PHD THESIS

Identification and characterization of the defensin-like antifungal protein produced by *Neosartorya fischeri*

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Introduction

The incidence of microbial infections has significantly increased over the past two decades in consequence of the increasing number of immunocompromised hosts, and the emergence and wide-spread of resistant (often multidrug-resistant) microbial strains arising from the inappropriate use of broad-spectrum antibiotics. Large increase occurred in the case number of opportunistic fungal infections, when a non-pathogenic fungi against human body appears as pathogen in a debilitated patient. The treatment of such infections are especially problematic: the number of available antifungal drugs are quite low, they often have narrow antimicrobial spectrum, they cause numerous side effects thus they are significantly harmful to the host. Another important aspect is that many filamentous fungi are destructive pathogens of plants and are thus responsible for enormous crop losses worldwide. Therefore there is a substantial demand for new compounds with antifungal activity.

The antifungal proteins with similar structure like defensins secreted by Ascomycetes are interesting from this respect. Their main characteristics correspond well to the key requirements of the development of new antifungal agents in medicine and in crop protection, therefore, they open up new horizons for the antifungal strategies. They have different mode of action and effective inhibitory potential against other filamentous fungi including medically and agriculturally harmful species. In antifungally effective concentration they do not exert toxic effects on plant and mammalian cells, and they do not provoke inflammation effect. They have high stability even in extreme environmental conditions. They can interact synergistically with other drugs and antimicrobial peptides.

The defensin-like antifungal proteins produced by filamentous Ascomycetes have very different amino acid sequences, but conserved

homologous regions can be identified. Based on these regions, these molecules can be divided into two groups: proteins with the *Penicillium chrysogenum* antifungal protein (PAF)-cluster and proteins with the *Penicillium brevicompactum* „bubble protein" (BP)-cluster. Proteins with PAF-cluster have been isolated and characterised from six different fungal species: *Aspergillus clavatus* (AcAMP, ACLA), *Aspergillus giganteus* (AFP), *Aspergillus niger* (ANAFP), *Fusarium polyphialidicum* (FPAP), *Penicillium chrysogenum* (PAF, PgAFP) and *Penicillium nalgiovense* (NAF). They are extracellular, small molecular mass, basic proteins containing 6-8 cysteines which form intramolecular disulfide bridges stabilizing the protein structure. The tertiary structure of PAF-cluster proteins is highly similar to the structure of β-defensins: 5-6 antiparallel β-sheet connected with three loop region, showing β-barrel topology. Their compact structure and the presence of the disulfide bonds of the molecules result in stability within extreme environmental conditions and in resistance against proteolytic degradation.

In the genome of *Neosartorya fischeri* isolate NRRL 181, a gene encoding a hypothetical defensin-like antimicrobial protein with PAF-cluster (*N. fischeri* antifungal protein, NFAP) was identified (NCBI ID: XM_001262585) by *in silico* analysis. In this study this protein was isolated and characterized.

Aims

The aims of the present study were the followings:

1. Isolation of the putative NFAP encoding gene from *N. fischeri* NRRL 181 isolate.
2. Isolation of the NFAP.
3. Investigation of the *in silico* structure and phylogenetic relationships of the NFAP.
4. Determination of the *in vitro* antimicrobial properties of NFAP.
5. Examination of mode of action of the NFAP.
6. Investigation of the biological role of the NFAP.

Methods

DNA and RNA techniques:

- DNA extraction
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- cDNA synthesis
- DNA and RNA gel electrophoresis
- Polimerase chain reaction (PCR), *double-joint*-PCR
- Cloning of DNA fragments
- Construction of plasmid and linear deletion fragment for transformation
- Plasmid rescue
- Southern hibridization

Analysis of nucleotide and amino acid sequence data:

- Analysis and comparison of nucleotide sequences
- Deduction of amino acid sequences from nucleotide sequences
- Determination of protein structure *in silico*
- Amino-acid based phylogenetic analysis

Protein techniques:

- Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)
- Size exclusion chromatography
- Ion exchange chromatography

In vitro antifungal susceptibility testing techniques:

- Agardiffusion method
- Microdilution method

Genetic transformation of filamentous fungi:

- Protoplast formation
- PEG-mediated protoplast transformation
- Isolation of monosporangial clones

In vitro antagonism test with filamentous fungi

Light-, fluorescence and scanning electron microscopy

Results

Isolation of *nfap* from *N. fischeri* NRRL 181 isolate (Kovács et al., 2011).

We isolated the *nfap* gene from *N. fischeri* NRRL 181 isolate via PCR experiments. Based on *in silico* investigations, a low molecular mass (6.6255 kDa), extracellular, cationic (pI=8.93), cysteine rich mature NFAP consists of 57 amino acid residues was deduced. The amino acid sequence of the hypotehtical mature protein shows 10.3-22.8% homology to similar proteins described in literature.

***In silico* determination of the structure of NFAP, and its phylogenetic analysis** (Kovács et al., 2011; Galgóczy et al., 2013a).

In silico investigations revealed that the tertiary structure of the mature NFAP is similar to the β-defensin-like molecules: 5 β-sheets connected with loops and stabilised by three disulfide bridges.

The phylogenetic relationships between the isolated and putative defensin-like proteins from Ascomycetes were investigated. Based on the primary protein structure, it seems that phylogenetically NFAP is located between the *P. chrysogenum* PAF and *A. niger* ANAFP and closely related to the *A. giganteus* AFP.

Isolation of the NFAP (Kovács et al., 2011).

Promoter region analysis of *nfap* revealed the presence of several putative regulatory elements that might be involved in the transcriptional regulation of *nfap* in response to environmental signals and stress. Considering these elements, a medium was optimized for the production of NFAP. After the purification of the ferment borth, a ~6.6 kDa protein was isolated from the antifungally active fractions. This protein was named as *N.*

fischeri antifungal protein (NFAP, EMBL ID: CAQ42994). The first 6 amino acid residues of this protein were identified with N-terminal sequencing, which corresponded well to the *in silico* determined sequence of the mature NFAP. The purification of NFAP was optimised. The final amount of the purified NFAP from 1000 ml ferment broth was 1250±123 µg.

Investigation of antifungal properties and effect of the NFAP (Kovács et al., 2011).

We examined the antifungal activity of the NFAP in 96-well microtiter plate bioassays. Among the tested five zygo- and five ascomycetous fungal strains , two isolates belonging to Ascomycetes (*A. niger* and *Aspergillus nidulans*) and one belonging to Zygomycetes (*Rhizomucor miehei*) proved to be susceptible to NFAP in the applied concentration range (12.5-200 µg/ml). NFAP acted in dose-dependent manner similar to the homologous proteins: In the case of *A. niger*, we demonstrated that the growth inhibitory effect of NFAP at sublethal concentration (100 µg/ml) turned into fungicidal at its higher dose (200 µg/ml). The manifestation of the antifungal effect of NFAP is similar to that was observed in the case of the related proteins *A. giganteus* AFP and *P. chrysogenum* PAF: NFAP also caused inhibition of spore germination and delayed, distorted and retarted hyphae lengthening. *Absidia corymbifera*, *Botrytis cinerea*, *Fusarium graminearum*, *Mortierella wolfii*, *Mucor piriformis*, *Rhizopus microsporus* var. *rhizopodiformis* and *Trichoderma longibrachiatum* were not susceptible in the applied *in vitro* antifungal tests.

Effect of different pH and temperature conditions on the antifungal activity of the NFAP was investigated in agar diffusion test against *A. niger*. NFAP showed the highest antifungal activity at pH 8.4-8.6, but it was maintained in different rate under all investigated pH conditions (pH 7.0-

9.0). NFAP proved to be stable even after a temperature treatment at 100 °C for 30 minutes, and the antifungal effect of the treated protein remained after 120 h of incubation at 25 °C. Antifungal activity after treatment at 100 °C for 40 minutes was not observed.

Investigation of mode of action of the NFAP (Galgózy et al., 2013b).

We carried out the heterologous expression of NFAP in *A. nidulans* CS2902 strain. The *A. nidulans* strain, which previously proved to be slightly susceptible to NFAP, produced the protein constitutively and in active form. The NFAP-yield of the expression system was similar to the native producer (1680±223 µg from 1000 ml ferment broth) due to the self-poisoning effect. Delayed and abnormal spore germination was observed in the case of NFAP-secreting transformants compared to the non-secreting *A. nidulans* CS2902 strain: conidiospores formed very short, swelled hyphae with multiple branches. This self-poisoning effect depended on the amount of conidia used for the inoculation: there were no significant differences in the hyphal growth up to 10^4 conidia, but at 10^5 conidia a significant reduction, and at 10^6 conidia a total self-killing effect was observed. Effect of the produced NFAP on the germination of *A. nidulans* CS2902 conidia was investigated with scanning electron microscopy (SEM), 4'-6-diamidino-2-phenylindole- (DAPI) and calcofluor white- (CFW) staining. 2',7'-Dichlorodihydrofluorescein diacetate-staining and Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) were used for revealing the accumulation of reactive oxygen species (ROS) and the possible apoptotic, necrotic effect. The impact of mono- and divalent cations on the antimicrobial activity of NFAP was also examined. The hyphal growth reduction effect of NFAP was absent in the presence of mono- and divalent cations (50 and 100 mM KCl, Mg_2SO_4, Na_2SO_4). These data suggest that NFAP can bind electrostatically to the fungal cell and this can be prevented

by cations. Damage in the organization of cell-wall, destruction of chitin filaments and accumulation of nuclei at the broken hyphal tips were detected by SEM, DAPI- and CFW-staining. Similar phenomenon was observed at the related peptide, *A. giganteus* AFP. Accumulation of ROS and more frequent apoptotic, necrotic events were also observed in the case of the NFAP-producing *A. nidulans* strain. Similar effect was previously described for the *P. chrysgenum* PAF treatment of *A. nidulans*.

Investigation of biological role of the NFAP

We created the *nfap*-deletion mutant strain of the *N. fischeri* NRRL 181, and we analysed its growth in minimal and complete medium compared to the wild-type. We also examined the *in vitro* antagonism ability (via calculation of *in vitro* antagonism index, IVAI) of the wild type and the Δ*nfap* mutant strains against possible competitor fungal isolates belonging to Zygo- and Ascomycetes.

We observed that the Δ*nfap* strain shows slower growth than the wild-type in minimal medium, indicating that NFAP could have an impact on the fungal growth in the presence of nutrient limitation. Compared to the Δ*nfap* strain, the wild-type effectively inhibited the growth of NFAP-sensitive fungal isolates. This difference in the *in vitro* antagonism ability is prevailed strongly in minimal medium. If both types of *N. fischeri* showed inhibition effect against the competitor fungal isolate, the IVAI value of the Δ*nfap* strain was lower. Based on these observations, NFAP could play a role in the emulation for nutrients and habitat against fungi with similar ecological niche and in the fungal growth in the presence of nutrient limitation.

Summary

1. We isolated a new antifungal peptide (NFAP) homologous to defensin-like proteins produced by ascomycetous fungi and its encoding gene from *N. fischeri* NRRL 181 isolate.
2. We determined *in silico* that the tertiary structure of NFAP is very similar to that of defensin-like proteins produced by ascomycetous fungi.
3. Our phylogenetic analysis revealed that NFAP is located between the *P. chrysogenum* PAF and *A. niger* ANAFP and closely related to *A. giganteus* AFP.
4. We demonstrated that NFAP inhibited the growth of filamentous fungi in a dose-dependent manner and it maintained its antifungal activity within broad pH and temperature ranges, furthermore it exhibited relevant resistance to proteolysis.
5. We carried out the heterologous expression of NFAP in *A. nidulans* CS2902 strain. Heterologous expression of a defensin-like protein from Ascomycetes in a filamentous fungi was firstly described in the literature by our research group.
6. We investigated the mode of action of NFAP. We have demonstrated that the manifestation of the antifungal effect of NFAP is similar to that was observed in the case of related proteins.
7. We proved that the presence of mono- and divalent cations decreased the antifungal effect of NFAP.
8. We created a Δ*nfap* mutant *N. fischeri* NRRL 181 strain.
9. We observed the possible role of NFAP in the growth of the producing fungus under nutrient limitation and in the emulation for habitat against fungi with similar ecological niche.

List of peer-reviewed articles directy related to the dissertation:

- **Kovács, L.**, Virágh, M., Takó, M., Papp, T., Vágvölgyi, Cs. and Galgóczy, L. (2011) Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP). Peptides. 32, 1724-1731. (IF_{2011}=2.434)
- Galgóczy, L., Virágh, M., **Kovács, L.**, Tóth, B., Papp, T. and Vágvölgyi, Cs. (2013a) Antifungal peptides homologous to the *Penicillium chrysogenum* antifungal protein (PAF) are widespread among Fusaria. Peptides. 39, 131-137. (IF_{2012}=2.522)
- Galgóczy, L., **Kovács, L**., Karácsony, Z., Virágh, M., Hamari, Zs. and Vágvölgyi, Cs. (2013b) Investigation of the antimicrobial effect of *Neosartorya fischeri* antifungal protein (NFAP) after heterologous expression in *Aspergillus nidulans*. Microbiology. 159, 411-419. (IF_{2012}=2.852)

List of other peer-reviewed articles:

- Galgóczy, L., **Kovács, L.**, Krizsán, K., Papp, T. and Vágvölgyi, Cs. (2009) Inhibitory effect of cysteine and cysteine derivatives on germination of sporangiospores and hyphal growth of different Zygomycetes. Mycopathologia. 168, 125-134. (IF_{2009}=1.728)
- Galgóczy, L., Papp, T., **Kovács, L.**, Ördögh, L. and Vágvölgyi, Cs. (2009) *In vitro* activity of phenothiazines and their combinations with amphotericin B against zygomycetes causing rhinocerebral zygomycosis. Med. Mycol. 47, 331-335. (IF_{2009}=2.133)
- Galgóczy, L., Virágh, M., **Kovács, L.**, Tóth, L. and Vágvölgyi, Cs. (2013) Potential applications of filamentous fungus derived β-defensin-like antifungal proteins in agriculture. Review on Agriculture and Rural Development. 2, Supplement CD of XII Wellmann International Scientific Conference. (IF2012=0.000)
- Virágh, M., Vörös, D., Kele, Z., **Kovács, L.**, Fizil, Á., Lakatos, G., Maróti, G., Batta, G., Vágvölgyi, C., Galgóczy, L. (2014) Production of a defensin-like antifungal protein NFAP from *Neosartorya fischeri* in *Pichia pastoris* and its antifungal activity against filamentous fungal isolates from human infections. Protein Expr. Purif. 94,79-84. (IF_{2012}=1.429)

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