PH.D. THESIS

ISOLATION AND CHARACTERIZATION OF *NEOSARTORYA FISCHERI* ANTIFUNGAL PROTEIN 2 (NFAP2)

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INTRODUCTION

In the last three decades, systemic fungal infections caused by different *Candida* spp. have become one of the most frequent healthcare-associated infections showing an increasing trend worldwide. The treatment is problematic because the currently used antifungal agents have narrow spectrum and can cause serious side-effects. A trend in resistance due to the misuse of antifungal drugs, especially azoles and echinocandins is recently reported moreover multidrug resistant strains are also occurring. There is therefore an urgent need to reduce the application of routinely administrated antifungal drugs by introducing novel, alternative agents and therapeutic strategies. Naturally occurring peptides, proteins and their synthetic derivatives with antifungal activity have been proposed as a potential source and templates of novel drugs to treat mycotic infections. The small, cysteine-rich and cationic antifungal proteins of filamentous ascomycetes have antifungal activity on several opportunistic human pathogenic filamentous fungi. Based on *in vitro* and *in vivo* interaction and toxicological studies the members of this protein group represent exceptionally suitable compounds of commercial drugs against fungi and offer an alternative, safely applicable solution for recent antifungal challenges in the human health.

AIMS

The aims of the present study were the followings:

1. Isolation, identification and investigation of *in silico* structure of an anti-yeast protein secreted by *Neosartorya fischeri* NRRL 181 isolate (NFAP2).
2. Investigation of the phylogenetic relationships of NFAP2.
3. Examination of the *in vitro* antifungal activity and antifungal mechanism of NFAP2.
4. Investigation of heat stability and structure of NFAP2.
5. Heterologous expression and bulk production of NFAP2 by *Penicillum chrysogenum.*
6. Chemical synthesis of NFAP2.
7. Investigation the antifungal activity of synthetic and recombinant NFAP2.
8. Functional mapping of NFAP2.
9. Structural investigation of recombinant and synthetic NFAP2.

METHODS

DNA Techniques

- Purification of genomic DNA
- Agarose gel electrophoresis
- Polymerase chain reaction (PCR)
- Construction of vectors for transformation
- Transformation of filamentous fungi

Protein Techniques:

- Heterologous expression
- Solid-phase peptide synthesis
- Cation-exchange chromatography
- Sodium-dodecyl-sulphate polyacrilamide gel electrophoresis (SDS-PAGE)
- Reversed-phase high-performance liquid chromatography (RP-HPLC)
- Electrospray ionization mass spectrometry (ESI-MS)
- Quadropole Time of Flight Mass Spectrometry (Q-TOF MS)
- Nuclear magnetic resonance (NMR)
- Electronic circular dichroism (ECD)
- *In silico* protein analyses (BioEdit, ExPAsy ProtParam tool, Protein Calculator, PSIPRED, DISULFIND, SignalP1)
- Phylogenetic analysis (BLAST, PRANK, FastGap, Maximum-Likelihood)

In vitro antifungal susceptibility testing

- Microdilution method (CLSI M27-A3)
- Checkerboard titration 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)

RESULTS

1. Isolation, identification and investigation of *in silico* structure of an anti-yeast protein secreted by *Neosartorya fischeri* NRRL 181 isolate (NFAP2)

At first, we isolated and identified a ~5.6 kDa antifungal protein with high anti-yeast activity from the supernatant of *Nesoartorya fischeri* NRRL 181 cultivated in a minimal medium. Based on mass spectrometric measurement, the monoisotopic molecular mass of this protein was 5555.5513 Da. Using the processed data from MS analysis of enzymatic digested NFAP2 we identified an uncharacterized, hypothetical protein that was termed as *Neosartorya fischeri* antifungal protein 2 (NFAP2).

Based on *in silico* investigations, the low molecular mass (average: 5564.3 Da), extracellular, basic (pI=9.02), cationic (net charge at pH 7.0 = +5.2), and cysteine-rich mature NFAP2 consists of 52 amino acid residues. The six cysteines form three intramolecular disulfide bridges showing "*abcabc*" bounding pattern. Furthermore, the NFAP2 is a hydrophilic (Grand Average of Hydropathy Value, GRAVY = -0.731) molecule.

2. Investigation of the phylogenetic relationships of NFAP2

The amino acid sequence of mature NFAP2 shows 11-23% identity to the isolated *Penicillium chrysogenum* antifungal protein (PAF)- and *Penicillium brevicompactum* DierckX *bubble protein* (BP)-cluster proteins from filamentous ascomycetes. BLAST searches yielded 32 protein sequences with significant similarity to NAFP2 in published Ascomycota genomes. The predicted mature forms of these putative proteins show 35-98% amino acid identity to NFAP2. Based on a phylogenetic analysis, NFAP2 and its putative homologs represent a new group of ascomycetous cysteine-rich antifungal proteins beside the described PAF- and BP-cluster proteins.

3. Examination of the *in vitro* antifungal activity and antifungal mechanism of NFAP2

The minimal inhibitory concentration (MIC) of NFAP2 for yeasts were in the range of 0.195-1.563 µg/ml, where *Saccharomyces cerevisiae* proved to be the most (MIC: 0.195 µg/ml), and *Candida krusei* the least (MIC: 1.563 µg/ml) susceptible in low cationic medium. MIC values varied between 0.391-1.563 µg/ml for clinically relevant *Candida* species, after 24 hours of incubation.

The manifestation of antifungal mechanism of NFAP2 on *S. cerevisiae* cells was investigated at its sublethal (1.56 µg/ml) and lethal (0.195 µg/ml) concentrations measured after 24 hours of incubation. Physiological changes in cells in the presence of an antifungal can be investigated at its sublethal concentrations which do not kill the fungus.

The two-colour fluorescent FUN1 stains the cytoplasm and metabolically inactive vacuoles green, while the metabolically active ones red. Based on the proportion of the red and green vacuoles between the treated and untreated samples, change in the metabolic activity of *S. cerevisiae* was not detected in the presence of sublethal NFAP2 concentration even after 16 hours-long NFAP2 treatment.

Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) dyes the apoptotic cells green, while the necrotic cells are counterstained red by the membrane impermeant, red-fluorescent nuclear and chromosome stain propidium iodide (PI), and living cells do not show any fluorescence. There was no significant difference between the proportion of green cells in the NFAP2-treated and untreated samples even after 16 hours. In short exposure time (10, 30 and 60 minutes) at sublethal NFAP2 concentration, same percent of the cells was counterstained with PI in the treated and untreated samples (ca. one percent of the total cell number), but after 16 hours of incubation though three times more red cells were counted in the treated sample than in the untreated control reaching a statistically significant difference ($p=0.00004$). Based on this last observation we suggested that NFAP2 cannot induce apoptosis in the yeast cells, but can disrupt the plasma membrane that was verified with a simple PI-staining.

After six hours of exposure to sublethal NFAP2 concentration, same percent of the total cell number showed red fluorescence as in the untreated control. In contrast to this, after 16 hours six percent of the total cell number were PI-positive in the untreated control compared to the sample treated with sublethal concentration of NFAP2 where it was 18%. When the cells were exposed to lethal concentration of NFAP2, significant differences were observed in the number of PI-positive cells between the NFAP2-treated sample and the untreated control already after 10 minutes of incubation. After 16 hours, viable cells were not observed in the NFAP2-treated sample.

4. Investigation of heat stability and structure of NFAP2

After continuous heating and five minutes exposure to 95 °C, NFAP2 maintained its antifungal activity against *S. cerevisiae* with a one dilution step shift in the 24 hours MIC from 0.195 μg/ml to 0.391 μg/ml.

To prove disulfide bond-stabilized compact tertiary structure of NFAP2 we applied a reversed-phase high performance liquid chromatography (RP-HPLC) method. Considering that NFAP2 eluted from the reversed-phase column as early as naturally folded PAF, the same interlocking disulfide bond pattern to it ("*abcabc*") seems to be the most probable for NFAP2.

Electronic circular dichroism (ECD) spectrum of NFAP2 at 25 °C shows features similar to spectra of the homologous PAF protein and other disulfide bridged, β-structured proteins. After the cooling of NFAP2 solution back to 25 °C moderate structural reorganization takes place, but this reorganization is incomplete even four weeks after the annealing. Based on thermal unfolding curves, the native fold remains intact up to 70 °C, although thermal denaturation is irreversible.

5. Heterologous expression and bulk production of NFAP2 by *Penicillum chrysogenum*

The generated *P. chrysogenum Δpaf/nfap2* strain produced recombinant NFAP2 (rNFAP2). After purification to homogeneity, only one protein band was detected by SDS-PAGE at the molecular weight of the native NFAP2 (nNFAP2). ESI-MS analysis confirmed that the monoisotopic molecular mass of this protein (5555.5 Da) corresponds well to the previously detected monoisotopic molecular mass (5555.6 Da) of nNFAP2 produced by *N. fischeri* NRRL 181. This result clearly indicated the heterologous production of correctly processed NFAP2 with three intramolecular disulfide bonds between the six cysteine residues. The final NFAP2 yield was 15±1.2 mg/l (n=2) compared to the native producer *N. fischeri* NRRL 181 where it was ony 368±19 μg/l (n=5).

6. Chemical synthesis of NFAP2

Peptide fragments of NFAP2 were prepared using manual solid-phase peptide synthesis and either Boc or Fmoc chemistry applying N,N'-dicyclohexylcarbodiimide / 1H-benzotriazol-1-ol hydrate (DCC/HOBt) coupling. For the chemical synthesis of NFAP2, the C-terminal half of the protein (Fragment 1) was prepared, and in addition to this, the N-terminal half of the protein was synthesized in thioester form (Fragment 2t, Fr-2t) to allow native chemical ligation with Fragment 1. Cysteine thiols of the ligation products were oxidized to disulfide bridges with glutathione redox buffer having the glutathione:glutathione disulfide (GSH:GSSG) ratio of 1:1. ESI-MS analysis revealed the expected molecular mass for synthetic NFAP2 (sNFAP2), having three intramolecular disulfide bonds (5554.7 Da).

7. Investigation of antifungal activity of synthetic and recombinant NFAP2

Native, recombinant and synthetic forms of NFAP2 totally inhibited the yeast growth after 48 hours in their investigated concentration range in low cationic medium, and there was no difference between their MICs. The MICs varied in the range of 1.56-12.5 μg/ml depending on the investigated species. Both the recombinant and the native forms of NFAP2 showed dose-dependent antifungal activity. These results propose the potential clinical applicability of the high-yield produced rNFAP2.

To determine MICs of rNFAP2 under standardized clinical microbiological conditions, its *in vitro* antifungal activity was investigated against human pathogenic *Candida* spp. in human serum mimicking RPMI 1640 medium based on the recommendation of Clinical and Laboratory Standards Institute-M27A3 susceptibility test method. *C. albicans* ATCC10231*, C. krusei* CBS 573*, C. lusitaniae* CBS 6936*,* and *C. tropicalis* CBS 94 showed higher MICs than 100 μg/ml; however, this applied dosage could decrease the fungal growth to 30% and 50% except of *C. krusei* CBS 573. The growth of *C. glabrata* CBS 138*, C. guilliermondii* CBS 566, and *C. parapsilosis* CBS 604 was totally inhibited in the investigated concentration range with MICs of 12.5, 3.125, and 100 μg/ml, respectively. These results sign the limited clinical applicability of NFAP2 as systemic monotherapeutic antifungal drug, thus its effectivity in combinatorial drug application was investigated.

C. albicans ATCC10231*, C. parapsilosis* CBS 604, and *C. krusei* CBS 573 were involved in the interaction tests of NFAP2 with fluconazole (FLC). To calculate the fractional inhibitory concentration index (FICI) to reveal the type of interaction, exact MICs of NFAP2 and FLC were determined a proved to be 200 μg/ml and 400 μg/ml for *C. albicans* ATCC 10231 and *C. krusei* CBS 573, respectively. Co-administration of NFAP2 and FLC showed synergistic interactions (FICI = 0.28 and 0.19) against *C. albicans* ATCC10231 and *C. parapsilosis* CBS 604, while it was indifferent (FICI = 0.52) to *C. krusei* CBS 573.

8. Functional mapping of NFAP2

Peptide motifs derived from a full-length antifungal protein allow the identification of putative antimicrobially active motifs. Considering this approach, we involved six synthetic peptide fragments of NFAP2 (Fragments 1-6; Fr 1-6) in antifungal activity assays to reveal its functionally active site(s). Solid-phase peptide synthesis was applied to prepare peptide fragments of NFAP2. Two halves (Fr-1 and Fr-2) and four quarters (Fr-3-6) were synthesized on a solid support applying either Boc or Fmoc chemistry. Besides this, shuffle variants of Fragments 2 and 4 (Sh-Fr-2 and Sh-Fr-4) were prepared using the same method.

In LCM, only Fr-2 and Fr-4 showed dose-dependent antifungal activity, and their MICs were higher than the full-length NFAP2. At their MICs they exerted the prompt plasma membrane disruption effect on *C. albicans* what we already observed at NFAP2 by applying PI staining. Taking into account that the Fr-4 is the C-terminal part of the Fr-2, and the N-terminal Fr-6 part of Fr-2 did not show antifungal activity, we assume that the mid-N-terminal part of the protein (Fr-4) influences the antifungal activity.

Previous studies demonstrated that synthetic peptide fragments of antifungal proteins and their rational-designed variants show remarkable inhibitory potential on fungi if they are hydrophilic and have high positive net-charge. Considering this, we were curious whether the observed antifungal activity of Fr-2 and Fr-4 depends on these features or the primary structure determines it. Hence, we also involved the shuffle variants of these two peptides (Sh-Fr-2 and Sh-Fr-4) in antifungal activity assays. Both shuffle variants showed the same inhibitory potential as Fr-2 and Fr-4, and the prompt plasma membrane disruption effect resulting in the conclusion that the antifungal activity of NFAP2 rather depends on the net charge and hydrophilicity of mid-N-terminal region than on its primary structure.

The C-terminal part of NFAP2 contains the consensus γ-core motif [GXC]-[X_{3-9}]-[C], which is important for the activity or folding of antifungal proteins in animals, humans, and antifungal plant defensins. Interestingly, the peptide fragment containing the γ-core motif of NFAP2 (*N*-GKCEWQGGQLNC-*C*) (Fr-3) was inactive against yeasts, and did not have plasma membrane disruption ability assuming that this specific motif alone has no anti-yeast function, but presumably needs the structural-functional support from other parts of NFAP2.

9. Structural investigation of recombinant and synthetic NFAP2

Formation of interlocking disulfide bridges characteristic for small, cysteine-rich antifungal proteins of filamentous ascomycetes results in decreased retention time on a reversed-phase column. RP-HPLC revealed the same retention time for the rNFAP2 and sNFAP2 as for the native one confirming right pairing of cysteines and native fold of the recombinant and synthetic proteins.

ECD spectra of all NFAP2 samples show features highly similar to that reported earlier for this class of proteins, with contributions emerging from β-conformation (200 nm, 212 nm) and disulfide bridges (228 nm). In fact, all NFAP2 samples are demonstrated to possess identical secondary structural elements regardless of their native, recombinant or synthetic origin. Thermal unfolding experiments followed by ECD indicated, that the native folds of NFAP2, rNFAP2 and sNFAP2 remain intact up to approximately 70 °C, and thermal denaturation is reversible. This contradicts previous observations taken for nNFAP2 samples purified by dialysis, where only partial structural reorganization was observed even four weeks after the annealing. This discrepancy may be attributed to higher integrity of samples purified by RP-HPLC as opposed to those purified by dialysis.

The preliminary nucleic magnetic resonance investigations were started with unlabelled sNFAP2 and $^{13}C/^{15}N$-labelled rNFAP2. First, we confirmed the structure identity

of sNFAP2 and rNFAP2 using the ^{13}C-heteronuclear single quantum coherence (HSQC) type fingerprint spectra of the two compounds. Though the signal intensities are diverse due to different relaxation behavior and the constant-time version of HSQC experiment necessary to remove ^{13}C-^{13}C couplings in the rNFAP2, it can be seen that nearly all HSQC peaks have a pair, a closest neighbor in the map. This observation strongly suggests that the two compounds have identical constitution and even more very similar, folded spatial structure.

SUMMARY

1. We isolated and identified a novel cysteine-rich anti-yeast antifungal protein from the supernatant of *Neosartorya fischeri* NRRL 181 culture, which was termed as *Neosartorya fischeri* antifungal protein 2 (NFAP2).
2. NFAP2 and its putative homologs represent a new group of the cysteine-rich antifungal proteins from filamentous ascomycetes. NFAP2 is the first isolated and described protein in this group.
3. We found that the protein effectively inhibits the growth of clinically relevant species of *Candida* at low concentrations.
4. NFAP2 has the same structure, disulfide pattern and thermal stability as previously isolated and characterized cysteine rich antifungal proteins.
5. We prepared a *Penicillium chrysogenum*-based expression system for bulk production of recombinant (rNFAP2). Furthermore, solid-phase peptide synthesis and native chemical ligation were applied to produce synthetic (sNFAP2).
6. rNFAP2 and sNFAP2 have very similar weight, secondary structure and antifungal activity to the native NFAP2.
7. We found that rNFAP2 shows higher MICs in RPMI 1640, but it interacts synergistically with fluconazole.
8. Functional mapping with synthetic peptide fragments of NFAP2 revealed that not the evolutionary conserved antimicrobial γ-core motif, but the mid-N-terminal part of the protein influences the antifungal activity that rather depends on the net charge and hydrophilicity of this region than on the primary and secondary structure.

PUBLICATIONS

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Publications in referred journals summarizing the results of this Ph.D. Thesis:

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