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Structural and Gene Characterization of a New Antifungal Peptide Obtained from *Penicillium crustosum* FP11 Strain

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Authors' contributions

This work was carried out in collaboration among all authors. Author JBM conducting of the research and investigation process, specifically performing the experiments and data collection. Authors JBM, MKK and JLCS participated in the coordination and designed the study, analyzed the data, wrote and edited the manuscript. Author LFR analyzed the amino acid sequence data and modeled the three-dimensional structure of the peptide. Authors RCGS and AM contributed in the preparation of the draft manuscript and ideas applied in the manuscript methodologies. All authors read and approved the final manuscript.

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ABSTRACT

A novel antifungal peptide, PcAFP (6.48 kDa, pI 8.83), was obtained from the culture supernatant of the fungus *Penicillium crustosum*. The gene encoding the PcAFP peptide was isolated based on its homologue in *Penicillium chrysogenum*, PgAFP. PcAFP is a small, cystine-rich peptide, and the mature peptide consists of 58 amino acid residues. The immature *P. crustosum* antifungal protein (AFP) showed 95.65% identity to the antifungal protein of *P. chrysogenum*, while the mature peptide showed 98.28% identity with PgAFP. Molecular modeling of the tertiary structure of the mature peptide revealed details of the conserved structure of the AFPs, such as the β-barrel motif stabilized by three disulfide bonds and the λ-core motif. Analysis of the extract by 16% tricine SDS-PAGE showed a 6.9 kDa peptide, which was close to the predicted molecular mass of the mature

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peptide of 6.48 kDa. Assays of antimicrobial activity, performed by broth microdilution using the crude extract obtained from the culture medium, showed activity against *Candida albicans*. These results demonstrate the conservation of the PcAPF gene and the high level of identity with the PgAFP antifungal protein of *P. chrysogenum*. Given these structural and biochemical characteristics, PcAFP could be a potential candidate for future investigations that may aid in the development of new antifungal compounds.

Keywords: AFP gene; Penicillium crustosum; antifungal peptide; polymerase chain reaction; antimicrobial activity.

1. INTRODUCTION

In recent years, the emergence of resistant microorganisms has caused great concern, and the number of cases of infection among immunocompromised patients has increased. The emergence of resistance in multidrug-resistant bacterial strains to the last generation of antibiotics is alarming, and this has occurred in parallel to the appearance of resistant filamentous fungi [1]. The increasing incidence of infections caused by filamentous fungi in immunocompromised patients has created new challenges in the search for bioactive molecules that are effective in combating resistant fungi [2].

Fungi produce a range of bioactive biomolecules, among which the defensin-like proteins stand out. These proteins are secreted by many taxonomically different species of fungi [3] and they exhibit potent antimicrobial activity [4].

These peptides differ in terms of their molecular size, potency and activity spectrum [4,5]. Antimicrobial peptides (AMPs) from *Ascomycetes* are bioactive molecules with molecular weights ranging from 1.3 to 30 kDa [5,6]. They have a basic character due to their high content of arginine and lysine residues [3], and other common features include the presence of six to eight cysteine residues and several disulfide bonds [4]. The defensin-like antifungal proteins that typically exist in filamentous fungi can be distinguished into two groups according to their conserved amino acid sequences: bubble proteins [6] and antifungal proteins [7].

Antifungal proteins (AFP) contain six to eight cysteines that form intramolecular disulfide bonds and stabilize the antiparallel β-sheet conformation flanked by a helical segment, also called the cysteine-stabilized α or β motif (CSα or CSβ) [8], forming a β-barrel topology [6,7]. These proteins are synthesized as pre-proteins, which have a molecular weight of 5.8 to 6.6 kDa after proteolytic processing. Despite the similar mechanisms of action and structural aspects of AFP proteins, they have low similarity in their amino acid sequence [4]. AFPs inhibit the growth of various pathogenic fungi, including *Aspergillus fumigatus, A. nidulans, A. niger, Botrytis cinerea* [9] and some species of dermatophytes [10,11].

The fungus *Penicillium chrysogenum* produces the antifungal protein PgAFP, which has potent antifungal activity [12]. PgAFP is a low-molecular-weight (6.5 kDa), basic, cysteine-rich protein whose N-terminal amino acid sequence shares homology with antifungal proteins produced by other fungi [12,13].

Penicillium crustosum was first described in 1930 by Thom. Since then, it has undergone taxonomic and nomenclature variations, but today it has a solid identification as *P. crustosum* [14]. This species produces a range of well-characterized secondary metabolites; however, no antifungal proteins have been described for *P. crustosum* so far.

In our work, we used the genome of *P. chrysogenum*, specifically the sequence of the PgAFP gene GQ911150.1, as a reference to use molecular biology techniques to identify a homologous protein in a fungal isolate of *P. crustosum* from the Atlantic forest region (FP11). This species has previously been reported to display antimicrobial activity.

2. MATERIALS AND METHODS

2.1 Microbial Strain

The *Penicillium crustosum* FP11 strain was isolated from soil from the Bela Vista Biological Refuge (Foz do Iguaçu, Paraná, Brazil) and previously identified (accession number KM065878) by Silva et al. [15]. The bacterial and yeast strains used in the antimicrobial activity tests were provided by the Microbiology Department of the Central Laboratory of the University Hospital of Western Paraná.

2.2 Growth Conditions for *Penicillium crustosum*

The filamentous fungus *P. crustosum* was cultured in the antifungal protein induction medium (AFPIM; 1.5% starch, 1% beef extract, 2% peptone, 0.5% NaCl, 1% ethanol) [4,6], subjected to a shaker rotation of 180 rpm at 28°C for 7 days, according to Marx [16] and Kovács et al. [4]. For control conditions, we used AFPIM medium that had not been inoculated with the tested fungus.

For comparison, malt extract broth (EMB) medium (2% malt extract, 2% glucose and 0.1% peptone) [12,17] was also tested under the same conditions described above.

2.3 Extraction of DNA from the Mycelium of *Penicillium crustosum*

For the extraction of genomic DNA from the mycelium, EMB culture medium was used, according to Acosta et al. [17]. The fungus was inoculated and maintained overnight at 28°C under agitation at 180 rpm. We followed the extraction protocol described by Bellemare et al. [18] with modifications. The genomic DNA was suspended in MilliQ water and quantified in a spectrophotometer at 260 nm [19].

2.4 Design and Synthesis of Primers for Polymerase Chain Reaction (PCR)

We used primers designed for the genome of *P. chrysogenum*, which expresses the antifungal peptide PgAFP [12]. Initially, the following PgAFP primers were tested: forward 5'-ATGCAGATCACCAGCATTGCC-3', reverse 5'-TCAAACTGGGGTCTGGCAGTC-3'.

Primers PR2 5'-CGCCAGTTACGAGACACACT-3', PR4 5'-CCGAACGAGTGTGGTGATAA-3' and PF3 5'-CCGTGGGGCAGATCAGTAG-3' were subsequently designed, taking into account the up- and downstream regions of the *P. chrysogenum* PgAFP gene to ensure that the PCR products covered the entire region of the *P. crustosum* PcAFP gene.

2.5 PCR Reaction and Purification of PCR Products

For PCR, the following reagents and conditions were used: 2.5 µL of 10X buffer, 1 µL of each primer (10 µmol), 1 µL of 100 ng/µL DNA, 1 µL of 50 mM $MgCl_2$, 0.5 µL of Taq polymerase, and 17 µL of 0.1% DEPC water (totaling 25 µL of sample). The thermocycler was programmed for one cycle of 95°C for 2 min, 35 cycles of 94°C for 1 min, 60°C for 30 s and 72°C for 1 min, followed by 72°C for the final extension and 4°C for the hold. The primers used for the PCR reactions were the forward and reverse PgAFP primers for amplification of the complete PcAFP gene, and primers PR2, PR3 and PR4 were optimized for the amplification of products to cover the upstream and downstream regions of the PcAFP gene. The results were visualized on a 1% agarose gel with 5 µL of each sample and the electrophoresis was performed with 1X TAE buffer at 100 V. Amplicons were visualized using a DigiDoc BioImaging Systems® UVP transilluminator.

The purification of amplicons was carried out with approximately 50 µL of PCR product loaded into a 2% agarose gel and electrophoresed in 1X TAE buffer at 100 V. PureLink-Quick Gel Extraction Kit (Invitrogen®) was used for purification, performed according to the manufacturer's protocol. PCR products were sent to the HELIXXA® and ACTGene® companies according to their specifications.

2.6 Analysis of Genomic DNA Sequence and PcAFP Peptide Modeling

The sequences obtained by capillary sequencing were submitted to alignment analysis with the Clustal Omega algorithm for comparison with the *P. chrysogenum* PgAFP gene for assembly of the complete PcAFP gene sequence. The nucleotide sequence was deposited in GenBank under accession number MN171487.

The protein predicted from the amino acid sequence was obtained using the translator tool (Expasy Bioinformatics Resource Portal) and the tertiary structure was modeled using PyMol software. The molecular weight and the pI of the mature peptide were predicted using the pI/Mw tool (Expasy Bioinformatics Resource Portal), and the signal peptide was screened using SignalP version 5.0.

2.7 Production of the PcAFP Peptide from the Culture Medium

Firstly, the supernatants of the fungal mycelium grown in AFPIM broth [4] or EMB broth [12,17] were separated under appropriate temperature and stirring conditions [6]. Separation of the extracellular filtrate was performed by vacuum filtration.

The crude extracellular extract was lyophilized (Terroni® LT-1000/8) and was precipitated with 60% ammonium sulfate overnight, centrifuged at 10000 × *g* for 30 min at 4°C. The precipitate was conditioned at -20°C and the supernatant was dialyzed overnight (benzoylated dialysis tubing, average flat width of 32 mm; Sigma-Aldrich). The dialyzed sample was lyophilized and resuspended in 500 µL of deionized water. Subsequently, it was passed through a 10 kDa molecular filter column (Sartorius®, Vivaspin®).

2.8 Protein Concentration and Tricine Polyacrylamide SDS-PAGE Gel Electrophoresis

Determination of the protein concentration of the samples was performed using the Bradford methodology [20]. Protein electrophoresis was performed in tricine SDS-PAGE 16% according to Schägger [21]. About 50 µg of the samples were boiled for 5 min and electrophoresed at 120 V. After electrophoresis the gel staining was performed with Coomassie Blue G-250.

2.9 Test of Antimicrobial Activity

The bacteria and yeasts used in the antimicrobial susceptibility tests were provided by the Laboratory of Microbiology of the University Hospital of Western Paraná. They included *Staphylococcus aureus* (SA), *Pseudomonas aeruginosa* (PA), *Escherichia coli* (EC), *Klebsiella pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* β-lactamase (ESBL), carbapenem-resistant *Klebsiella pneumoniae* (KPC) and the yeast *Candida albicans* (Cand).

The methodology used for this experiment was the standardized by Clinical and Laboratory Standards Institute (CLSI) (2003) protocol [22].

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Extraction of *Penicillium crustosum* DNA and isolation of the PcAFP gene

After extraction of the fungal DNA under optimum growth conditions, the genomic DNA was visualized on 1% agarose gel to confirm the quality and purity (Fig. 1A). In Fig. 1B, we show the amplification of the PcAFP gene, represented by the 405-bp band on the gel. Fig. 1C shows amplification of fragments with the primers designed upstream and downstream of the PgAFP gene to cover the entire PcAFP gene.

3.1.2 Analysis of the PcAFP gene sequence and comparison to the AFP family

Alignment of the PcAFP gene with the PgAFP gene of *P. chrysogenum* (GQ911150.1) was performed to assess the homology. The 405-bp genomic sequence of *P. crustosum* was submitted to a Blastn search for evidence of homology with the *P. chrysogenum* gene. This search revealed 96.30% identity of PcAFP to the PgAFP gene of *P. chrysogenum*, with an e-value of 0.0, demonstrating a high level of identity with this gene. The AFPs are structurally conserved but exhibit variability in their primary amino acid sequence of 31.6–91.4% [4]. This result shows that the PgAFP and PcAFP genes are almost identical at the gene level.

In Fig. 2, it can be seen that the PcAFP gene is interrupted by introns with conserved splicing sites. After splicing, a 279-base mRNA is generated that will encode a protein with 92 amino acids, which is almost identical to the PgAFP gene [12]. This sequence was translated with the Expasy translate tool (https://web.expasy.org/translate/), which produced the open reading frame for the PcAFP gene. These proteins are generally pre-proteins that contain a signal peptide for secretion and a pro-peptide that is cleaved after secretion [4].

The PcAFP protein has different amino acid residues in the signal sequence (1–18) and two different residues in the pro-sequence (19–34). In the mature peptide, residues 34–92 show only one distinct residue, replacing histidine with asparagine. When the pre-pro-protein sequence was analyzed with the Balstp algorithm, the complete *P. crustosum* AFP protein showed 95.65% identity with the antifungal protein of *P. chrysogenum*. However, when compared to the mature PgAFP peptide of *P. chrysogenum*, the identity was 98.28%. In Fig. 2, the three characteristic disulfide bonds of the AFP can be observed between the cysteine residues 8-36, 15-43 and 28-54.

3.1.3 Predicted tertiary structure of PcAFP of *Penicillium crustosum*

In silico prediction experiments revealed that the PcAFP peptide has similar characteristics to those of antifungal peptides, such as β-defensins belonging to the AFP group. Some of these similarities include the presence of disulfide bonds, a high isoelectric point (pI) and the β-barrel conformation [6].

Fig. 3 shows the tertiary structure of the PcAFP protein of *P. crustosum*, with the conserved structure of the disulfide bridges and the five β-sheets which determine the β-barrel structure, as previously reported [1,4,8,12,23], with the AFPs presenting this tertiary structure. Fig. 3A shows the structure of the pre-protein which, after secretion, undergoes processing into its mature form, shown in Fig. 3B. In Fig. 3C, the λ-core motif conserved in the AFPs of Eurotiomycetes is highlighted, which has demonstrated activity against human pathogenic yeast *Candida albicans* [24].

3.1.4 Biochemical characteristics of the PcAFP peptide

The crude extract obtained by incubating the fungus *P. crustosum* in the AFPIM medium for 7 days was freeze-dried, followed by precipitation with 60% ammonium sulphate. The supernatant was dialyzed and lyophilized, then the sample

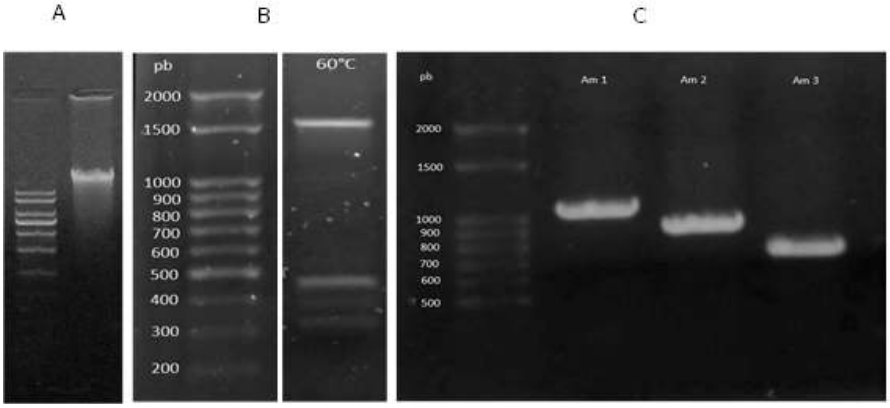

Fig. 1. Genomic DNA extracted from *Penicillium crustosum* FP11 and PCR reactions. (A) Genomic DNA extracted from *Penicillium crustosum* FP11. (B) Fragment of the amplified 405-bp PcAFP gene produced with the forward and reverse primers of the PgAFP gene. (C) Amplified PCR samples with primers Am1 (PR1/PF3), Am2 (PR4/PF3) and Am3 (Prev/PF3). The 900-bp Am2 sample was sequenced to complete the 5' and 3' regions of the PcAFP gene. Standard λ/HindIII in A; Ludwig ladder 100 bp in B and C

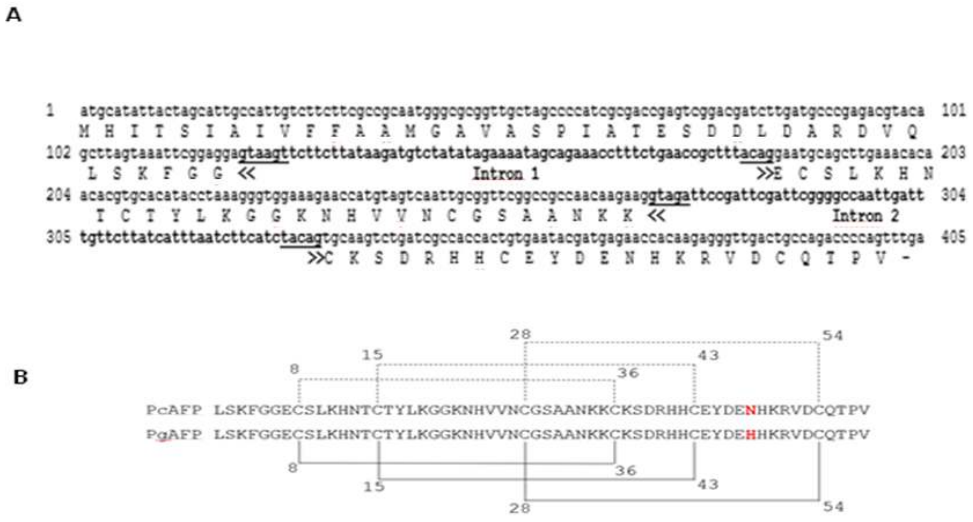

Fig. 2. Complete sequence of the *Penicillium crustosum* PcAFP gene. A- Open reading frame has been translated into amino acids. Intron consensus sequences are underlined. B- Mature PgAFP protein from *Penicillium chrysogenum* and PcAFP from *P. crustosum*, showing the disulfide bridges formed between the cysteine residues. The only different amino acid residue is highlighted in red

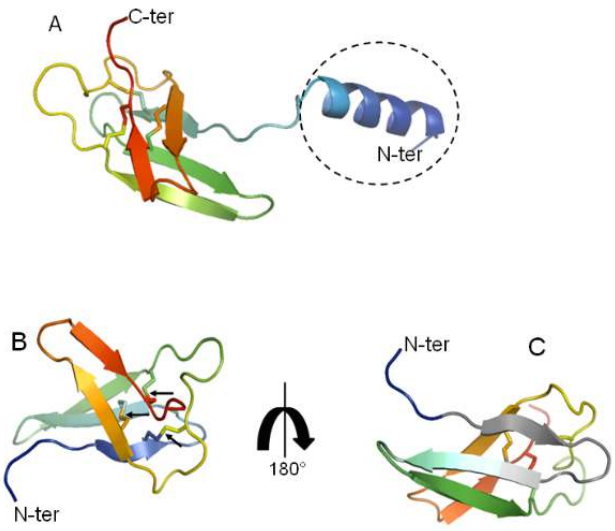

Fig. 3. Three-dimensional structure generated by molecular modeling using PyMol software. (A) Representation of the type of the AFP model. The N-terminal (N-ter) is shown in blue, and the C-terminal (C-ter) is shown in red. (B) Representation of the tape of the PAF model. The N-ter is shown in blue, and C-ter is shown in red. There are three disulfide bonds, which are indicated by arrows. (C) Orientation highlighting the γ-core motif (gray)

was frozen and loaded into the tricine SDS-PAGE 16% for electrophoresis (Fig. 4, sample 3). A second test with the fungus incubated in EMB medium was also run on the gel (sample 4). As a negative control, only AFPIM culture medium without inoculum of the fungus was used.

A prominent band less than 10 kDa was visualized in the gel (lane 3) obtained from sample incubated in AFPIM medium, indicated by an arrow in the Fig. 4, which shows the presence of the peptide in the sample. This was not observed when the EMB medium was used. The estimated molecular weight of the PcAFP peptide is approximately 6.9 kDa, as shown in Fig. 4, obtained from another electrophoresis gel with the same sample. From the predicted amino acid sequence, we obtained the theoretical pI (8.83) and molecular weight (6.48 kDa) of the mature peptide using the Expasy tool.

3.1.5 Antimicrobial activity

According to results, growth inhibition was observed for *Staphylococcus aureus* and *Candida albicans* (Table 1). The first dilution that obtained a greater difference in absorbance than the difference for the positive control was the first to show bacterial growth, thus the previous dilution is considered the minimal inhibitory dilution (MID). Thus, the MID for SA was 1/16 and for Cand it was 1/8.

Although there was no effective inhibition of the other microorganisms tested, as expected, we observed a change in growth in a gradual manner according to the dilution of the crude extract tested. This demonstrates that there was a low concentration of the antimicrobial peptide in the crude extract used for the assay. However, these results suggest that purification of the peptide will certainly increase its antimicrobial activity.

3.2 Discussion

The PcAFP gene that encodes the investigated peptide was isolated from the fungus *Penicillium crustosum* FP11 KM065878. For the initial amplification of the PcAFP gene, primers were extracted from the 2010 study by Rodríguez-Martín et al. [12]. Additional primers covering the upstream and downstream regions were designed and synthesized from the *Penicillium chrysogenum* genome, which resulted in a 405-bp complete genomic sequence of the PcAFP gene. Alignment with the *P. chrysogenum* PgAFP gene was performed using the Clustal Omega platform, and the sequences were compared to identify the regions of introns and exons and to obtain the coding sequence of the

peptide. The small size of these introns compared to mammals is a typical feature of fungal genes [25].

The coding segment obtained was 279 bp, which is similar to other antifungal proteins produced by fungi [16,26], and the open reading frame for the PcAFP gene encodes an immature protein of 92 amino acids. The first 18 amino acids correspond to a predicted signal sequence [12]. Likewise, the 16 amino acids of residues 19–34 constitute a pre-protein that would be removed prior to or as the mature PcAFP is being released [13]. Pro-sequences play an important role by ensure the protein is inactive prior to secretion. As proposed for PAF [27], the mature protein should adopt its active conformation after the pre-sequence is cleaved. The antifungal proteins produced as pre-pro-proteins of ascomycetes include PAF, AFP and AcAFP [13,26,28].

The mature PcAFP has 58 amino acid residues, and the molecular mass of PcAFP on the polyacrylamide gel was 6.9 kDa, which was higher than the 6.48 kDa predicted using the Expasy tool. This difference is probably due to anomalous peptide migration in SDS-PAGE, as many characteristics of the protein can cause atypical migration. This may occur in SDS-PAGE due to other reasons that do not involve post-translational modifications. Proteins with extreme isoelectric points may have a higher apparent molecular weight (about 25% higher) due to electrostatic repulsions, meaning that less SDS can bind, consequently decreasing the migration speed [29].

This cationic characteristic, common to fungal antifungal proteins [16], has been directly related to a strong antimicrobial activity [25]. As the mass and isoelectric point are very close to those of other small antifungal proteins produced by fungi [16,26], PcAFP appears to belong to this group of ascomycetes antifungal proteins.

The high percentage of basic amino acids in the sequence (27.6%) is consistent with the pI of 8.8 estimated by the Expasy tool. Although this prediction tool may not be accurate for small and highly basic proteins [30], the pI value suggests a net positive charge under physiological conditions, which is a common feature among fungal antifungal proteins.

Using the PyMol tool, a predicted model of this protein was obtained, which showed a structure with five antiparallel β-chains forming a compact β-barrel. The structure of PcAFP is stabilized by internal disulfide bonds formed by cysteine residues [31,32]. In fact, PcAFP contains six conserved cysteines, as previously reported for this group of antifungal proteins. Therefore, a pattern of disulfide bonds between cysteine pairs 8-36, 15-43 and 28-54 is suggested.

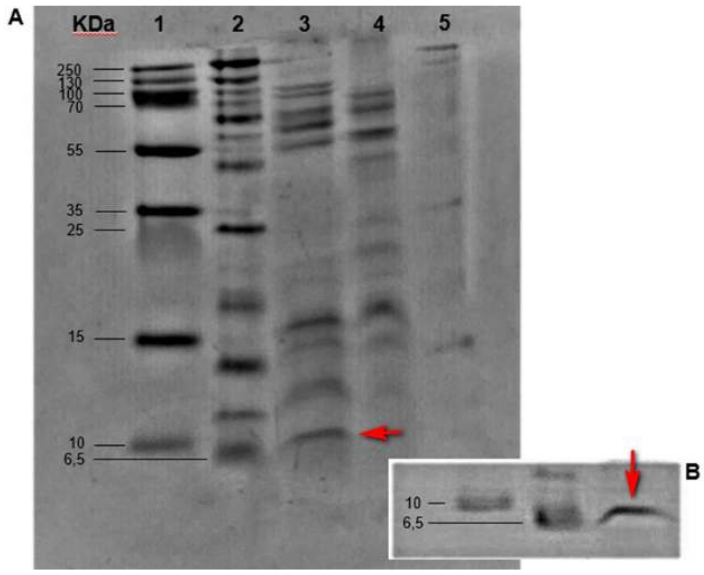

Fig. 4. Tricine SDS-PAGE 16% stained with Coomassie Blue: (1) Thermo Scientific® PageRuler® molecular weight marker, (2) Amersham Biosciences® molecular weight marker, (3) AFPIM sample, (4) EMB sample, and (5) AFPIM sample without fungus inoculum

Table 1. Difference in absorbance values before and after incubation of microbial strains with extract AFPIM in Minimum Inhibitory Dilution (MID) assay

				1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Microbial strains		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	SA	CP 0,269	EST 0,0	0,000	0,000	0,008	0,029	0,453	0,564	0,602	0,663	0,700	0,777
B	PA	CP 0,730	MHB 0,0	0,860	0,861	0,884	0,885	0,924	0,994	1,063	1,426	1,503	1,769
C	EC	CP 0,566		0,593	0,697	0,735	0,807	0,810	0,847	0,935	0,968	0,969	0,991
D	KP	CP 0,518		0,901	0,940	0,947	0,971	0,990	1,015	1,016	1,177	1,224	1,227
E	MRSA	CP 0,192		0,372	0,408	0,503	0,528	0,635	0,827	0,925	0,947	1,006	1,027
F	ESBL	CP 0,303		0,625	0,650	0,732	0,756	0,811	0,858	0,872	0,882	0,994	1,099
G	KPC	CP 0,474		0,536	0,612	0,678	0,760	0,782	0,799	0,810	0,935	0,951	0,965
H	Cand	CP 0,073		0,031	0,069	0,071	0,075	0,100	0,129	0,205	0,531	0,583	0,629

CP = Positive Control; EST = Extract Sterility Control; MHB = Mueller-Hinton broth; Staphylococcus aureus (SA); Pseudomonas aureginosa (PA); Escherichia coli (EC); Klebsiella pneumoniae (KP); Staphylococcus aureus methicillin resistant (MRSA); Escherichia coli β-lactamase Extended Spectrum (ESBL); Klebsiella pneumoniae Carbapenem Resistant (KPC); Candida albicans (Cand)

In the three-dimensional model of the PcAFP peptide, it was also possible to detect the presence of the λ-core motif present in AFP proteins, which confers activity against *Candida albicans* [24]. In our broth microdilution assay, we observed activity against *Candida albicans* at the 1/2 to 1/8 dilution, despite considerable activity at the 1/2 dilution.

Recently a natural peptide called MCh-AMP1 from *Matricaria chamomilla* L. flowers proved to be an inhibitor of *C. albicans* [33]. In addition, newly was reported that antifungal protein 2 (NFAP2) from *Neosartorya fischeri* effectively inhibits the growth of *Candida* spp [22]. Supporting these data, our work reports for the first time a new member of the antifungal peptide family in *P. crustosum* and reveal the biological effect against *Candida albicans* from an extract obtained from the supernatant of the cultivation of fungus *P. crustosum*. This biological activity reinforces the functional characterization of PcAFP as an antifungal peptide, with potential application in the development of new antifungal compounds.

4. CONCLUSION

Expression of the PcAFP peptide by *P. crustosum* was confirmed by biochemical techniques. Moreover, use of the Clustal W, PyMol and Blastn tools allowed the identification of the PcAFP gene in the *P. curstosum* genome, in addition to its structural characteristics.

Peptides of the PAF family, as described in this work, have previously been reported to be produced by other microorganisms. In *P. crustosum*, the purification and biochemical characterization of this peptide will provide a better understanding of its secretion and its mechanism of action. This peptide antifungal should be considered for possible use in the fight against fungal and bacterial resistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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