COMPARISON OF ANTI-CRYPTOCOCCAL KILLER TOXIN PRODUCING AND NON-PRODUCING STRAINS OF *FILOBASIDIUM CAPSULIGENUM,* AND CHARACTERIZATION OF THE TOXINS

A thesis submitted by

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Introduction

In nature competition for territories and food had forced organisms to develop different mechanisms to their successful spreading and living. Among these are the antagonistic relationships, where a given organism represses the growth of its competitors. Processes like this can be found in every level of living organisms from the prokaryotes to higher animals. The suppression of growth can have common effect, or can be specific to different organisms. Killer toxins or mycocins secreted by fungi and active against only strains related to the producing strain represent metabolites with specific effect. Killer toxins were first described forty years ago in *Saccharomyces cerevisiae.* Since then many strains with killer-phenotype were found. Information from these toxins helped scientists in many ways to better understand several biological processes. Besides, antifungal property of toxins raises the possibility of many agricultural and industrial applications. Some of these has overcome experimental phase and is in practical use. There are ongoing tests of medical application of killer toxins active against pathogen yeasts (*Candida* and *Trichosporon* species, *Cryptococcus neoformans*) and antifungal agents developed based on their characteristics. In this work we screened for strains having the ability to produce killer toxin active against the opportunistic human pathogen yeast *Cryptococcus neoformans*. IFM 40078 strain of *Filobasidium capsuligenum* produces a highly effective anti-cryptococcal killer toxin. As it is known that killer-phenotype is strain and not species dependent, we tested further isolates of *F. capsuligenum* on toxin production. We compared other characteristics of toxin producer and non-producer strains. Their phylogenetical relationships were also examined by different molecular methods suitable for distinguishing species and taxons under this level. These methods included comparison of variable rDNA sequences, PCR-fingerprinting, RAPD and mtDNA-PFLP patterns.

It is also known, that not all stains of a given species are able to produce killer toxin and producer strains do not always secret the same mycocin. In some cases one strain can produce more than one toxin. Thus, we aimed to compare the toxins of the producing strains of *F. capsuligenum*. Besides, we also attempted to identify and partially purify FC-1 toxin of strain IFM4078. Known toxins can have several different

mechanisms which leads to their lethal effect. They can disorganise the membrane, induce cell cycle arrest or repress synthesis of the cell wall. All of these toxins have receptors on the cell wall and membrane of the sensitive cells, which are needed to ensure their specific effect. Better understanding of the mechanisms and receptors underlying the lethal effect of the toxins is especially important in the case of toxins active against pathogenic yeasts, as this knowledge can lead to the development of new and efficient antifungal drugs. Thus, in our work we also aimed to identify the cell wall receptor and characterize the mechanism of action of FC-1 toxin.

Aims of the thesis:

- Screening for anticryptococcal toxin producing strains
- Identification of *F. capsuligenum* strains with killer activity, characterization of killer-sensitive relationships
- Comparison of killer and non-killer strains of *F. capsuligenum* strains, characterisation of their phylogenetic relationships
- Comparison of killer toxins secreted by the producing strains, description of their specificity and biochemical characters
- Characterization, identification and partial purification of FC-1 toxin of the IFM 40078 strain
- Identification of the cell wall receptor and characterization of the mechanism of action of FC-1 toxin

Methods

- Classical microbiological methods
  - Maintenance of the strains
  - Production of the toxins
  - Determination of mating types
  - UV mutagenesis
- Molecular methods
  - Analysis of ITS/D1D2 region
  - RAPD-analysis
  - PCR-fingerprinting
  - mtDNA RFLP
- Phylogenetic analysis
  - Sequence alignment CLUSTALW program
  - Generation of phenograms
    - Phylogenetic Computer Tools Version 1.32
    - PHYLIP (Phylogeny Inference Package) 3.65
- Protein based techniques
  - SDS gel electrophoresis
  - Affinity chromatography
- Methods for characterization of the mechanism of action of FC-1 toxin
  - Competition analysis
  - FITC (Fluorescein isothiocyanate) staining
  - Laser scanning cytometry

Results

1. Screen for strains with killer-phenotype, characterization of killer-sensitive relationships

In this work yeast strains were screened for the production of anti-cryptococcal toxin. Only *Filobasidium capsuligenum* IFM 40078 proved to be able to kill *C. neoformans* cells. In order to explore the rate of occurrence of killer toxins within the *F. capsuligenum* species, we collected nine more strains from strain collections - originating from different geographical and environmental sources. A relatively high percentage of them (40%) had the ability to produce anti-cryptococcal toxin, and all the non-killers were sensitive to them. No neutral (not producing and not sensitive) strains were among the ten isolates indicating that no strains evolved that had lost their ability to produce toxin, but had retained their immunity. This might suggest that the gene(s) responsible for toxin production is/are encoded on chromosomal DNA, as extrachromosomal fragments are less stable than nuclear ones.

2. Characterization of killer and non-killer *F. capsuligenum* strains and their phylogenetic relationships

To reveal the relationships among producing and non-producing *F. capsuligenum* strains, first we identified their mating types. With one exception (NCAIM Y-0472) all non-producing strains had mating type **a** and all the producing ones had α; indicating a possible link between the killer-phenotype and α mating-type.

Electrophoresis of total nucleic acid extracts of the producing strains showed no other bands than the nuclear DNA and the ribosomal RNAs, which supports our earlier suggestion, that the toxins are encoded on chromosomal DNA. Thus, the difference between producing and non-producing strains is at the chromosomal level, and not the presence of DNA plasmids or RNA viruses is responsible for the phenotypic alteration. Accordingly we examined whether the two groups also differ in other regions of their nuclear DNAs. Sequences of variable rDNA regions such as ITS and D1/D2 were

compared and RAPD-analysis was performed. The strains belonging to the producing groups were identical in their nucleotide sequences in the D1/D2 region, and slightly differed (0.4%) in their ITS regions. These were also true for the members of the non-producing group. However, high rate of divergence (1.6-2.24%) was observed between the two groups in both rDNA regions. The phenogram based on PCR-fingerprinting and RAPD data also confirmed the divergence of the two groups. Analysis of mtDNA-RFLP patterns gave similar results, indicating that these strains differ not only in their chromosomal DNA, but also at the extrachromosomal level.

This high rate of difference could raise the possibility of the proposal for two varieties of *F. capsuligenum*.

3. Characterization and comparison of the toxins produced by the strains with killer-phenotype

After revealing the relationships between the producing and non-producing groups we compared the toxins of the strains with killer-phenotype. According to our results they were identical both in their specificity and in their biochemical properties. All four toxin-producing strains were active against numerous *C. neoformans* isolates, independently from their serotypes and molecular subtypes as well as from their source. There was only a single exception (WM 161) among the tested *C. neoformans* strains which showed resistance to all four toxins. This resistance can be resulted from differences in cell wall or membrane receptors, which are known to be responsible for resistance in many cases. The four toxins also showed identical specificities when other yeast strains were tested.

As the toxins were indistinguishable by their specificity, their biochemical characteristics were compared. However, to further study these toxins, the optimal parameters for toxin production had to be obtained, as according to the literature, mycocins are usually extracted at low concentration. Growing the killer strains in different media revealed that only complete media are suitable for toxin production. In the absence of peptone or malt extract, the addition of yeast extract or the 21 essential amino acids was not sufficient for gaining biologically active substance. No toxin production was detectable in the presence of dead, sensitive *C. neoformans* either. Thus, the question, which components of the complete media are necessary for successful toxin

production, remains open. According to our results the growing cells must reach the early stationary phase before they can produce detectable amounts of toxin. Toxins are the most active against young sensitive cells in their logarithmic phase suggesting that the natural the role of killer toxins is to defend the occupied niche from new invasive strains. However, probably because of the increasing pH value or the cumulative protease production of the ageing cells, no killer activity could be detected in the late stationary phase of the culture.

The toxins also share common heat and pH optima. They are inactive above 30 °C and pH 5.5-6. As toxins do not reactivate with reestablishment of the low pH value, this process is probably irreversible. This also suggests that the role of the low pH is not in helping the interaction with the cell wall of the sensitive cells; rather it has function in the stability of the toxin.

Toxins can be proteins or glycoproteins, but other compounds such as glycolipids can also have antifungal effects. To prove the proteinaceous nature of antifungal agents produced by *F. capsuligenum* strains, they were treated with different proteinase enzymes, and their residual activity was measured in bio-assay. Proteinase E treatment resulted in significant loss of activity which confirms that these toxins are peptides, proteins or glycoproteins. The effectiveness of the other enzymes can be resulted from the absence of their recognition site but the reason also can be that low pH is not optimal for the function of these enzymes.

As no difference was detectable between the toxins of the four producing strains by different approaches, it can be concluded that these toxins are very similar, if not identical agents.

4. Characterization of FC-1 toxin of IFM 40078 strain

After these experiments, we intended to study the FC-1 toxin of IFM 40078. To examine the specificity of FC-1, we isolated and further tested *Cryptococcus* isolates from environmental and clinical sources. Besides, several other yeasts and some filamentous fungi were studied for toxin sensitivity. Except for the above mentioned WM 161, all strains that proved to be *C. neoformans* were sensitive to FC-1. Within the genus *Cryptococcus* one strain out of two *C. laurentii* and *C. podzolicus* were sensitive while all strains of *C. albidus* showed resistance. Regarding that besides *C. neoformans*, *C. albidus* is more and more frequently infecting immunocompromised patients; its toxin resistance can be used for exact identification of the causative agent of cryptococcosis. Two *Schizosaccharomyces cerevisiae* mutants also showed resistance, but this is most probably caused by random mutations arising from UV mutagenesis. The sensitivity of one strain of *Candida quilliermondii* – another opportunistic human pathogen – is unique, because the other five examined *C. guilliermondii* strains and several other strains belonging to the genus *Candida* showed resistance. The sensitivity of a member of *Filobasidiaceae – Paratorulopsis pseudaeria* – is not surprising due to its close relationship to *F. capsuligenum*.

The size of the FC-1 toxin was determined by filtering the crude toxin through membranes with different pore size; revealing that the size of the toxin is between 30 and 50 kDa, which is in accordance with the size of other toxins in the literature. To identify the toxin, *F. capsuligenum* IFM 40078 was cultured in different media where the toxin activities varied. SDS gel electrophoresis was performed on the extracellular proteins of these cultures, but no bands could be observed with altered density according to the toxin activity. *F. capsuligenum* cultured in synthetic minimal medium had no toxin activity, and in its extracellular protein pattern several bands were missing compared to the YM4 control medium. Thus, with these methods we could not identify the FC-1 toxin. Nevertheless, non-producing mutants were generated by UV mutagenesis and their extracellular protein profiles were compared to the active control. The non-producing VKM 1513 strain was also involved in this comparison. One protein with approximately

40 kDa in molecular weight was missing from these non-producing strains. However, sequencing of this band showed that it contains two or more proteins.

As no exact identification was possible with the methods mentioned above, we aimed to purify the toxin. For this, we intended to exploit the interaction between the toxin and the cell wall of the sensitive cells. Binding directly to the sensitive cells was not effective to purify the toxin. However, active fractions could be separated when the possible cell wall receptor (pustulan) was used as a ligand for affinity chromatography. SDS electrophoresis of these fractions showed three bands, one in the size region that we determined earlier as the possible size of FC-1. The *N*-terminal of this protein showed no significant homology to other toxins or proteins. However, regarding that homology between toxins is quite rare, the possibility that this protein is responsible for the killing activity can not be excluded.

In the last part of this study we intended to reveal the mechanism of action of the FC-1 toxin. Toxins described in the literature all have a receptor on the cell wall of sensitive cells. This receptor can be any of the main cell wall components such as mannan, laminarin, β-glucans and chitin. As competition assays revealed, the possible cell wall receptor of FC-1 is β-1,6-glucan (pustulan). Studies on the kinetics of killing effect showed that the toxin has cytocydal effect. The numbers of dead cells was counted using FITC stain, which could penetrate through the disorganized cell membrane of the dead cells. This staining also indicates that the toxin acts by disorganizing the cell membrane or the cell wall. To explore whether FC-1 has an effect on cell cycle the DNA contents of the sensitive cells were measured. Following the analysis of the proportions of n and 2n state DNA it was revealed that the toxin has no effect on the cell cycle. As sensitive cells were not protected by isoosmotic environment, it can be presumed, that FC-1 – as several other toxins – expresses its killing effect by disorganizing the cell membrane of the sensitive cells.

Conclusions

In our work we screened yeast strains of SZMC (Szeged Microbial Collection) for strains that are able to produce killer toxin with anticryptococcal effect. Only IFM 40078 strain of *F. capsuligenum* had the ability to repress the growth of *C. neoformans*. The result of testing nine further strains of *F. capsuligenum* indicate that killer-phenotype is relatively common in this species. Non-producer strains were all sensitive to the toxins of the producer strains. Our results on comparison of toxin producing and non-producing strains suggested that these groups differ significantly both in their nuclear and mitochondrial genomes. This divergence raises the possibility of the existence of two varieties in the species. Comparison of the toxins of the producer strains showed that they share common specificity and biochemical characteristics. They are most likely encoded in the nuclear DNA, are active only in a narrow, acidic pH range; are heat labile and have proteinous nature.

Size of FC-1 toxin of IFM 40078 strain appeared to be approximately 30-50 kDa. Growing IFM 40078 strain in different media results in altered toxin activity. Comparing the extracellular protein profiles of the crude toxin extract of these cultures there was no band observed with altered density according to the toxin activity. Thus, non-killer mutants of IFM 40078 were generated by UV mutagenesis and their extracellular protein profiles were compared to the crude toxin preparation of wild type IFM 40078 culture. A protein, 40-45 kDa in size, was identified, which was missing from the profiles of the mutants. As purification using sensitive cells was not successful, the possible cell wall receptor (pustulan) of FC-1 was used as ligand for affinity chromatography. Active fractions were collected, and separated by SDS gel electrophoresis. Three protein band could be identified, one in the size region that we determined earlier as the possible size of FC-1. The *N*-terminal sequence of this protein was determined, and no significant homology to other toxins or proteins was found in databases.

Our results show that the possible cell wall receptor of FC-1 toxin is β-1,6-glucan (pustulan); and toxin has citocydic effect. It probably does not arrest cell cycle, and has

no effect on the cell wall of the sensitive cells. Most likely the killing effect of the toxin is expressed by disorganization of the cell membrane.

Many interesting questions arose during our work that has to be answered by further studies. Investigation of the possibility of a linkage between toxin production and mating type could add interesting and new results to our knowledge of killer toxins and antagonistic relationships between these strains. Taking an advantage from the specificity of the FC-1 toxin it could be applied – in combination with other toxins, or molecular methods – to identify species or strains with epidemiological interest. Besides, further purification of the toxin followed by generation of antiidiotypic antibodies can lead to a new, antifungal therapy effective against *C. neoformans*.

Publications on the thesis

Full papers:

Keszthelyi A, Hamari Z, Pfeiffer I, Vagvolgyi Cs, and Kucsera J
Comparison of killer toxin producing and non-producing strains of *Filobasidium capsuligenum*, proposal for two varieties, Microbiological Research, In Press

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Abstracts:

Keszthelyi A
Characterisation of anticryptococcal FC-1 toxin of *Filobasidium capsuligenum,* Acta Biologica Szegediensis Volume 50(3-4):155, 2006

Posters:

35th Annual Conference on Yeasts, 2007, Smolenice, Slovakia,
Poster: **Andrea Keszthelyi,** Zoltan Farkas, Zsuzsanna Hamari, Ilona Pfeiffer, Csaba Vágvölgyi, Judit Kucsera
Comparison of killer toxin producing and toxin non-producing *Filobasidium capsuligenum* strains

34th Annual Conference on Yeasts, May 10-12, 2006, Smolenice, Slovakia
Poster: **Andrea Keszthelyi**, Zoltán Farkas, Zsuzsanna Hamari, Judit Kucsera, Ilona Pfeiffer
Killer toxins against pathogenic yeasts

3rd Hungarian Mycological Conference: May 26 - 27, 2005, Mátraháza, Hungary
Poster: **Andrea Keszthelyi**, Ilona Pfeiffer, Judit Kucsera
Sensitivity of *Cryptococcus neoformans* strains of clinical and environmental origin to killer toxins produced by *Filobasidium capsuligenum* (IFM 40078) and *Cystofilobasidium bisporidii* (VKM Y-2700)

Annual meeting of the Hungarian Society for Microbiology, October 07-09, 2004, Keszthely, Hungary
Poster: **Andrea Keszthelyi**, Mária Kovács, István Palatinus, Zsuzsanna Buzás, Judit Kucsera
Isolation and purification of a killer toxin produced by *Filobasidium capsuligenum* IFM 40078 strain

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Poster: Kucsera J., **Keszthelyi, A**., Kovács, M., Palatinus, I., and Pfeiffer, I.: Molecular characterisation of the anti-cryptococcal toxin producing *Filobasidium capsuligenum*