Purification and Identification of a Novel Antifungal Protein Secreted by *Penicillium citrinum* from the Southwest Indian Ocean

Chao Wen†, Wenbin Guo†, and Xinhua Chen*

Key Laboratory of Marine Biogenetic Resources, and Collaborative Innovation Center of Deep Sea Biology, Third Institute of Oceanography, State Oceanic Administration, Xiamen 361005, P. R. China

Introduction

Fungal infections have become severe problems in the medical and agricultural fields. Around 300 fungal species are reported to be origins of major diseases, whereby the filamentous fungus *Aspergillus fumigatus* and the dimorphic yeast *Cryptococcus neoformans* and *Candida albicans* are the three predominant causative agents of human diseases [15]. Approximately 25% (about 1.7 billion) of the worldwide population suffers from superficial fungal infections of the skin and nails [2]. Other fungal species, particularly filamentous fungi, are pathogenic to plant species and cause crop losses of major economic significance each year [21]. Moreover, fungal contamination led to substantial losses of postharvest food in storage [17]. Many antifungal drugs have low efficacy rates, show severe side effects, and can even be toxic to humans. This makes it necessary to develop new antifungal compounds [22].

Antimicrobial proteins that are ubiquitous in nature are suitable as fungicide candidates because of their natural origin and reduced side effects [3, 28]. Both prokaryotes and eukaryotes can produce antimicrobial proteins [18, 25, 31]. Among them, a number of different antifungal proteins have been derived from ascomycetes, such as AFP from *Aspergillus giganteus* [19], PAF from *Penicillium chrysogenum* [14], NAF from *Penicillium nalvogense* [7], and AnAFP from *Aspergillus niger* [8]. All these proteins exclusively exhibit antifungal activity against filamentous ascomycetes at micromolar concentrations, including opportunistic plant and animal pathogens, such as *Fusarium* sp., *Botrytis* sp., and *Aspergillus* spp. [15]. Although direct evidence is lacking, it is believed that the toxicity of many of these antifungal proteins is based on their interaction with molecules/receptors situated in the cell wall and/or the plasma membrane of target fungi from where signalling cascades are activated [9]. The expression of certain antifungal proteins in transgenic plants has increased resistance to disease, suggesting a promising means for plant protection [16, 20].

As interests have turned to marine microorganisms,
marine fungi have proved to be rich and promising sources of novel bioactive natural products [5]. These fungi were obtained from every possible marine habitat, including sediments, the water column, algae, and marine invertebrates [6]. Most of these microorganisms grow in a unique and extreme habitat and therefore have the capability to produce unique and unusual secondary metabolites. It is believed that the metabolites possibly act as a chemical defense adaptation of fungi competing for substrates. The production of these unique secondary metabolites by marine fungi is most likely due to their adaptation to a very distinct set of environmental pressures [1].

In this study, a novel antifungal protein (PcPAF) from the fungal strain P. citrinum W1, which was isolated from a Southwest Indian Ocean sediment sample, was purified, identified, and characterized. The antifungal activity of the antifungal protein PcPAF was evaluated against several pathogenic fungi, and its physicochemical properties were also studied.

Materials and Methods

Tested Strains and Media

Antifungal activity was assayed against different phytopathogenic fungal species, including Paecilomyces variotii (CGMCC 3.776, China General Microbiological Culture Collection Center), Colletotrichum gloeosporioides (ACCC 31200), Agricultural Cultural Collection of China), Fusarium oxysporum (ACCC 31352), Trichoderma viride (ACCC 30902), and Alternaria longipes (ACCC 30002). All fungi were purchased from the Agricultural Cultural Collection of China, except for P. variotii, which was obtained from CGMCC, Institute of Microbiology, Chinese Academy of Sciences. All fungi were grown on potato dextrose agar (PDA) plates at 28°C.

Isolation and Identification of an Antifungal Strain

The sample used for strain isolation was collected from a sediment sample from the Southwest Indian Ocean (S 38.1329°, E 48.5975°). Based on the gradient dilution method, the sample was diluted with sterilized seawater to build a serial concentration gradient. Approximately 50 µl of the diluted sample was spread on plates containing different types of medium such as modified YTM (yeast extract 0.5%, tryptone 0.3%, Mannitol 2.5%, glucose 1%) [5], TSB (Trypsin 1.5%, Peptone 0.5%, NaCl 0.5%), and GTY (Glu 1%, Tryptone 0.2%, Yeast extract 0.1%). Plates were incubated at 28°C for growth. The strains were selected based on their morphological feature and inoculated into corresponding liquid media for further growth to evaluate their antifungal potential.

To further identify strain P. citrinum W1 in terms of the ribosomal internal transcribed spacer (ITS) DNA sequence, its ITS gene was amplified using the primers ITS5 (5'-GGAAGTAAA AGTCGTAACAAGG-3') and ITS4 (5'-TCCCTCGCTTATGATAT GC-3'). The ITS gene was amplified as described previously [1]. The amplified ITS products were sequenced at Sangon Biotech (Shanghai, China). The sequences were analyzed for similarity to other known sequences found in the GenBank database using BLAST.

Purification of Antifungal Protein

For large-scale culture, strain P. citrinum W1 was primarily inoculated on PDA medium. Spores were precultured in 200 ml of modified YTM liquid medium (yeast extract 0.5%, tryptone 0.3%, mannitol 2.5%, glucose 1%) in 1 L Erlenmeyer flasks at 28°C in a shaker for 1 day. Seed cultures were transferred to 4.5 L of modified YTM liquid medium in a BIOSTAT B plus fermenter (B. Braun, Germany) and cultured at 28°C with agitation at 200 rpm for 3 days. Supernatant was collected after vacuum filtration. Solid ammonium sulfate was added to the supernatant to 100% relative saturation. After storing at 4°C overnight, the precipitate was collected by centrifugation (12,000 × g for 30 min). Precipitates were redissolved in distilled water, and dialyzed extensively against distilled water for 2 days to remove the ammonium sulfate. The solution was centrifuged at 10,000 × g for 20 min at 4°C to remove the insoluble components, before freeze-drying to yield crude proteins. The crude proteins were dissolved in a small volume of distilled water to prepare a crude protein solution.

Ion-exchange chromatography was carried out to isolate active compounds from the crude protein solution. The antifungal activity of crude proteins and fractions separated by ion-exchange chromatography was tested against Trichoderma viride. Ion-exchange chromatography was performed with an AKTA FPLC system (GE Healthcare, USA). The crude protein solution was loaded onto a DEAE Sepharose Fast Flow column (GE Healthcare), which was previously equilibrated with starting buffer A (pH 8.1, 20 mM Tris–HCl) for the primary purification step. The column was first eluted with starting buffer A to yield unabsorbed proteins, the fraction A, and then with elution buffer B (pH 8.1, 20 mM Tris–HCl, 1 M NaCl) to desorb a large amount of absorbed proteins. The bioactive fraction A was concentrated by ultrafiltration in a Vivaspin 15R (molecular weight cutoff 5,000; Sartorius, Germany) concentrator. Fraction A was then applied to a CM Sepharose Fast Flow column (GE Healthcare) that had been equilibrated with starting buffer A. After elution of unabsorbed proteins with the starting buffer A, the column was eluted with a linear gradient of 0–1 mol/l NaCl in the same buffer to desorb the absorbed proteins. The unabsorbed fraction B (washed with starting buffer A) and absorbed proteins (eluted with a 0–100% linear gradient of elution buffer B) were pooled and the antifungal activity was tested.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The purity of the active component (fraction C) was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% gel concentration. The concentration of fraction C was 1.22 µg/µl, and 10 µl of fraction C was loaded for SDS-PAGE. After electrophoresis under reducing conditions, the
The antifungal protein was determined by comparing its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences.

N-Terminal Amino Acid Sequence
To analyze the N-terminal amino acid sequences of the active protein PcPAF, the purified protein was subjected to SDS-PAGE under reducing conditions and then electroblotted onto a polyvinyl difluoride (PVDF) membrane. Proteins, transfer, was performed during 1 h at room temperature. After the membrane was stained with 0.1% Coomassie brilliant blue R-250, the protein bands on the membrane were cut off and rinsed with 50% and 100% methanol (three times each) to eliminate the staining reagent. The NH$_2$-terminal end of the antifungal protein was sequenced by automated Edman’s degradation at Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and the resulting N-terminal amino acid sequences were compared with the protein sequences in the NCBI databases and Antimicrobial Peptide Database (http://aps.unmc.edu/AP/).

Assay of Antifungal Activity
The assay for antifungal activity toward the phytopathogenic fungal species was carried out in 90 x 15 mm petri plates containing 10 ml of PDA medium. One 0.6 cm diameter piece of tested phytopathogenic fungal strains’ cylinder agar with mycelial growth was placed on the center of a PDA plate. After the mycelial colony had developed, sterile blank paper discs of 0.65 cm diameter were placed at a distance of 0.8 cm away from the rim of the growing mycelial colony. Approximately 40 µl of the antifungal protein (1.22 µg/µl) was added to each paper disc. Forty microliters of starting buffer A, which dissolved the antifungal protein, was used as a blank control. The plates were incubated at 28°C until mycelial growth enveloped discs containing the control disc, or formed crescents of inhibition around discs containing samples with antifungal activity.

MIC Determination of PcPAF
The minimum inhibitory concentration (MIC) of PcPAF against different pathogenic fungi was determined by the paper disc dilution method [3, 28]. Two-fold serial dilutions of the active protein solution ranging from 9.73 to 0.019 µg/µl were prepared, and 40 µl of each diluted solution was added onto paper discs placed 0.8 cm from the growing mold on a PDA plate. The plates were left at 28°C for several days, depending on the tested pathogen. The MIC was determined as the lowest concentration of active protein that could inhibit visible mold growth and was calculated as the total protein added on each paper disc (microgram per disc). Starting buffer A without active protein was used as a blank control.

Effect of Heat Treatment on the Antifungal Activity of PcPAF
To evaluate the thermostability of the purified antifungal protein, the PcPAF protein (1.22 µg/µl) was treated at 40°C, 60°C, 80°C, 100°C, and 121°C (autoclaved) for 20 min. After cooling the protein samples to room temperature, the residual antifungal activity of the pure protein was tested against sensitive fungi. Approximately 40 µl of the heat-treated antifungal protein was used for the antifungal tests. PcPAF without heat treatment and starting buffer A were used as the positive and blank controls, respectively.

Effect of pH on the Antifungal Activity of PcPAF
To evaluate the pH stability of the purified antifungal protein, the PcPAF protein (1.22 µg/µl) was treated within a pH range from pH 1 to pH 11 with an interval of 2. Reaction mixtures were incubated at room temperature for 1 h, and then adjusting the pH value of different protein solutions back to pH 7. The residual antifungal activity of the treated protein was tested against sensitive fungi. Approximately 40 µl of the treated antifungal protein was used for the antifungal tests. PcPAF without pH treatment and starting buffer A were used as the positive control and blank control, respectively.

Effect of Metal Ions on the Antifungal Activity of PcPAF
To examine the effect of metal ions on PcPAF activity, selected metal ions such as Na$^{+}$, K$^{+}$, Ca$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ were dissolved in starting buffer A at the concentration of 20 mM. The PcPAF protein (1.22 µg/µl) was treated with different ion solutions at room temperature for 1 h before testing for antifungal activity. Approximately 40 µl of the treated antifungal protein was used for the antifungal tests. The PcPAF protein without ion solution treatment was used as the positive control, and starting buffer A and ion solutions were used as blank negative controls.

Effects of Proteases and Surfactants on the Antifungal Activity of PcPAF
To understand sensitivity of PcPAF to proteases, the purified PcPAF protein (1.22 µg/µl) was subjected to treatment with 1 mg/ml proteinase K (BBI, USA), papain (Worthington, USA), and trypsin (Worthington, USA) at 50°C for 24 h. In a similar way, in order to understand the sensitivity of PcPAF to surfactants, the purified protein was treated with 0.5% SDS, 0.5% Triton X-100, and 1% Tween-20 at 28°C for 24 h. The treatment effect was analyzed by observing the residual antifungal activity of PcPAF. Approximately 40 µl of the treated antifungal protein was used for the antifungal tests. The PcPAF without treatment with the proteases and surfactants was used as a positive control. The proteases, surfactants and starting buffer A were used as blank controls.

Results
Strain Identification and Detection of Antifungal Activity
A total of 37 strains were purified from the sediment samples from the Southwest Indian Ocean. After detecting
their antifungal activity, the supernatant of fungal strain W1 cultured in modified YTM liquid medium showed remarkable inhibitory activity against various pathogenic fungi. Spores of this strain, grown on modified YTM agar plates, were washed with 40% glycerol and preserved at -80°C.

Mycelia of fungal strain W1 could grow very fast on PDA plates at 28°C. Many green spores would be produced and finally turned dark brown. A light yellow-green substance was produced on PDA plates. Its colony characteristics were similar to *Penicillium* sp. according to the Fungal Identification Manual. Moreover, the ITS DNA sequence of strain W1 was similar to the ITS sequence (GenBank Accession No. KF530863.1) from *Penicillium citrinum* strain IARI-RPF-7 with an identity of 100%. Its ITS DNA sequence has been submitted to NCBI GenBank with an accession number KP986248. Based on the morphological and ITS identification, fungal strain W1 was determined to be *Penicillium citrinum*. The fungal strain *P. citrinum* W1 has been deposited at the China Center for Type Culture Collection under the preservation number CCTCC M 2013205 (Wuhan, China).

The supernatant of *P. citrinum* W1 was added on paper discs at a distance of 0.8 cm from the rim of each growing mycelium of *Trichoderma viride*, *Fusarium oxysporum*, *Paecilomyces variotii*, and *Alternaria longipes*. The appearance of a crescent mycelium rim around paper discs indicates the mycelium inhibitory activity of the supernatant of *P. citrinum* W1.

**Purification of PcPAF**

Preliminary analysis of *P. citrinum* W1 antifungal components revealed that the precipitate obtained by saturating the culture supernatant with ammonium sulfate exerted antifungal activity. Then the crude extract was used for further purification. Further purification of the bioactive protein was performed using ion-exchange chromatography and was monitored with the antifungal activity assay. The precipitate with antifungal activity was first loaded onto a DEAE Sepharose Fast Flow column. Only fractions A and B were isolated, and fraction A was found to possessed antifungal activity (Fig. 1A). Subsequently, the partially purified fraction A was loaded onto a CM Sepharose Fast Flow column. Two major fractions, peaks C and D, were generated (Fig. 1B). The results of the antifungal activity assay indicated that only fraction C contained a bioactive component, which could inhibit several pathogenic fungi, especially *Trichoderma viride*, *Fusarium oxysporum*, *Paecilomyces variotii*, and *Alternaria longipes* (Fig. 2). The active component showed only a single band on a SDS-PAGE gel with an estimated molecular mass of 10 kDa (Fig. 3). The active protein was named PcPAF.

**N-Terminal Amino Acid Sequence of PcPAF**

The N-terminal amino acid sequence of purified PcPAF was determined to be AGNRDPDFPRRHHPGG and compared with other protein sequences in the NCBI databases. Comparison result showed that there are some resemblance between the N-terminal amino acids of PcPAF and some proteins from different organisms, such as the hypothetical protein from *Gluconobacter morbifer*, the hypothetical protein from...
from *Coccidioides immitis* RS, and the drug-transport integral membrane protein from *Mycobacterium avium*. A BLAST search of the Antimicrobial Peptide Database showed that there is no resemblance between the N-terminal amino acids of PcPAF and any antifungal proteins encoded by *Penicillium* sp., such as PAF, PgAFP, and Pc-Arctin.

**MIC Determination of PcPAF**

The purified PcPAF protein was tested against different pathogenic fungi with the paper disc dilution method. PcPAF showed strong antifungal activity against *Trichoderma viride*, *Fusarium oxysporum*, *Paecilomyces variotii*, and *Alternaria longipes* with a MIC of 1.52, 6.08, 3.04, and 6.08 µg/disc, respectively, indicating that different pathogenic fungi exhibited different sensitivity to PcPAF.

**Physiochemical Properties of PcPAF**

The activity of the purified PcPAF did not decrease at all when incubated at the temperature of 40°C, 60°C, and 80°C for 20 min, whereas the activity of PcPAF was lost after being treated at 100°C or 121°C (autoclaved) for 20 min (Fig. 4A). Meanwhile, the PcPAF activity was retained after being treated at 100°C for 1 and 5 min. However, when PcPAF was incubated at 100°C for 10 or 15 min, its antifungal activity was partly or completely lost (Fig. 4B).

The antifungal activity of PcPAF was not obviously affected at pH 3 and 7, slightly weakened at pH 1, and rapidly inactivated at pH above 9 at 28°C for 60 min. The highest antifungal activity of PcPAF was found to be at pH 5, where the antifungal activity of PcPAF was increased by 28.9% compared with the control (Fig. 5).

In the presence of 20 mM Na\(^+\), K\(^+\), and Ca\(^{2+}\), the PcPAF activity was well retained. The inhibition activity of PcPAF remained above 75% of control activity when it was treated with 20 mM Mn\(^{2+}\). However, the activity was severely inhibited by 20 mM Zn\(^{2+}\), Mg\(^{2+}\), and Cu\(^{2+}\). The effects of metal ions on the antifungal activity of PcPAF are shown in Fig. 6.

The antifungal activity of PcPAF was not obviously affected after being treated with trypsin and papain at 50°C for 60 min. The inhibition activity of PcPAF remained above 90% of control activity (Fig. 7). Besides this, the antifungal activity of PcPAF could be inhibited by...
0.5% SDS, where the antifungal activity of PcPAF was decreased by 35% comparing with the control (Fig. 8). Moreover, 0.5% Triton X-100 and 1% Tween-20 showed no inhibiting effects on PcPAF activity (Fig. 8).

Discussion

In the present study, we have characterized an antifungal protein, named PcPAF, which was secreted by *P. citrinum* W1 and exhibited antifungal activity against pathogenic fungi. During the purification process, PcPAF could not be absorbed by the cation exchanger CM-Sepharose, which is different from PAF and Pc-Arctin [3, 4, 11]. Initially, the

![Fig. 4. Analysis of the thermostability of PcPAF following exposure to high temperatures, using *Trichoderma viride* as the tested strain. (A) Effect of temperature on the antifungal activity of PcPAF against *Trichoderma viride*. (B) Effect of heating time on the antifungal activity of PcPAF against *Trichoderma viride.*](image)

![Fig. 5. Effect of pH on the antifungal activity of PcPAF against *Trichoderma viride.*](image)

![Fig. 6. Effect of metal ions on the antifungal activity of PcPAF against *Trichoderma viride.*](image)

![Fig. 7. Effect of proteases on the antifungal activity of PcPAF against *Trichoderma viride.*](image)
A novel antifungal protein secreted by *P. citrinum* was identified by MALDI-TOF-TOF-MS, whose data showed no resemblance to any known proteins in the NCBI databases. The N-terminal amino acid sequence analysis and BLAST showed that there were some resemblance between PcPAF and some unrelated proteins from different organisms in partial sequence, although their characteristics were largely different (Table 1) [10, 12, 27]. The N-terminal amino acid sequence of PcPAF also did not collectively match to a single peptide or protein encoded by *Penicillium sp.*, such as PAF, PgAFP, and Pc-Arctin [3, 4, 22]. These differences indicate that PcPAF might be a novel antifungal protein.

During the purification and identification of PcPAF, a number of physical and chemical properties were investigated. Previously, it was found that the purified protein displayed thermostability in its antifungal activity, retaining almost 100% activity at 80°C for 20 min, and even being active at 100°C for 10 min (Fig. 4). It appears that many small antifungal proteins are thermostable owing to the formation of disulfide bridges [3, 13, 15, 23]. PcPAF exhibited the strongest antifungal activity at weak acid environment, and its antifungal activity was significantly reduced at pH 11 (Fig. 5). This feature of PcPAF was similar to some reported antifungal proteins, such as lysozymes and chitinases from plant origin [29, 30]. It was presumed that the weak acid environment may facilitate the folding of active PcPAF. In addition, some metal ions such as Mn$^{2+}$, Zn$^{2+}$, and Cu$^{2+}$ decreased the antifungal activity (Fig. 6). One possible reason is that certain types of ions may interact with specific receptors that are targeted by antifungal proteins. The occupation of these receptors with ions could reduce the interaction between the receptors and the proteins [3, 11, 24, 26]. Moreover, the activity of PcPAF was not affected after being treated with different proteases, except that proteinase K could weakly inhibit the antifungal activity of PcPAF (Fig. 7). The stability of PcPAF against proteases may be due to the specific digestion character of these proteases; for example, Pc-Arctin was not especially susceptible to proteases [3]. On the other hand, some antifungal proteins isolated previously did show protease inhibitory activities [4].

In summary, a novel antifungal protein, PcPAF, was purified from a fungal strain, *P. citrinum* W1, isolated from a sediment sample of Southwest Indian Ocean origin. PcPAF could effectively inhibit the growth of pathogenic fungi, including *Trichoderma viride*, *Fusarium oxysporum*, and others.

### Table 1. Comparison of the N-terminal amino acid sequence of PcPAF with that of other proteins.

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<th>Reference</th>
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<td><em>P. citrinum</em> W1</td>
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Figure 8. Effect of surfactants on the antifungal activity of PcPAF against *Trichoderma viride*.
Paecilomyces variotii, and Alternaria longipes. It was able to keep its antifungal activity after being subjected to treatment with some proteases, some metal ions, and common surfactants. The good stability and antifungal activity of PcPAF indicate that it has good potential for application in controlling plant pathogenic fungal infection. Therefore, the PcPAF characterized here may be considered as a promising candidate in the biological control of plant pathogenic fungal infection.

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