



Yeast antimicrobial proteins

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Summary

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 carbohydrates (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, thus stimulating their excretion from the intestines of infected mammals. It is supposed that the above interactions could be important for therapeutic applications, especially for 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 biologically active yeast protein.

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

Key words:

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

Adres do korespondencji

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1. Introduction

1.1. 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* (1-8). 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. entering the cytosol and attacking its essential constituents), 2) inhibition of protein synthesis, or 3) arrest of the G₁ phase of the cell cycle (6, 9-13).

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 the initial contact of the toxin with the cell surface (15). Moreover, the γ toxin subunit of *Kluyveromyces lactis* is transported inside the cell and it 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. Therefore, they may kill various microorganisms, 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 (K₁ killer) of *Saccharomyces cerevisiae* is mediated by two linear dsRNA plasmids, M₁dsRNA 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, toxin (α and β -heterodimeric subunits of 9.0 and 9.5 kD, respectively) and glycosylated γ peptide (which remains in the cell wall during the secretion of the α - β heterodimer). The toxin of the above-mentioned *Saccharomyces cerevisiae* is an unglycosylated protein (18 kD) and it kills sensitive strains of the same or related species (6).

Other types of toxins (K₂, K₃, K₂₈) have also been found in the genus of *Saccharomyces*. These are distinguishable from K₁ killer, and from each other, by their killing and immunity specificities (3). Generally speaking, K₁ toxin is normally found in laboratory strains (6,10,18), K₂ and K₂₈ occur in wine yeast (19,20) whilst K₃ 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 exam-

ined, however, the linear dsDNA-plasmids from *Kluyveromyces lactis* and *Pichia acaciae* are responsible for the killer activity only (1,12,21-24). *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, pPac1-1 and pPac1-2 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, 25-27). These killer toxins are produced as single peptides or proteins, or glycoproteins.

Nevertheless, our knowledge about the above genera, i.e. toxins from some killer strains, including those *Williopsis* (or *Hansenula*) sp., is still insufficient. 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 the lack of sufficient 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, 30-36), bacteria (37-39) 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 a variety of pathogenic microorganisms.

1.2. 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 increasingly important in today's medical field. Therefore, biotechnology is turning to the natural product to find new biotherapeutic 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 therefore they offer greater opportunities to find novel low molecular weight lead peptides or proteins 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 biotherapeutic agent for the improvement of the human or animal health (29,37,42-47) as well as for environmental control (28,48).

The first application of the yeast killer system seems to have been noted as an epidemiological marker for intraspecific differentiation of opportunistic microorganisms (4,29-34,38-40,49-51). 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*, *Microsporium canis* (2,28,30,37,42-47,49).

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 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 the action of lectin). Study of the mechanisms of antimicrobial actions is 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 the whole-cell functioning.

A preliminary requirement would be the knowledge about the influence of some genetic determinants on the biosynthesis and secretion of killer proteins/glycoproteins. A previous study (52,53), relative to the frequency of electrotransformation of dsRNA killer plasmids into the *Saccharomyces cerevisiae* protoplasts (strains *rho*/*rho*⁰ and *rho*⁺), showed that their genotype (haploid, MAT α , *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 mutant strains of *Saccharomyces cerevisiae* have recently been carried out in the laboratory of Gunge and co-workers (1).

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 toward various yeast species and therefore they would be useful for breeding in wine making and would find a wide range of applications 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 two toxins (K-I and K-II) into culture media. 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 the 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 25°C. They showed identical killer actions (disrupting the permeability of the cell membrane and ATP leakage) and killed yeasts which belonged 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) through the 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 the other (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 (35°C) than K-II. Both toxins were active between pH 2.5-5.0 (at 5°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 K₁-K₁₁.

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

2. Methods

2.1. Methods of separation and purification of killer proteins

2.1.1. Ion exchange chromatography (IEX): Fast Flow on Sepharose SP

Some killer proteins were preliminarily characterised by Salek [Report III, 1995/1996; confidential material]. They are most stable at low pH and have an acidic (positive charge) isoelectric point, pI. For these applications in the present study a

strong cation exchange medium, Sepharose SP (Fast Flow), was chosen. Purification was performed in the following way.

2.1.1.1. 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, more than 500 ml of the concentrate was again concentrated by freeze-drying (till 200-fold).

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

2.1.1.2. Medium for IEX chromatography Fast Flow

SP Sepharose Fast Flow medium was used as all the characterised killer proteins have pI in the range of acidic pH , positive charge, which has previously been reported [Salek, Report III, 1995/1996; 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 do not change with changes in the ionic strength or pH) is used to give the medium great physical and chemical stability. SP Sepharose Fast Flow ion exchanger is highly substituted with strong ion exchange groups ($-\text{O}-\text{CH}_2-\text{CHOH}-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2 - \text{CH}_2\text{SO}_3$, 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 it has an exclusion limit of approximately 4×10^6 . These conditions allowed for the selection of the pH value and the buffer, appropriate for the properties of the sample with killer proteins. For the separation of killer proteins from yeast culture in the dialysed concentrates (min. 200x) the pre-swollen SP Sepharose Fast Flow beads (in deionized water, 1:2) was used. Column 11A with 1cm bed volume was prepared.

2.1.1.3. 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 the above-mentioned matrix up to 18 ml of buffer (during 2 hours) was used and the same volume of buffer was used for loading (bonding) of sample and washing of ion exchanger.

2.1.1.4. Elution of killer protein in salt gradient

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

2.1.1.5. Regeneration of matrix in column 11A

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

2.1.1.6. 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, 1995/1996; confidential material], i.e. 1 arbitrary/lethal Unit of killer protein activity is 10 mm² of clear zone surrounded by dead cells (a field w/o of well area). The clear zone was such an effect of killer activity of toxin (in volume 20 μl) against the sensitive strain (e.g. *Saccharomyces cerevisiae* S · 6/1) in the test medium.

Therefore, 1 Unit, 10 mm², is $\varnothing = 1.37$ mm of clear zone w/o live 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 $7.71 \cdot 10^3$ cells of *Saccharomyces cerevisiae* S · 6/1 or $7.71 \cdot 10^4$ cells of *Pseudomonas fluorescens* DSM 50106 (or eventually other bacteria strains). In practice, the clear zone with a diameter smaller than 2 mm (i.e. 1.6 U) was not considered due to non-precise measurements.

1 arbitrary Unit of killer activity = $7,7 \cdot 10^3$ killed sensitive yeast cells or $7,7 \cdot 10^4$ 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/6 ρ^- .

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. Samples were stored at -20°C for a long time. The above-mentioned preparations were necessary for SDS-PAGE electrophoresis to avoid the problem of concentrated buffer salt and for visible purity of separated protein by IEX chromatography.

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

For separation (purification) of killer proteins the method of ion exchange chromatography with the following column was also used:

- 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 column 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 -CH₂-SO₃⁻. Ionic capacity of the gel is 0.13-0.18 mmoles/ml. Separations of substances with molecular weights up to 10⁷ are 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 (C₁-C₄ alcohols).

In the case of killer protein separation (Column Resource Mono S®) manually run programme B₁ (way 2) was used:

Specification 1

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.

2.1.2.1. Buffer systems and gradient separation

For the equilibration of Resource Mono S[®] column and for the 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 the 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 the above buffer system Resource Mono Q column, killer toxin 250 and 50x concentrated.

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

2.1.3. Gel filtration chromatography (GF)

2.1.3.1. Column and matrix

Separation of killer proteins presented in concentrated (min. 200x) and dialysed samples of yeast cultures was 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.

2.1.3.2. Killer protein elution

Samples were eluted with equilibration buffer, i.e. by 50 mM citrate/phosphate at pH 4.7 and 4°C. The volume of fraction was 600 µl, collected over 12 minutes. The total time of separation was max. 17 hours.

2.1.3.3. Molecular weight standards

In this case, cytochrom c (MW 12.5 kD), chymotrypsinogen A (25 kD), albumin fraction V (66 kD) and aldolase (158 kD) were used as standards.

2.1.3.4. Biological test for killer activity in fractions after gel filtration chromatography

All fractions which showed peaks were tested for killer activity and then active fractions were collected, dialysed (10 kD MWCO membrane, for 24 hours, in water

at 4°C) and next concentrated by freeze-drying method (till 2000x). These samples were ready for SDS-PAGE electrophoresis, and storage at -20°C for a long time.

2.1.4. Molecular weight determination

2.1.4.1. 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 the first estimation of the bands in samples obtained after IEX or gel filtration chromatography of concentrated cultures. For sample preparation simultaneous desalting and concentration in "Centricon-3" (firm "Amicon") was used.

2.1.4.2. SDS-PAGE electrophoresis (10% T)

The method of polyacrylamide gel electrophoresis in reducing conditions, i.e. with sodium dodecyl sulphate (10% polyacrylamide gel matrix, suitable for protein in the range of 20-100 kD) was used for the determination of the approximate molecular weight of studied killer proteins. The following markers were used: High-Range Marker, M_r 39.2-200 kD, firm Boehringer-Mannheim.

Below is presented the protocol of gel preparation (thickness 0.75 mm) for small system (vertical set up), i.e. Midget Electrophoresis Unit, LKB 2050.

Specification 2

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: 0.5 M Tris-HCl at pH 6.8 with 0.4% SDS and 0.4% TEMED. Separating gel buffer 4x conc. contained: 1.5 M Tris-HCl at pH 8.8 with 0.4% SDS and 0.4% TEMED. For sample preparation (min. 0.7 µg protein/ml)

sample buffer 2x conc. was used, which contained: 0.12 M Tris-HCl at pH 6.8 with 4% SDS, 20% glycerol, 2 mM EDTA and 0.02% bromophenol blue. Samples (15 μ l/band) were diluted with the above buffer (as 1:1) and heated at 100°C for 4 minutes, cooled and centrifuged for a short time before loading (to remove any debris). For running the gel, a tank buffer with final concentration of 0.025 M Tris, 0.19 M glycine and 0.1% SDS was prepared.

2.1.4.3. 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 the 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 the 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 μ l for 2-D and 50 μ l for electroblotting (selective detection of proteins) were used.

Running conditions for the first-dimensional IEF with IPG gel strips were as follows:

- 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 **not** 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-Weight 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 as follows:

- 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).

2.1.4.4. Silver stain

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

- Fixation I: 50% methanol, 12% TCA, 2% CuCl₂ 5 minutes
- Fixation II: 10% ethanol, 5% acetic acid 5 minutes
- Oxidation: 0.01% KMnO₄ 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% AgNO₃ 5 minutes
- Development: 2% K₂CO₃, 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).

2.1.4.5. Molecular Weight Standard: High Range Boeringer-Mannheim:

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

2.1.5.1. Isoelectric point determination

2.1.5.2. Separation and isolation of killer protein: preparative scale of isoelectric focusing

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 cell-free culture after dialysis and the twice freeze-dried 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 they were analysed for protein content (absorbance, A_{280}). After dialysis, for removal of 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, Servalyte ("Serva") at pH 3-7, was used.

2.1.5.3. Analytical scale

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

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% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ (Starter)	50 μl

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

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.

After separation the gels were silver stained, like in SDS-PAGE and finally washed in the solution of 5% glycerol and protected by cellophane. After air-drying they were stored in a plastic bag.

2.1.6. Protein assay

2.1.6.1. 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 25 µg 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.) they were re-dissolved in 60 µl deionized water. For spectrophotometric measurements 40 µl of this solution was taken.

Samples with less than 25 mg per ml of proteins were mixed with RNA (25 µl soluble RNA at 5 mg/ml in 1 ml of sample) and after incubation (45 min. in bath-ice) and centrifugation (for 10 min. at the 27 000 g, 20°C) pellets were dissolved in 0.1 N NaOH. Proteins were assayed according to Lowry (22), but with twice the concentration of sodium tartrate and copper sulphate. For spectrophotometric measurements 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 ml of reaction solution).

2.1.6.2. 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 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.

3. Results

3.1. 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⁻) was performed on the media described previously [Salek, Report II 1994/1995; confidential material]. They were developed after a number of experimental cultures, tested for their killer activity against a few different sensitive strains.

For biochemical characterisation of killer proteins, *peptide-free medium* from the examined strains (also described in Report II) was used. It was used for the purpose of escape of numerous non-killer polypeptides/proteins from the medium. These

proteins usually cause some difficulties upon interpretation of the results. Contents of total proteins in the above-mentioned yeast killer cultures are shown in Table 1.

Table 1

A content of total proteins and specific killer activity

Supernatant of killer strain	Medium	Protein ($\mu\text{g/ml}$)	Activity (U/ml culture)	Specific activity (U/mg protein) $\times 10^5$	Specific activity (dead cells/ml cult.) $\times 10^6$
<i>Williopsis mrakii</i> AS/15 ρ^-	YNBglu2	0.8	255	3.2	1.96
	YPG	1.2	455	3.8	3.51
<i>Pichia anomala</i> USCS 25F	YNBglu2	0.9	410	4.6	3.16
	YPG	1.1	600	5.5	4.63
<i>Saccharomyces globosus</i> BKM γ -438	YNBYEglu2	0.6	190	3.2	1.47
	YPG	1.3	330	2.5	2.54
<i>Pichia subpelli culosa</i> NCYC 16	YNBglu2	0.5	130	2.6	1.00
	YPG	0.9	220	2.4	1.70
<i>Hansenula anomala</i> NCYC 435	YNBglu2	0.4	105	2.6	0.81
	YPG	0.8	160	2.0	1.23
<i>Hanseniaspora valbyensis</i> 13cs/6 ρ^-	B _m	0.3	80	2.7	0.62
	MRS	1.2	160	1.3	1.23

In order to assay of proteins in a very low amount ($> 25 \mu\text{g/ml}$), especially in the culture of killer yeast on *peptide-free medium*, it was necessary to use a modification of Lowry method (with RNA, see Methods). Biochemically examined strains contained protein on *peptide-free medium* in the range of 0.3-0.9 $\mu\text{g/ml}$. Therefore, for further purification, (using ion exchange or gel filtration chromatography), dialysed concentrates, in about 200-fold concentration (i.e., 0.60-0.18 mg/ml) were used.

Upon the first isoelectric focusing separation (analytical scale, on 10% polyacrylamide gel), many bands with different isoelectric points were identified.

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

The next step of protein purification was a preparative large-scale separation of proteins in solution based on the differences of isoelectric points (pI) (for instance see Fig. 1).

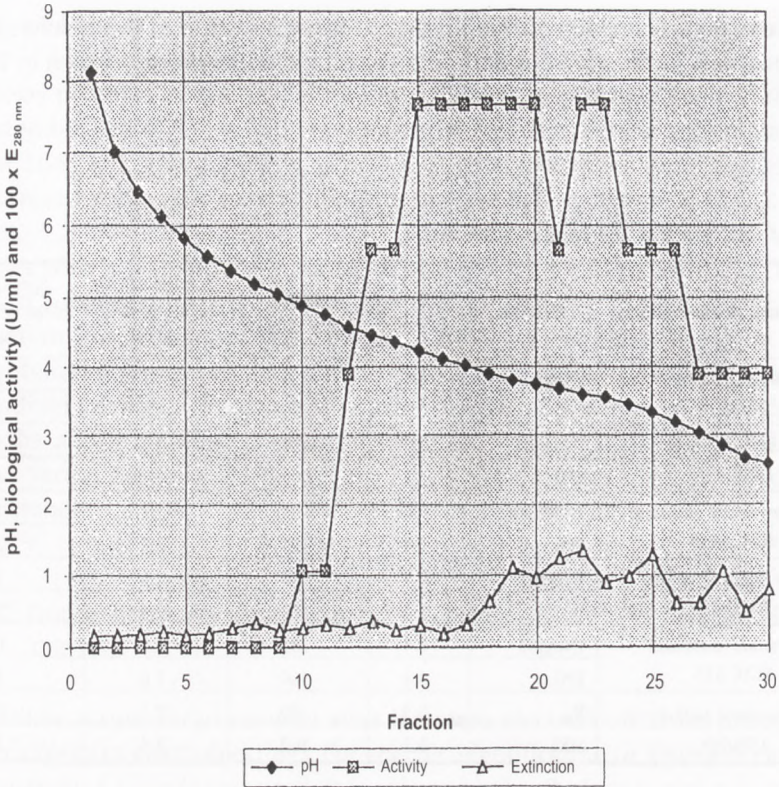


Fig. 1. Isoelectric focusing (preparative scale) of killer protein in RF-3 Recycling Free Flow Focusing Apparatus. Conditions of separations:
 - a volume of 110 ml, incl. sample, with 1% Servolyte (pH 3-4) and 10% glycerol;
 - electrolytes: 0.1 N H₃PO₄ (positive charge) and 0.1 N NaOH (negative charge), at 2°C.
 Legend: a) pH of fractions; b) killer activity of obtained fractions; c) absorbance (A₂₈₀) of proteins (incl. killer) in fractions.

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

Biological test applied for the determination of killer activity in over 30 fractions, obtained after gel chromatography separation, showed that all the analysed concentrated samples had killer activity in a very big range of fractions, e.g. from no. 8 till fraction no. 25 (for instance see Fig. 2). This means that the molecular weight of all the analysed proteins should be between 45 kD and 60 kD, but not smaller than 45 kD. For that reason, 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

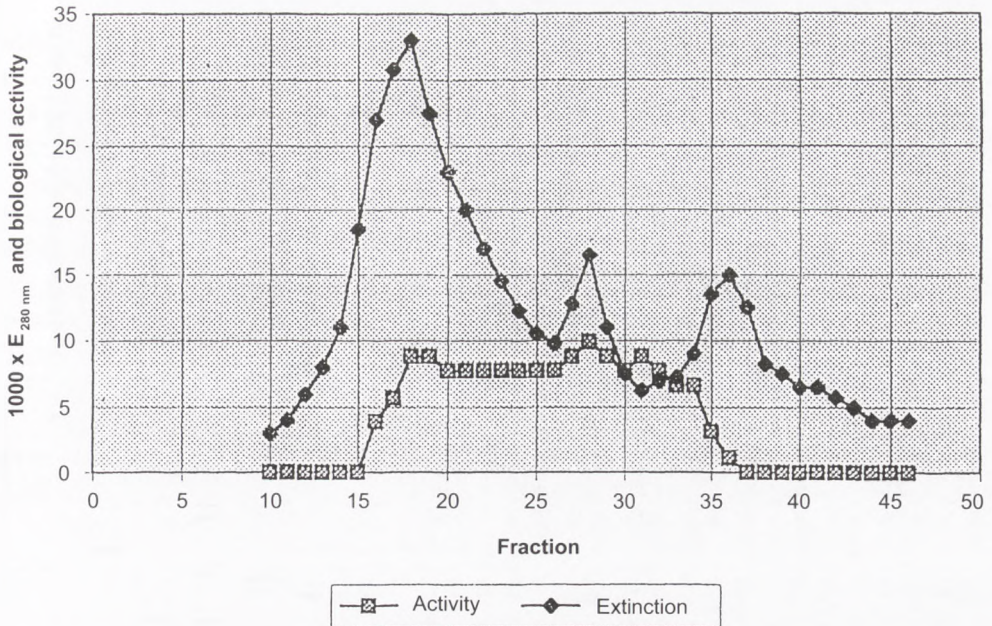


Fig. 2. Gel filtration chromatography (GF) on column TSK G 2000 SW of killer protein from *Williopsis mrakii* AS/15 ρ^- – the first separation. The second separation of protein (as a last polishing/purification method of killer active fractions) was performed using the ion exchange chromatography Fast Flow on Sepharose SP.

as for the 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, higher than expected, when they were compared with the elution of standards (cytochrom c, chymotrypsinogen A, egg albumin). Therefore, it was hypothesized that killer toxins (often glycoproteins, detected by the concanavalin A-peroxidase method after blotting to nitrocellulose) might have undergone subunit aggregations. That effect has also been previously reported by Kagiya (56). Moreover, in many cases (e.g. *W. mrakii* AS/15 ρ^- , *P. anomala* USCS 25F) on PAGE-native one-big-wide spot was shown, which seems to come from proteins with different glycosylation level. Following the isoelectric focusing separation on PAGE (analytical-scale), minimum 2 proteins bands with different the pI were identified (see summarization in Fig. 3).

That problem was partly resolved by using IPG-Dalt method for 2-D electrophoresis as the specific conditions of sample preparation ensured some dissociation of toxin-carbohydrate complexes.

Afterwards, it was much easier to interpret the results and to determinate the real killer proteins. Dissociative conditions for the toxin-carbohydrate complex (w/o inactivation of it) in this separation method (sample soluble in the buffer contained: urea, DTT, detergent) showed that killer-active fractions of some strains possessed

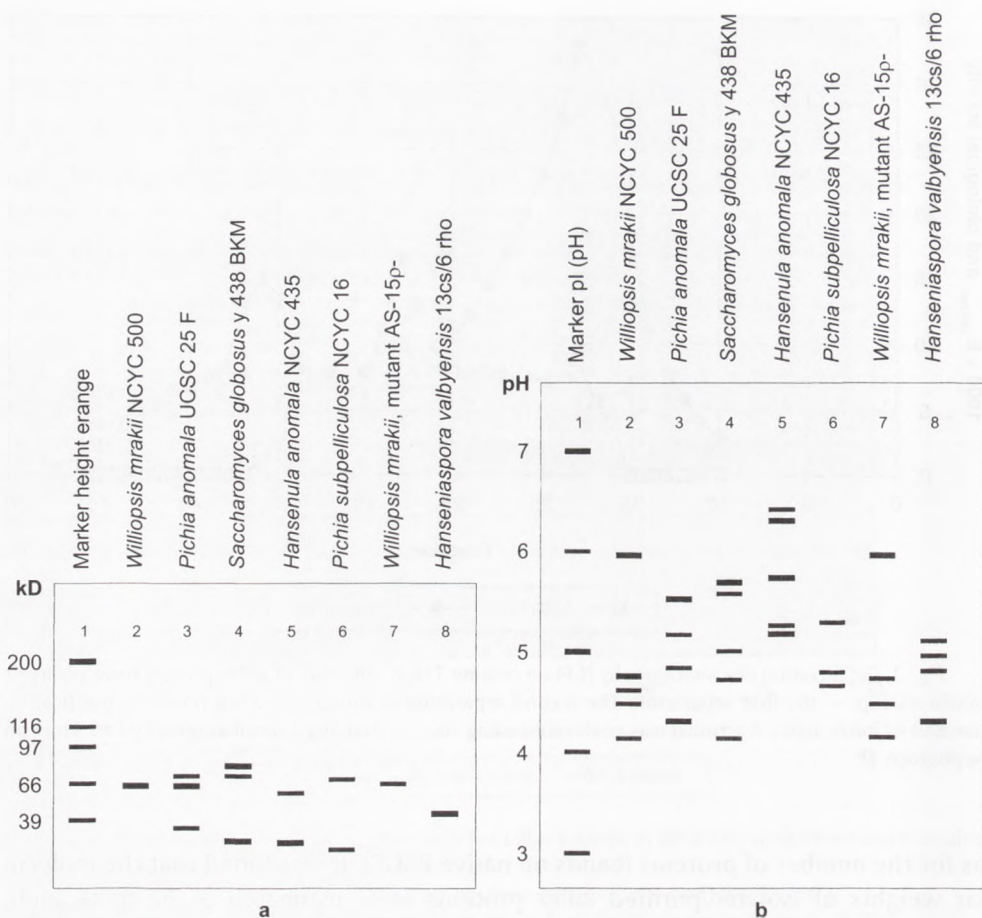


Fig. 3. Biochemical parameters purified killer proteins. Scheme, prepared by using a statistical (satisfactory) number of PAGEs for representative killer proteins: a) Molecular weight of killer proteins, purified by IEX chromatography; b) Isoelectric point of killer proteins, separated by gel filtration chromatography.

agglomerated proteins of different origin (some of glycosylated), molecular weight and the pI. However, when killer toxin from *Williopsis mrakii* AS/15p⁻ was collected after gel filtration and subjected to IPG-SDS, the obtained protein bands did not show any detectable carbohydrate moieties (data not shown).

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

Therefore IEX chromatography separations of proteins from concentrated samples (20-60-times) of studied yeast cultures was performed. Each fraction from indi-

vidual 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). The concentration of killer toxin was 250x.

After elution in gradient of salt all fractions with the highest killer activity (No. 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. 15,6 mg powder with 5.42 mg of killer proteins was obtained (according to Lowry method). That powder was dissolved in 5 ml MilliQ sterile water (preparation – a pure killer protein) and its purity was confirmed using SDS-PAGE or IEF technique.

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

Table 2 shows the biochemical characteristics of killer proteins, i. e. their molecular weight and isoelectric point, resulting from the experiments recently performed.

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

3.2. Characteristic of killer proteins from 6 strains of yeast

3.2.1. *Williopsis mrakii* AS/15ρ⁻

Williopsis mrakii AS/15ρ⁻, the yeast with saturn-shaped ascospores, show quite strong killer activity toward various yeast species [Salek, Report II, 1994/1995; 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/15 ρ^- possesses a better extracellular secretion of killer toxins, which was testified using sensitive strain of *Saccharomyces cerevisiae* S · 6/1 in biological test.

For optimal production of killer protein (type K₉) one should use YPG medium (pH 5.0) and 3 days cultivation at 25°C with vigorous shaking on the 1st day, gentle shaking on the 2nd day and w/o shaking on the 3rd day.

Table 3

Molecular weights and isoelectric focusing points of killer proteins

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

* G – glycoprotein

Table 3 (or Fig. 3) presents the purified killer toxins from above strain:

– polypeptide of a molecular weight (M_r) about 14 kD and some of less than 10 kD,

– glycoprotein of a molecular weight 67 kD.

They had the pI at pH 4.87 and pH 5.1, respectively. Moreover, they were very stable against stomach digesting enzymes (pepsin, pancreatin and bromelin), they showed 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, 1992/1993; confidential material].

3.2.2. *Pichia anomala* UCSC 25F

Pichia anomala USCS 25F is also a yeast with saturn-shaped ascospores, secreting very active killer toxins against quite a wide range of microorganisms, including pathogens [Salek, Report II, 1994/1995; confidential material].

YPG medium (pH 5.0) and the same cultivation conditions as for *Williopsis mrakii* AS/15p⁻ should be used for optimal production of killer protein.

The purified killer toxins are (Tab. 3 or Fig. 3): a polypeptide with M_r smaller 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 show distinct killer activity in the range of pH 3-5 and at the temperature up to 35°C as well as they do not lose all the activity during the operation of digesting enzymes.

3.2.3. *Saccharomyces globosus* BKM y-438

The genetic origin of killer proteins from the 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 produce killer toxin on *peptide-free medium* YNBglu2-medium (w/o amino acids). That yeast needed a small amount of yeast extract (YNBYEglu2-medium). Optimal medium for the production of the killer toxin was YPG-medium and propagation according to Report II [Salek, Report II, 1994/1995; confidential material].

Purified and separated killer proteins (type K₄) were active in the pH range of 3.0-4.7 and at temperature 25-37°C [Salek, Report I, 1992/1993; 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 pI, e.g., at pH 4.7 and 5.43.

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

3.2.4. *Pichia subpelliculosa* NCYC 16

This strain showed optimal growing and production of killer toxins (type K₅) on YPG-medium and secreted three proteins:

- protein with M_r 20.2 kD and pI at pH 4.86,
- protein with M_r 67 kD, pI at pH 4.75 and
- glycoprotein with M_r 67 kD but with pI at pH 5.34.

The above proteins showed activity against tester strain of *Pseudomonas fluorescens* DSM 50106 between pH 2-4.7 at 25°C [Salek, Report I, 1992/1993; confidential material]. Digested enzymes had no destructive influence on killer proteins.

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

3.2.5. *Hansenula anomala* NCYC 435

Type of toxin K₈. For optimal production of killer protein *YPG-medium*, and conditions as described in Report II [Salek, Report II 1994/1995; confidential material] are needed. They were represented by:

- glycoprotein with M_r 51 kD and pI at pH 5.22,
- protein with M_r 25 kD and pI at pH 5.15.

Killer proteins from *Hansenula anomala* NCYC 435 were active between pH 3-5 and at temperature not higher than 35°C. It was found that *Hansenula anomala* NCYC 435 was particularly active against pathogenic bacteria, such as *Micrococcus* sp., and *Escherichia coli* [Salek, Report II, 1994/1995; confidential material].

3.2.6. *Hanseniaspora valbyensis* 13cs/6p⁻

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

Nevertheless, anti-microbial activity of these proteins appeared highly efficient in the killing of pathogenic bacteria and fungi [Salek, Report II, 1994/1995; confidential material] which was probably the result of specific receptors and the presence of organic acids (at quite a high level), lactic acid or acetic acid in the tested samples (data not shown).

4. Discussion

4.1. 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 mean that changes in chromatographic conditions may have a profound effect. However, in chromatography the balance between the preservation of biological activity and protein stability must be maintained.

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

Ion exchange chromatography belongs to the most precise methods for the fractionation of biological substances and it is based on differences in charge char-

acteristics (cationic/anionic). Thus, it is 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, 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 as proteins) showing even small differences in the surface charge characteristics may be separated by ion exchange chromatography. Very high resolution is usually 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 for 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 the majority of proteins. Most protein separation schemes involve 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 the negatively charged target molecules (anions), or a negative charge (cation exchange), used to separate the 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. It is worthy of 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) binds 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 the stability and solubility of both the target protein molecule itself and the other molecules in the sample. To maximize the binding strength, an operating pH range must be selected 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 was performed according to differences in their molecular weight and sizes as they passed through a column packed with a chromatographic medium which was gel. The 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 at 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, an additional method was necessary, such as **IEF, isoelectric focusing** on PAGE, for identifying one or more critical impurities that later were removed from the target. In all cases, however, the screening assays were limited to the information needed to identify "good" results and to move forward with the chromatographic development.

As it has been mentioned in the Results, the molecular weight of the 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 expected. In some cases, after the isoelectric focusing separation on PAGE (analytical-scale), minimum 2 proteins bands with different pI were detected in most killer-active fractions.

It was only possible to resolve such a 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 of different origin (some of glycosylated), molecular weight and isoelectric point.

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

4.2. Biological activity of killer proteins

The killing effect is mediated by diffusible toxins since sensitive cells are killed/inhibited when 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 of 5-6 units/ml, respectively.

Moreover, the killing of sensitive cells (e.g. *Saccharomyces cerevisiae* S · 6/1) was strongly dependent upon the physical parameters of the environment (i. e. buffered medium, where the toxin function and stability depends 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 the number of specific receptors on the surface of the cell wall in sensitive acceptor strains (e.g. in pathogenic fungi such as *Candida albicans* or bacteria, such 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 it was detected that killer toxin of 1 arbitrary unit (3-4 ng killer protein) killed $7,7 \cdot 10^3$ yeast cells or $7,7 \cdot 10^4$ bacteria.

It was also found that the adsorption of killer toxin to the yeast cell wall (because of 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/15 ρ^- , *Pichia anomala* UCSC 25F and *Saccharomyces globosus* BKM y-438 many debris cells were observed (as a dark-blue front of the clear zone on the test-medium).

Furthermore, cell-excretion was very important for killer activity. For all examined toxins it was detectable from the middle exponential phase and it was maximal in the 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 was quite complex (see also (60)).

Since the discovery of killer toxins from yeast was made over 30 years ago and that phenomenon has been the subject of numerous papers – purification of toxins has appeared difficult (18-20).

As reported previously by some authors (61), the toxin (protein) of killer *Saccharomyces cerevisiae* was absorbed by Sephadex and thus it was not fractionated by gel filtration with Sephadex G-150. Due to a broad distribution of the killer toxin in many fractions after gel filtration on Sephadex 4B, it was assumed that the toxin consisted of a multicomponent complex, which could polymerize at highly concentrated protein (62).

As it 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 this type of preservation was used. Moreover, even glycerol was used, for an ultrafiltration technique through "Amicon" membrane PM10 or PM30 showed evident disruptions of killer proteins, especially for *Williopsis mrakii* AS/15p⁻, *Pichia anomala* USCS 25F and *Saccharomyces globosus* BKM y-438. Freeze-drying technique or "Centricon-3" concentrator was much more protective.

The instability of killer activity was also observed during purification of proteins by ion-exchange chromatography, using a cation exchange batch method with "Source™ 30Q" (Pharmacia Biotech AB). Probably it was caused by the separation conditions which were not optimal for the activity of 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). At the temperature above 35°C they 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 the purification process, and
- demands for production of killer proteins – were toxins from *Williopsis mrakii* AS/15p⁻, *Pichia anomala* USCS 25F and *Saccharomyces globosus* BKM y-438. Their killer activity with lethal effect on many pathogens and as a model system for the fungal viruses may also appear to be a model for latent viral infection of higher eukaryotic organisms, as in mammalian cells the toxicity effect of such proteins was not observed (data not shown).

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