Production of a defensin-like antifungal protein NFAP from Neosartorya fischeri in Pichia pastoris and its antifungal activity against filamentous fungal isolates from human infect...

ARTICLE *in* PROTEIN EXPRESSION AND PURIFICATION · NOVEMBER 2013

Impact Factor: 1.7 · DOI: 10.1016/j.pep.2013.11.003

Protein Expression and Purification 94 (2014) 79–84

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

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

Máté Virágh [a], Dóra Vörös [a], Zoltán Kele [b], Laura Kovács [a], Ádám Fizil [c], Gergely Lakatos [d], Gergely Maróti [d], Gyula Batta [c], Csaba Vágvölgyi [a], László Galgóczy [a,*]

[a] Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, Szeged H-6726, Hungary
[b] Department of Medical Chemistry, Faculty of Medicine, University of Szeged, Dóm tér 8, Szeged H-6720, Hungary
[c] Department of Organic Chemistry, University of Debrecen, Egyetem tér 1, Debrecen H-4010, Hungary
[d] Institute of Biochemistry, Hungarian Academy of Sciences, Biological Research Centre, Temesvári krt. 62, Szeged H-6726, Hungary

ARTICLE INFO

Article history:
Received 21 June 2013
and in revised form 4 November 2013
Available online 19 November 2013

Keywords:
Neosartorya fischeri antifungal protein
Pichia pastoris
Antimicrobial susceptibility
Clinical fungal isolates
Ascomycetes
Zygomycetes

ABSTRACT

Neosartorya fischeri NRRL 181 isolate secretes a defensin-like antifungal protein (NFAP) which has a remarkable antifungal effect against ascomycetous filamentous fungi. This protein is a promising antifungal agent of biotechnological value; however in spite of the available knowledge of the nature of its 5′-upstream transcriptional regulation elements, the bulk production of NFAP has not been resolved yet. In this study we carried out its heterologous expression in the yeast *Pichia pastoris* and investigated the growth inhibition effect exerted by the heterologous NFAP (hNFAP) on filamentous fungal isolates from human infections compared with what was caused by the native NFAP. *P. pastoris* KM71H transformant strain harboring the pPICZαA plasmid with the mature NFAP encoding gene produced the protein. The final yield of the hNFAP was sixfold compared to the NFAP produced by *N. fischeri* NRRL 181. Based on the signal dispersion of the amide region, it was proven that the hNFAP exists in folded state. The purified hNFAP effectively inhibited the growth of fungal isolates belonging to the *Aspergillus* and to the *Fusarium* genus, but all investigated zygomycetous strain proved to be insusceptible. There was no significant difference between the growth inhibition effect exerted by the native and the heterologous NFAP. These data indicated that *P. pastoris* KM71H can produce the NFAP in an antifungally active folded state. Our results provide a base for further research, *e.g.*, investigation the connection between the protein structure and the antifungal activity using site directed mutagenesis.

Introduction

The increasing incidence of fungal infections both in medicine and agriculture urges the development of novel safely applicable compounds with extensive antifungal activity. The extracellular, defensin-like antifungal proteins secreted by filamentous ascomycetes are interesting in this respect both in medicine and agriculture. These proteins have potent antifungal activity against potential human, plant and food-borne pathogenic fungal species [1–3], they could not have any toxic effects on plant and mammalian cells *in vitro* and *in vivo* [2,4,5], they can interact synergistically with other antifungal drugs and peptides [6–8], and they have high stability against protease degradation, high temperature and that they are active within broad pH range [1]. These facts and their low costs of production could make them promising candidates as a base of commercial biopesticides, medicines and food preservatives against harmful filamentous fungi [1,2].

In our previous work we demonstrated that the *Neosartorya fischeri* (anamorph: *Aspergillus fischerianus*) NRRL 181 isolate secretes a novel representative of this protein group, the *N. fischeri* antifungal protein (NFAP)[1] which effectively inhibits the growth of numerous filamentous Ascomycetes including potential plant and human pathogenic isolates [9]. Its antifungal spectrum and mechanism show similarities, but also show differences to that of the well characterized related proteins, such as the *Aspergillus giganteus* antifungal protein (AFP) and the *Penicillium chrysogenum* antifungal protein (PAF) [9,10]. NFAP interferes with the organization of the cell wall, destroys the chitin filaments as it was observed in case of the AFP, and triggers apoptotic-necrotic event through ROS

* Corresponding author. Tel.: +36 62 544005; fax: +36 62 544823.
E-mail address: galgoczi@gmail.com (L. Galgóczy).

[1] *Abbreviation used:* AFP, *Aspergillus giganteus* antifungal protein; hNFAP, heterologous *Neosartorya fischeri* antifungal protein; NFAP, *Neosartorya fischeri* antifungal protein; PAF, *Penicillium chrysogenum* antifungal protein.

1046-5928/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pep.2013.11.003

accumulation as described for PAF [10]. Manifestation of the antifungal effect of NFAP on the hyphal morphology of a sensitive fungus is similar to those previously described for PAF and AFP [9]: protein treated spores develop very short, swelled and curved hyphae with multiple branches. Its antifungal effect also shows a dose-dependent characteristic and it highly depends on the extracellular mono- and divalent cation concentration [10].

In spite of the promising features, the practical application of defensin-like antifungal proteins from Ascomycetes is still limited as a consequence of their low yield production despite the available knowledge of the nature of their 5′-upstream transcriptional regulation elements in response to environmental signals and stress [1,2]. Based on our previous investigation the maximum yield of the NFAP produced by *N. fischeri* NRRL 181 was 1250 ± 123 μg/l [9]. For the future investigations and the practical applications of NFAP it is important to produce the protein in higher amount by a non-sensitive, easily fermentable, "generally recognized as safe" fungus.

For this purpose in this study we carried out the heterologous expression of NFAP in the yeast *Pichia pastoris*, and we investigated the antifungal activity of heterologous NFAP (hNFAP) on filamentous fungal isolates from human infections compared to what was exerted by the native NFAP from *N. fischeri*.

Materials and methods

Strains and media

The heterologous expression of NFAP was carried out in *P. pastoris* KM71H (*arg4 aox1::ARG4*). This strain was maintained on yeast extract peptone dextrose medium (YPD: 1% yeast extract, 2% peptone, 2% dextrose, 2% agar) slants at 4 °C. Fourteen asco- (representing 3 genera and 12 species) and 5 zygomycetous (representing 3 genera and 5 species) clinical isolates from different human infections were involved in the *in vitro* antifungal susceptibility tests (Table 1). These strains were maintained on malt extract slants (ME; 0.5% malt extract, 0.5% yeast extract, 0.5% glucose, 1% KH_2PO_4, 1.5% agar) at 4 °C. For production of native NFAP the previously described antifungal protein induction medium (AFPIM; 1.5% starch, 1% beef extract, 2% peptone, 0.5% NaCl, 1% ethanol) was inoculated with *N. fischeri* NRRL181 (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois USA) based on the method described previously [9]. Antifungal susceptibility tests were performed in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA; with L-glutamine, without sodium bicarbonate powder, buffered with 0.165 M 4-morpholinepropanesulfonic acid at pH = 7.0).

Cloning and transformation procedure

NFAP encoding cDNA was amplified by *Pfu* polymerase (Fermentas, Glen Blunie, MD, USA) based on the method as described previously [9], it was cloned to *Eco*RV (Fermentas, Glen Blunie, MD, USA) digested pBSK(−) plasmid. This construction (pBSK(−)*nfap*) was transformed into *Escherichia coli* TOP 10F⁻ [11].

P. pastoris transformation and expression procedure was achieved based on the instructions of the EasySelect *Pichia* Expression Kit (Invitrogen-Life Technologies, Carlsbad, CA, USA). The cDNA encoding the mature NFAP was amplified from pBSK(−)*nfap* by using of NFAPF (5′-GGC CCT CGA GAA AAG ACT TGA GTA TAA AGG AGA ATG-3′) and NFAPR (5′-GGC CTC TAG ATC AAT GGC GGA AGT CAC ACT TG-3′) primers with the following parameter: an initial denaturation step at 94 °C for 2 min; 35 cycles, with 1 cycle consisting of 10 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C; and a final extension at 72 °C for 5 min. NFAPF carries the recognition site of *Xho*I (CTC GAG) and the Kex2 (GAG AAA AGA) signal cleavage site at its 5′-end. NFAPR has the recognition site of *Xba*I (TCT AGA) at its 3′-end. The amplified cDNA was purified (GenElute™ PCR Clean-Up Kit, Sigma–Aldrich, St. Louis, MO, USA) and then was digested with *Xho*I and *Xba*I (Fermentas, Glen Blunie, MD, USA) and cloned to the corresponding sites of the pPICZαA plasmid. This vector construction (pPICZα*Anfap*) was transformed into *E. coli* TOP 10F⁻ [11]. After the purification of a high amount of pPICZα*Anfap* from *E. coli* TOP 10F⁻ transformants with Mini Plus Plasmind DNA Extraction System (Viogene BioTek Corp., New Taipei, Taiwan) it was digested with *Sac*I (Fermentas, Glen Blunie, MD, USA) and used to transform *P. pastoris* KM71H cells by electroporation using a MicroPulser apparatus (Bio-Rad Laboratories, Hercules, CA, USA) based on the manufacturer's instructions.

Protein production, optimization and purification

NFAP was produced by *N. fischeri* NRRL181 as described previously [9], and was purified from the ferment broth according to the above described method in case of hNFAP.

Production of hNFAP and its optimization was achieved by following the instructions of the EasySelect *Pichia* Expression Kit (Invitrogen-Life Technologies, Carlsbad, CA, USA). hNFAP was isolated from the supernatant of *P. pastoris* KM71H culture after incubation for 5 days at 30 °C under continuous shaking (180 rpm). After harvesting the cells by centrifugation (5 min, 4000*g*, 25 °C), the extracellular protein fraction of the supernatant was precipitated with 80% of ammonium sulfate at 4 °C for 24 h then the precipitated proteins were collected by centrifugation (30 min, 10,000*g*, 4 °C) and resuspended in 50 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.2). This protein solution was dialyzed (Snake Skin dialysis tubing, 3,5 K MWCO, Thermo Scientific, Logan, UT, USA) against 50 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.2) in 1:100 volume ratio based on the instruction of manufacturer at 4 °C for 48 h with buffer changing after 24 h. The <10 kDa molecular weight protein fraction of the fivefold concentrated sample was separated with centrifugal ultrafiltration (Corning® Spin-X® UF 20 mL Centrifugal Concentrator, 10,000 MWCO Membrane, Corning, Tewksbury MA, USA), then it was purified by ion-exchange chromatography on a Bio-ScaleTM Mini Macro-Prep High S column (Bio-Rad Laboratories, Hercules, CA, USA) using the Duo Flow system (Bio-Rad Laboratories, Hercules, CA, USA). The column was equilibrated with 50 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.2) and eluted with NaCl gradient (0.0–1.0 M) prepared in the equilibrating buffer at a flow rate of 2 ml/min. The quality of the hNFAP fractions was checked with SDS–PAGE (NuPAGE Novex 4–12% Bis–Tris Gel, 1.0 mm, 10 well; Invitrogen-Life technologies, Carlsbad, CA, USA). Protein bands were visualized with Coomassie Brilliant Blue R-250 and silver staining. The pool of the pure hNFAP fractions was dialyzed against 50 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.2), then it was sterilized by filtration (Millex-HP, pore size: 0.45 μm; Millipore, Billerica, MA, USA).

All production and purification experiments were repeated three times.

Characterization and identification of the heterologous NFAP

To determine the proper cleavage of the alpha signal sequence, first five amino acids of the purified hNFAP were identified with N-terminal amino acid sequencing by Edman degradation method using an ABI Procise Model 492 Edman Micro Sequencer that is online connected to an ABI Model 140C PTH Amino Acid Analyzer at the Innsbruck Medical University (Innsbruck, Austria).

Molar mass measurement of mature hNFAP was performed on a Micromass Q-TOF Premier mass spectrometer (Waters MS

Table 1
The investigated fungal strains in the *in vitro* antifungal susceptibility test and their sources.

Species	Strain	Clinical source
Zygomycetes		
Absidia corymbifera	SZMC 95033	Human lung infection
Rhizomucor miehei	CBS 360.92	Human mycosis
Rhizomucor pusillus	ETH M4920	Human tracheal discharge
Rhizopus microsporus var. *rhizopodiformis*	CBS 102.277	Human rhinocerebral infection
Rhizopus oryzae	CBS 146.90	Human palatum molle infection
Ascomycetes		
Aspergillus awamori	SZMC 2390	Human keratitis
Aspergillus flavus	SZMC 2521	Human keratitis
Aspergillus fumigatus	SZMC 2394	Human keratitis
Aspergillus niger	SZMC 2402	Human keratitis
Aspergillus nomius	SZMC 2441	Human keratitis
Aspergillus tamarii	SZMC 2482	Human keratitis
Aspergillus terreus	SZMC 2535	Human keratitis
Aspergillus tubingensis	SZMC 2503	Human keratitis
Fusarium incarnatum	SZMC 11403	Human keratitis
Fusarium solani	SZMC 11412	Human keratitis
Fusarium solani	SZMC 11427	Human keratitis
Fusarium sporotrichioides	SZMC 11421	Human keratitis
Trichoderma longibrachiatum	UAMH 7955	Human sinus
Trichoderma longibrachiatum	UAMH 9515	Human peritoneal effluent

Culture collection abbreviations: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; ETH, Swiss Federal Institute of Technology Culture Collection, Zurich, Switzerland; SzMC, Szeged Microbiological Collection, University of Szeged, Szeged, Hungary; UAMH, Devonian Botanic Garden, University of Alberta Herbarium and Microfungus Collection, Edmonton, Alberta, Canada.

Table 2
The yield of NFAP secreted by *Neosartorya fischeri* NRRL 181 and hNFAP by *Pichia pastoris* KM71H.

Protein/yield of production	NFAP by *N. fischeri* NRRL 181 (μg/l)	hNFAP by *P. pastoris* KM71H (μg/l)
1st Purification	802	6205
2nd Purification	1197	5734
3rd Purification	934	5935
Mean of the three yields (±SD)	978 ± 201	5958 ± 236***

Significant difference (*p*-value) of the hNFAP yield was determined based on the comparison with the yield of NFAP. ***$p < 0.0001$. SD: standard deviation.

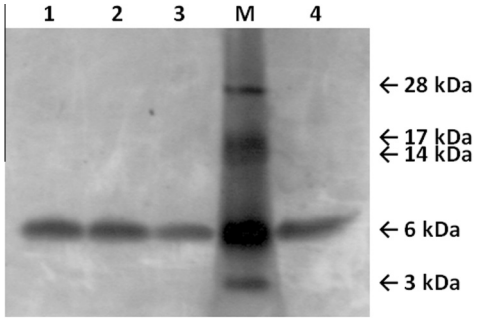

Fig. 1. Purity of the *Pichia pastoris* KM71H produced hNFAP after the ion-exchange chromatography, checked with 4–12% Bis–Tris SDS PAGE in MES buffer. Protein bands were detected with silver staining. M: molecular weight marker (SeeBlue Plus2 Pre-Stained Standard, Invitrogen-Life Technologies, Carlsbad, CA, USA); lane 1–3: fraction 17–19 from hNFAP purification; lane 4: purified NFAP.

Technologies, Manchester, UK) equipped with a nanoelectrospray ion source. In order to confirm the previous results, a mass spectrometric method was used, which was based on enzymatic digestion of the mature hNFAP [12]. Digested samples were analyzed on a Waters NanoAcquity UPLC (Waters MS Technologies, Manchester, UK) system coupled with a Micromass Q-TOF premier mass spectrometer. LC conditions were the followings: flow rate: 350 nl/min; eluent A: water with 0.1% formic acid, eluent B: acetonitrile with 0.1% formic acid; gradient: 40 min, 3–40% B eluent; column: Waters BEH130 C18 75 μm*250 mm column with 1.7 μm particle size C18 packing (Waters Inc., Milford, MA, USA). The mass spectrometer was operated in MSE mode with lockmass correction (standard: Glu-1-Fibrinopeptide M+2H+m/z = 785.842). Acquired data were processed by the WATERS Biopharmalynx software (Waters Inc., Milford, MA, USA).

To decide whether the purified hNFAP has a folded structure or not, ^{1}H NMR was applied. One hundred microgram protein was dissolved in 280 microliter TRIS buffer with 5% D_2O, and filled into a Shigemi tube (Shigemi Inc., Allison Park, PA, USA). ^{1}H NMR spectrum was obtained with excitation sculpting water suppression sequence using watergate W5 pulse sequence with gradients [13]. Due to the low protein concentration 1600 scans were acquired for a proper spectrum with adequate signal to noise ratio. Spectrum was acquired on a Bruker Avance II 500 MHz equipped with a 5 mm Z-gradient triple resonance probe head (Rheinstetten, Germany). Topspin 3.0 software (Bruker GmbH, Rheinstetten, Germany) was used for data acquisition, processing and plotting of the spectrum as well.

In vitro antifungal activity of NFAP and heterologous NFAP

The *in vitro* antifungal effect of NFAP and hNFAP was investigated on filamentous fungal isolates derived from human infections (Table 1) in a 96-well microtiter plate bioassay based on the slightly modified instructions of the Clinical and Laboratory Standards Institute M38-A2 broth microdilution method [14]. One hundred microliter of purified NFAP or hNFAP (12.5–200 μg/ml) was mixed with 100 μl of spore suspension (10^5 spores/ml),

LEYKGECFTKDNTCKYKIDGKTYLAKCPSAANTKCEKDGNKCTYDSYNRKVKCDFRH

Fig. 2. Identified peptides (underlined) by the mass spectrometric analysis of enzyme digested hNFAP. They cover 89.5% of the sequence of NFAP.

Table 3
Growth percentages of the susceptible fungal isolates in presence of different concentrations of NFAP and hNFAP in *in vitro* microdilution test after 48 h of incubation at 37 °C.

Concentration of protein/isolate	200 μg/ml		100 μg/ml		50 μg/ml		25 μg/ml		12.5 μg/ml		0 μg/ml	
	NFAP (%)	hNFAP (%)	NFAP (%)	hNFAP (%)	NFAP (%)	hNFAP (%)	NFAP (%)	hNFAP (%)	NFAP (%)	hNFAP (%)	NFAP (%)	hNFAP (%)
Aspergillus awamori (SZMC 2390)	53 ± 9.9***	47 ± 5.7***	62 ± 9.4**	67 ± 7.3**	74 ± 4.5**	75 ± 2.0**	84 ± 3.8ns	83 ± 1.8ns	92 ± 1.6ns	100	100	100
Aspergillus fumigatus (SZMC 2394)	58 ± 2.9***	55 ± 3.2***	77 ± 1.8*	74 ± 2.9**	82 ± 1.4*	82 ± 1.8*	90 ± 3.4ns	94 ± 5.6ns	93 ± 2.9ns	100	100	100
Aspergillus niger (SZMC 2402)	50 ± 8.0***	42 ± 3.4***	61 ± 7.0***	62 ± 5.8***	68 ± 2.1**	67 ± 3.3**	73 ± 1.5**	76 ± 2.3**	80 ± 6.4*	83 ± 6.8ns	100	100
Aspergillus nomius (SZMC 2441)	46 ± 2.6***	47 ± 0.9***	65 ± 3.8**	67 ± 2.2**	75 ± 8.0*	77 ± 4.4*	81 ± 8.7*	83 ± 7.7ns	94 ± 0.4ns	96 ± 2.3ns	100	100
Aspergillus tamarii (SZMC 2482)	53 ± 2.8***	54 ± 5.4***	70 ± 4.6**	67 ± 3.6**	78 ± 7.0*	79 ± 6.4*	86 ± 4.8ns	84 ± 2.9ns	92 ± 0.8ns	98 ± 1.2ns	100	100
Aspergillus tubingensis (SZMC 2503)	31 ± 4.6***	20 ± 3.6***	42 ± 2.6***	36 ± 4.3***	56 ± 3.5***	56 ± 6.3***	63 ± 4.5**	67 ± 5.8**	76 ± 2.3**	76 ± 3.1**	100	100
Fusarium incarnatum (SZMC 11403)	74 ± 2.2**	59 ± 7.9***	82 ± 0.7*	83 ± 3.1*	90 ± 1.8ns	93 ± 2.0ns	100	100	100	100	100	100
Fusarium solani (SZMC 11412)	78 ± 4.3*	72 ± 1.2**	86 ± 1.9ns	84 ± 4.3ns	92 ± 2.0ns	96 ± 3.7ns	100	100	100	100	100	100

The untreated control is taken as 100% of growth. The mean growth percentages and their standard deviations from three replicates ($N = 3$) are indicated in the cells. Significant differences (p-values) were determined based on the comparison with the untreated samples (0 μg/ml NFAP or hNFAP). ***$p < 0.0001$, **$p < 0.005$, *$p < 0.05$, ns: no significant differences. There were no significant differences between the antifungal effect exerted by NFAP and that caused by hNFAP.

both were prepared in RPMI 1640 medium. The plates were incubated for 0, 24 (in case of Zygomycetes) and 48 h (in case of Ascomycetes) at 37 °C, and then the absorbances (OD_{620}) were measured with microtiter plate reader (SPECTROstar nano, Ortenberg, Germany). Fresh medium was used as background for the spectrophotometric calibration. For calculation of the inhibition rates, the absorbances of the untreated control cultures were referred to 100% of growth, in each case.

All susceptibility tests were repeated three times.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). The significant differences between sets of data were determined by One-way analysis of variance with Bonferroni's multiple comparison posttest according to the data.

Results and discussion

NFAP production in P. pastoris

Several antimicrobial proteins serve as the basis for the design of new synthetic proteins and analogs used as active ingredients of commercial biopesticides, medicines and food preservatives [1,15,16]. In this study a promising antifungal protein, the NFAP was successfully produced by *P. pastoris*. Production of similar proteins, AFP and PAF by this yeast was discussed previously in the literature [17,18].

P. pastoris KM71H transformants harboring the pPICZαA*nfap* plasmid produced the hNFAP. After purification the final yield of the hNFAP from the three productions was 5958 ± 236 μg/l which is sixfold compared to the native producer *N. fischeri* NRRL 181 where it was 978 ± 201 μg/l (Table 2). After purification to homogeneity, presence of any other proteins was not detected beside the 6.6 kDa hNFAP (Fig. 1). This result was also confirmed by MALDI-TOF analysis. Mass spectrometric molar mass measurement of mature hNFAP resulted 6615.5 Da which shows good correlation with calculated value, which is 6615.2 Da. The peptides identified by the mass spectrometric analysis of enzyme digested sample cover 89.5 % of the total sequence (Fig. 2). The sixfold increased availability of pure hNFAP achieved in this study will permit investigation of the mode of action and targeting specificity of NFAP by using site-directed mutagenesis approaches in the future.

Poor information is available in the literature about the structure–effect relationship in case of NFAP related proteins (such as AFP, PAF) from a structural point of view. It was demonstrated that the proper signal cleavage and folding of these proteins are required for their optimal antifungal activity [17–20]. The cleavage of the alpha signal 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 mature NFAP (GenBank ID: CAQ42994.1) [9]. Based on the NMR signal dispersion of the amide region (6–10 ppm), it is proven that hNFAP exists in folded state as it was demonstrated in the case of NFAP too (data not shown); but tertiary structure determination by further NMR investigations using isotope-labeled NFAP and hNFAP are required to prove their structural identity.

In vitro antifungal activity of NFAP and hNFAP

Purified hNFAP was antifungally active against all human pathogenic fungal isolates which proved to be susceptible to NFAP. There were no significant differences between the growth inhibi-

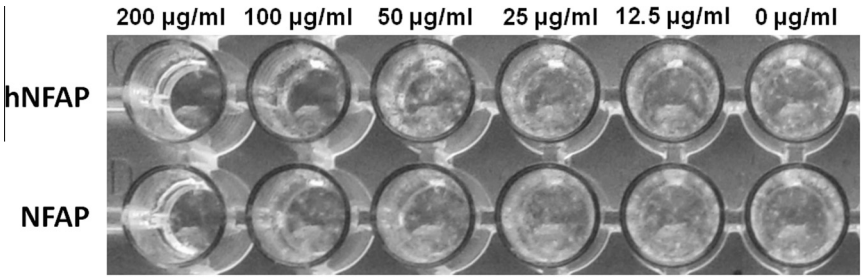

Fig. 3. Comparison of the fungal growth inhibition exerted by hNFAP and NFAP on *Aspergillus tubingensis* (SZMC 2503) after 48 h of incubation at 37 °C.

tion effects exerted by NFAP and hNFAP (Table 3), they caused similar reduction on the growth of the susceptible fungus (Fig. 3).

NFAP and hNFAP exerted antifungal activity against 8 ascomycetous fungal isolates belonging to the genera of *Aspergillus* and *Fusarium*. Two hundred microgram/mililiter of the NFAP had a growth inhibition effect on six *Aspergillus* isolates (*Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus nomius*, *Aspergillus tamarii*, *Aspergillus tubingensis*), and two *Fusarium* isolates (*Fusarium incarnatum* and *Fusarium solani* SZMC 11412). At this concentration their growth reduced to about fifty percent (Table 3). *A. tubingensis* proved to be the most sensitive investigated *Aspergillus*, its growth was inhibited significantly even in the presence of 12.5 µg/ml protein: compared to the untreated control, 76 ± 2.3% and 76 ± 3.1% growth were detected in case of NFAP and hNFAP, respectively (Table 3). The strongest growth inhibition was also detected at this isolate: 68 ± 5.4% and 79 ± 6.4% growth inhibition was observed at 200 µg/ml NFAP and hNFAP, respectively (Table 3). The *F. incarnatum* and the *F. solani* SZMC 11412 isolate was slightly sensitive to NFAP and hNFAP, significant growth reduction was observed only at 200–100 and 200 µg/ml proteins, respectively (Table 3). The antifungal activity of NFAP and hNFAP was maintained against the sensitive isolates even after 96 h of incubation (data not shown). All investigated zygomycetous fungi (*Absidia corymbifera*, *Rhizomucor miehei*, *Rhizomucor pusillus*, *Rhizopus microsporus* var. *rhizopodiformis*, *Rhizopus oryzae*), and *Aspergillus flavus*, *Aspergillus terreus*, *F. solani* SZMC 11427, *Fusarium sporotrichioides*, and both *Trichoderma longibrachiatum* isolates were completely insensitive to NFAP and hNFAP within the investigated concentration range of the proteins. They showed 100% growth at every concentration of the NFAP and hNFAP. This result is easily comparable with our previous finding, where all investigated Zygomycetes proved to be insusceptible to NFAP, except for *R. miehei* where slight growth inhibition was detected at a relative high concentration of NFAP [9]. In contrast to this result the PAF effectively inhibits the spore germination and growth of many isolates belonging to Zygomycetes at different concentrations [21].

Based on the antifungal susceptibility data found in the literature the antifungal spectrum differs between the defensin-like antifungal proteins secreted by Ascomycetes and differences exist in the drug sensitivity within members of the same genus and in the minimal inhibitory concentrations determined for same species [1,3,21–29]. By comparing the antifungal effectivity of NFAP and hNFAP to these data it becomes evident that their antifungal spectrum differs from that of the other defensin-like antifungal proteins, and these exert more potent antifungal activity on same species as the NFAP and hNFAP. However the previous susceptibility results are not easily comparable with those of the present study, because of the differences of the applied test method and the involved fungal strains.

Isolates belonging to the *Aspergillus* genus proved to be the most susceptible to NFAP and hNFAP in our study. *Aspergillus* spp. represent one of the most frequently isolated filamentous fungi from different human infections, and the number of conventional antifungal agent-resistant strains from clinical sources is continuously increasing among the members of this genus [30]. Based on our susceptibility data and the previously described features of the NFAP (*viz.* high stability against protease degradation, antifungal activity within broad temperature and pH ranges) [9], this protein could be a promising alternative compound for the treatment of fungal infections caused by antimycotic resistant *Aspergillus* spp. This hypothesis is supported by a recent study where the successful *in vitro* application of the NFAP-related PAF in a pulmonary aspergillosis animal model experiment is reported [5].

Based on the susceptibility tests and that the hNFAP exists in a folded structure, we suppose that the hNFAP has the same tertiary protein structure as NFAP, but further investigations (*e.g.*, nuclear magnetic resonance investigations and thermal unfolding experiments) are needed to confirm this hypothesis. Considering that the antimicrobial spectrum and efficacy of defensin-like antifungal proteins secreted by filamentous Ascomycetes is different [1,3,9], NFAP could be an applicable compound in therapy, pest control and food preservation beside the other members of this protein group.

Acknowledgments

The research of Á.F., G.B. and L.G. was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4.A/2-11-1-2012-0001 'National Excellence Program'. The relating research groups were also supported by the Hungarian Scientific Research Fund (OTKA; grant reference number PD 83355) and by the EU ERC AdG grant "SYMBIOTICS" providing infrastructure and research equipment.

References

[1] F. Marx, Small, basic antifungal proteins secreted from filamentous ascomycetes: a comparative study regarding expression, structure, function and potential application, Appl. Microbiol. Biotechnol. 65 (2004) 133–142.
[2] V. Meyer, A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value, Appl. Microbiol. Biotechnol. 78 (2008) 17–28.
[3] L. Galgóczy, L. Kovács, C. Vágvölgyi, Defensin-like antifungal proteins secreted by filamentous fungi, in: A. Méndez-Vilas (Ed.), Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, vol. 1, Formatex, Badajoz, 2010, pp. 550–559 (Microbiology book series-number 2).
[4] F. Marx, U. Binder, É. Leiter, I. Pócsi, The *Penicillium chrysogenum* antifungal protein PAF, a promising tool for the development of new antifungal therapies and fungal cell biology studies, Cell. Mol. Life Sci. 65 (2008) 445–454.
[5] Z. Palicz, Á. Jenes, T. Gáll, K. Miszti-Blasius, S. Kollár, I. Kovács, M. Emri, T. Márián, É. Leiter, I. Pócsi, É. Csősz, G. Kalló, C. Hegedűs, L. Virág, L. Csernoch, P. Szentesi, *In vivo* application of a small molecular weight antifungal protein of *Penicillium chrysogenum* (PAF), Toxicol. Appl. Pharmacol. 269 (2013) 8–16.
[6] A.B. Moreno, Á.M. del Pozo, M. Borja, B.S. Segudo, Activity of the antifungal protein from *Aspergillus giganteus* against *Botrytis cinerea*, Phytopathology 93 (2003) 1344–1353.
[7] L. Galgóczy, T. Papp, G. Lukács, É. Leiter, I. Pócsi, C. Vágvölgyi, Interactions between statins and *Penicillium chrysogenum* antifungal protein (PAF) to

inhibit the germination of sporangiospores of different sensitive Zygomycetes, FEMS Microbiol. Lett. 270 (2007) 109–115.
[8] L. Galgóczy, T. Papp, I. Pócsi, N. Hegedüs, C. Vágvölgyi, *In vitro* activity of *Penicillium chrysogenum* antifungal protein (PAF) and its combination with fluconazole against different dermatophytes, Anton. Leeuw. Int. J. G. 94 (2008) 463–470.
[9] L. Kovács, M. Virágh, M. Takó, T. Papp, C. Vágvölgyi, L. Galgóczy, Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP), Peptides 32 (2011) 1724–1731.
[10] L. Galgóczy, L. Kovács, Z. Karácsony, M. Virágh, Z. Hamari, C. Vágvölgyi, Investigation of the antimicrobial effect of *Neosartorya fischeri* antifungal protein (NFAP) after heterologous expression in *Aspergillus nidulans*, Microbiology – SGM 159 (2013) 411–419.
[11] J. Sambrook, D.W. Russell, Molecular Cloning: A Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press, Plainview, 2001.
[12] U. Hellman, C. Wernstedt, J. Góñez, C.H. Heldin, Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing, Anal. Biochem. 224 (1995) 451–455.
[13] M. Liu, X. Mao, C. He, H. Huang, J.K. Nicholson, J.C. Lindon, Improved WATERGATE pulse sequences for solvent suppression in NMR spectroscopy, J. Magn. Reson. 132 (1998) 125–129.
[14] Clinical and Laboratory Standards Institute, Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, in: Approved Standard CLSI Document M38–A2, Clinical and Laboratory Standards Institute, Wayne, 2008.
[15] T. Theis, U. Sthal, Antifungal proteins: targets, mechanisms and prospective applications, Cell. Mol. Life Sci. 61 (2004) 437–455.
[16] E. Montesinos, Antimicrobial peptides and plant disease control, FEMS Microbiol. Lett. 270 (2007) 1–11.
[17] G. Batta, T. Barna, Z. Gáspári, S. Sándor, K.E. Kövér, U. Binder, B. Sarg, L. Kaiserer, A.K. Chhillar, A. Eigentler, E. Leiter, N. Hegedüs, I. Pócsi, H. Lindner, F. Marx, Functional aspects of the solution structure and dynamics of PAF-a highly-stable antifungal protein from *Penicillium chrysogenum*, FEBS J. 276 (2009) 2875–2890.
[18] B. López-García, A.B. Moreno, B. San Segundo, V. De los Ríos, J.M. Manning, J.G. Gavilanes, A. Martínez-del-Pozo, Production of the biotechnologically relevant AFP from *Aspergillus giganteus* in the yeast *Pichia pastoris*, Protein Expr. Purif. 70 (2010) 206–210.
[19] F. Marx, W. Salvenmoser, L. Kaiserer, S. Graessle, R. Weiler-Gröz, I. Zadra, C. Oberpairleiter, Proper folding of the antifungal protein PAF is required for optimal activity, Res. Microbiol. 156 (2005) 35–46.
[20] G. Váradi, K.G. Tóth, Z. Kele, L. Galgóczy, Á. Fizil, G. Batta, Synthesis of PAF, an antifungal protein from *P. chrysogenum* by native chemical ligation: disulfide pattern and fold recovered in oxidative refolding, Chem. Eur. J. 19 (2013) 12684–12692.
[21] L. Galgóczy, T. Papp, É. Leiter, F. Marx, I. Pócsi, C. Vágvölgyi, Sensitivity of different Zygomycetes to the *Penicillium chrysogenum* antifungal protein (PAF), J. Basic Microbiol. 45 (2005) 136–141.
[22] G.D. Lee, S.Y. Shin, C.Y. Maeng, Z.Z. Jin, K.L. Kim, K.S. Hahm, Isolation and characterization of a novel antifungal peptide from *Aspergillus niger*, Biochem. Biophys. Res. Commun. 63 (1999) 646–651.
[23] R. Geisen, *P. nalgiovense* carries a gene which is homologous to the *paf* gene of *P. chrysogenum* which codes for an antifungal peptide, Int. J. Food Microbiol. 62 (2000) 95–101.
[24] L. Kaiserer, C. Oberparleiter, R. Weiler-Görz, W. Burgstaller, É. Leiter, F. Marx, Characterization of the *Penicillium chrysogenum* antifungal protein PAF, Arch. Microbiol. 180 (2003) 204–210.
[25] T. Theis, M. Wedde, V. Meyer, U. Stahl, The antifungal protein from *Aspergillus giganteus* causes membrane permeabilization, Antimicrob. Agents Chemother. 47 (2003) 588–593.
[26] H. Skouri-Gargour, A. Gargouri, First isolation of a novel thermostable antifungal peptide secreted by *Aspergillus clavatus*, Peptides 29 (2008) 1871–1877.
[27] M. Hajji, K. Jellouli, N. Hmidet, R. Balti, A. Sellami-Kamoun, M. Nasri, A highly thermostable antimicrobial peptide from *Aspergillus clavatus* ES1: biochemical and molecular characterization, J. Ind. Microbiol. Biotechnol. 37 (2010) 805–813.
[28] U. Binder, M. Bencina, A. Eigentler, V. Meyer, F. Marx, The *Aspergillus giganteus* antifungal protein AFP_{NN5353} activates the cell wall integrity pathway and perturbs calcium homeostasis, BMC Microbiol. 11 (2011) 209.
[29] L. Galgóczy, M. Virágh, L. Kovács, B. Tóth, T. Papp, C. Vágvölgyi, Antifungal peptides homologous to the *Penicillium chrysogenum* antifungal protein (PAF) are widespread among Fusaria, Peptides 39 (2013) 131–137.
[30] P.H. Chandrasekar, E.K. Manavathu, Antifungal resistance: *Aspergillus*, in: D.L. Mayers (Ed.), Antimicrobial Drug Resistance, Humana Press-Springer, New York, 2009, pp. 953–965 (Chapter 65).