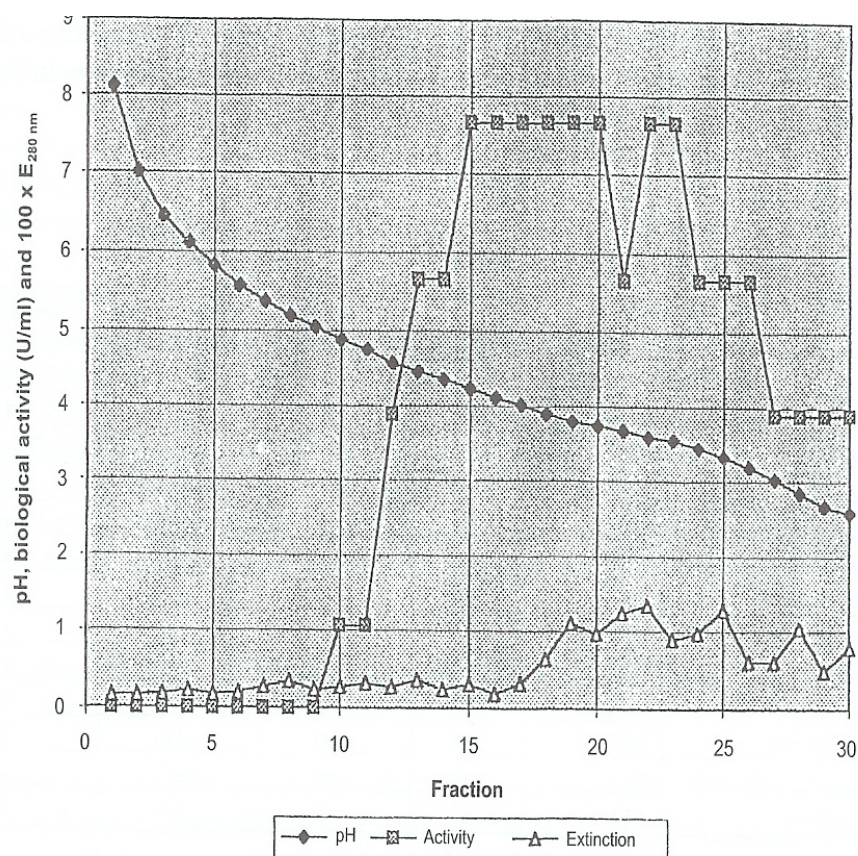*Address for correspondence:*
e-mail: Anna.Salek@T-Online.de

Fig. 1. Isoelectric focusing (preperative scale) of killer protein in RF-3 Recycling Free Flow Focusing Apparatus. Conditions of separations:
  − a volume of 110 ml, incl. sample, with 1% Servolyte (pH 3-4) and 10% glycerol;
  − electrolites: 0.1 N H_3PO_4 (positive charge) and 0.1 N NaOH (negative charge), at 2°C.
Legend: a) pH of fractions; b) killer activity of obtained fractions; c) absorbance (A_{280}) of proteins (incl. killer) in fractions.
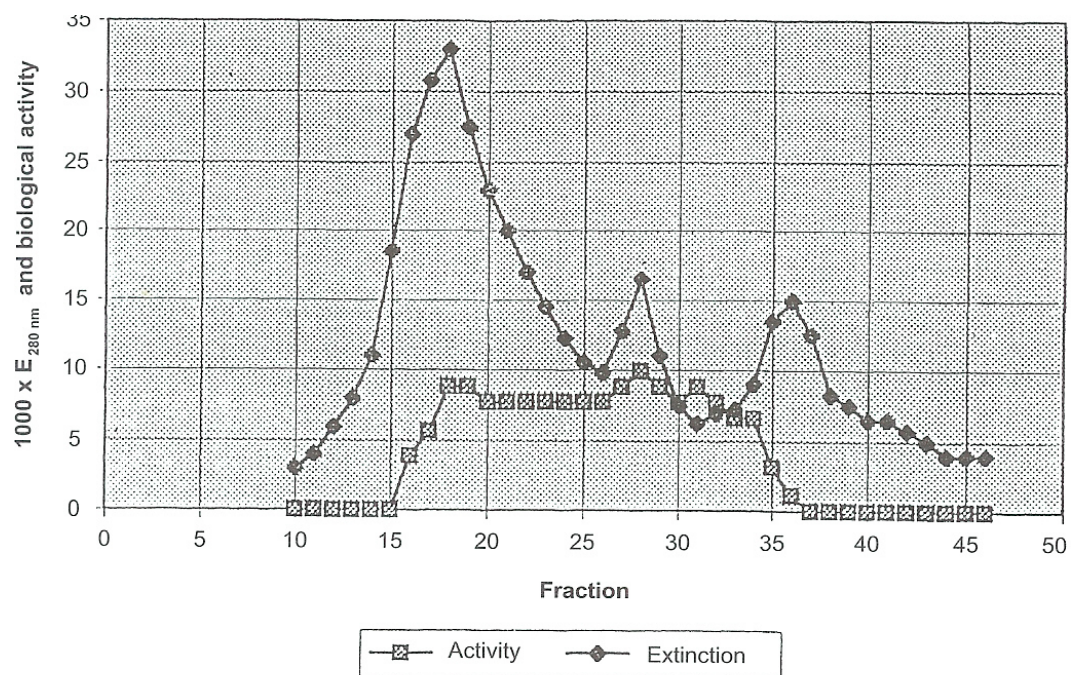
Fig. 2. Gel filtration chromatography (GF) on column TSK G 2000 SW of killer protein from *Williopsis mrakii* AS/15ρ⁻ – the first separation. The second separation of protein (as a last polishing/purification method of killer active fractions) was performed using the ion exchange chromatography Fast Flow on Sepharose SP.
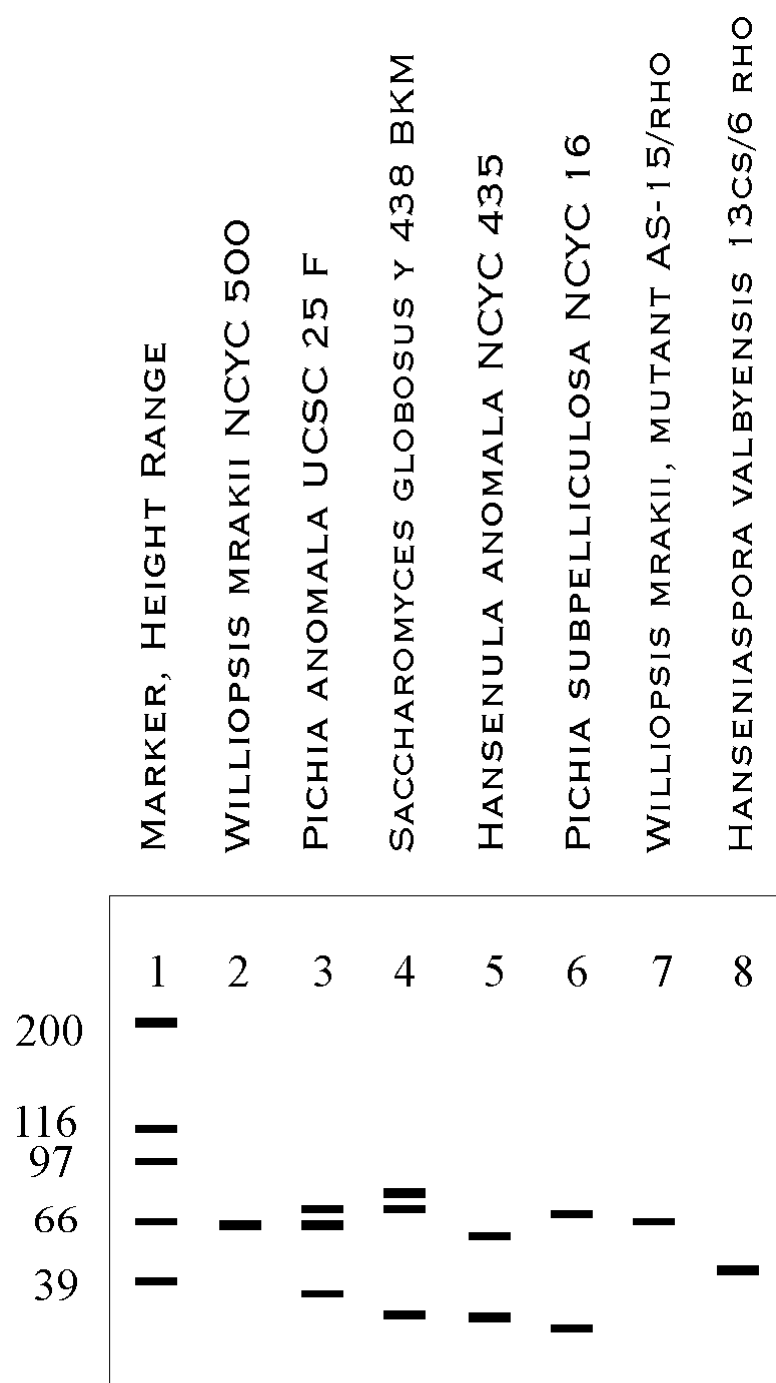
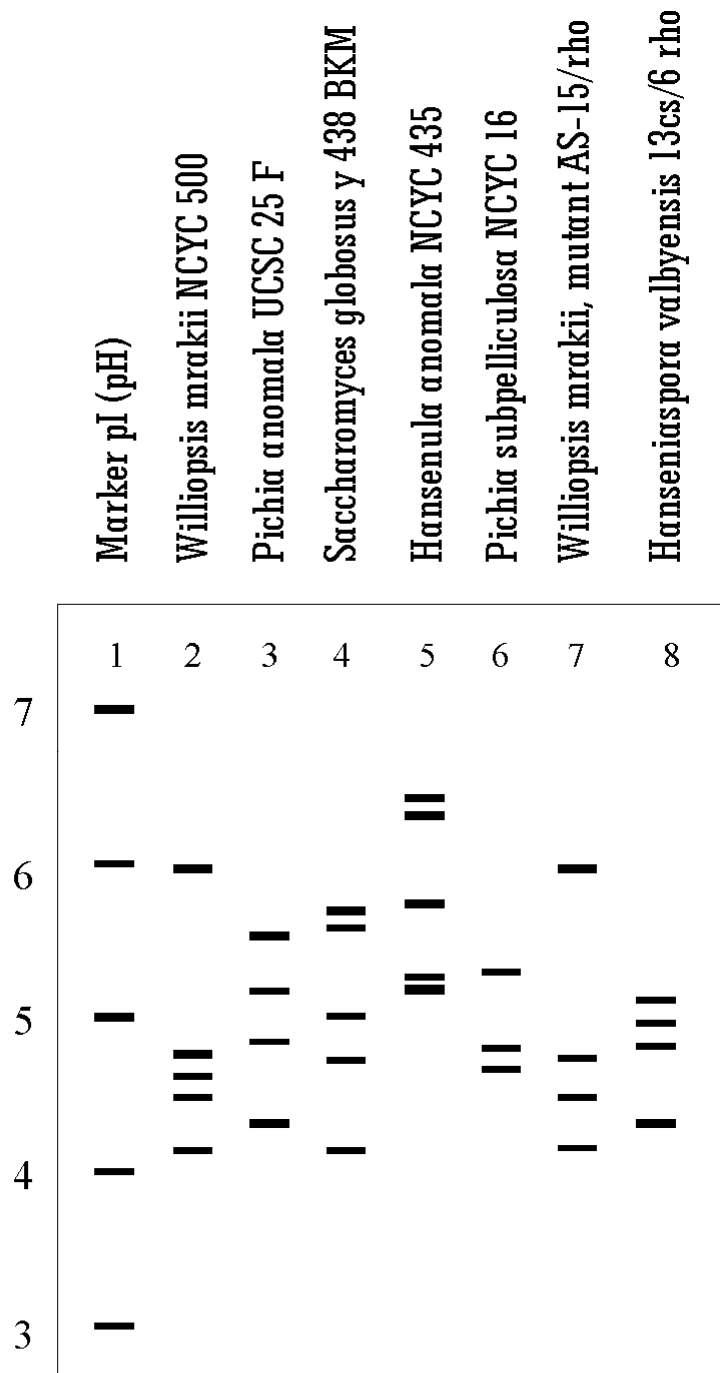Fig. Molecular weight of killer proteins,
purified by IEX chromatography

Fig ꜝ pI (isoelectric point) of killer proteins separated by gel filtration chromatography (GF)

Figure legends

Fig. 1. Isoelectric focusing (preperative scale) of killer protein in RF-3 Recycling Free Flow Focusing Apparatus. Conditions of separations:
- a volume of 110 ml, incl. sample, with 1% Servolyte (pH 3-4) and 10% glycerol;
- electrolites: 0.1 N H_3PO_4 (positive charge) and 0.1 N NaOH (negative charge), at 2°C.

Legend: a – pH of fractions; b – killer activity of obtained fractions; c – absorbance (A_{280}) of proteins (incl. killer) in fractions.

Fig. 2. Gel filtration chromatography (GF) on column TSK G 2000 SW of killer protein from *Williopsis mrakii* AS/15ρ^-:

a – the first separation;

b – the separation as a last polishing method of killer active fractions obtained after purification by ion exchange chromatography Fast Flow on Sepharose SP.

Fig. 3. Biochemical parameters purified killer proteins:

a – Molecular weight of killer proteins, purified by IEX chromatography;

b – Isoelectric point of killer proteins, separated by gel filtration chromatography.

Table 2.   Contents and activity of separated killer proteins.

Nr. fraction	Absorbtion (at 280 nm)	Protein (µg/ml)	Killer activity (Units/ml)
Elution:			
5	0.223		0
6	0.159		0
7	0.140		0
8	0.292		±
9	0.722	235	52
10	0.629	206	69
11	0.481		52
12	0.421		±
13	0.330		±
14	0.330	102	21

Table 3.   Molecular weights and isoelectric focusing points of killer proteins

Strains	Character	K-1	K-2	K-3
Williopsis mrakii AS/15ρ⁻	pI at pH	5.10	4.87	
	M_r in kD	67	14	
Pichia anomala UCSC 25 F	pI at pH	5.50	5.23	
	M_r in kD	60 (G*)	14	
Saccharomyces globosus BKM y-438	pI at pH	4.70	5.43	5.00
	M_r in kD	62 (G*)	62 (G*)	14
Pichia subpelliculosa NCYC 16	pI at pH	4.75	5.34	4.86
	M_r in kD	67	67 (G*)	20.2
Hansenula anomala NCYC 435	pI at pH	5.22	5.15	
	M_r in kD	51	25	
Hanseniaspora valbyensis 13cs/6ρ⁻	pI at pH	4.36		
	M_r in kD	40-52 (G*)		

* G - glycoprotein