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Isolation and characterization of *Neosartorya fischeri* antifungal protein (NFAP)

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ABSTRACT

A novel 6.6 kDa antifungal peptide (NFAP) from the culture supernatant of the mold, *Neosartorya fischeri* (anamorf: *Aspergillus fischerianus*), and its encoding gene were isolated in this study. NFAP is a small, basic and cysteine-rich protein consisting of 57 amino acid residues. It shows 37.9–50% homology to similar proteins described in literature from *Aspergillus clavatus*, *Aspergillus giganteus*, *Aspergillus niger*, and *Penicillium chrysogenum*. The *in silico* presumed tertiary structure of NFAP, e.g. the presence of five antiparallel β-sheet connected with filaments, and stabilized by three disulfide bridges, is very similar to those of the defensin-like molecules. NFAP exhibited growth inhibitory action against filamentous fungi in a dose-dependent manner, and maintained high antifungal activity within broad pH and temperature ranges. Furthermore, it exhibited relevant resistance to proteolysis. All these characteristics make NFAP a promising candidate for further *in vitro* and *in vivo* investigations aiming at the development of new antifungal compounds.

1. Introduction

The increasing incidence of infections caused by various filamentous fungi generates new challenges for both medicine and agriculture. In the background of this phenomenon, there are root causes like the increasing number of immunocompromised hosts and the emergence of antibiotic resistant fungal strains [23]. Therefore, there is a substantial demand for new compounds with extensive antifungal activity. Some secreted proteins of filamentous fungi possessing defensin-like structure are interesting in this respect, because they have potent antimicrobial activity on taxonomically different filamentous fungi [13,24,35,41].

Until now, eight different defensin-like antifungal proteins have been isolated from ascomycetous fungal species (*Penicillium chrysogenum*, *Penicillium nalgiovense*, *Aspergillus clavatus*, *Aspergillus giganteus* and *Aspergillus niger*) [16,20,21,24,25,30–32]. These proteins have different mode of actions and specificities, albeit, their structures is similar. Low molecular mass (5.8–6.6 kDa), basic character and the presence of six to eight cysteine residues and several disulfide bonds are their main common features. At the same time, they show relatively low similarity. Their primary amino acid sequences have only 31.6–91.4% identity, with the exception of the antifungal proteins of *P. chrysogenum* (PAF) and *P. nalgiovense* (NAF), which are completely identical. The mature forms of *A. clavatus* antimicrobial peptide (AcAMP), *A. giganteus* antifungal protein (AFP), *A. niger* antifungal protein (ANAFP) and PAF show 25.4–96% identity. Conserved homologous amino acid positions can be found mainly in the flanking regions of cysteines (Fig. 1) [24].

PAF and AFP are the most intensively studied fungus-derived defensin-like antifungal proteins. They are synthesized as pre-proteins, which contain a signal sequence for secretion and a prosequence that is removed before or during their release into the supernatant [24]. It was found that the production of AFP and PAF is primarily activated by environmental factors and, in the case of AFP, co-cultivation with other molds [24,26]. Indeed, the 5′-upstream regions of their genes carry several putative binding sites of transcription factors that might be involved in the regulation in response to environmental signals and stresses [24,25]. Although antifungal effect of AFP and PAF generate similar symptoms in the susceptible organisms, such as inhibition of spore germination and hyphal growth, retardation of the hyphae lengthening, membrane perturbation, the induction of intracellular oxidative stress and an apoptosis-like phenotype, it was revealed that they have different modes of actions. AFP disturbs the polarised growth of the hyphae by interfering directly or indirectly with the cell wall biosynthesis, while PAF acts via a G-protein signal transduction pathway and exerts programmed cell death through the release of reactive oxygen species and its other multiple detrimental effects [6,7,15,18,20,22,36]. Similarly to the plant- and animal-derived antifungal proteins, presence of the mono- and divalent cations decreases their inhibitory potential [24]. AFP and PAF are relatively stable in various environmental conditions, and until now, they have not displayed any toxic effect on plant or mammalian cells

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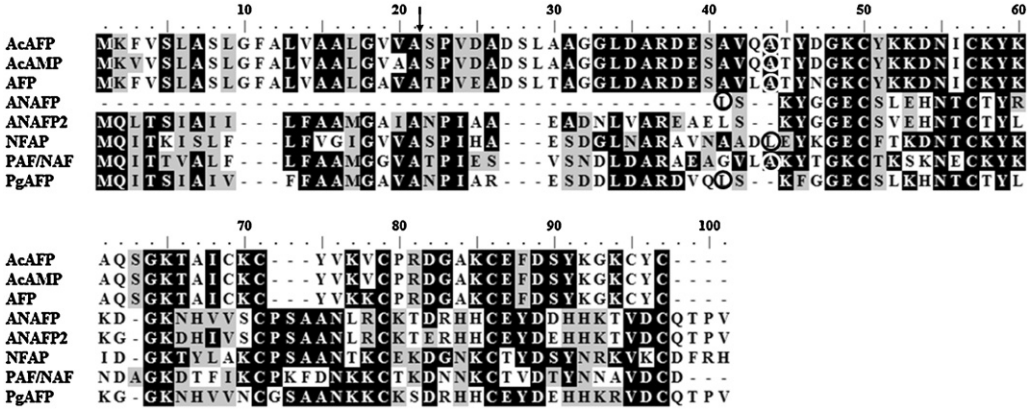

Fig. 1. Alignment of the isolated and putative defensin-like antifungal proteins from filamentous fungi. *Abbreviations*: AcAFP, *Aspergillus clavatus* antifungal peptide (ABR10398.1); AcAMP, *Aspergillus clavatus* antimicrobial peptide (ADC55278.1); AFP, *Aspergillus giganteus* antifungal protein (X53432.1); ANAFP1, *Aspergillus niger* strain KCTC 2025 antifungal protein [25]; ANAFP2, *Aspergillus niger* strain CBS 513.88 antifungal protein (putative, accession no. XP_001391221); NFAP, *N. fischeri* antifungal protein (putative, AM983570.1); PAF, *Penicillium chrysogenum* antifungal protein (AAA92718.1); NAF, *Penicillium nalgiovense* antifungal protein [24]; PgAFP, antifungal protein from *Penicillium chrysogenum* [30]. The first amino acid of the mature AFP, ANAFP, NFAP and PAF proteins is circled; the predicted secretion signal cleavage site is marked by an arrow.

[3,4,14,27–29,32–34,38]. All these features nominate them to be suitable for practical applications in the future.

In the genome of the *Neosartorya fischeri* isolate NRRL 181 [9], a gene encoding a hypothetical protein was identified (NCBI ID: XM_001262585), which proved to be a putative defensin-like antimicrobial protein (*N. fischeri* antimicrobial protein, NFAP) based on *in silico* investigations (Fig. 1) [25]. Here, we report on the isolation of NFAP and the characterization of its antifungal activity.

2. Materials and methods

2.1. Strains and media

An antifungal protein induction medium (AFPIM; 1.5% starch, 1% beef extract, 2% pepton, 0.5% NaCl, 1% ethanol) was used for production of NFAP [24].

The antifungal effect of NFAP was examined against five ascomycetous and five zygomyceteous fungal isolates as follows: *Aspergillus nidulans* (Szeged Microbial Collection, University of Szeged, Szeged, Hungary; SZMC 0307), *A. niger* (SZMC 601), *Botrytis cinerea* (National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary; NCAIM F00751), *Fusarium graminearum* (SZMC 11030), *Trichoderma longibrachiatum* (Devonian Botanic Garden, University of Alberta Herbarium and Microfungus Collection, Edmonton, Alberta, Canada; UAMH 7955), *Absidia corymbifera* (SZMC 95033), *Mucor piriformis* (SZMC 12078), *Rhizomucor miehei* (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CBS 360.92), *Rhizopus microsporus* var. *rhizopodiformis* (CBS 102.277), and *Mortierella wolfii* (CBS 651.93).

All fungal strains, including *N. fischeri*, were maintained on malt extract medium (MEA: 0.5% malt extract, 0.25% yeast extract, 1% glucose, 2% agar) at 4 °C.

To test the inhibition of spore germination and hyphal growth, strains were cultivated in yeast extract–peptone–glucose broth and agar medium (SPEC; 0.1% yeast extract, 0.05% peptone, 2% glucose, solidified with 2% agar if necessary).

2.2. In silico investigations

The BioEdit program [17] was used to examine the *nfap* sequence; similarity searches in the NCBI databases were performed using the Basic Local Alignment Search Tool [1]. The physical and chemical properties of the mature NFAP were examined with the primary structure analysis software of the ExPASy Proteomics Server [38]. The signal sequence, the structure and 3D model of the mature peptide were predicted using the Signal P 3.0 [5], the SCRATCH Protein Predictor [8], and the SWISS-MODEL automated protein structure homology-modeling [2,39] servers, respectively.

2.3. Isolation of nfap gene

Genomic DNA was extracted with the MasterPure Yeast DNA Purification Kit (Epicentre) according to the instructions of the manufacturer. A primer pair PRIMER2F (5′-CAT CGC CCA CCA AAC ATG GTT AT-3′) and PRIMER2R (5′-TGC TTT GCC TGA AGA GTC TTT TGA AG-3′) corresponding to the up- and downstream regions (from the position −801 to −823 and from 1108 to 1136, respectively) of the hypothetical NFAP encoding gene was designed for identification and isolation of the *nfap* gene and its promoter and terminal region. The PCR program was settled to perform the following reaction conditions: an initial denaturation step at 94 °C for 3 min; 30 cycles, with 1 cycle consisting of 40 s at 94 °C, 30 s at 58 °C, and 40 s at 72 °C; and a final extension at 72 °C for 7 min.

To determine the coding region of the hypothesized gene, total RNA was purified with the E.Z.N.A. Fungal RNA Kit (Omega Bio-tek, USA) from the harvested mycelia of the induced fungal culture and cDNA was synthesized using the Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the instructions of the manufacturer. PRIMER1F (5′-ATG CAG ATC ACT AAG ATT TCC C-3′) and PRIMER1R (5′-TCA ATG GCG GAA GTC ACAC-3′) were used to amplify the cDNA with the following parameters: an initial denaturation step at 94 °C for 2 min 30 s; 30 cycles, with 1 cycle consisting of 30 s at 94 °C, 1 min at 48 °C, and 1 min at 72 °C; and a final extension at 72 °C for 7 min.

All PCR reaction mixtures contained 5 pg template DNA, 2.5 μl 10× *Pfu* Buffer with 20 mM $MgSO_4$ (Fermentas), 0.3 μl *Pfu* polymerase (2.5 U/ml, Fermentas) 5 μl of dNTP Mix (each 2.5 mM, Fermentas) and 2 μl of each primer (100 pmol/ml) in a final volume of 25 μl.

PCR products were checked by gel electrophoresis in 0.8% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen) and sequenced commercially by LGC Genomics (Berlin, Germany).

2.4. Isolation of NFAP

NFAP was isolated from the supernatant of *N. fischeri* NRRL 181 culture, which was grown in AFPIM for 7 days at 28 °C under

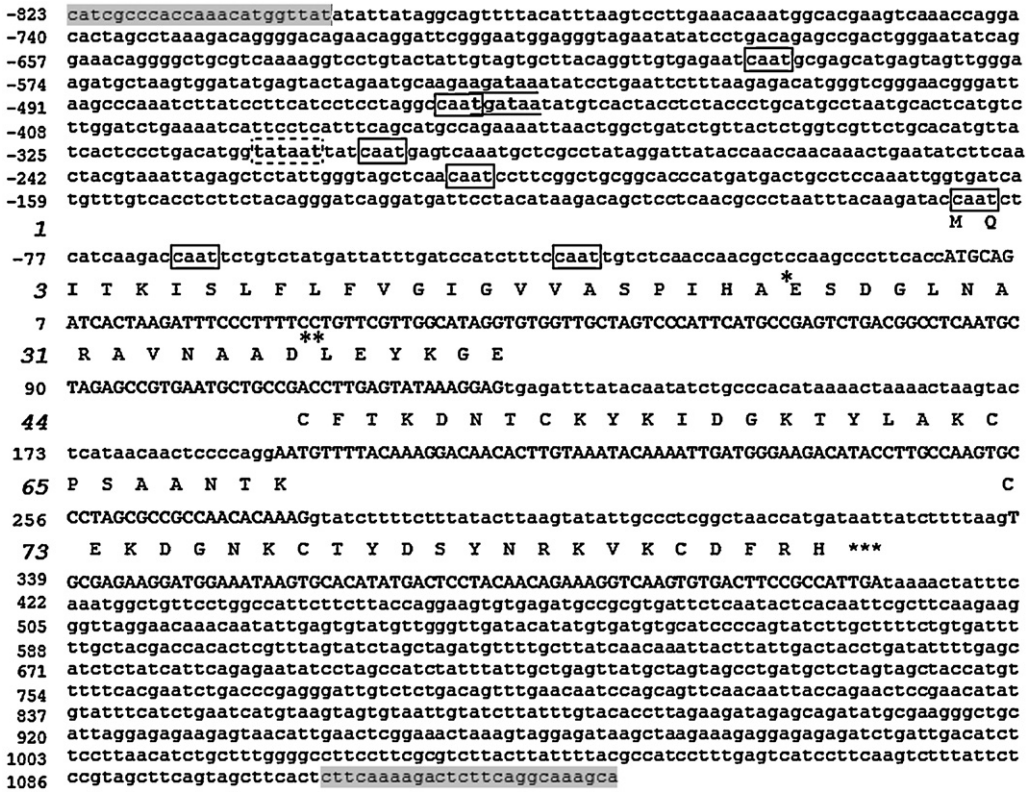

Fig. 2. Nucleotide sequence of the *nfap* gene and its up- and downstream regions. The amino acid residues 1–23 comprise a putative signal peptide; the cleavage site is indicated by an asterisk. Another cleavage site for the proprotein is marked by two asterisks. TATA sequence is boxed by broken lines; the putative CAAT sequences for binding of HAP-like complexes are boxed and the putative GATA-factor binding sites are underlined. The stop codon is marked by three asterisks. Noncoding sequences are shown in lower case letters. The highlighted nucleotides are the sequences used to design primers.

continuous shaking (180 rpm). After harvesting of the mycelia by centrifugation (30 min, 10,000 × *g*, 4 °C), the extracellular protein fraction of the supernatant was precipitated with 70% of ammonium sulfate at 4 °C and dialyzed (Dialysis tubing, benzoylated Avg. flat width 32 mm, 1.27 in., Sigma–Aldrich) against 20 mM Tris–HCl/OH buffer (pH 7.2).

The low molecular weight protein fraction of the five-fold concentrated sample was purified with size exclusion chromatography. The column (Sephadex G-50 superfine, 27 mm × 400 mm, exclusion range of 1.5–30 kDa, GE Healthcare) was equilibrated with 20 mM Tris–HCl/OH buffer (pH 8.4). The protein fractions were eluted (flow rate: 2 ml/min) with the above mentioned buffer. Protein fractions which showed high antifungal activity in agar diffusion tests were collected, concentrated (6×), and separated with centrifugal ultrafiltration (15 ml, Amicon Ultra-15 10K, Millipore). The <10 kDa fraction was dialysed again against 20 mM Tris–HCl/OH buffer (pH 7.2), then purified with ion-exchange chromatography on a CM Sepharose Fast Flow (Sigma–Aldrich) containing column (13 mm × 60 mm, equilibrated with 20 mM Tris–HCl/OH buffer (pH 7.2)) at a flow rate of 1 ml/min. NFAP was eluted with a NaCl gradient (0.1–1.0 M) prepared in the equilibrating buffer. The purified protein was dialysed against 20 mM Tris–HCl/OH buffer (pH 7.2) and prepared in same buffer at pH 8.4; finally, it was sterilized by filtration (MILLEX-HP, pore size: 0.45 μm, Millipore). The quality of the purified NFAP was checked with SDS-PAGE (NuPAGE Novex 4–12% Bis–Tris Gel, 1.0 mm, 10 well, Invitrogen). Protein bands were visualized with Coomassie Brilliant Blue R-250 and silver staining.

The N-terminal amino acid sequence was determined by Edman degradation method using an ABI Procise Model 492 Edman Micro Sequencer which is online connected to an ABI Model 140C PTH Amino Acid Analyzer at the Innsbruck Medical University (Innsbruck, Austria).

2.5. Investigation of antifungal properties

The *in vitro* antifungal effect of NFAP against different filamentous fungi was examined in 96-well microtiter plate bioassays by measuring the optical density of the fungal cultures. One hundred microliter of purified NFAP (12.5–200 μg/ml in twofold dilution) diluted in SPEC medium was mixed with 100 μl of spore (10^5 spores/ml) suspension prepared also in SPEC. The plates were incubated for 0, 24, 48 and 72 h at 25 or 20 °C (in case of *M. piriformis*), and the optical densities were then measured at 620 nm with a microtiter plate reader (ASYS Jupiter HD-ASYS Hitech). Fresh SPEC medium was used as background for the spectrophotometric calibration. To calculate the inhibition rates, optical densities of the untreated control cultures were referred to 100% of growth, in each case.

Effect of NFAP on the germination efficiency of *A. niger* (SZMC 601) conidia was examined in SPEC medium. Conidia (10^5 conidia/ml) were incubated in presence of NFAP (100 and 200 μg/ml) in a total volume of 400 μl of SPEC liquid culture media for 24 and 48 h at 25 °C. Untreated conidia were used as controls. The germinated spores and morphological changes of the developing mycelia were investigated with light microscopy.

The pH- and temperature-dependence of the antifungal activity of the purified NFAP was investigated against *A. niger* (SZMC 601) in SPEC medium using an agar diffusion technique. Solid SPEC medium were inoculated with 10^5 conidia/ml, and 150 μl of the treated protein solution was filled into the wells. The diameters of the inhibition zones were documented after incubation for 24, 48,

72, 96 and 120 h at 25 °C. The purified protein (200 μg/ml) diluted in 20 mM Tris–HCl/OH buffers at different pHs (from pH 7.0 to 9.0) were used in the pH-dependence investigations. NFAP (200 μg/ml, diluted in 20 mM Tris–HCl/OH buffer, pH 8.4) treated at different temperatures (from 30 °C to 100 °C for 30 min) were used for the temperature-dependence investigations. Sterile buffers without protein were used as negative controls in these experiments.

Investigation of the antifungal effect of the NFAP against *A. niger* (SZMC 601) after proteinase K treatment was performed also in SPEC medium using the described agar diffusion technique. The purified protein (200 μg/ml, diluted in 20 mM Tris–HCl/OH buffer, pH 8.4) was treated with 1.25, 2.5, 5 and 10 mg/ml proteinase K (Sigma–Aldrich) at 20 °C for 16 h based on the instruction of the manufacturer. Sterile buffers with proteinase K without protein were used as negative control in this experiment.

All experiments were repeated three times.

2.6. Microscopy

Drops of NFAP-treated suspension of *A. niger* (SZMC 601) were stained with lactophenol cotton blue and were examined and photographed using a light microscope (LR 66238C, Carl Zeiss, Axiolab) and a digital camera (Nikon, Coolpix 4500).

2.7. Statistical analysis

All the statistical analysis was performed using GraphPad Prism version 5.02 for Macintosh (GraphPad Software, San Diego, CA, USA). The significant differences between sets of data were determined by Kruskal Wallis test, with Dunn's multiple comparison posttest, or ANOVA with Dunnett's multiple comparison posttest according to the data.

3. Results

3.1. Isolation of nfap

A fragment of 1954 bp containing the 409 bp length *nfap* gene and its flanking regions was amplified from the DNA of the *N. fischeri* strain NRRL 181 by PCR and sequenced. The sequence was deposited to the EMBL Nucleotide sequence database (EMBL ID: AM922334). The amplified gene showed 100% homology to the hypothetical antifungal protein-encoding gene found previously in the *N. fischeri* genome database (NCBI ID: XP_001262586). Based on the investigation of the cDNA sequence, *nfap* contains two introns between the positions 128 and 190 and the positions 277 and 337 (Fig. 2).

Several putative regulatory elements that might be involved in the transcriptional regulation of *nfap* in response to environmental signals and stress were observed in the promoter region, such as seven CAAT-boxes for binding of HAP-like complexes, and two HGATAR-boxes for binding of GATA factors (Fig. 2).

3.2. In silico investigation

Based on the *in silico* examinations, changes in the primary structure of NFAP during its maturation can be hypothesized. According to this, a 23 aminos acids length extracellular signal sequence and additional 14 amino acids are cleaved from the N-terminal end of the protein (Figs. 1 and 2).

The predicted mature NFAP was found to be a small basic protein. It consists of 57 amino acids and has a calculated molecular mass of 6625.5 Da and a pI of 8.93. The putative protein contains two domains constituted by the amino acids 1–24 and 25–57 and is stabilized by three disulfide bridges between the cysteines in the positions 7 and 14, 27 and 42 and 35 and 53. The amino acid sequence of the mature protein shows 50%, 44.8%, 43.1%, 37.9% homology to PAF, AFP, AcAMP and ANAFP, respectively (Fig. 1). Based on the 3D structure of NFAP predicted by homology modeling, the tertiary structure of the protein is very similar to the defensin-like molecules; it contains five β-sheet connected with filaments [19,37,40] (Fig. 3).

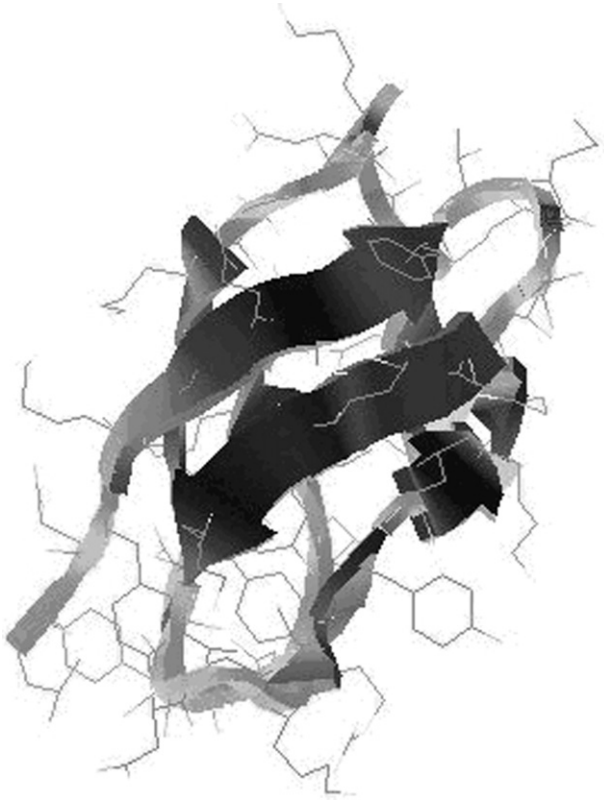

Fig. 3. Hypothetical 3D model of the mature NFAP predicted by the SWISS-MODEL automated protein structure homology-modeling server [2].

3.3. Purification of NFAP

Gel electrophoresis revealed the presence of a small protein (with a molecular mass of approximately 6.6 kDa) in the purified fractions of the ferment broth, which had antifungal activity. This 6.6 kDa-protein was named as *N. fischeri* antifungal protein (NFAP, EMBL ID: CAQ42994). The N-terminal sequencing experiments revealed that the first 6 amino acid residues of the purified mature protein is LEYKGE (Figs. 1 and 2), which corresponds well to the *in silico* determined amino acid sequence of the mature NFAP.

The final concentration of the purified AAFP from 1000 ml ferment broth was 1250 ± 123 μg/ml in 1 ml end volume.

3.4. Antifungal properties of NFAP

Five zygo- and five ascomycetous fungal strains were tested for growth inhibition in the presence of different concentrations of NFAP in 96-well microtiter plate bioassays (Table 1). Two isolates belonging to Ascomycetes (*A. niger* and *A. nidulans*) and one belonging to Zygomycetes (*R. miehei*) proved to be susceptible to NFAP in its administered concentration range (12.5–200 μg/ml) (Table 1). Among them, *A. niger* was the most sensitive to the peptide, 200 μg/ml NFAP completely inhibited its growth after 48 h of incubation and 12.5 μg/ml NFAP was still partially inhibitory (32 ± 5% growth inhibition). The half maximal inhibitory concentration (IC_{50}) was 200 μg/ml after 48 h of incubation and 25,

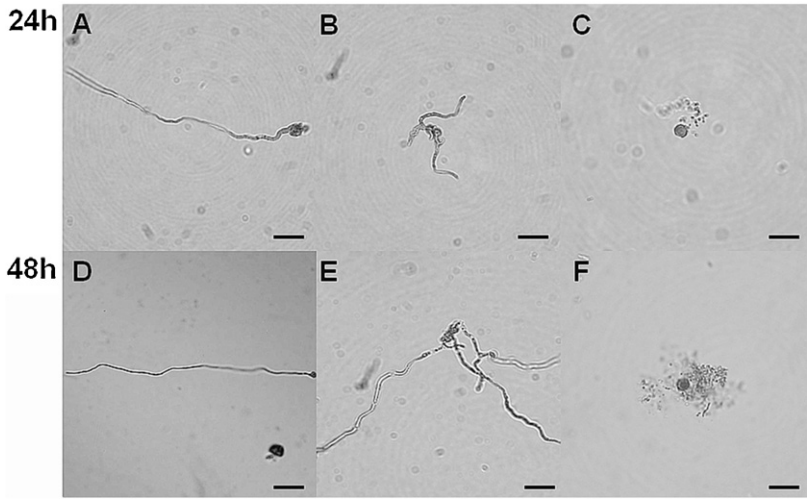

Fig. 4. Germination of *A. niger* conidia in presence of 100 μg/ml (B and E) and 200 μg/ml (C and F) NFAP compared to the untreated control (A and D) after 24 h (A–C) and 48 h (D–F) of incubation at 25 °C. Scale bars represent 20 μm (A–C, E, F) and 10 μm (D).

Table 1
Growth percentages in presence of different concentrations of NFAP.

	NFAP (μg/ml)					
	200	100	50	25	12.5	0
Ascomycetes						
Aspergillus nidulans						
48 h	42 (±4.5)%***	66 (±3.9)%***	76 (±5.3)%***	85 (±2.7)%***	93 (±1.3)%	100%
72 h	77 (±3.2)%***	85 (±3.39)%***	100%	100%	100%	100%
Aspergillus niger						
48 h	0%***	18 (±5.2)%***	40 (±1.5)%***	52 (±1.7)%***	68 (±5.4)%***	100%
72 h	32 (±5.0)%***	44 (±3.3)%***	70 (±3.6)%***	76 (±4.8)%***	88 (±5.9)%*	100%
Zygomycetes						
Rhizomucor miehei						
24 h	76 (±1.5)%***	85 (±2.0)%***	93 (±3.6)%**	100%	100%	100%
48 h	78 (±6.7)%***	88 (±4.9)%**	100%	100%	100%	100%
72 h	85 (±5.7)%***	91 (±1.3)%**	100%	100%	100%	100%

The untreated control is taken as 100% of growth. Standard deviations of three replicates ($N = 3$) are indicated in the brackets. Significant differences (p-values) were determined based on the comparison with the untreated samples (0 μg/ml NFAP).

* $p < 0.05$.
** $p < 0.005$.
*** $p < 0.0001$.

100 μg/ml after 48 and 72 h of incubation in case of *A. nidulans* and *A. niger*, respectively (Table 1). IC_{50} value of NFAP was not detected in the administered concentration range of NFAP at *R. miehei*. The antifungal activity of NFAP was maintained after 96 h of incubation (data not shown). *A. corymbifera*, *B. cinerea*, *F. graminearum*, *M. wolfii*, *M. piriformis*, *R. microsporus* var. *rhizopodiformis* and *T. longibrachiatum* were not susceptible in the applied *in vitro* antifungal tests.

Microscopic observation revealed that germination tubes and hyphae forming from conidiospores of *A. niger* exhibited severe changes in morphology when the fungus was cultivated in presence of 100 μg/ml NFAP. Compared to the untreated control, treated conidiospores displayed abnormal and delayed germination developing very short, swelled and curved hyphae with multiple branches and fragmented cytoplasm. Moreover, 200 μg/ml of NFAP totally destructed the germination tubes after 24 h of cultivation (Fig. 4).

Effect of different pH and temperature conditions on the biological activity of the purified protein 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 (Table 2). The protein proved to be stable even after a temperature treatment at 100 °C for 30 min, and the antifungal effect of the treated protein was remained after 120 h of incubation at 25 °C (Table 3). Antifungal activity after treatment at 100 °C for 40 min was not observed.

Antifungal effect of NFAP was maintained after the treatments with different concentrations of proteinase K at 30 °C for 16 h (Fig. 5) and the growth inhibition effect was observable even after 120 h of incubation at 25 °C (Table 4). Without NFAP, inhibition zones were not detected in presence of proteinase K.

4. Discussion

A defensin-like protein with antifungal activity was isolated and investigated from *N. fischeri* NRRL181 in this study. In spite of that its amino acid sequence differs from other antifungal proteins investigated so far, conserved homologous regions can be identified at the flanking amino acids of cysteines, which might play an important role in the development of the antifungally active protein structure [4,24,25].

Table 2
Antifungal activity of the partially purified NFAP (200 μg/ml) at 25 °C in agar diffusion test against *A. niger* SZMC 601 under different pH conditions in 20 mM Tris–HCl/OH buffer.

pH	The diameters (mm) of the inhibitory zones			
	48 h	72 h	96 h	120 h
7.0	16.67 (±1.53)***	15.33 (±0.58)***	12.00 (±2.65)***	12.00 (±2.60)
7.2	18.00 (±3.00)**	15.67 (±1.15)***	15.00 (±2.00)**	14.67 (±1.53)
7.4	20.67 (±2.08)	17.33 (±1.53)*	17.33 (±1.53)	17.00 (±1.00)
7.6	21.33 (±2.08)	18.00 (±1.73)	17.67 (±1.15)	17.67 (±1.15)
7.8	21.67 (±0.58)	18.33 (±0.58)	17.33 (±1.15)	17.67 (±1.53)
8.0	23.00 (±3.00)	18.67 (±1.15)	18.00 (±1.00)	17.33 (±0.58)
8.2	23.00 (±0.00)	19.67 (±0.58)	19.00 (±1.00)	18.33 (±0.58)
8.4	24.33 (±0.58)	20.33 (±0.58)	19.67 (±0.58)	19.33 (±0.56)
8.6	24.17 (±0.76)	20.67 (±0.58)	20.00 (±1.00)	19.67 (±1.15)
8.8	23.50 (±0.50)	19.67 (±0.58)	19.00 (±1.00)	18.67 (±0.58)
9.0	23.33 (±1.15)	19.00 (±1.73)	18.67 (±1.53)	18.67 (±1.73)

Standard deviations of three replicates ($N = 3$) are indicated in the brackets. Significant differences (p-values) were determined based on the comparison with the samples treated at the optimal pH (pH = 8.4).
* $p < 0.05$.
** $p < 0.005$.
*** $p < 0.0001$.

It was previously demonstrated that nutrient-limitation and different type of stress enhanced the expression of AFP and PAF [24]. The upstream region of *nfap* carries several putative regulatory elements in response to environmental signals and stress similarly to the AFP and PAF encoding genes [24].

Similarly to the antifungal proteins derived from other filamentous fungi, the antifungal effect of NFAP showed a dose-dependent characteristic on the sensitive fungal isolates tested [18,24]. It exhibited growth retardation in sublethal concentrations (100 μg/ml) and this effect became fungicidal in higher concentration (200 μg/ml). The manifestation of its antifungal effect (swollen, short, curved hyphae and fragmented cytoplasm) was similar to those described for AFP and PAF [24,25].

The growth inhibitory effect of filamentous fungal defensin-like proteins is mainly prevailed against other filamentous fungi [24]. ANAFP, AFP, PAF and NAF have a strong inhibitory potential in different concentrations (MIC = 1–200 μg/ml) on the germination and growth of fungal species belonging to Ascomycetes and Zygomycetes [3,10–12,18,20,21,24,25,27,28,36]. In this study NFAP reduced the growth of *A. nidulans*, *A. niger* and *R. miehei* in different rates depending on the applied concentrations. The previously investigated antifungal proteins secreted by filamen-

Table 3
Antifungal activity of NFAP (200 μg/ml) at 25 °C in agar diffusion test against *A. niger* SZMC 601 after treatment with different temperature conditions in 20 mM Tris–HCl/OH buffer (pH = 8.4).

Temperature	The diameters (mm) of the inhibitory zones			
	48 h	72 h	96 h	120 h
30 °C	21.00 (±0.58)	18.00 (±0.58)	17.50 (±1.73)	17.50 (±1.15)
40 °C	21.33 (±1.15)	17.33 (±1.73)	17.17 (±0.58)	17.00 (±1.00)
50 °C	21.00 (±1.53)	16.50 (±1.53)	16.40 (±2.00)	16.50 (±1.15)
60 °C	22.00 (±1.73)	17.00 (±1.00)	17.33 (±1.53)	17.00 (±1.53)
70 °C	21.67 (±0.58)	17.50 (±1.15)	17.50 (±1.15)	17.67 (±1.00)
80 °C	19.33 (±1.15)*	17.50 (±1.73)	17.50 (±0.58)	17.00 (±0.58)
90 °C	18.00 (±0.58)***	16.00 (±0.58)**	16.33 (±0.76)	16.17 (±0.73)*
100 °C	16.00 (±0.29)***	14.50 (±1.15)***	14.00 (±0.50)***	14.00 (±0.33)***
Control (25 °C)	22.00 (±0.58)	19.67 (±0.58)	18.67 (±1.15)	18.67 (±1.00)

Standard deviations of three replicates ($N = 3$) are indicated in the brackets. Significant differences (p-values) were determined based on the comparison with the samples treated at control degree (25 °C).
* $p < 0.05$.
** $p < 0.005$.
*** $p < 0.0001$.

Table 4
Antifungal activity of NFAP (200 μg/ml) after treatment with different concentrations of proteinase K for 16 h at 20 °C in agar diffusion test against *A. niger* SZMC 601 at 25 °C.

Proteinase K	The diameters (mm) of the inhibitory zones			
	48 h	72 h	96 h	120 h
10 mg/ml	14.50 (±0.50)***	13.83 (±0.76)***	12.33 (±0.58)***	12.17 (±0.29)***
5 mg/ml	15.67 (±0.58)***	16.00 (±0.00)***	13.83 (±0.29)***	12.83 (±0.76)***
2.5 mg/ml	16.83 (±0.29)***	16.83 (±0.29)***	15.67 (±0.58)***	15.00 (±0.50)***
1.25 mg/ml	18.50 (±0.50)**	17.67 (±0.58)***	17.33 (±0.29)**	16.83 (±0.29)***
Control (0 mg/ml)	20.67 (±0.58)	20.17 (±0.29)	19.17 (±0.29)	19.33 (±0.58)***

Standard deviations of three replicates ($N = 3$) are indicated in the brackets. Significant differences (p-values) were determined based on the comparison with the untreated samples (control, 0 mg/ml proteinase K).
** $p < 0.005$.
*** $p < 0.0001$.

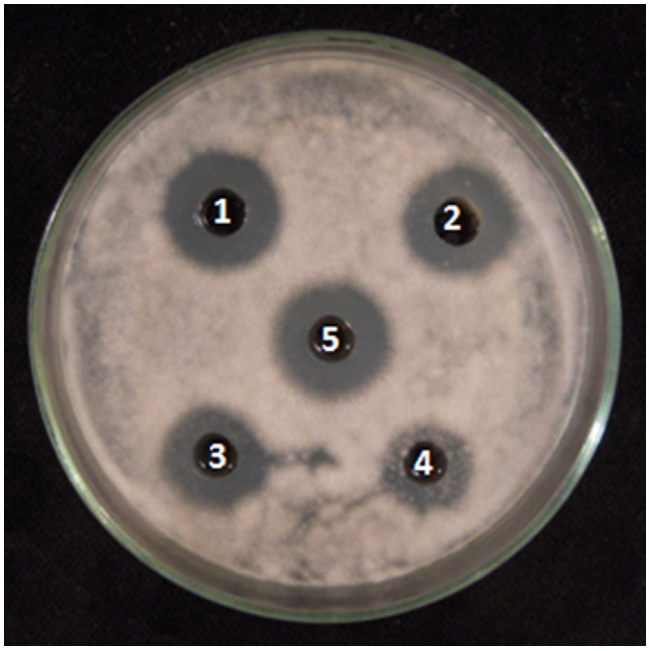

Fig. 5. Antifungal activity of NFAP (200 μg/ml) against *A. niger* SZMC 601 in agar diffusion test after treatments with 1.25 mg/ml **(1)**, 2.5 mg/ml **(2)**, 5 mg/ml **(3)**, 10 mg/ml **(4)**, 0 mg/ml **(5)** proteinase K for 16 h at 20 °C. Plate was incubated for 48 h at 25 °C.

tous fungi have a narrow antimicrobial spectrum, but their species specificity is different. This specificity is depend on the applied culture medium for the antifungal susceptibility tests [24,25].

Previous studies demonstrated the stability of AFP and PAF in extreme pH and thermal conditions and their resistance against protein degradation [4,38]. In our study, NFAP showed similar features. It maintained its antifungal activity within broad pH and temperature ranges, furthermore it exhibited relevant resistance to proteolysis. This feature of NFAP could be accounted for its tertiary structure, which is stabilized by three disulfide bridges.

The observed characteristics of NFAP could make it suitable for a practical purpose and/or for design of new synthetic analogs in the future after the investigation of its antimicrobial spectrum, mode of action and the connection between its structure and efficacy.

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References

[1] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
[2] Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modeling. Bioinformatics 2006;22:195–201.
[3] Barna B, Leiter É, Hegedűs N, Bíró T, Pócsi I. Effect of the *Penicillium chrysogenum* antifungal protein (PAF) on barley powdery mildew and wheat leaf rust pathogens. J Basic Microbiol 2008;48:516–20.
[4] Batta GY, Barna T, Gáspári Z, Sándor SZ, Kövér KE, Binder U, et al. Functional aspects of the solution structure and dynamics of PAF-a highly-stable antifungal protein from *Penicillium chrysogenum*. FEBS J 2009;276:2875–90.
[5] Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 2004;340:783–95.
[6] Binder U, Chu M, Read ND, Marx F. The antifungal activity of the *Penicillium chrysogenum* protein PAF disrupts calcium homeostasis in *Neurospora crassa*. Eukaryot Cell 2010;9:1374–82.
[7] Binder U, Oberparleiter C, Meyer V, Marx F. The antifungal protein PAF interferes with PKC/MPK and cAMP/PKA signalling of *Aspergillus nidulans*. Mol Microbiol 2010;75:294–307.
[8] Cheng J, Randall AZ, Sweredoski MJ, Baldi P. SCRATCH: a protein structure and structural feature prediction server. Nucleic Acids Res 2005;33:W72–6 [Web Server issue].
[9] Fedorova ND, Khaldi N, Joardar VS, Maiti R, Amedeo P, Anderson MJ, et al. Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. PLoS Genet 2008;11:e1000046.
[10] Galgóczy L, Papp T, Leiter É, Marx F, Pócsi I, Vágvölgyi CS. Sensitivity of different Zygomycetes to the *Penicillium chrysogenum* antifungal protein (PAF). J Basic Microbiol 2005;45:136–41.
[11] Galgóczy L, Papp T, Lukács Gy, Leiter É, Pócsi I, Vágvölgyi CS. Interactions between stains and *Penicillium chrysogenum* antifungal protein (PAF) to inhibit the germination of sporangiospores of different sensitive Zygomycetes. FEMS Microbiol Lett 2007;270:109–15.
[12] Galgóczy L, Papp T, Pócsi I, Hegedűs N, Vágvölgyi CS. In vitro activity of *Penicillium chrysogenum* antifungal protein (PAF) and its combination with fluconazole against different dermatophytes. Antonie Van Leeuwenhoek 2008;94:463–70.
[13] Ganz T. Defensins antimicrobial peptides of innate immunity. Nat Rev Immunol 2003;3:710–20.
[14] Girgi M, Breese WA, Lörz H, Oldach KH. Rust and downy mildew resistance in pearl millet (*Pennisetum glaucum*) mediated by heterologous expression of the *afp* gene from *Aspergillus giganteus*. Transgenic Res 2006;15:313–24.
[15] Hagen S, Marx F, Ram AF, Meyer V. The antifungal protein AFP from *Aspergillus giganteus* inhibits chitin synthesis in sensitive fungi. Appl Environ Microbiol 2007;73:2128–34.
[16] Hajji M, Jellouli K, Hmidet N, Balti R, Sellami-Kamoun A, Nasri M. A highly thermostable antimicrobial peptide from *Aspergillus clavatus* ES1: biochemical and molecular characterization. J Ind Microbiol Biotechnol 2010;37:805–13.
[17] Hall TA. BioEdit a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999;41:95.
[18] Kaiserer L, Oberparleiter C, Weiler-Görz R, Burgstaller W, Leiter É, Marx F. Characterization of the *Penicillium chrysogenum* antifungal protein PAF. Arch Microbiol 2003;180:204–10.
[19] Korneva EA, Kokryakova VN. Defensins: Antimicrobial peptides with a broad spectrum of biological activity. Neuro Immun Biol 2003;3:451–62.
[20] Lacadena J, Martinez del Pozo A, Gasset M, Patino B, Campos-Olivas R, Vazquez C, et al. Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus*. Arch Biochem Biophys 1995;324:273–81.
[21] Lee GD, Shin SY, Maeng CY, Jin ZZ, Kim KL, Hahm KS. Isolation and characterization of a novel antifungal peptide from *Aspergillus niger*. Biochem Biophys Res Commun 1999;63:646–51.
[22] Leiter É, Szappanos H, Oberparleiter C, Kaiserer L, Csernoh L, Pusztahelyi T, et al. Antifungal protein PAF severely affects the integrity of the plasma membrane of *Aspergillus nidulans* and induces an apoptosis-like phenotype. Antimicrob Agents Chemother 2005;49:2445–53.
[23] Marcos JF, Gandía M. Antimicrobial peptides: to membranes and beyond. Expert Opin Drug Discov 2009;4:659–71.
[24] Marx F. Small, basic antifungal proteins secreted from filamentous ascomycetes: a comparative study regarding expression, structure, function and potential application. Appl Microbiol Biotechnol 2004;65:133–42.
[25] Meyer V. A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value. Appl Microbiol Biotechnol 2008;78:17–28.
[26] Meyer V, Stahl U. The influence of co-cultivation on expression of the antifungal protein in *Aspergillus giganteus*. J Basic Microbiol 2003;43:68–74.
[27] Moreno AB, del Pozo ÁM, Borja M, Segudo BS. Activity of the antifungal protein from *Aspergillus giganteus* against *Botrytis cinerea*. Phytopathology 2003;93:1344–53.
[28] Moreno AB, del Pozo ÁM, Segundo BS. Antifungal mechanism of the *Aspergillus giganteus* AFP against the rice blast fungus *Magnaporthe grisea*. Appl Microbiol Biotechnol 2006;54:245–59.
[29] Oldach KH, Becker D, Lörz H. Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. Mol Plant Microbe Interact 2001;14:832–8.
[30] Rodríguez-Martín A, Acosta R, Liddell S, Núñez F, Benito MJ, Asensio MA. Characterization of the novel antifungal protein PgAFP and the encoding gene of *Penicillium chrysogenum*. Peptides 2010;31:541–7.
[31] Skouri-Gargouri H, Gargouri A. First isolation of a novel thermostable antifungal peptide secreted by *Aspergillus clavatus*. Peptides 2008;29:1871–7.
[32] Skouri-Gargouri H, Ben Ali M, Gargouri A. Molecular cloning, structural analysis and modeling of the AcAFP antifungal peptide from *Aspergillus clavatus*. Peptides 2009;30:1798–804.
[33] Szappanos H, Szigeti GyP, Pál B, Rusznák Z, Szűcs G, Rajnavölgyi É, et al. The antifungal protein AFP secreted by *Aspergillus giganteus* does not cause detrimental effects on certain mammalian cells. Peptides 2006;27:1717–25.
[34] Szappanos H, Szigeti GyP, Pál B, Rusznák Z, Szűcs G, Rajnavölgyi É, et al. The *Penicillium chrysogenum*-derived antifungal peptide shows no toxic effects on mammalian cells in the intended therapeutic concentration. Naunyn Schmiedebergs Arch Pharmacol 2005;371:122–32.
[35] Theis T, Sthal U. Antifungal proteins: targets, mechanisms and prospective applications. Cell Mol Life Sci 2004;61:244–437.

[36] Theis T, Wedde M, Meyer V, Stahl U. The antifungal protein from *Aspergillus giganteus* causes membrane permeabilization. Antimicrob Agents Chemother 2003;47:588–93.
[37] Thomma BP, Cammue BP, Thevissen K. Plant defensins. Planta 2002;216:193–202.
[38] Vila L, Lacadena V, Fontanet P, Martínez del Pozo A, San Segundo B. A protein from the mold *Aspergillus giganteus* is a potent inhibitor of fungal plant pathogens. Mol Plant Microbe Interact 2001;14:1327–31.
[39] Watanabe K, Harayama S. SWISS-PROT: the curated protein sequence database on Internet. Tanpakushitsu Kakusan Koso 2001;46:80–6 [in Japanese].
[40] White SH, Wimley WC, Selsted ME. Structure, function, and membrane integration of defensins. Curr Opin Struct Biol 1995;5:521–7.
[41] Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharm Rev 2003;55:27–55.