Ph.D. Thesis

Heterologous expression and the investigation of the antifungal mechanism of *Neosartorya fischeri* antifungal protein

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2015

INTRODUCTION

The incidences of human infections caused by filamentous fungi have been increasing continuously in the past two decades as a consequence of the growing number of immunocompromised hosts and the occurrence of antimycotic resistant fungal strains. Despite the therapeutic application of novel, recently introduced antifungal drugs, the mortality rate of mould infection among the immunocompromised patients is between 14% and 100% depending on the casual agent and the degree of immunosuppression. Due to similar cellular, physiological and metabolic properties between hosts and invading fungi, the application of conventional antifungal agents is seriously limited by their severe side effects, especially, in the case of prolonged antifungal treatments. Emerging infectious diseases caused by fungi also threaten animals. Another important aspect is that filamentous fungi endanger agriculture and food industry, they are responsible for enormous crop losses worldwide. The occurrence of mycotoxin producing pre- and postharvest pathogenic fungi and the number of mycotoxin contaminated feeds and foods are also continuously increasing in Europe in the last years. Filamentous fungi are also known as the most important bio-deteriorative agents of cultural heritages in museums, storage rooms, libraries, collections and restoration studios. They destroy papers, parchments, paintings and wooden statues. The protection of these heritages against mould contamination is problematic because the applied biocides and disinfection methods can be toxic for humans and/or can cause further damages to the infected object. Therefore

there is a substantial demand for new, safely applicable compounds with antifungal activity and fungal target selectivity.

In this respect, antifungal proteins secreted by filamentous Ascomycetes are promising candidates (broad antifungal spectra, high stability in extreme environmental conditions, limited toxicity towards mammalian and plant cells). After the investigation of their mode of action and their structure, they can be suitable compounds for new preservatives, bio-pesticides and drugs against filamentous fungi.

The *Neosartorya fischeri* NRRL 181 strain produces an antifungal protein, named *N. fischeri* antifungal protein (NFAP), which effectively inhibits the growth of some ascomyceteous filamentous fungi. Despite the application of stress conditions enhancing the expression of NFAP, the native producer secretes the protein in a very low amount; therefore the bulk production of NFAP has not been achieved yet. During our work we carried out the heterologous expression of NFAP (hNFAP) by *Pichia pastoris*, predicted the structure of NFAP and hNFAP using *in silico* methods, compared the antifungal spectra of NFAP and hNFAP, investigated the morphological changes caused by short- and long-term NFAP-treatment, and investigated the antifungal mechanism of hNFAP.

AIMS

The increased incidence of severe fungal infections and the fast development of drug resistant filamentous fungi causing mycoses, plant infections or damage to cultural heritages strongly demand for

the development of new antifungal strategies. Small, cysteine-rich, highly stable, defensin-like antifungal proteins secreted by filamentous Ascomycetes have great potential for application in these fields.

The *Neosartorya fischeri* antifungal protein (NFAP) produced by *N. fischeri* NRRL 181 is a novel member of this protein group. Features of NFAP (such as effective inhibitory potential against filamentous fungi, pH- and thermal stability, resistance to degradation by proteases) render it exceptionally suitable as potential commercial preservative, biopesticide and drug against moulds. In spite of the available knowledge of the nature of 5'-upstream transcriptional regulation elements, the bulk production of analitically pure NFAP has not been resolved yet and a detailed study of antifungal mechanism of NFAP is still missing, however, they represent essential prerequisites for the practical application in the future.

Therefore, int he present study, we aimed:

1. The production of NFAP by a *Pichia pastoris*-based heterologous expression system in a high amount.
2. The investigation of the structure of NFAP by *in silico* methods.
3. The comparison of the antifungal spectrum of the native NFAP and the heterologous NFAP produced by *P. pastoris*.
4. The investigation of the phenotypic changes induced by hNFAP in a susceptible fungi
5. The investigation of the antifungal mechanism of hNFAP in *A. nidulans*

METHODS

DNA Techniques

- Plasmid DNA extraction
- Agarose gel electrophoresis
- Polymerase chain reaction (PCR)
- Purification of DNA fragments
- DNA sequencing
- Constraction of vectors for transformation
- Transformation of bacteria
- Electroporation of yeast

Protein Techniques

- Heterologous expression (Pichia Easy Select Expression Kit)
- Ion exchange chromatography
- Sodium-dodecyl-sulphate polyacrilamide gel electrophoresis (SDS-PAGE)
- Mass Spectrometry
- Quadropole Time of Flight Mass Spectrometry
- N-terminal protein sequencing
- Antisera production against hNFAP
- Western-blot

Analysis of nucleotide and amino acid sequence data

- Alignment of nucleotide and amino acid sequences (Clustal W)

- *In silico* prediction of protein structure (MODELLER, Procheck, Disulfind)
- *In silico* investigation of protein structure (Swiss PDB Viewer, Chimera, PyMol)

***In vitro* antifungal susceptibility tests**

- Microdilution method

Microscopic investigation

- Light- and fluorescent microscopic investigations
  - FUN-1 staining (FUN-1 viability staining kit)
  - Propidium iodide staining
  - Detection of apoptotic/necrotic events (*Annexin V-FITC Apoptosis Detection Kit*)
  - Investigation of an *A.nidulans* strain expressing actin conjugated with green fluorescent protein
  - Calcofuor white staining
  - Immunofluorescent staining

RESULTS

Heterologous expression, purification and identification of NFAP

Investigation of protein structure and antifungal mechanism require pure protein in the range of milligrams. For this purpose we carried out the heterologous expression of NFAP in the yeast *P. pastoris* KM71H. *P. pastoris* KM71H transformant strain harboring the pPICZαA plasmid with the mature NFAP encoding gene

produced the protein. The final yield of the hNFAP from three independent productions was 5958±236 µg/l which is six-fold compared to the native producer *N. fischeri* NRRL 181 where it was 978±201 µg/l. After purification to homogenity with cation-exchange chromatography, presence of any other proteins was not detected beside the 6.6 kDa hNFAP. Mass spectrometric molar mass measurement of mature hNFAP resulted in a monoisotopic molecular weight of 6615.1 Da which correlates with the calculated molecular weight of NFAP. The peptides identified by the mass spectrometric analysis of enzyme-digested sample cover 89.5 % of the total sequence. The cleavage of the *P. pastoris* extracellular signal (α-factor) during the expression of hNFAP was efficient, because N-terminal sequencing experiments revealed that the first five amino acid residues of the purified hNFAP is LEYKG, which corresponds well to the first five N-terminal amino acids of the native NFAP. Based on these results we concluded that *P. pastoris* KM71H can produce hNFAP in a properly maturated form in amino acid sequence level.

In silico predicted structure of hNFAP and NFAP

The 3D structure of NFAP was predicted with *in silico* methods. The putative 3D structure of NFAP is very similar to the other defensin-like antifungal proteins produced by filamentous Ascomycetes. hNFAP adopts a β-barrel structure consisting of five antiparallel β-strand. Three lysins in the positions of 10., 34. and 37. form a positively charged surface region, which, according to the

literature could play a role in the antifungal mechanism. Unlike PAF and AFP, NFAP has a negatively charged C-terminal tail region (F55-H57) and a part of its large internal loop region (T33-D38) elongates from the β-barrel topology. We also predicted the disulfide bond pattern of hNFAP, which shows the *abcabc* motif.

Comparison of the antifungal spectrum of hNFAP and NFAP

The purified hNFAP effectively inhibited the growth of fungal isolates belonging to the genus *Aspergillus* (*A. fumigatus, A. niger, A. nomius, A. tamarii, A. tubingensis, A. welwitschiae*) and *Fusarium* (*F. incarnatum, F. solani* species complex), but all investigated zygomycetous strains (*Absidia corymbifera*, *Rhizomucor miehei*, *Rh. pusillus*, *Rhizopus microsporus* var. *rhizopodiformis*, *R. oryzae*) and six ascomyceteous isolates (*A. flavus*, *A. terreus*, *F. solani* species complex SZMC 11427, *F. sporotrichioides*, *Trichoderma longibrachiatum*) proved to be unsusceptible. There was no significant difference between the growth inhibition effect exerted by the native NFAP and the hNFAP. These data indicated that *P. pastoris* KM71H can produce the NFAP in an antifungally active form.

Short- and long-term phenotypic changes in the presence of hNFAP in a sensitive mold

After short-term exposure (30 minutes) to sublethal concentration of hNFAP (25 μg/ml), reduced cellular metabolism was detected with FUN-1 staining in *A. nidulans* FGSC A4 hyphae

compared to the untreated control. This phenomenon was also observed after 60 minutes and 16 hours of NFAP-treatment.

The membrane-disrupting activity of hNFAP was also investigated by applying propidium iodide (PI) stain. Most of the *A. nidulans* FGSC A4 hyphae did show intact membrane after a 30 and 60 minutes-long incubation in the presence of hNFAP (25 μg/ml). After 16 hours almost all of the hyphae showed massive membrane disruption in the hNFAP-treated sample presumably as a consequence of the long-time cell killing effect of hNFAP.

In order to decide if the loss of the membrane integrity of *A. nidulans* FGSC A4 is the consequence of apoptosis or necrosis, we investigated the possible apoptotic/necrotic events in the presence of hNFAP (25 μg/ml) by the application of the Annexin V-FITC Apoptosis Detection Kit. After 30 and 60 minutes a significant increase in the number of apoptotic germlings was observed in the hNFAP-treated samples compared to the untreated control. Only few cells with disrupted membrane integrity were detected in the untreated and hNFAP-treated samples after 30 and 60 minutes. These results suggest that hNFAP exerts its antifungal effect through induction of apoptosis. After 16 hours, however, almost all germlings were stained by propidium iodide, which suggests, that the loss of the membraneintegrity is the consequence of the late phase of apoptosis.

To study the morphological aberrations of hNFAP-exposed *A. nidulans* hyphae, we analysed the actin distribution and the deposition of chitin at the hyphal tips. Actin-GFP expressing *A.*

nidulans showed typical actin patch distribution at the hyphal tips clustered near the apical region and scattered behind the tips. In contrast, actin patches were disturbed in hyphae that were treated with 25 µg/ml of hNFAP for 30 minutes only. Calcofluor white staining (CFW) revealed delocalized chitin deposition at hyphal tips of *A. nidulans* FGSC A4 after hNFAP treatment (25 µg/ml) for 30 minutes. In contrast, the untreated control sample exhibited a characteristic cap-like CFW fluorescence. These effects in actin and chitin delocalization were also observed after 60 minutes and 16 hours of incubation with hNFAP. These results indicate that in the presence of hNFAP the normal actin polarization/localization and chitin distribution are disturbed in *A. nidulans*.

Localization of hNFAP in a susceptible mold

An indirect immunofluorescence staining method was applied to study the localization of hNFAP in *A. nidulans* FGSC A4. hNFAP did not enter the fungal cell in detectable concentration levels after 30 and 60 minutes of exposure, but after 16 hours hNFAP-specific fluorescence signals accumulated at hyphal fractures, twists and in cell-wall bubbles. To clarify whether the hNFAP internalization was the consequence of an endocytotic mechanism, the indirect immunofluorescence staining was repeated in the presence of latrunculin B (latB). LatB selectively inhibits the actin polymerization and therefore disturbs endocytosis. hNFAP-specific fluorescence signals appeared in hyphae only after 16 hours of incubation with hNFAP and latB, but not after the exposure for 30

or 60 minutes. Based on these results we conclude that hNFAP is presumably not internalized by endocytosis. Instead, the accumulation of NFAP after 16 hours is possibly a consequence of a passive diffusion at disrupted sites of the cell wall and plasma membrane, that we proved with PI staining.

Antifungal mechanism of hNFAP

The growth inhibition effect of hNFAP was studied on *A. nidulans* strains carrying mutations in the guanidine nucleotide binding domain of heterotrimeric G-protein α-subunit, cAMP/protein kinase A (Pka) and protein kinase C (Pkc)/mitogen-activated protein kinase (Mpk) signal transduction pathways. Differences in the susceptibility of these mutants towards hNFAP in *in vitro* broth microdilution tests allowed us to postulate the following mode of action: hNFAP binds to a G-protein coupled receptor in the fungal membrane and activates the cAMP/Pka A pathway via G-protein signalling, which leads to inhibition of polar growth and ultimately triggers apoptosis. However, NFAP does not have any influence on the Pkc/Mpk cell wall integrity pathway, but a so far unknown cell wall integrity pathway-independent MpkA-activated target is assumed to be involved in the cell death induction.

SUMMARY

Summarizing our results we can conclude:

1. The applied *Pichia pastoris*-based heterologous expression system can produce an antifungally active, properly maturated hNFAP in a high amount.
2. Based on *in silico* investigations, NFAP/hNFAP adopts a β-barrell structure which shows similarities, but also differences to defensin-like proteins produced by filamentous Ascomycetes.
3. There are no signifficant differences in the antifungal efficiency of the hNFAP and the native NFAP.
4. After short term exposure (30 min) to sublethal concantration (25 μg/ml) of hNFAP, reduced cellular metabolism and apoptotic events were detected in *A. nidulans* FGSC A4. After 16 hours, int he late apoptotic phase the loss of membrane integrity is also detectable indicating the death of the cells.
5. After short term exposure (30 min) to sublethal concantration (25 μg/ml) of hNFAP disturbed actin patches and delocalized chitin deposition were detected in *A. nidulans* FGSC A4.
6. After 16 hours, hNFAP localizes intracellularly at the hyphal fructures and „cell wall bubbles" by passive diffusion through the damaged cell-wall and membrane.
7. hNFAP activates the cAMP/Pka signalling pathway through heterotrimer G-protein coupled signalling and disturbs polarized growth and induces apoptosis.

8. An unidentified, MpkA-activated target is also assumed to be involved in in the cell death induction ability of hNFAP.

PUBLICATIONS

Results summarized in this Ph.D.thesis were published in the following articles:

Publications in referred journals

Virágh M, Marton A, Vizler C, Tóth L, Vágvölgyi C, Marx F, Galgóczy L. Insight into the antifungal mechanism of *Neosartorya fischeri* antifungal protein. Protein Cell. 2015;6:518-28. (IF_{2014}=3.247)

Virágh M, Vörös D, Kele Z, Kovács L, Fizil Á, Lakatos G, Maróti G, Batta G, Vágvölgyi C, Galgóczy L. 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. 2014;94:79-84. (IF_{2015}=1.695)

Congress abstracts summarizing the results of this Ph.D. Thesis

Galgóczy L , **Virágh M** , Marx F , Tóth L , Marton A , Vízler C , Vágvölgyi C. (2014) Insight into the antifungal mechanism of *Neosartorya fischeri* NFAP. In: Cotoraci C , Ardelean A (Ed.) 16th Danube-Kris-Mures-Tisa (DKMT) Euroregion Conference on Environment and Health: Book of Abstracts. Supplement, 24.

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Other publications in referred journals

1. Galgóczy, L., Kovács, L., Karácsony, Z., **Virágh, M.**, Hamari, Zs. and Vágvölgyi, Cs. (2013) Investigation of the antimicrobial effect of *Neosartorya fischeri* antifungal protein (NFAP) after heterologous expression in *Aspergillus nidulans*. Microbiology. 159, 411-419. (IF_{2012}=3,173)

2. Galgóczy, L., **Virágh, M.**, Kovács, L., Tóth, B., Papp, T. and Vágvölgyi, Cs. (2013) Antifungal peptides homologous to the *Penicillium chrysogenum* antifungal protein (PAF) are widespread among Fusaria. Peptides. 39, 131-137. (IF_{2012}=2,522)

3. Galgóczy, L., Tóth, L., **Virágh, M.**, Papp, T. and Vágvölgyi, Cs. (2012) In vitro interactions of amantadine hydrochloride, R-(-)-deprenyl hydrochloride and valproic acid sodium salt with antifungal agents against filamentous fungal species causing central nervous system infection. Acta Biol. Hung. 63, 490-500. (IF_{2012}=0,504)

4. 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)

5. Galgóczy, L., Bácsi, A., Homa, M., **Virágh, M.,** Papp, T. and Vágvölgyi, Cs. (2011) In vitro antifungal activity of phenothiazines and their combination with amphotericin B against different *Candida* species. Mycoses. 54, 737-743. (IF_{2011}=2,247)

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Cumulative impact factor: 16,082