Peptides 30 (2009) 1798–1804

Contents lists available at ScienceDirect

Peptides

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

Molecular cloning, structural analysis and modelling of the AcAFP antifungal peptide from *Aspergillus clavatus*

Houda Skouri-Gargouri [a], Mamdouh Ben Ali [b], Ali Gargouri [a,*]

[a] Laboratoire de Génétique Moléculaire des Eucaryotes, Centre de Biotechnologie de Sfax, Route Sidi Mansour, BP "K" 3038-Sfax, Tunisia
[b] Laboratoire d'Enzymes et de Métabolites des Procaryotes, Centre de Biotechnologie de Sfax, BP "K" 3038-Sfax, Tunisia

ARTICLE INFO

Article history:
Received 18 March 2009
Received in revised form 25 June 2009
Accepted 26 June 2009
Available online 8 July 2009

Keywords:
Aspergillus clavatus
Antifungal peptide
Gene organization
Cellular morphology
3D structure

This work is dedicated to the memory of our friend and colleague Amira Romdhani and Professor Mohamed Marrakchi.

ABSTRACT

An abundantly secreted thermostable peptide (designed AcAFP) with a molecular mass of 5777 Da was isolated and purified in a previous work from a local strain of *A. clavatus* (VR1). Based on the N-terminal amino acid (aa) sequence of the AcAFP peptide, an oligonucleotide probe was derived and allowed the amplification of the encoding cDNA by RT-PCR. This cDNA fragment encodes a pre-pro-protein of 94 aa which appears to be processed to a mature product of 51 aa cys-rich protein. The deduced aa sequence of the pre-pro-sequence reveals high similarity with ascomycetes antifungal peptide. Comparison of the nucleotide sequence of the genomic fragment and the cDNA clone revealed the presence of an open reading frame of 282 bp interrupted by two small introns of 89 and 56 bp with conserved splice site. The three-dimensional (3D) structure modeling of AcAFP exhibits a compact structure consisting of five anti-parallel β barrel stabilized by four internal disulfide bridges. The folding pattern revealed also a cationic site and spatially adjacent hydrophobic stretch. The antifungal mechanism was investigated by transmission and confocal microscopy. AcAFP cause cell wall altering in a dose-dependent manner against the phytopathogenic fungus *Fusarium oxysporum*.

1. Introduction

A wide variety of living organisms produce antimicrobial peptides to protect themselves from infectious microbial agents that might act as nutrient competitors in the same environment. The promising properties of these peptides have prompted a whole line of research that probe into natural antimicrobial agents that may act as effective alternatives for the conventional chemical ones. In fact, peptides with antimicrobial properties were first isolated from plants [3]. Proteins with similar functions have then been found in a wide array of organisms ranging from prokaryotes to lower and higher eukaryotes [9,10,16,23–26,28].

Filamentous fungi have been reported as valuable sources of small-size antifungal peptides. A limited number of them have so far been isolated from this restraint family. *Aspergillus giganteus* was the first filamentous fungus to be described as a potent producer of an antifungal peptide called AFP [12,15,23,34]. A *Penicillium chrysogenum*-derived antifungal peptide, named PAF, was then isolated from *Penicillium chrysogenum* [17], and, later, another antifungal peptide termed as AnAFP was identified from the mould *Aspergillus niger* [13]. More recently the NAF peptide was isolated from *Penicillium nalviogense* that was identical to PAF [7]. As a matter of fact, AFP showed 35.6 and 47.1% homology to AnAFP and PAF/NAF, respectively.

Despite their moderate amino acid similarities, antifungal peptides share common structural properties. They are highly basic, with a large number of cysteine residues, present a compact structure, possess thermal stability and potent antimicrobial activity [16,20]. The *A. giganteus* antifungal peptide exhibits an extra pair of Cys in addition to the common six canonical Cys residues described for PAF, NAF and AnAFP [14,22]. As a whole, the peptides identified so far have been reported to act effectively against a wide range of phytopathogenic fungi, and AnAFP was particularly prominent among them for it was found to inhibit the growth of yeast strain, however none of these peptides has been reported to exhibit any antibacterial activity.

Accordingly, the present study is particularly concerned with the individual characteristics and activities of a recently identified antifungal peptide termed as AcAFP that was isolated and purified from a local *Aspergillus clavatus* strain in a previous study [25]. It was undertaken with the aim of further investigating the molecular cloning, encoding gene organization, predicted structure and potential biological activity of this promising antifungal peptide.

* Corresponding author. Tel.: +216 74 874 449; fax: +216 74 874 449.
E-mail address: faouzi.gargouri@cbs.rnrt.tn (A. Gargouri).

0196-9781/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2009.06.034

2. Materials and methods

2.1. Microorganism strains, plasmids and growth conditions

The producer strain *Aspergillus clavatus* VR1 was grown in LBG broth (Luria–Bertani broth (0.5% yeast extract, 1% peptone and 0.5% NaCl) supplemented with 2% glucose) and antagonist strain *Fusarium oxysporum* was grown on YPG broth: yeast-peptone-glucose (0.5% yeast extract, 1% peptone, 2% glucose) [25]. *Escherichia coli* TOP10F': *F'[lacI^q Tn10 (Tet^R) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1araD139Δ(ara-leu) 7697galKrps (STR^R)endA1nupG* (Invitrogen) was used as a host for plasmid propagation. The *pGEM®-T* vector (Promega) was used for cloning PCR fragments.

2.2. Peptide purification and MIC assay

A. clavatus was grown for 96 h at 30 °C in 1L Erlen-meyer flasks, each containing 200 ml of LBG culture medium. Both, the purification of AcAFP from the culture supernatant of *A. clavatus* and the quantification of its antifungal activity based on the determination of the minimal inhibitory concentrations (MIC) using *F. oxysporum* as a test strain, were performed as previously described [25].

2.3. DNA and RNA isolation

The genomic DNA and RNA were isolated from the mycelia of 4-day-old *A. clavatus* cultures, following the method described by Trigui Lahiani and Gargouri [33].

2.4. PCR and RT-PCR reactions

The isolated genomic DNA was used as a template for the amplification of the DNA fragment that carried the AcAFP gene. Two oligonucleotide primers were designed; O_1: 5′-AAATGCTA-CAAGAAGGATAA-3′a specific primer in the sense direction was deduced from the N-terminal sequence of the purified protein AcAFP, and O_2: 5′-ACACGCCACATCTTGCTCGG-3′a non-specific primer in the anti-sense direction was obtained according to the 3′-UTR end of the published sequence of the homologous *AFP gene* from *Aspergillus giganteus*, available in the NCBI database. PCR was carried out under the following conditions: 95 °C for 5 min, addition of 1 U of Taq polymerase (GoTaq® Promega), 45 cycles of denaturation at 94 °C for 30 s; annealing at 52 °C for 30 s; elongation at 72 °C for 60 s with a final elongation step at 72 °C for 7 min. A typical PCR reaction mixture contained: 200 ng template DNA, 1 U of Taq polymerase, 10 μl of 5× Taq polymerase buffer, 2 mM of $MgCl_2$, 0.2 mM dNTP, and 20 pmol of each primer; the final volume of the reaction mixture was set to 50 μl. PCR products were visualized on a 1% agarose gel.

For RT-PCR reaction, 10 μg of total RNA was used as template. The RT reaction was conducted using the AMV Reverse Transcriptase, according to the manufacturer's instructions (Promega). The synthesized cDNA was used as template for PCR to screen the AcAFP-encoding cDNAs. Subsequent PCR was conducted with O_1 and O_2 under the PCR reaction conditions described above.

2.5. DNA and cDNA sequencing

The PCR and RT-PCR products were purified from agarose gel after electrophoresis using the Wizard® SV Gel and PCR Clean-up System (Promega). These fragments were ligated into pGEM-T easy vector (Promega); the resultant ligation products were transferred into Top10 *Escherichia coli* (Invitrogen) competent cells. Plasmids DNA were isolated from bacterial clones using the alkaline lysis method [2,24,33]. The nucleotide sequences were determined automatically through the BigDye® Terminator v3.1 Cycle Sequencing Kit in the ABI PRISM 3100-Avant Genetic Analyser using universal and reverse primers. The analysis and comparison of nucleotides sequences were performed using the Blast and Bio-edit programs.

2.6. Sequence alignments and nucleotide sequence access number

Nucleic acid and amino acid sequence alignments were performed using the bio-edit program. The GeneBank database was scanned for other antifungal peptide encoding genes. The nucleotide sequence determined in this study has been deposited in the Gene Bank database under access number EF600065.

2.7. Computer-aided model building of the tertiary structure of AcAFP

The automated protein structure homology-modelling Geno3D server (http://geno3dpbil.ibcp.fr) was used to generate the 3D model. The molecular modelling of the AcAFP from *A. clavatus* was analyzed based on the NMR structure of the AFP (pdb accession code 1AFP) from *Aspergillus giganteus* [4]. Finally, PyMOL (http://www.pymol.org) was used to edit the figures [5].

2.8. Analysis of the fungicidal and fungistatic effect of AcAFP

The conidia of *F. oxysporum* AcAFP-sensitive fungus were cultivated in YPG media containing different AcAFP concentrations that ranged from 3 to 200 μg mL^{-1}. After 48 h of incubation, the AcAFP solution was removed by washing the conidia and mycelia three times with the YPG medium. Afterwards, fungi were incubated for an additional 72 h in fresh YPG media. For negative control, the conidia of *F. oxysporum* were cultivated for 48 h in AcAFP-free YPG media then washed and further cultivated in fresh YPG as described above. A fungistatic effect of AcAFP was assumed if the growth of the *F. oxysporum* was reduced during AFP treatment but became comparable to that of the negative control after the replacement of the medium. The growth inhibitory effect was considered fungicidal if no growth could be detected after 72 h incubation both in fresh AcAFP-free YPG media and on PDA plates.

2.9. Microscopy

In order to visualize the morphological changes that were induced by AcAFP, the mycelia from the AcAFP-treated group and those from the AcAFP-untreated (control) group were centrifuged, the supernatant was removed and the tested fungi were twice-washed with double distilled water before microscopic observation. A hyphal growth and morphology analysis was performed and the samples were photographed and examined using an Olympus BX 51 microscope equipped with a 35 mm camera. The hyphal auto-fluorescence was recorded in a 510 LSMS Pascal (Carl Zeiss) confocal laser scanning microscope. Stacks of images were taken through a *z*-axis of the fungal cells at intervals of 0.3 μm three-dimensional projections of the *Z*-axis series were then processed using LSM 510 software. Appropriate filter sets for excitation/emission at 488/530 nm were also used.

2.10. Reducing sugars analysis

The dinitrosalicylic acid (DNS) method was used to measure liberated reducing sugars, determined as glucose equivalents by a colorimetric method [21].

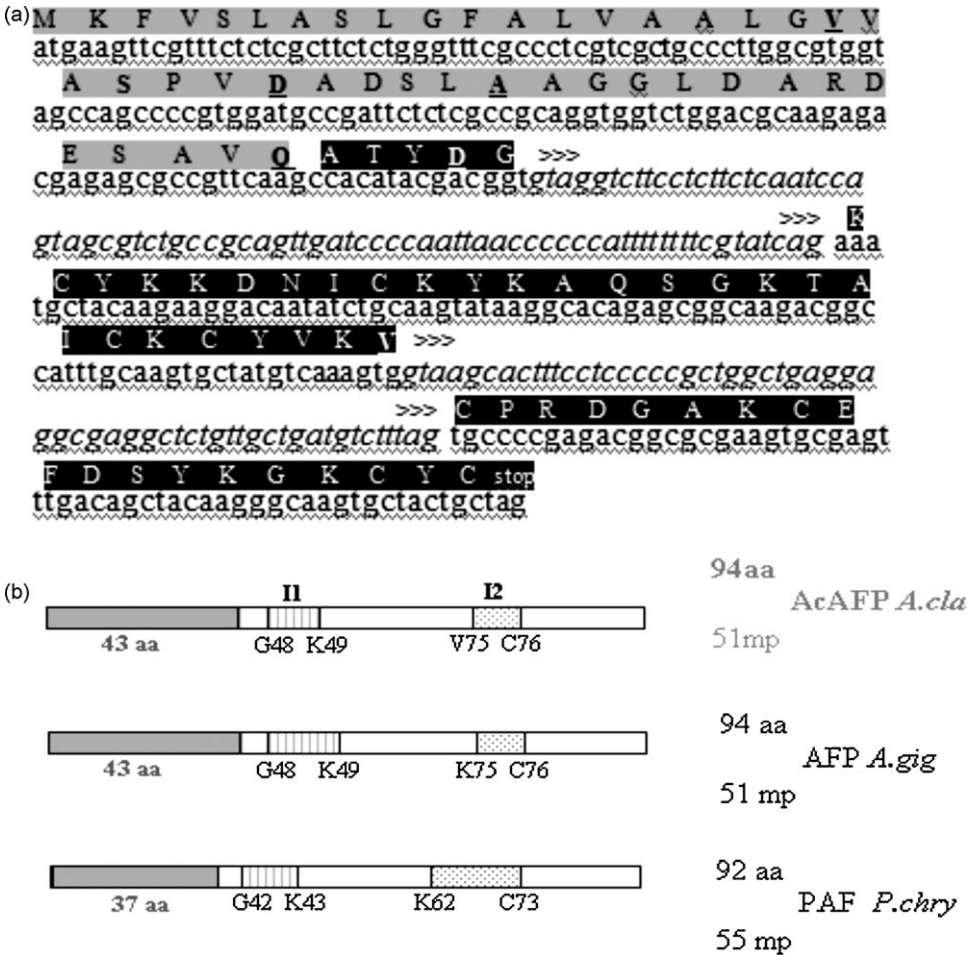

Fig. 1. (a) Nucleotide sequence of the gene encoding *AcAFP*. The deduced aa sequence of the gene is shown below the corresponding codons. Signal peptide (light grey); mature peptide (dark gray). Intron sequences are in lower characters, >>> represent 5′ and 3′ splice sites, respectively. The residues underlined and indicated in bold represent the differences between *AcAFP* and *AFP* of *A. giganteus*. This sequence has been deposited in the gene bank nucleotide sequence database under the accession number EF 600065. (b) Schematic overview of ascomycetes antifungal peptide genes indicating the introns positions (I1, I2), lengths of the total (aa) and mature protein (mp).

2.11. Thin Layer Chromatography

Hydrolysis products of cell wall polymers induced by AcAFP on sensitive fungous were visualized by Thin Layer Chromatography. TLC was performed on a pre-coated Silica Gel TLC aluminium cards (Fluka) and developed with a solvent system consisting of acetic acid/chloroform/H_2O (7:6:1, v/v/v). 1 μl of each standard and 10 μl of each samples were loaded onto the plate. The developed plate was stained by spraying with a solution of ethanol, H_2SO_4 (9:1, v/v) and 0.1% orsinol and heated at 120 °C for 5 min.

3. Results

3.1. Cloning and nucleotide sequence of the AcAFP encoding gene

Based on the N-terminal amino acid sequence of AcAFP and the C-terminal amino acid sequence of a homologous peptide AFP from *A. giganteus*, pair of degenerate primers was designed for the amplification of the genomic fragment and the cDNA performed with PCR and RT-PCR, respectively. Both strands of selected clones that harbored fragments corresponding to the cDNA and the AcAFP-encoding part of the gene were sequenced. The genomic nucleotide sequence revealed an open reading frame length of 282 bp and a 3′ non-coding region length of 253 bp. The translation of the cDNA sequence confirmed that the cloned fragment encoded the purified peptide, since the aa Ala_{44}–Thr_{66} perfectly matched those that were derived from the direct sequencing of the N-terminal domain [25]. In other words, the comparison between cDNA and genomic sequences revealed the presence of two introns; intron 1 was 80 pb long and intron 2 was 59 pb long (Fig. 1). The boundary junctions (*GTA...YAG*) and the putative Lariat branch point sequence (RCTRAC) were approximately similar to those found in other fungal introns [6,11] (Table 1).

3.2. Comparison of AcAFP to other fungal "antifungal peptides"

Using basic local alignment search tool (Blast) and Bio-edit programs, the nucleotide sequence and the predicted amino acid of the coding region were compared to other ascomycetes antifungal peptide sequences. The open reading frame of the AcAFP gene encoded a polypeptide composed of 94 amino acids, including the mature AcAFP sequence. The precursor of the AcAFP peptide was

Table 1
Comparison of *A. clavatus AcAFP* gene intron. a: consensus sequences found for filamentous fungi. The underlined letters refer to invariable nucleotides among all known introns [11].

Intron	5′ junction	Lariat structure	3′ junction
1	GTA	TCTC**A**A	CAG
2	GTA	GCTG**A**T	TAG
Concensus	**GTA**	**RCTRAY**	**YAG**

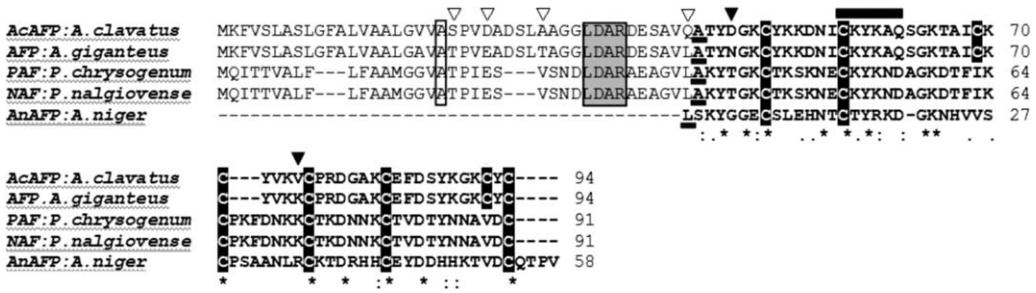

Fig. 2. Alignment of the protein aa sequences of AcAFP (ABR10398), AFP (CAA43181.1), AnAFP and PAF (AAA 92718). The predicted signal cleavage site is boxed and the putative consensus sequence for the cleavage of the pro-sequence is boxed and highlighted in grey. The first aa of the mature protein is underlined. Identical aa of the mature protein are marked by an asterisk. The shaded boxes indicate conserved Cys residues and the arrowhead indicate the two supplementary Cys residues in the aa sequences of *A. clavatus* and *A. giganteus*. The putative chitin-binding motif of AcAFP (CKYKAQ) is indicated with an open bar.

found to be organized into a signal peptide sequence of 17 aa followed by a pro-sequence of 21 aa. The mentioned structural organization is quite similar to other ascomycetes antifungal peptide precursors such as AFP [12], PAF [17] and NAF [7].

The secreted form of the predicted AcAFP peptide, which was found through the culture supernatant of *A. clavatus*, was a 51 aa long peptide that comprised the N terminal domain encompassing the first 22 aa of the mature form whose content was highly rich in cystein (8), tyrosine (6) and lysine (11) residues (Fig. 1). Based on an amino acid analysis by Protparam software in Expasy (http://ca.expasy.org/cgi-bin/protparam), the isoelectric point was estimated at 9.3. Under physiological conditions, therefore, the AcAFP peptide was positively charged.

A BLAST (http://www.ncbi.nlm.nih.gov/BLAST) comparison revealed a 36–92% identity similarity between the amino acid sequence of the deduced AcAFP pre-pro-protein and other ascomycetes antifungal proteins. The multiple sequence alignment of AcAFP to the reported ascomycetes antifungal peptides is given in Fig. 2. A more advanced search revealed a high degree of identity at amino acid levels with AFP from *A. giganteus*; both contained a high amount of hydrophobic and cationic residues, and 8 cys residues, namely Cys7, Cys14, Cys26, Cys28, Cys33, Cys40, Cys49 Cys51 (numbering refers to the mature AcAFP). Nevertheless, there were 7 differences that existed between the AFP and AcAFP peptides: one in the pre-sequence (A19V), four in the pro-sequence (T22S; E25D, T30A and L43Q) and two in the mature peptide (N4D and K32V). It was particularly noted that PAF/NAF and AnAFP contained only 6 Cys residues (with Cys33 and Cys49, which were present in AFP and AcAFP, being missing).

3.3. Prediction of the three-dimensional structure of AcAFP peptide

The 3D model obtained revealed that the AcAFP structure was similar to that of AFP peptide, and thus suggested that they shared a common fold structure consisting of five anti-parallel β strands that define a small and compact β barrel stabilized by four internal disulfide bridges between the eight conserved cysteine residues [12,14,20]. The structure superposition of AcAFP showed that, in spite of the minor differences found between the two amino acid sequences, the latter fitted perfectly with a slight but significant difference (Figs. 2 and 3A). In fact, the two amino acid changes (N4D and K32V) disturbed the length of the β barrels and the two loops, as depicted in Fig. 3A and B. Interestingly, the AcAFP adopted an amphipathic structure that could be attributed to the presence of a hydrophobic domain (Y29, V30, Y45, Y50) and a cationic domain (K9, K10). In addition, AcAFP, harbored a putative chitin-binding domain (CKYKAQ) at its N-terminal similarly to the AFP antifungal peptide which might have enabled protein to bind to chitin under *in vitro* conditions [20,8].

3.4. Fungicidal activity of AcAFP

The application of AcAFP concentrations, ranging from 3.4 to 50 μg mL^{-1}, was found to inhibit the growth of the sensitive fungus *Fusarium oxysporum* [25]. A normal growth that was obtained when moving on an AcAFP-free medium indicated that, when applied at the mentioned concentrations, AcAFP exhibited an activity that was fungistatic in nature (compare conditions a and b to the control c, Fig. 4A). Nevertheless, at concentrations higher than 70 μg mL^{-1}, AcAFP was able to act fungicidally (see conditions d in Fig. 4A). The treatment of hyphae for 24 h with the indicated fungicidal dose was observed to completely inhibit fungal growth, and no further mycelial growth was detected after 72 h incubation both in fresh AcAFP-free YPG media and on PDA plates (Fig. 4A, d).

3.5. Morphological changes induced by AcAFP

When cultivated in a liquid AcAFP-rich medium and when compared to the control, the hyphae of *Fusarium oxysorum* were observed to exhibit severe morphological alterations. The tested fungus reacted in a dose-dependent way, showing that when directly added to conidia, the increase in AcAFP concentration was paralleled with a decrease in biomass production. The *F. oxysporum* hyphae, which had germinated in the absence of AcAFP and were subsequently treated for 24 h with 50 μg mL^{-1} of AcAFP, showed an abnormal cellular morphology. A confocal analysis that was performed brought about a remarkable finding. Interestingly, it was noted that in the absence of treatment the mycelium of the sensitive strain *Fusarium oxysporum* auto-fluoresces, in the same way that many fungal cell walls do. A 24-h treatment with 50 μg mL^{-1} of AcAFP, brought about two noticeable changes namely that the part of the mycelium swelled and that each cell was detached. This lead to a "protoplasts-like" appearance and the fluorescence in this case was located in the extracellular fluid, which indicated the release of the fluorescent molecules from the cells that became colorless (Fig. 4B).

As possible explanation for the lost of the cell wall rigidity would be the deterioration and hydrolysis of cell wall polysaccharides induced by AcAFP. To verify this hypothesis, firstly total reducing sugars were determined as glucose equivalents. An increase in the liberated reducing sugars estimated to 2.287 mg mL^{-1} was observed after treatment with 50 μg mL^{-1} of AcAFP for 24 h, in comparison to the control which was estimated to 1.07 mg mL^{-1}. In a second step, hydrolysis products of cell wall polymers induced by AcAFP on sensitive fungus were visualized by Thin Layer Chromatography (Fig. 5). TLC analysis showed clearly the release of soluble mono and polysaccharide in the extracellular fluid. The nature of these compounds is not yet determined.

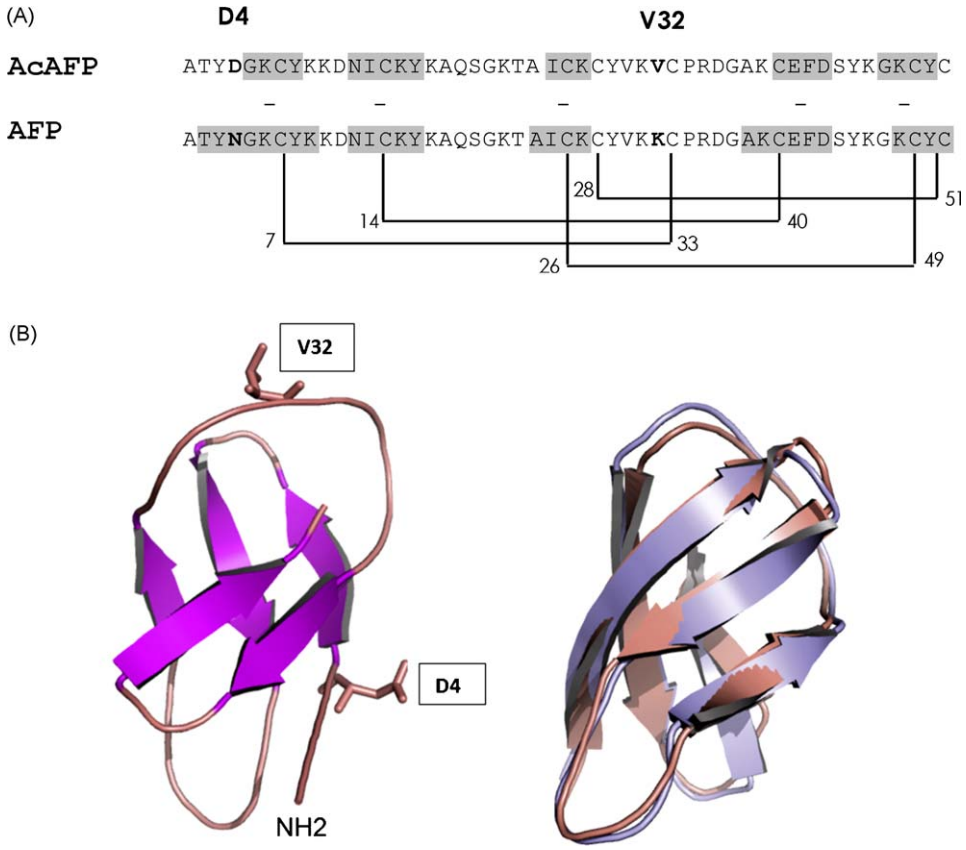

Fig. 3. (A) Amino acid sequence alignment of AcAFP and AFP. The β strands are indicated by grey shading; disulfide bridges of AFP are indicated as lines according to Campos-Olivas et al. [4]. (B) The protein structure homology-modelling of the Ac.AFP. β-sheet structure is shown as arrows; boxed residues (V32, D4) indicate the modified aa; compared to the mature protein of AFP, N marks the amino-terminal end of AcAFP (left); superposition of AcAFP (in purple) and AFP structures from *A. clavatus* and *A. giganteus*, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The presence of the fluorescence in the extracellular fluid was observed only after the treatment with AcAFP. In this context, major cell wall compounds as Chitin: poly-N-acetylglucosamine from Crab shells (Sigma C-3387) and β-glucan from barley (Sigma G-6513) was observed by confocal microscopy at the same conditions described for samples showed in Fig. 4B. Based on this observation, cell wall fluorescence seems to be provided by chitin (Fig. 4B).

4. Discussion

The present paper is a continuation of a previous study that reported on the isolation and purification of a novel thermostable antifungal peptide with a molecular mass of 5777 Da (named AcAFP) from the imperfect filamentous fungi *A. clavatus* [25]. Considering its promising properties, the current study aimed to further explore AcAFP in terms of the molecular characterization of its encoding gene, its three-dimensional (3D) structure and the impact of the amino acid sequence on its function and activity.

Molecular analysis revealed that AcAFP was expressed by *A. clavatus* as a 94 amino acid long pre-pro-protein that contained a predicted signal secretion sequence of 21 amino acids and a pro-sequence of 22 aa residues located N-terminally to the mature peptide (51 aa). In addition to the classical Ala rich pre-sequence, a putative consensus sequence, "XZAR" (X = L; Z = D), was also present in the AcAFP sequence and other related peptides.

In fact, the LDAR motif resembles the kex2p cleavage sites of various Killer toxins from *S. cerevisiae*, *Ustilago madis* and *Kluyveromyces lactis*, and might represent a similar protease cleavage site [18,27]. It has also been reported that for the PAF peptide, the function of the pro-sequence is to prevent any premature activity of the antifungal peptide until it has passed the plasma membrane [19]. As far as the AcAFP gene under investigation is concerned, it was interrupted by two introns that were, compared to mammalian introns, small in size which is a typical feature of fungal genes [6,11]. The 5′- and 3′-ends of both introns were very similar and in agreement with the consensus splice sequences of fungal introns and the internal putative lariat formation element. The number and the positions of the two AcAFP introns were identical to those found in the AFP [34,35] and PAF [18] genes. The introns insertion positions were, in fact, well conserved and always occurring between the amino acids G and K for intron 1, and the V/K and C for intron 2 (Fig. 1b), which is, again, a typical feature of the AFP [34,35] and PAF [17] genes. It should be noted in this context that, for AnAFP, the gene has not yet been isolated and that, so far, only the amino acid sequence of the mature peptide has been identified [13]. Nevertheless, the *A. clavatus* NRRL 1 genome has been completely sequenced under the accession number of XP 001267787. Moreover, although a putative antifungal peptide sequence has been identified, its functional characterization and gene organization have not been provided. This sequence, termed as AFPAc by the research team mentioned above, actually differed from the AcAFP under investigation by a single change at the third residue, namely that the Phe in AcAFP is a Val in AFPAc.

Moreover, the mature form of AcAFP shares common properties with the AFP of *A. giganteus*, namely the presence of 8 Cys residues at the same positions. The PAF/NAF and AnAFP antifungal peptides, however, contain only 6 Cys residues. Furthermore, the predicted tertiary structure of AcAFP seems to be similar to that of AFP, i.e. it

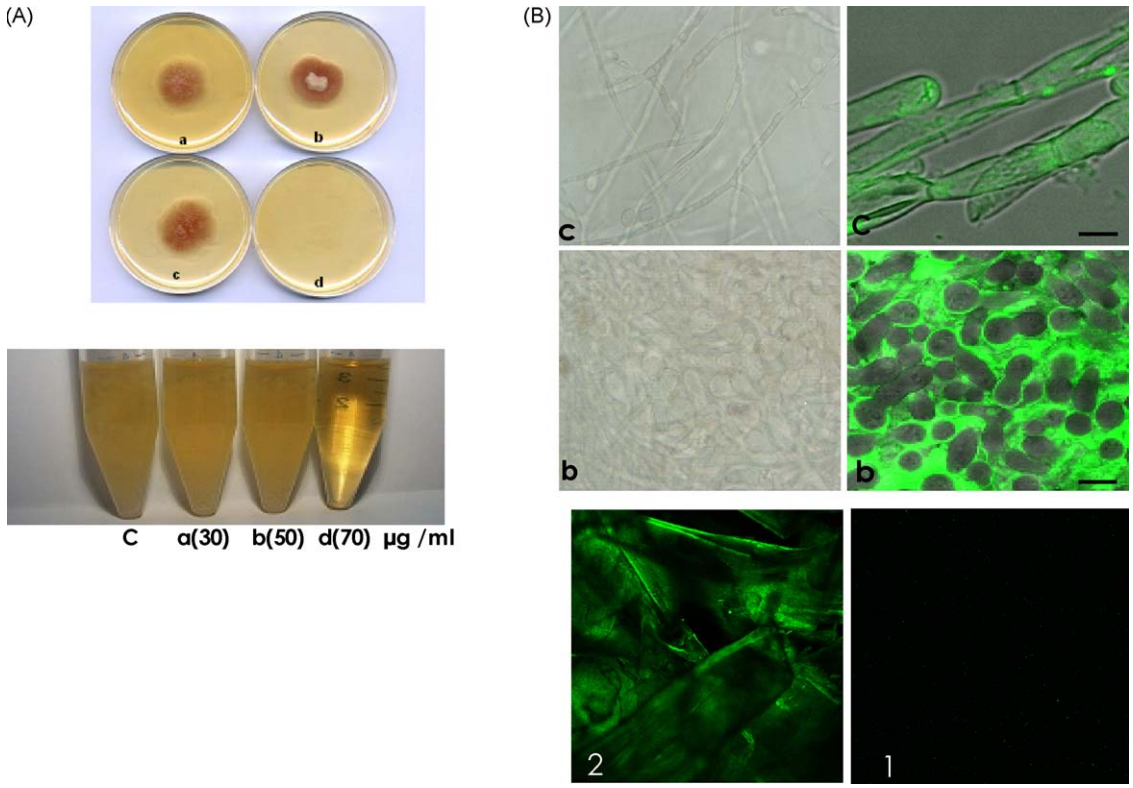

Fig. 4. (A) Fungicidal effect of AcAFP with C: control, treated with ($\mu g\ mL^{-1}$) a: 30; b: 50; d: 70; c: control. (B) Morphological changes induced by AcAFP on the sensitive strain *Fusarium oxysporum*. c. Control, b. Treatment with 50 $\mu g\ mL^{-1}$ of AcAFP observed with transmission and confocal microscopy without any staining. Confocal laser scanning microscopical analysis of 1: Chitin (Sigma C-3387); 2: β-glucan (Sigma G-6513).

has five anti-parallel β strands, defining a small and compact β barrel which is stabilized by 4 internal disulfide bridges [12]. This folding pattern could explain their high stability against protease degradation, high temperature and broad pH range. AcAFP takes an amphipathic structure that can be attributed to the presence of a hydrophobic (Y29, V30, Y45, Y50) and a cationic (K9, K10) domain. In the latter, the substitution of K32 in AFP by V32 in AcAFP could reduce the AcAFP cationic charge and might explain the differences between the two antifungal peptides described [26]. In fact, and as has been pointed out earlier, both antifungal peptides exhibit high thermostability. The findings of the present study demonstrate that AcAFP has the advantage of being more thermostable. In fact, while AcAFP retained a significant antifungal activity after incubation for 3 h at 100 °C, AFP lost approximately 90% of its antifungal activity after incubation for 15 min at 100 °C [30]. Actually, among fungal-produced antifungal peptides, AcAFP seems to exhibit a fairly good stability. AcAFP and AFP, on the other hand, were not equally effective against the different fungi tested [25]. The reduction of the AcAFP cationic charge compared to AFP peptide might explain this differences in species-specificity of antifungal peptides, since it was reported that AFP promoted *in vitro* aggregation of large unilamellar vesicles of the acidic phospholipid dimyristoylphosphatidylserine, which suggests an interaction between the basic AFP and the negatively charged phospholipid [14,18].

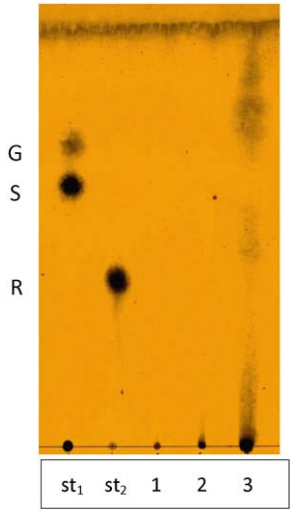

Fig. 5. Thin Layer Chromatography of hydrolytic products, from cell wall polymers, induced by AcAFP. Lane st_1: glucose (G as a monosaccharide) sucrose (S as a disaccharide); st_2: Raffinose (as a trisaccharide); 1: purified AcAFP; 2: control: culture supernatant of *Fusarium oxysporum*; 3: culture supernatant of *Fusarium oxysporum* after AcAFP treatment for 24 h.

In fact, the majority of the antimicrobial peptides presented in the literature have been reported to proceed by interacting with fungal plasma membranes and their physiological activities have often been described as heavily related to membranolytic properties [20,29]. Moreover, the plant defensin family is reported to encompass small basic cys-rich peptides that are quite conserved in terms of 3D structure but that display diverse biological activities for they are not equally effective against different microorganisms [31,32]. It was shown that their specific interaction requires a putative receptor in the outer layers of sensitive fungi [32] and that the binding of the fungal peptides to this molecule is cation-sensitive.

Overall, the findings from the preliminary study of AcAFP's mode of action against the phytopathogenic fungus *Fusarium oxysporum* indicate that AcAFP acted in a fungicidal fashion at high concentration (more than 70 $\mu g\ mL^{-1}$) by inhibiting the hyphal

growth and spore germination (Data not shown). The application of fungistatic concentrations (less than 50 $\mu g\ mL^{-1}$) induced a number of morphological changes, mainly the conferring of a protoplast-like appearance to the cells, the swelling of the mycelium and the detachment of each cell, were inevitably caused by the deterioration of the rigidity of cell wall. The determination of a total released reducing sugars and TLC of hydrolytic products from sensitive fungous cell wall obtained after AcAFP treatment, showed clearly the release of soluble oligo and polysaccharide in the extracellular fluid. It was previously shown that the predominant effect of the antifungal peptide AFP1 from *Streptomyces tendae* Tü901 on *P. variotii* interfered with polarized growth at hyphal tips, leading to abnormal branching and swollen hyphae with weakened walls that did not resist internal turgor pressure upon mechanical stress [1]. The AcAFP peptide can, therefore, be safely hypothesized to induce the destabilization of sensitive fungi's cell wall as a first step target to combat antagonist fungi.

A detailed study with AFP by indirect immunofluorescence showed that while in sensitive fungi the AFP was accumulated in the outer cell layers, including the cell wall and plasma membrane, however it was internalized by the resistant ones [34]. It is, therefore, that the chitin binding, cationic, and hydrophobic domains participate in the interaction of AcAFP with the cell wall of target organisms. This could be accomplished by the presence of more defined elements required for specific-interaction, such as the putative receptors in the outer layer of sensitive fungi, or by the inhibition of other events during the cellular growth and division.

Considering the findings presented in the present study, AcAFP can be considered as a promising "new born" in the restraint family of ascomycete antifungal peptides that might productively contribute to the decryption of their mode of action. This antifungal peptide could open new opportunities for the alleviation of currently troublesome concerns, particularly those associated with the spread of pathogenic microorganisms. For this reason, further studies are currently under way in our laboratories to further explore its mode of action, to improve its production under different culture conditions and to make this process suitable for potential future industrial applications.

Acknowledgments

The authors thank Mohamed Jaoua for nucleotide sequencing, Salma Zouari for confocal microscope manipulations and Anouar Smaoui for manuscript English reviewing. This work was supported by the "Ministère de l'Enseignement Supérieur, de la Recherche Scientifique et de la Technologie".

References

[1] Bormann C, Baier D, Hörr I, Raps C, Berger J, Jung G, et al. Characterization of a novel, antifungal, chitin-binding protein from *Streptomyces tendae* Tü901 that interferes with growth polarity. J Bacteriol 1999;4:7421–9.
[2] Brinboim HC, Doly JA. Rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res 1979;7:1513–23.
[3] Broekaert WF, Cammue BPA, De Bolle MFC, Thevissen K, De Samblanx GW, Osborn RW. Antimicrobial peptides from plants. Crit Rev Plant Sci 1997;16: 297–323.
[4] Campos-Olivaz R, Brux M, Santoro J, Lacadena J, Martinez del Pozo A, Gavilanes JG, et al. NMR solution structure of the antifungal protein from *Aspergillus giganteus*: evidence for cysteine pairing isomerisme. Biochemistry 1995;34: 3009–21.
[5] De Lano WL. The PyMOL molecular graphics system. De Lano Scientific LLC, San Carlos, CA, 2002, http://www.pymol.org.
[6] Deutsh, Long M. Intron-exon structures of eukaryotic model organisms. Nucleic Acids Res 1999;27:3219–28.
[7] Geison R. *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 2000;62:95–101.
[8] Hagen S, Marx F, Ram AF, Meyer V. The antifungal protein AFP from *Aspergillus giganteus* inhibits chitin synthesis in sensitive fungi. Appl Environ Microb 2007;37:2128–32.
[9] Kaiserer L, Oberparleiter C, Weiler-Gôler R, Burgstaller W, Leiter E, Marx F. Characterization of the *Penicillium Chrysogenum* antifungal protein PAF. Arch Microbiol 2003;180:204–10.
[10] Kastin A. Handbook of biologically active peptide, 1st ed., Academic Press; 2006.
[11] Kupfer DM, Drabenstot SD, Buchanan KL, Lai H, Zhu H, Dyer DW, et al. Introns and splicing elements of five diverse fungi. Eukaryot Cell 2004;3:1088–100.
[12] Lacadena J, Martinez del Pozo A, Gasset M, Patino B, Campos-Olivaz R, Vazquez C, et al. Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus*. Arch Biochem Biophys 1995;324:273–81.
[13] Lee DG, Shin SY, Maeng CY, Jin ZZ, Kim KL, Ham KS. Isolation and characterization of a novel antifungal peptide from *Aspergillus niger*. Biochem Biophys Res Commun 1999;263:646–51.
[14] Martinez Del Pozo A, Lacadene J, Mancheno JM, Olmo N, Onaderra M, Gavilanes JG. The antifungal protein AFP OB *Aspergillus giganteus* in an OB fold-containing protein that produces condensation of DNA. J Biol Chem 2002;277:46179–83.
[15] Martinez-Ruiz A, Martinez del Pozo A, Lacadena J, Mancheno JM, Onaderra M, Gavilanes G. Characterization of a natural larger form of the antifungal protein (AFP) from *Aspergillus giganteus*. Biochim Biophys Acta 1997;1340:81–7.
[16] Marx F, Binder U, Leiter E, Pocsi I. 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 2008;65:445–54.
[17] Marx F, Haas H, Reindl M, Stoffler G, Lottspeich F, Redl B. Cloning, structural organization and regulation of expression of the *Penicillium chrysogenum paf* gene encoding an abundantly secreted protein of *Aspergillus giganteus*. Gene 1995;167:17–71.
[18] Marx F. Small, basic antifungal proteins secreted from filamentous ascomycetes: comparative study regarding expression, structure, function and potential application. Appl Microbiol Biotechnol 2004;65:33–42.
[19] Marx F, Salvenmoser W, Kaiserer L, Grassessle S, Weiler-Gotz, Zadra I, et al. Proper folding of the antifungal protein PAF is required for optimal activity. Res Microbiol 2005;156:35–46.
[20] 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.
[21] Miller GL. Use of dinitosalicylic acid reagent for determination of reducing sugars. Anal Chem 1959;31:426–8.
[22] Nakaya K, Omata K, Okahashi I, Nakamura Y, Kolekenbrok H, Ulbrich N. Amino acid sequence and disulfide bridges of an antifungal protein isolated from *Aspergillus giganteus*. Eur J Biochem 1990;193:31–8.
[23] Olsen BH, Goerner GL. Alpha sarcin, a new antitumor agent. I. Isolation, purification, chemical composition and the identity of a new amino acid. Appl Microbiol 1965;13:314–21.
[24] Sambrook J, Fritsh EF, Maniatis T. Molecular cloning A laboratory manual. Spring Harbor, NY (Ed). 1989.
[25] Skouri Gragouri H, Gargouri A. First isolation of a novel thermostable antifungal peptide secreted by *Aspergillus clavatus*. Peptides 2008;29:1862–70.
[26] Ng TB. Peptides and proteins from fungi. Peptides 2004;25:1055–73.
[27] Tao J, Ginsberg I, Banerjee N, Held W, Koltin Y, Bruenn JA. *Ustalgo madis* KP6 Killer toxin: structure, expression in *Saccharomyces cerevisiae* and relationship to other cellular toxins. Mol Cell Biol 1990;10:1373–81.
[28] Theis T, Stahl A. Antifungal proteins: targets, mechanisms and prospective applications. Cell Mol Life Sci 2004;61:437–55.
[29] Theis T, Wedde M, Meyer M, Stahl U. The antifungal protein from *Aspergillus giganteus* causes membrane permeabilization. Antimicrob Agents Chemother 2003;47:588–93.
[30] Theis T, Marx F, Salvenmoser W, Stahl U, Meyer V. New insights into the target site and mode of action of the antifungal protein of *Aspergillus giganteus*. Res Microbiol 2005;156:47–56.
[31] Thevissen K, Ferket EJA, François IEJA, Cammue BP. Interactions of antifungal plant defensins with fungal membrane components. Peptides 2003;24: 1705–12.
[32] Thevissen K, Warnecke DC, François IE, Leiplt M, Heinz E, Ott C, et al. Defensins from insects and plants interact with fungal glucosylcermides. J Biol Chem 1998;36:533–6.
[33] Trigui Lahiani H, Gargouri A. Cloning, genomic organization and mRNA expression of a pectin lyase gene from a mutant strain of *Penicillium Occitanis*. Gene 2007;288:54–60.
[34] Wenedt S, Ulbrich N, Stahl U. Molecular cloning sequence analysis and expression of the gene encoding an antifungal-protein from *Aspergillus giganteus*. Curr Genet 1994;25:519–23.
[35] Wenedt S, Ulbrich N, Stahl U. Cloning and nucleotide sequence of a cDNA encoding the antifungal-protein of *Aspergillus giganteus* and preliminary characterization of the native gene. Nucleic Acids Res 1990;18(13):3987.