

Defensin-like antifungal proteins secreted by filamentous fungi

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The incidence of fungal infections has increased continuously during recent years in consequence of the increasing number of immunocompromised hosts and the occurring of antibiotic resistant strains. Another important aspect is that many filamentous fungi are destructive pathogens of plants and are thus responsible for enormous crop losses worldwide. Therefore, there is a substantial demand for new compounds with extensive antifungal activity. The proteins with similar structure like defensins secreted by filamentous fungi are interesting from this respect. These proteins secreted by taxonomical distinct species have different mode of action and species specificity, nevertheless, their structure is very similar. They are promising compounds in medical and agricultural fields; and their features (high stability and efficacy, safety application, limited toxicity and low costs of production) could make them suitable for use in practicable respects in the future.

Keywords filamentous fungi; defensin-like antifungal proteins, susceptibility, mode of the action, protein expression

1. Introduction

Defensins are a family of evolutionary related antimicrobial peptides [1]. They are part of the second defence system, innate immunity that was discovered in the early 1980s in the higher organisms. These small molecular weight cationic peptides have a characteristic β -sheet rich fold and a framework of disulfide-linked cysteines [1-3]. They have a broad antimicrobial spectrum and different mode of action [1, 2, 4, 5]. Similar proteins are widely distributed in the nature; they are not limited to higher organisms, their presence is confirmed in insects, plants and fungi [2, 5, 6]. Their antimicrobial effects are exerted on three major targets in the sensitive organisms: cell wall, cell membrane and intracellular organelles [2, 4].

The features of extracellular, defensin-like antifungal proteins secreted by filamentous fungi are a low molecular mass (5.8-6.6 kDa), a basic character, and the presence of 6-10 cysteine residues and several disulfide bonds. Proteins with such properties with antifungal activity have been isolated and investigated from 4 ascomycetous fungal species (*Penicillium chrysogenum*, *Penicillium nalgiovense*, *Aspergillus giganteus* and *Aspergillus niger*). Furthermore, *in silico* investigation of genomic databases has been revealed a putative protein with high homology to them in *Gibberella zeae*, *Aspergillus clavatus* and *Neosartorya fischeri* [7, 8]. A highly homologous putative protein is deduced in *A. niger* CBS 518.88 strain with 7 differences in amino acid sequence of the mature protein compared to *A. niger* antifungal protein (ANAFP) [9] (Fig. 1.). Only one similar protein with an antibacterial activity has been described in the literature so far in a zygomycetous fungus. The *Rhizopus microsporus* var. *oligosporus* antibiotic peptide (ABP) encoded by two genes with one amino acid difference in the signal sequences (*abp1* and *abp2*) has an antibacterial effect only against some Gram-positive bacteria [9].

Their safety, sustainability, high efficacy, limited toxicity and low costs of production could make them favourable compounds in medicine and agriculture in the near future [7, 8, 10]. *P. chrysogenum* antifungal protein (PAF) and *A. giganteus* antifungal protein (AFP) are the most intensively studied fungus-derived antifungal proteins.

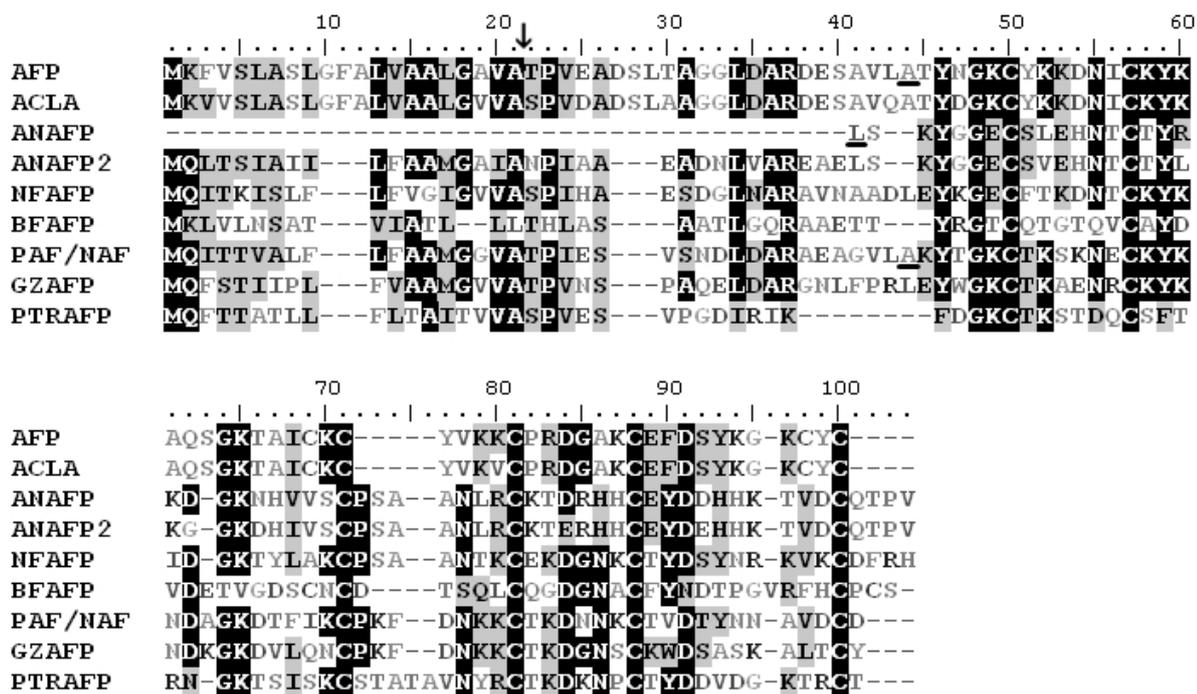


Fig. 1 Alignment of the isolated and putative defensin-like antifungal proteins from filamentous fungi. Abbreviations: AFP, *Aspergillus giganteus* antifungal protein; ACLA, *Aspergillus clavatus* antifungal protein (putative, accession no. ABR10398); ANAFP1, *Aspergillus niger* strain KCTC 2025 antifungal protein; ANAFP2, *Aspergillus niger* strain CBS 513.88 antifungal protein (putative, accession no. XP_001391221); NFAFP, *Neosartorya fischeri* antifungal protein (putative, accession no. XP_001262586); BFAFP, *Botryotinia fuckeliana* antifungal protein (putative, accession no. XP_001548954); PAF, *Penicillium chrysogenum* antifungal protein; NAF, *Penicillium nalgiovense* antifungal protein; GZAFP, *Gibberella zeae* antifungal protein (putative, accession no. XP_384921); PTRAFP, *Pyrenophora tritici-repentis* antifungal protein (putative, XP_001934325). The first amino acid of the mature AFP, ANAFP and PAF proteins is underlined. The predicted secretion signal cleavage site is marked by an arrow.

Table 1 Physical and chemical properties of the mature identified and putative defensin-like antifungal proteins derived from filamentous fungi.

Protein	Fungus	Number of amino acids	Molecular weight (kDa)	Number of Cys	Number of Lys/Arg	Theoretical pI	Reference(s)
Antifungal effect							
AFP	<i>Aspergillus giganteus</i>	51	5.8	8	12/1	9.27	47
ANAFP	<i>Aspergillus niger</i>	58	6.6	8	5/3	7.14	20
ACLA	<i>Aspergillus clavatus</i>	n.d.	n.d.	n.d.	n.d.	n.d.	8
NAF	<i>Penicillium nalgiovense</i>	55	6.3	6	13/0	8.93	28
PAF	<i>Penicillium chrysogenum</i>	55	6.3	6	13/0	8.93	15
GAMA	<i>Gibberella zeae</i>	55	6.4	6	12/1	9.1	7

NFAP	<i>Neosartorya fischeri</i>	57	6.6	6	11/2	8.93	8, Galgóczy et al. unpublished
BFAP	<i>Botryotinia fuckeliana</i>	n.d.	n.d.	n.d.	n.d.	n.d.	Galgóczy et al. unpublished
PTRAFP	<i>Pyrenophora tritici-repentis</i>	n.d.	n.d.	n.d.	n.d.	n.d.	Galgóczy et al. unpublished

Antibacterial effect

ABP 1,2	<i>Rhizopus microsporus</i> var. <i>oligosporus</i>	49	5.1	10	4/3	8.6	9
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n.d.: no data

2. Amino acid sequences and protein structure

Defensin-like antifungal proteins have been isolated from 4 ascomyceteous fungal species and their putative homologs have been revealed in further 5 species (*A. clavatus*, *Botryotinia fuckeliana*, *G. zaeae*, *N. fischeri* and *Pyrenophora tritici-repentis*) based on investigation of genomic databases (Fig. 1., Table 1., Galgóczy et al. *unpublished data*) [7, 8]. The primary amino acid sequences of the antifungal proteins show 14.2-91.4% identity, whereby *P. nalgiovensis* antifungal protein (NAF) and PAF are 100% identical. With respect to the mature proteins of AFP, ANAFP and PAF show 25.4-43.6% identity to each other. In spite of that, they are very different in their amino acid sequences, conserved homologous regions can be identified at the flanking amino acids of cysteines (Fig. 1.). The length of the mature proteins varied between 51-58 amino acids, and the molecular weight 5.8-6.6 kDa. Further general feature is the basic character due to the presence of a high amount of arginine and lysine residues [7]. The high cysteine content (6-8) and the several disulfide bonds (3-4) between them contributed the stability of the molecules against protease degradation, high temperature and within a broad pH range [11-13].

The tertiary structure has been resolved in case of AFP and PAF [8, 13]. AFP show a barrel topology in consequence of the five highly twisted antiparallel β -strands. This protein contains a hydrophobic core surrounded with the remaining hydrophobic, aromatic, and polar residues on the surface resulting in a high solubility of the protein. The tertiary structure of AFP is stabilised by four disulfide bridges between the cysteines residues in position of 7, 14, 26, 28, 33, 40, 49 and 51 in the amino acid sequence of the mature protein. AFP has an amphipathic structure in consequence of the presence of a cationic domain in conjunction with a hydrophobic stretch [8]. PAF contains five β -strands connected by three small loops which create two orthogonally packed β -sheets. PAF also has an amphipathic surface alternating the positively- and negatively-charged patches. The six conserved cysteines in position of 7, 14, 28, 36, 43 and 54 in the amino acid sequence of the mature protein form three disulfide bonds surrounded by the two orthogonal β -sheets, creating a hidden central core. It is demonstrated using site-directed mutagenesis that the highly-conserved and positively-charged lysine-rich surface region plays a central role with respect to the toxicity of PAF on target organisms [13].

The architecture of AFP resembles an oligonucleotide/oligosaccharide binding (OB)-fold domain that presents a binding face for different ligands such as RNA, single-stranded DNA, oligosaccharides and proteins [8]. In *in vitro* experiments it has been demonstrated that AFP binds to DNA via this OB motif and causes the chromatin to condense [14]. AFP also can attach to chitin under *in vitro* conditions with its N-terminal chitin binding domain [8]. These effects and similar binding domain have not been revealed in case of PAF [14].

3. Transcriptional regulation and expression

AFP and PAF synthesised as a preprotein which contain both a signal sequence for secretion and a prosequence that is removed before or during their release into the supernatant (Fig. 1.). The maximum protein yield is reached during the stationary growth phase after 70-90 h of cultivation in liquid culture and it is affected by different environmental factors and substances [7, 15, 16]. Rhythmic *afp* expression was observed during growth on solid medium and the expression occurred exclusively in the vegetative mycelium at the developmental stage when the colony was competent to form

aerial hyphae, but it was not found in conidiophores or conidia. Based on these observations it is suggested that *afp* expression is regulated during asexual development [16]. It is demonstrated that the carbon catabolite repression has no influence on the expression of AFP in contrast to nitrogen metabolite repression. Stress conditions (alkaline pH, salt, heat shock, carbon and phosphate starvation) and presence of other fungi during co-cultivation enhance the transcription of *afp*, and it is inhibited by acidic pH and presence of phosphate [7, 8, 16, 17]. It was found that the transcription of the *paf* gene was induced by carbon starvation, limited glucose concentration and in the presence of secondary carbon sources. In contrast to AFP the heat shock and the phosphate concentration do not influence the yield of PAF. Glutamate repress, but NaNO_3 induce the *paf* mRNA synthesis. The pH dependent gene regulation in case of *paf* has not been investigated yet [7, 15].

According to the above mentioned observations, the 5'-upstream regions of *afp* and *paf* carry several putative regulatory elements that might be involved in the transcriptional regulation of both genes in response to environmental signals and stress [7, 8].

The transcription of *afp* is under control of different transcription factors. It is hypothesised that a calcineurin signalling pathway, the phosphate starvation, and an asexual developmental regulation mechanism play a central role in transcription of *afp*. Five putative Crz1p (stress response element) binding sites can be found within the *afp* promoter. Crz1p is activated by calcineurin in response to environmental stress (salt, pH, heat shock). Heat shock and low phosphate levels enhance *afp* transcription and AFP secretion. Expression of *afp* might be repressed by phosphate through the PalcA transcription factor (phosphate regulation element), but it has been waiting for experimental validation. It is observed that the alkaline pH generates phosphate starvation in fungi, suggesting that the pH and phosphate dependent regulation of the gene might be interconnected. In spite of the presence of two putative pH responsive PacC transcription factor (pH-responsive element) binding sites in the upstream region of the gene, they are not relevant for the up-regulation of *afp* under alkaline conditions and strongly inhibits the expression of *afp* under acidic pH and in medium containing excess of phosphate. The expression of AFP is positively regulated by RlmA transcription factor (cell wall integrity element), which amount is increasing after the disruption of cell wall integrity. Carbon catabolite (CreA-mediated) and nitrogen metabolite repression (AreA-mediated) have not a regulatory effect on the transcription of the *afp* gene. It is suggested that *afp* gene is also under developmental control by PalcA and StuA protein. StuA positively or negatively regulate the asexual development of filamentous fungi, and its transcript levels are significantly induced in vegetative hyphae at the time when the competence for asexual development is acquired [8].

Some different transcription factors might have influence on the expression of *paf* based on the analysis of its promoter region. CreA (carbon catabolite repressor element) might repress the gene in the presence of glucose, and the two putative AreA (nitrogen metabolite repression element)-binding sites might play a role in nitrogen metabolite repression. A pH-dependent mechanism and stress-regulatory protein regulation is hypothesised by the presence of four PacC (pH-regulatory element) and two Stre (stress-regulatory element) consensus motifs in the *paf* promoter region [7].

Summarizing, PAF and AFP are under control of complex regulatory systems, some of which might even be interconnected, and the expression of both genes is differently regulated [7, 8].

4. Antifungal effect and susceptibility

Previous studies suggest that defensin-like antifungal proteins secreted by filamentous fungi have different species specificity; nevertheless the manifestation of their antifungal effect is very similar [7]. The main symptoms are the inhibition of spore germination and hyphal growth, retardation of the lengthening of the hyphae, membrane perturbation, the induction of intracellular oxidative stress and an apoptosis-like phenotype [7, 12, 18, 19]. Treated mycelia are generally characterized with swollen, short hyphae with multiple branches, fragmented cytoplasm and accumulation of nuclei at broken hyphal tips [7]. Their antifungal effect show dose-dependent characteristic: They exhibit growth retardation as long as they are applied in sublethal concentrations; and they become fungicidal with increasing concentrations [7, 12, 18]. Presence of mono- and divalent cations decreases their inhibitory potency. This effect was observed at $>20\text{mM}$ KH_2PO_4 , 50-100 mM KCl/NaCl ; and $>20\text{mM}$ MgCl_2 , 50-100 mM Na_2SO_4 in case of AFP and PAF, respectively [7]. Similar inhibitory effect on their antimicrobial potency of antimicrobial peptides was observed in case of plants and animals [7].

The antifungal activity of filamentous fungal defensin-like proteins is prevailed against other filamentous fungi [7]. Their inhibitory effect against yeasts (*Candida albicans*, *Saccharomyces cerevisiae*, and *Trichosporon beigelii*; MIC=8-15 μM) was demonstrated only in the case of the ANAFP [20]. ANAFP, AFP, PAF and NAF strongly inhibits the germination and growth of fungal species belonging to Ascomycetes and Zygomycetes in different concentrations (MIC=1-200 $\mu\text{g/ml}$) [7, 8, 11, 18-26]. Information about the susceptibility of basidiomyceteous fungi is available in case of *Rhodotorula mucilaginosa*, which showed resistance to AFP; *T. beigelii* and *Puccinia recondita* f.sp. *tritici*, which proved to be sensitive to ANAFP and PAF, respectively [11, 20, 26]. The antifungal spectrum of fungus derived defensin-like antifungal proteins is different, but there is similarity among the sensitive species to them [7]. They have strong inhibitory potency against important opportunistic human and/or plant pathogens [7, 8, 11, 17, 20-28]. Antifungal effect of the proteins depends on the culture medium. In a previous study, the highest inhibitions with PAF against different Zygomycetes were recorded on minimal culture medium compared to complete media [21]. It can be

explained with the different demands for nutrients of fungi as well as with the presence of certain constituents in the media, which may interfere with the activity of PAF [7, 18]. Similar effect was demonstrated in co-cultivation experiments with AFP-secreting *A. giganteus* isolate [27]. The susceptible fungal species are listed in the Table 2.

Previous studies demonstrated that defensin-like antifungal peptides can be able to interact with other drugs and antimicrobial peptides and exert a stronger inhibitory effect on the fungus, than applied alone. PAF acts synergistically with statins and fluconazole, and exhibit potent antifungal activity against different Zygomycetes and dermatophyte isolates [22, 23]. An additive effect against the plant pathogenic fungus *Botrytis cinerea* was observed when AFP was combined with an antifungal peptide isolated from a *Cecropia* moth, cecropin A [24].

Table 2 Investigated sensitive fungal species to ANAFP, AFP, PAF, and NAF.

Antifungal protein	Sensitive fungal species	Reference(s)
ANAFP	Ascomycetes <i>Aspergillus</i> spp. (<i>A. flavus</i> ^O , <i>A. fumigatus</i> ^O); <i>Candida albicans</i> ^O ; <i>Fusarium</i> spp. (<i>F. oxysporum</i> ^{O,PP} , <i>F. solani</i> ^{O,PP}); <i>Saccharomyces cerevisiae</i> Basidiomycetes <i>Trichosporon beigelii</i> ^O	20
AFP	Ascomycetes <i>Aspergillus</i> spp. (<i>A. awamorii</i> , <i>A. nidulans</i> , <i>A. niger</i> ^O); <i>Botrytis cinerea</i> ^{PP} , <i>Erysiphe graminis</i> ^{PP} , <i>Fusarium</i> spp. (<i>F. aquaeductum</i> ^{PP} , <i>F. bubigenum</i> ^{PP} , <i>F. culmorum</i> ^{PP} , <i>F. equiseti</i> ^{PP} , <i>F. lactis</i> ^{PP} , <i>F. lini</i> ^{PP} , <i>F. moniliforme</i> ^{O,PP} , <i>F.</i> <i>oxysporum</i> ^{O,PP} , <i>F. poae</i> ^{PP} , <i>F. proliferatum</i> ^{PP} , <i>F. solani</i> ^O , <i>F.</i> <i>sporotrichioides</i> ^{PP} , <i>F. vasinferctum</i> ^{PP}); <i>Magnaporthe grisea</i> ^{PP} ; <i>Penicillium</i> <i>purporogenum</i> , <i>Phytophthora infenstans</i> ^{PP} , <i>Trichoderma</i> spp. (<i>T.</i> <i>harzianum</i> ^O , <i>T. koningii</i> ^O)	8, 11, 19, 24, 25
PAF	Zygomycetes <i>Absidia corymbifera</i> ^O ; <i>Micromucor ramanniana</i> ; <i>Mortierella elongata</i> ^O ; <i>M. nanthalensis</i> ^O ; <i>M. wolfii</i> ^O ; <i>Mycotypha africana</i> ; <i>Rhizomucor miehei</i> ^O ; <i>R. pusillus</i> ^O ; <i>Rhizopus microsporus</i> var. <i>oligosporus</i> ^O ; <i>R. oryzae</i> ^O ; <i>Thamnostylum piriforme</i> ; <i>Umbellopsis isabellina</i> ; <i>Zygorhynchus</i> <i>macrocarpus</i> Ascomycetes <i>Aspergillus</i> spp. (<i>A. flavus</i> ^O , <i>A. fumigatus</i> ^O , <i>A. giganteus</i> , <i>A. nidulans</i> , <i>A.</i> <i>niger</i> ^O , <i>A. terreus</i> ^O); <i>Blumeria graminis</i> f. sp. <i>hordei</i> ^{PP} ; <i>Botrytis cinerea</i> ^{PP} ; <i>Cochliobolus carbonum</i> ^{PP} ; <i>Fusarium oxysporum</i> ^{O,PP} ; <i>Gliocladium</i> <i>roseum</i> ^{PP} ; <i>Hypocrea orientalis</i> ^O ; <i>Neurospora crassa</i> , <i>Trichoderma</i> spp. (<i>Trichoderma atroviride</i> ^{*O} , <i>T. citrinoviride</i> ^{*O} , <i>T. harzianum</i> ^{*O} , <i>T.</i> <i>inhamatum</i> ^{*O} , <i>T. koningii</i> ^O , <i>T. longibrachiatum</i> ^{*O}); <i>Trichophyton</i> spp. (<i>T.</i> <i>mentagrophytes</i> ^{PH} , <i>T. rubrum</i> ^{PH} , <i>T. tonsurans</i> ^{PH}); <i>Microsporum</i> spp. (<i>M.</i> <i>canis</i> ^{PH} , <i>M. gypseum</i> ^{PH}); Basidiomycota <i>Puccinia recondita</i> f.sp. <i>tritici</i> ^{PP}	7, 18, 21, 22, 23, 26
NAF	Zygomycetes <i>Mucor</i> sp. Ascomycetes <i>Aspergillus flavus</i> ^O ; <i>Byssochlamys</i> sp., <i>Fusarium solani</i> ^{O,PP} , <i>Geotrichum</i> <i>candidum</i> ^O ; <i>Penicillium</i> spp. (<i>P. digitatum</i> , <i>P. italicum</i> , <i>P. roquefortii</i>)	28

*: unpublished data, Galgóczy et al., 2007
^O: opportunistic pathogenic fungus
^{PH}: potentially human pathogenic fungus
^{PP}: potentially plant pathogenic fungus

5. Mode of action

In spite of that the amino acid sequence, protein structure, attributes and the symptoms of the antifungal effect is very similar, different mode of actions are hypothesised in case of AFP and PAF, which were studied in this aspect.

In susceptible organisms AFP disturbs the polarised growth of hyphae by interfering directly or indirectly with the cell wall biosynthesis [19]. It is supported by the observation of Hagen et al., that a protein kinase C (Pkc)-dependent cell wall integrity pathway was induced in the AFP-sensitive *A. niger* after the AFP administration [30]. In the sensitive fungal species small proportion of the applied AFP binds electrostatically to the cell membrane and induces membrane permeabilization and pore formation [19]. A large amount of AFP accumulates in the cell wall outer layer and the cell wall, and cause the loss of cell wall integrity [12]. In the insensitive fungi (as investigated by *P. chrysogenum*), the large amount of AFP accumulates intracellularly, and degraded by the cell [12, 19]. In the ten- to hundredfold higher concentration than the minimal inhibitory concentration, AFP can be detected intracellularly in sensitive organisms also [12, 26]. In this case AFP disintegrates the intracellular structures and the protein binds via its OB-fold or cationic domain to intracellular remnants [12]. Based on these results, it is hypothesised that AFP has a specific binding structure in the outer layer and cell wall of the sensitive fungi. The membrane permeabilization effect of AFP is cation-sensitive (as like as the inhibitory effect) suggesting the electrostatical binding mechanism of AFP to the cell membrane [7, 19]. Hagen et al. demonstrated that AFP can bind to the chitin by its putative N-terminal chitin-binding motifs *in vitro*, and inhibits the cell wall chitin biosynthesis by the specific inhibition of the chitin synthase III and V [29]. These enzymes are unique among fungi and essential to maintenance the polarised growth and virulence of pathogenic fungi. Presence of chitin synthase III and V is confirmed in the AFP-sensitive fungi, and the insensitive fungal species do not have these types of this enzyme. There is some possible explanation for the cell wall destruction effect of AFP: It is possible that AFP interferes with the chitin synthases and inhibits the transport and insertion of the enzyme into the plasma membrane; or AFP interacts with a membrane component, which play a role in the anchoring of chitin synthases to the membrane. Irrespectively of the truth, the two possible explanation result in the loss of cell wall integrity. Sphingolipid membrane components in the sensitive fungi might be severe as secondary receptors for the AFP. It is confirmed by preliminary investigation of Hagen and Meyer, who found that depletion of cellular glucosylceramide levels in AFP-sensitive fungal species (*Aspergillus fumigatus* and *A. niger*) resulted in reduced AFP susceptibilities [8]. This supplemented with the observation that the sphingolipids are necessary to maintain the polarised hyphal growth, elucidates the mechanism of polarised growth degeneration effect of AFP [8, 30]. The species specificity of AFP might be connected with the sphingolipid profile of the sensitive fungi [8].

It has been shown that in sensitive fungi, PAF is internalized by receptor-mediated endocytosis and localized in the cytoplasm, but not co-localized with the nucleus [18, 31, 32]. PAF does not show nucleotid- and chitin-binding activity [7, 13]. It is hypothesized that PAF acts via a G-protein signal transduction pathway, but the exact mechanism has not yet been elucidated [32]. G-protein coupled activity of PAF was confirmed by investigation of *fadA* mutant of *Aspergillus nidulans*, which proved to be less sensitive to PAF treatment compared to the wild type. The *fadA* gene encodes the heterotrimeric G-protein α subunit, which dissociation is inhibited from the $G_{\beta\gamma}$ complex in case of *fadA* mutant *A. nidulans* [31]. These results indicate that PAF toxicity requires active heterotrimeric G-protein signalling [31, 32]. Based on these observations Marx et al. hypothesised that PAF interacts directly or indirectly with G-protein signal transduction pathways [32]. In the sensitive organisms PAF exerts multiple detrimental effects: induction of plasma membrane polarization, intracellular reactive oxygen species (ROS) and apoptosis-like phenotype [18, 31, 32]. The significant elevation in the potassium ion concentration in the extracellular space and the hyperpolarization of fungal membranes at the hyphal tips after the administration of PAF indicates the induction of selective potassium permeability, which leads the collapse of the intracellular homeostasis in sensitive fungal species [32]. These phenomena are parallel with the changes in the morphology. Oxidative damage of cell organelles in PAF-treated hyphae of sensitive fungal species indicates that PAF induces generation of ROS [18, 31, 33]. It is manifest as mitoptosis: Mitochondria show aberrant ultrastructural morphology, and they have discontinuous or missing outer membranes [31, 33]. PAF induces a strictly regulated programmed cell death (PCD) through the ROS [32]: The investigation of PAF-sensitive *A. nidulans* protoplast after treatment with PAF revealed the increased exposure of phosphatidylserine on the cell surface, DNA fragmentation, membrane blebbing and cell shrinking. Based on the above mentioned investigations and observations, Marx et al. hypothesised the possible mechanism of mode of action in case of PAF: Cell wall could be a selective barrier for PAF, but *in vivo* chitin-binding activity of PAF has not been revealed yet. A PAF-mediated G-protein signalling results the hyperpolarization of the membrane and increase the potassium ion permeability. After receptor-mediated endocytosis, PAF induces ROS formation in the cytoplasm, which induces PCD. The interacting components of PAF in the cell layers (cell wall, cell membrane) and in the cytoplasm have not been identified yet, but the active internalization and the cytotoxic effect of the protein suggest their existence [32]. In a recent study Binder et al. revealed the possible interfering components of PAF: PAF interferes with Pkc/mitogen-activated protein kinase (Mpk) and cAMP/protein kinase A (Pka) signalling in *A. nidulans*. They proposed that the apoptosis, the defective actin polymerization and apolar growth are resulted by the PAF activated cAMP/PkaA signalling cascade possibly via G-protein signalling. Further PAF inhibits the activating of MpkA and probably PkcA, disrupting basal resistance of *A. nidulans* towards antifungal activity [34].

6. Biological role

Most filamentous fungi actively secrete proteins with antimicrobial activity into their environment during vegetative growth against different groups of microbes [35]. Their structure, mode of action and antimicrobial spectrum are very different [2, 6, 10, 36]. There is no information in the literature about the biological function of defensin-like antimicrobial proteins secreted by filamentous fungi. These proteins have been identified from agriculturally and medically harmful fungal species yet (Table 1) [7, 8]. It is supposed that they play a role in the emulsion for nutrients and habitat against microorganisms with similar ecological niche [37].

This assumption might be supported by previous investigations: it was demonstrated that co-cultivation of *A. giganteus* with *Fusarium oxysporum* resulted in elevated AFP concentration in the culture broth [38]. Meyer and Stahl used a reporter system based on the β -glucuronidase gene for support their assumption that *A. giganteus* is able to sense the presence of a putative nutrient competitor hereby enhance the transcription of the *afp* gene, which provide a growth advantage against the competitor species [27]. They found that the biological function of AFP is more complex, than they supposed: Some bacterial (*Pseudomonas aeruginosa*) and yeast (*S. cerevisiae*, *C. albicans*) species enhanced the transcription of *afp* gene in their experiments. These microorganisms are not competitor species of *A. giganteus* and the AFP does not inhibit their growth [7, 11]. They hypothesised that the transcription factors which activated in consequence of the changed pH, nutrient-limitation and different type of stress enhance the expression of the AFP in this case. Their binding sites were revealed in the promoter region of the *afp* gene [7, 8, 11, 19]. The vital cell-to-cell contact with the competitor microorganisms might be necessary for induction and enhancing the transcription of *afp* gene. It was confirmed by Meyer and Stahl by the investigation of *A. giganteus*-*F. oxysporum* co-cultivation. They found that the AFP transcription was not induced; when they culture supernatant of *F. oxysporum* and autoclaved mycelia of *F. oxysporum* was applied in the tests instead of vital *F. oxysporum* culture. The *afp* promoter induction was highly depended on the nutrient composition of the media in vital cell-to-cell contact also. The increasing concentration of peptone in the medium implicates the strengthening expression of AFP. Based on these data they speculated that only in the presence of high peptone concentrations, *F. oxysporum* produces an unknown signal, which can be sensed by *A. giganteus* [27]. In conclusion, defensin-like antifungal proteins might play a role in the competition for nutrients and habitat against fungi with similar ecological niche, but it shows a high dependence on their availability and the physiological condition of the competitor species.

Based on their investigation of a *paf*-deletion *P. chrysogenum* strain, Hegedűs et al. hypothesised that PAF is a signalling molecule also and has a role in differentiation. This strain displayed a delay in conidiation under different stress conditions. It was most pronounced in the presence of osmotic and oxidative stress. The conidiation, germination efficiency in the deletion strain was not affected in surface cultures. The deletion strain showed accelerated autolysis under carbon starvation in submerses culture compared to the PAF-secreting wild type, where this phenomenon was not observable under these conditions. This observation suggested that PAF play a role as a molecule for promoting vegetative growth under starvation in submerge cultures. The lack of the delaying in germination and conidiation in surface cultures of the deletion strain (where the O₂ availability is higher than in the submerge culture), indicates the probable oxidative stress-sensing role of PAF, and its different functions in surface and submerge cultures [37]. These assumption is confirmed by the observations of Galgóczy et al., who demonstrated by agar-diffusion technique that PAF-treated sensitive *Trichoderma* and *Hypocrea* strains showed strong inhibition of the germination of spores and the hyphal extension for 48 h cultivation after administration of PAF, than sprawling and overconidiated hyphae were observed in the region of earlier inhibition zones compared to the untreated control (Fig. 2., Galgóczy et al. *unpublished data*).

Further investigations are needed for demonstration of the above mentioned hypotheses and for reveal their connection with the stress-element regulation of *afp* and *paf*.



Fig. 2 Effect of PAF on hyphal extension on the growth of *Hypocrea orientalis* (UAMH 9573) on complete medium (CM: 0,2% peptone, 0,1% yeast extract, 0,1% NZ-amine A, 2% glucose, 0,05% KCl, 0,04% MgSO₄·7H₂O, 0,15% KH₂PO₄, pH=6.5) after incubation of 24, 48, 72, 96 h, at 25 °C. The wells contain 100 µl PAF diluted in CM broth in the following concentrations: 50 µg/ml (A), 25 µg/ml (B), 12.5 µg/ml (C) and 6.25 µg/ml (D). 100 µl CM broth without PAF was used as control (E).

7. Potential application

Based on the previous studies several features of the defensin-like antifungal proteins secreted by filamentous fungi correspond to the requirement of the novel antifungal drugs in medicine and agriculture: They are effective inhibitors of germination of spores and hyphal extension [7, 8, 21-23, 27]. They do not have any toxic effects on plant and mammalian cells [24-26, 39-45]. They are stable molecules in various environmental conditions [13, 24]. Their ability to act synergistically with other antifungal drugs and peptides has been revealed [22-24]. They are secreted in high abundance into the supernatant of easily fermentable molds, and their low costs of production could make them favourable [2, 7, 8]. All these characteristics are supported by the fact that several antimicrobial peptides and their synthetic analogues were used in transgenic plants, and their application as biopesticides and pharmaceutical agents have been intensively studied [2, 8, 10].

It was demonstrated previously that fungus derived defensin-like antifungal peptides inhibited the germination of spore and growth of agriculturally and medically harmful fungal species (Table 1) [7, 8, 21-26].

AFP might be a useful peptide in the crop protection in the near future based on its specific inhibition of chitin synthase III and V. These enzymes play crucial role in the pathogenicity of plant pathogen fungi [8]. Direct application of AFP on rice and *Pelargonium* plants leaves protected them against *Magnaporthe grisea* and *B. cinerea* after still two or six weeks of infection [24, 43]. Pre-incubation of tomato roots with AFP protected the plants against *F. oxysporum* [12]. Barakat et al. observed that secondary growth of different *Fusarium* species is also inhibited by AFP on barley in post harvest conditions; furthermore they detected a reduction in levels of the mycotoxin deoxynivalenol after the AFP treatment compared the untreated control [46]. Similar preservative effects of NAF have been suggested by Geisen [28]. Expression of AFP by transgenic plants has been performed successfully involved in wheat, rice plant, and pearl millet. These plants proved to be less susceptible against their potential pathogens. Significant reduction of disease symptoms were observed for transgenic wheat, rice plants and pear millet infected with *Erysiphe graminis*, *P. recondita*, and *M. grisea*, and *Sclerospora graminicola*, *Puccinia substriata*, respectively [39-42]. Detrimental effects on the host organisms were not observed in case of the above mentioned external and internal application and expression of AFP. The transgenic plants showed normal growth behaviour, morphology and they were fertile [8]. In the effective concentration ranges AFP does not provoke cytotoxic and harmful effects on human endothelial cells, neurons, astrocytes and skeletal muscle myotubes, and does not activate the immune system [45]. Its additive effects with other antifungal peptides (e.g. cercopin A) could make the AFP promising antifungal agent in the agriculture. [24].

Previous studies demonstrated that PAF does not have any toxic effect on mammalian cells and does not induce the production of proinflammatory cytokines. It is also demonstrated that PAF does not modify the ion channels of mammalian neurons, skeletal muscle, and astrocytes in the potential therapeutic concentrations [44]. Thus PAF would be a potential antifungal agent in the medicine. Its synergistic interactions with statins (which are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase) against potential opportunistic human pathogen zygomyceteous fungi; and with fluconazole against dermatophytes (*Trichophyton* spp. and *Microsporum* spp.) confirm this assumption [22, 23]. PAF expressing transgenic plants have not been described in the literature yet.

As mentioned above AFP proved to be stable molecule in leaf surfaces protecting experiments in common environmental conditions, but the stability of AFP and PAF against extreme conditions is also demonstrated. This feature of molecules is accounted for their tertiary structures, which are stabilised by disulfide bridges [13, 24, 43]. AFP exhibits relevant resistance to proteolysis and a remarkably high denaturation temperature, furthermore it is relative stability in wide pH-range. Lacadena et al. demonstrated that AFP was not degraded by SV-8 protease, trypsin, pepsin, or thermolysin under non-denaturing conditions and at 37 °C, although very extreme conditions were used in some cases. Only pronase and proteinase K treatment resulted partial degradation, but peptides remained bounding by the disulfide bridges. It was demonstrated that thermal transition are not detected in the 25-80 °C temperature range, and the AFP relative stable in pH ranging from 2 to 12 [43].

In a previous study PAF proved to be stable over the pH range 1.5-11. The antifungal activity of the protein decreased with ~20 % after treatment with 10-60 min at 60-80 °C. Significant reduction of the protein activity with the exposure time was observed in extreme temperature conditions (60 min at 95-100 °C). The loss of protein activity was not reversible, indicated a permanent change in PAF structure and activity. Pepsin, proteinase K, pronase digestions for 3-9 h did not have an effect on the activity of PAF, but long exposure to pronase and proteinase K (12 and 24 h) reduced the protein activity significantly. This inactivation was accompanied by protein degradation [13].

The above mentioned features of filamentous fungal defensin-like proteins could make them favourable in agricultural and medical respect in the future, but it is needed further studies to prove their practical efficiency, for example in animal and plant model experiments.

8. Conclusion

The incidence of fungal infections has increased continuously during recent years in consequence of the increasing number of immunocompromised hosts and the occurring of antibiotic resistant strains. The most widely used antifungal agents (e.g. amphotericin B) are quite toxic and have serious side-effects. Another important aspect is that many

filamentous fungi are destructive pathogens of plants and are thus responsible for enormous crop losses worldwide. Therefore there is a substantial demand for new compounds with antimicrobial activity.

The proteins with similar structure to defensins secreted by filamentous fungi are interesting from this respect, as they have effective inhibitory potential against other filamentous fungi. Their narrow antifungal spectrum is a limitation factor in their practical application, but it is hypothesised that combination of different species-specific proteins represents a promising novel broad-spectrum antifungal agent. Furthermore, they could serve as the basis for the design of new synthetic analogs after the understanding of the structure-effect-efficacy connection. Both the peptides and their analogs expression in transgenic plants to confer disease protection or their secretion by microorganisms to be used as active ingredients of commercial biopesticides and medicines could be possible in the near future [2, 8, 10].

After the evaluation of their low costs of production by an easily fermentable, “generally recognized as safe” yeast or filamentous fungi (e.g. *Pichia pastoris*, *A. niger*, *P. chrysogenum*) could make them favourable in medicine and agriculture by their safety, sustainability, high efficacy, stability and limited toxicity [7, 8, 10].

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