ELSEVIER

Enzyme and Microbial Technology 25 (1999) 439–446

enzyme and microbial technology

Purification and characterization of a new antifungal compound produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium

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Received 12 August 1998; received in revised form 12 April 1999; accepted 4 May 1999

Abstract

A new antifungal compound, which was named pafungin, was purified form culture broth of *Pseudomonas aeruginosa* K-187 grown aerobically in a shellfish waste-containing medium. The molecular weight of pafungin was 66 000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Pafungin's composition was similar to that of complex carbohydrate. Pafungin was stable at pH 5–7, and its thermal stability was 100°C for 40 min. The role of purified pafungin in the suppression of *Fusarium oxysporum* that causes damping-off disease was studied. Suppression of swelling and lysis of the hyphae were observed in the presence of pafungin. When covalently immobilized on an enteric coating polymer (hydroxypropyl methylcellulose acetate succinate), which has reversible soluble-insoluble characteristics with pH change, the pafungin retained its antifungal activity completely. This is the first report of a high-molecular-weight nonenzymatic antifungal compound from a prokaryote.

Keywords: Shrimp and crab waste; *Pseudomonas aeruginosa*; Antifungal; Chitinase; Biofungicide

1. Introduction

Synthetic chemical fungicides have long served as agents for reducing the incidence of plant disease; however, they are costly, can cause environmental pollution, and may induce pathogen resistance. Considering the limitations of chemical fungicides, it seems appropriate to search for a supplemental control strategy. Biological control, the use of a micro-organism or its secretion to prevent disease, offers an attractive alternative or supplement to pesticides and genetic resistance for the management of plant disease, without the negative effects of chemical control. Therefore, it becomes an important aspect of sustainable agriculture.

Recent biochemical research on plant disease control focused on two prime objectives: first, to identify micro-organisms with antifungal activities and to isolate and characterize the specific antifungal factors from these micro-organisms, and, secondly, to determine their mechanisms of action. In the past few years, numerous micro-organisms with antifungal activities and their active factors have been identified [1–6], and the mechanisms by which antifungal factors inhibit growth of potentially pathogenic fungi have been demonstrated [2,5,6,7–10].

Pseudomonas aeruginosa K-187, which was isolated from soil in northern Taiwan, has been shown in our previous studies [11–13] to produce chitinase in a shrimp and crab shell powder (SCSP) medium. The purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by this micro-organism in SCSP medium has also been demonstrated recently [14]. However, the bifunctional chitinase/lysozyme thus produced showed no antifungal activity [15]. Recently, we have shown that culture broth of *P. aeruginosa* K-187 grown aerobically in an SCSP medium displayed antifungal activity, and inhibited growth of many pathogenic fungi. Maximum antifungal activity was attained in a medium composition of 4.75% SCSP, 0.75% carboxymethyl cellulose, 0.1% K_2HPO_4, 0.05% $MgSO_4$ $7H_2O$, 0.3% NaCl, 0.35% yeast extract, pH 6 [15]. To further identify the specific factors responsible for the antifungal activity, as well as

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0141-0229/99/$ – see front matter
PII: S0141-0229(99)00069-1

elucidate its antifungal mechanism, we carried out the isolation and characterization of an antifungal compound named pafungin from culture broth of *P. aeruginosa* K-187, and studied its antifungal mechanism with biochemical tests and microscopic observation. Its antifungal characteristics were also evaluated as seed treatment to prevent damping off of alfalfa caused by *Fusarium oxysporum*.

2. Materials and methods

2.1. Materials

The SCSP used in these experiments was purchased from Chya-Pau Co., Taiwan. Dimethylaminoethyl (DEAE)-Sepharose CL-6B was from Pharmacia, and Econo Pac Q was from Bio-Rad. Alfalfa seeds were purchased from a local health food store (Yunlin, Taiwan). Hydroxypropyl methylcellulose acetate succinate, molecular weight 93 000, was manufactured by Shin-Etsu Chemical Company, Tokyo, Japan. Ethylene glycol chitin, glycol chitin, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride, and lyophilized cells of *Micrococcus lysodeikticus*, were products of Sigma (St. Louis, MO, USA). Colloidal chitin was prepared from powdered chitin (Wako Bio-Chemicals, Osaka, Japan) by the method of Jeniaux [16]. All other reagents used were of the highest grade available.

2.2. Micro-organism and pafungin production

P. aeruginosa K-187 was isolated from the soil in Taiwan [11] and maintained on nutrient agar plates at 37°C. For the production of pafungin, *P. aeruginosa* K-187 was grown in 150 ml of liquid medium in an Erlenmeyer flask (250 ml) containing 4.75% (w/v) SCSP, 0.75% (w/v) carboxymethyl cellulose, 0.1% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4\ 7H_2O$, 0.3% (w/v) NaCl, 0.35% (w/v) yeast extract, pH 6. Two milliliters of the seed culture was transferred into 150 ml of the same medium and grown in an orbital shaking incubator for 48 h at 37°C. The culture broth was centrifuged at 4°C for 20 min at 8200 $\times$ g, and the supernatant was used for the purification of the pafungin.

2.3. Purification of pafungin

2.3.1. DEAE-Sepharose CL-6B chromatography

To the cell-free culture broth (300 ml), 183 g of $(NH_4)_2SO_4$ was added. The resultant mixture was kept at 4°C overnight and the precipitate formed was collected by centrifugation at 4°C for 20 min at 9200 $\times$ g. The precipitate was dissolved in a small amount of 50 mM sodium phosphate buffer (pH 6), and dialyzed against the buffer. The resultant dialysate (100 ml) was loaded onto a DEAE-Sepharose CL-6B column (5 $\times$ 30 cm) pre-equilibrated with 50 mM sodium phosphate eluting buffer (pH 6). The unadsorbed materials were washed from the column with the same eluting buffer, and the rest was fractionated with a linear gradient of 0–1 M NaCl in the same buffer at a flow rate of 75 ml/h. The eluted fractions were assayed for antifungal activity. The antifungal fractions (224 ml) were combined and concentrated with ammonium sulfate precipitation (80%). The resultant precipitate was collected by centrifugation and dissolved in 9 ml of 50 mM sodium phosphate buffer (pH 6), followed by dialysis against the same buffer.

2.3.2. Econo-Pac Q chromatography

The dialysate (34 ml) was chromatographed on a column of Econo-Pac Q that had been equilibrated with 50 mM phosphate buffer. After application of the dialysate and washing of the column with 50 mM phosphate buffer (pH 6), the column was eluted with a linear gradient of 0–1 M NaCl in the same buffer at a flow rate of 75 ml/h. The antifungal fractions were combined and lyophilized to yield 59.3 mg of solid.

2.4. Determination of molecular weight

The molecular weight of purified pafungin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn [17] with phosphorylase B (molecular weight, 94 600), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and lactalbumin (14 480) as standard proteins. Before electrophoresis, the proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing 2-mercaptoethanol. The gels were stained with Coomassie brilliant Blue R-250 in methanol-acetic acid water (5:1:5, v/v), and decolorized in 7% acetic acid. The molecular weight of the purified pafungin was determined to be 66 000.

2.5. Amino acid and total sugar analysis

The purified pafungin was hydrolyzed with 6 N HCl at 110°C for 24 h in a sealed and evacuated tube, and the amino acid compositions were determined with a Beckman system 6300E. The total sugar content of pafungin was determined by the phenol-sulfuric acid method [18]. A sample solution was prepared by dissolving a suitable amount of purified pafungin in water. Twenty-five μl of 5% aqueous phenol was added to 1 ml of the sample solution. The solution was mixed, then 2.5 ml of concentrated sulfuric acid was added and mixed. After the reagents had been allowed to stand for 10 min to allow color to develop, the absorbance was measured at 420 nm (Beckman UV-S70). A standard curve with 10–40 μg of glucose as standard was prepared to obtain the content of the pafungin.

2.6. In vitro antifungal activity tests

The antifungal activity for the purified pafungin was estimated by using a growth inhibition assay described

earlier [2,5]. Fungal spores were grown on Petri plates filled with potato dextrose agar. After 10 days of incubation at 25°C, the fungal colonies were removed with sterile water containing 0.1% (v/v) Tween 80. The resulting suspension was filtered aseptically through 0.45-μm pore-size membrane filters. The filtrate was adjusted with sterile water to a concentration of 1×10^6 spores/ml, and stored at 4°C. The pafungin purified as described above by DEAE-Sepharose CL-6B and Econo-Pac Q column chromatography was dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7), followed by dialysis against the same buffer. The resultant dialyzate was used for testing. To test the antagonistic effect of the inhibitor produced by *P. aeruginosa* K-187, Petri plates were filled with standard amounts of molten potato dextrose agar precooled to 45°C, and divided into two groups (triplicate for each). To each protein in the experimental group (E) was added the appropriate amount of pafungin dialyzate, and to those of the control group (C) was added an equal amount of sterilized water. After the plates were cooled, the fungal inoculum was placed on the agar surface. Both groups were incubated for 72 h at 25°C. The diameters of the largest and smallest fungal colonies were recorded, and the inhibition ratios were calculated with the following formula:

$$\text{Inhibition ratio } (\%) = (C - E)/C \times 100\%$$

C = the average diameter of largest and smallest colonies of the control groups;
E = the average diameter of largest and smallest colonies of the experimental groups.

The test fungi used were *Aspergillus fumigatus* CCRC 30099, *Aspergillus parasiticus* CCRC 30117, *A. parasiticus* CCRC 30170, *A. parasiticus* CCRC30423, *Aspergillus clavatus* CCRC 31116, *Aspergillus sydowii* CCRC 32141, *Aspergillus flavofurcatis* CCRC 32128, *Aspergillus candidus* CCRC 31543, *Aspergillus nomius* CCRC 30109, *Aspergillus oryzae* CCRC 30101, *A. oryzae* CCRC 30428, *Aspergillus penicilloides* CCRC 32309, *Aspergillus flavus* CCRC 30021, *Aspergillus restrictus* CCRC 31894, *A. parasiticus* CCRC 30228, *A. parasiticus* CCRC 30132, *Candida albican*, *Cladosporium oxysporum* CCRC 32121, *F. oxysporum* CCRC 32121, *Fusarium graminer*, *Fusarium moniliforme*, *Fusarium sporotrichoiodes*, *Geotrichum candidum*, *Penicillium islandicum* CCRC 31560, *Penicillium corylophilum*, *Penicillium urticase*, *Penicillium rugulosum* CCRC 31518, *Peniciilium italicum*, *Penicillium cyclopium*, *Penicillium viridicatum*, *Penicillium purpurogeman* CCRC 31680, *Penicillium citrinum* CCRC 31620, *Monascus pilosus*, *Moniliella acetoabutans* CCRC 31999, *Mucor* spp, *Neosartorya fischeri*, *Rhizopus microsporus* CCRC 31750, *Saccharomyces cerevisiae* (wine), *S. cerevisiae (chablis).* All test strains were generously provided by professor K. J. Tsai, Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan.

2.7. Measurement of enzyme activity

Chitinase activity was measured with collodial chitin as a substrate [14]. The amount of reducing sugar produced was measured by the method of Imoto and Yagishita [19] with *N*-acetylglucosamine as a reference compound. The lysozyme activities on ethyleneglycolchitin and *M. lysodeikticus* cells were assayed by the procedures as described previously [14].

2.8. pH and thermal stability of pafungin

The pH stability of purified pafungin was determined by the measurement of the residual inhibitory activity at pH 7 as described above after dialyzing the samples against 50 mM buffer solution of various pHs (pH 3–11) at 4°C for 18 h. The buffer systems used were glycine-HCl (50 mM, pH 3), acetate (50 mM, pH 4.5), phosphate (50 mM, pH 6–8), Na_2CO_3-$NaHCO_3$ (50 mM, pH 9–11). The thermal stability of pafungin was studied by heating the samples at 100°C for various time periods, and the residual inhibitory activity was measured as described above.

2.9. Hyphal morphology affected by pafungin

Fungal spores were grown in test tubes (ϕ 16 × 100 mm) containing 2 ml of potato dextrose broth; 100 μl of sample solution of purified pafungin was added simultaneously, or 24 h later, so that the final concentration of pafungin was 9.3 mg/ml. The resultant solution was incubated at 25°C for 72 h, and the hyphae were observed by a light microscope.

2.10. Plate assay for disease of alfalfa seedlings

Alfalfa seeds obtained from local health-food supplier were first sterilized by immersion in a solution containing 2% sodium hypochlorite, followed by sterile water washing and filtering aseptically according to a previous method [20]. The sterilized seeds were germinated as described previously [21]. Approximately 1 g of seed was placed in 10 ml of sterile distilled water in a 125 ml flask and shaken 6 days at 25°C and placed on water agar plates at a density of 10 seedlings/plate. A 10–μl mixture of conidia of *F. oxysporum* and inhibitor/purified pafungin (experimental group) or water (control group) was applied to each seedling. The plates were incubated at room temperature, and scored for symptoms of disease after 4 or 5 days. Diseased seedlings were characterized by browning of the stems [6].

3. Results

3.1. Purification of pafungin

In the presence of SCSP as a major carbon source, *P. aeruginosa* K-187 released pafungin into the culture fluid.

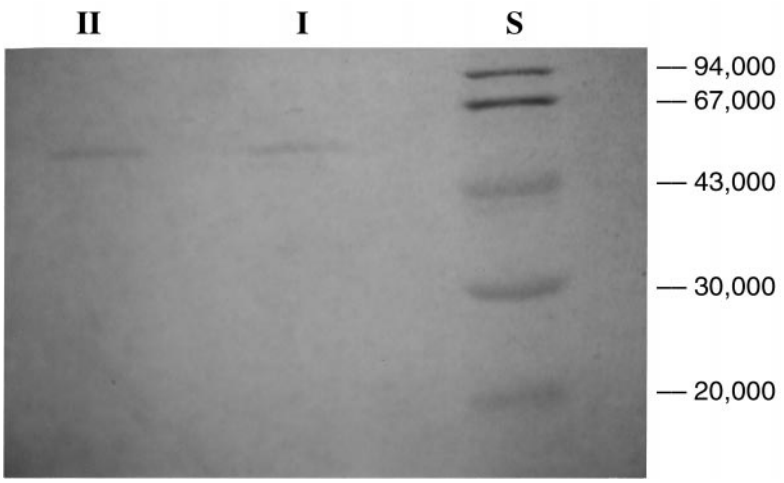

Fig. 1. SDS-PAGE of the purified pafungin. Lane S, low molecular-weight. standards (Pharmacia); Lane I and II, pafungin.

This was purified from the culture supernatant (300 ml) of *P. aeruginosa* K-187 as described in Section 2. In the process of purification, the antifungal fractions were monitored by bioassay because the antifungal portions did not contain chromophores that could be detected spectrophotometrically. The final amount of pafungin obtained was 59.3 mg after DEAE-sepharose CL-6B and Econo-Pac Q column chromatography, and it was confirmed to be homogeneous by SDS-PAGE (Fig. 1) as well as gel filtration on a column of Sephacryl S-200 (data not shown). After dialysis in a high salt solution and gel filtration, we found that the pafungin remained intact without any dissociation or loss of activity.

3.1.1. Molecular weight, amino acid composition, and sugar content

The molecular weight of purified pafungin was calculated on the basis of semilogarithmic plots of the mobilities of the bands on SDS-PAGE by using a standard curve ($Y = -1.24\ X + 5.6336$) established with proteins of known molecular weight. The molecular weight of the pafungin was estimated to be 66 000. A purified pafungin sample (2.9 mg) was used for amino acid analysis, and the amino acid contents account for 17% of the sample. Table 1 summarizes the data from amino acid analyses of the purified pafungin, in which large amounts of proline (13.3%), alanine (16.1%), valine (11.1%), isoleucine (10.1%), and leucine (12.0%) were shown; they accounted for 62.6% of total amino acids. The total sugar content of pafungin was determined by the phenol-sulfuric acid method. The absorbance was measured at 420 nm, and a standard curve using glucose as standard was established. The total sugar content was determined to be 62%.

Table 1.
Amino acid composition of pafungin

Amino acid	Mol %
Asx	5.0
Thr	0.6
Ser	ND[a]
Glx	0.8
Pro	13.3
Gly	5.4
Ala	16.1
Cys	ND[a]
Val	11.1
Met	0.9
Ile	10.1
Leu	12.0
Tyr	3.1
Phe	2.5
His	1.7
Lys	7.7
Arg	6.7
Trp	3.2

[a] ND, not detected.

3.2. Properties of pafungin

The purified pafungin was found effective against all 39 strains of fungi tested. The effect of pH on the inhibitory

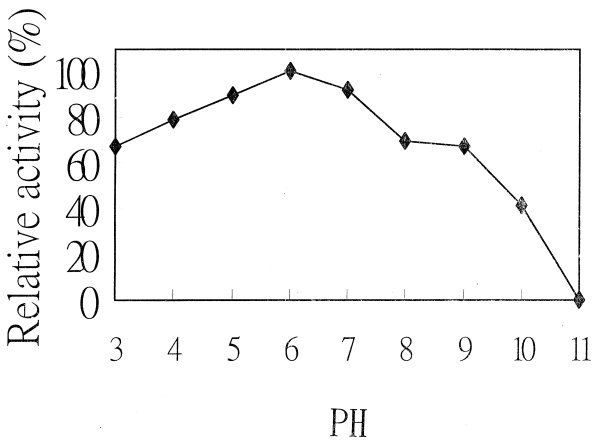

Fig. 2. The pH stability of pafungin. The inhibition activity of *F. oxysporum*.

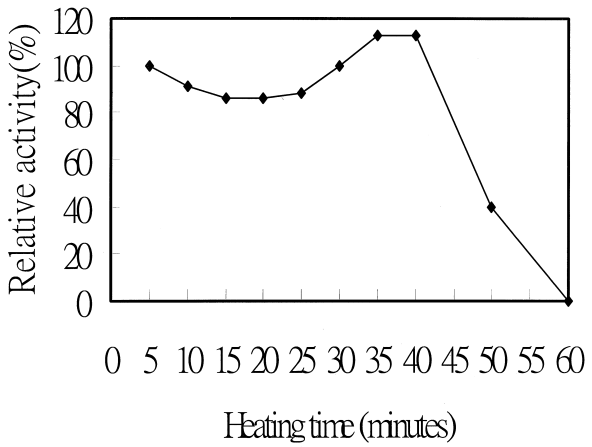

Fig. 3. The thermal stability of pafungin. The inhibition activity of *F. oxysporum*.

activity of pafungin to *F. oxysporum* was studied at various pHs. The results showed (Fig. 2) that the inhibitory activity was highly retained at pH 5–7 with a maximum value at pH 6. It was noticeable that pafungin still maintained about 66% of the inhibitory activity even at pH 3, whereas the inhibitory activity dropped to 0% at pH 11. We concluded that pafungin was less vulnerable in acidic condition. The thermal stability of pafungin was studied by heating the samples at 100°C for various time periods, and measuring residual inhibitory activity to *F. oxysporum*. The results (Fig. 3) showed that pafungin was thermostable, and the inhibitory activity gradually increased within 20–30 min and reached to the maximum in about 35–40 min. It was obvious that it retained 40% of the inhibitory activity even after heating at 100°C for 50 min. However, longer heating decreased its inhibitory activity drastically.

3.3. Hyphal morphology affected by pafungin

The effect of purified pafungin on hyphal growth was examined by light microscopy after incubating with pafungin (9.3 mg/ml) at 25°C for 72 h. The effects of purified pafungin from *P. aeruginosa* K-187 on morphology of *F. oxysporum* and *A. fumigatus* were shown in Figs. 4 and 5, respectively, in which it was obvious that pafungin caused extensive hyphal swelling of both organisms; however, no lysis was observed. On the other hand, the mycelia grew normally in the absence of pafungin.

3.4. Damping-off suppression of alfalfa seedlings

The disease suppression activity of pafungin on alfalfa seedling damping off caused by *F. oxysporum* was shown in Fig. 6. The results showed that the alfalfa seedling survival was 100% after treatment of sterilized water (A), whereas the survival of seedlings was 0% in the *F. oxysporum* conidia treatment (B), in which the browning and subsequent damping off of stem was usually observed. On the other hand, treatment of seeds with a mixture of *F. oxysporum* and purified pafungin (10 μl, 1:1) resulted in a survival of 80% (C). To demonstrate that pafungin did not cause alfalfa damping off, the seedling was treated with 10 μl of pafungin solution without the presence of *F. oxysporum*. Under such circumstance, the survival of seedling was 100% (D), and the alfalfa grew normally without any browning and subsequent damping off of stem.

3.5. Antifungal activity of immobilized pafungin

Immobilized pafungin was prepared by the procedures described previously [22]. The immobilized pafungin was filter-sterilized through a 0.45-μm membrane prior to testing its antifungal activity. We found that immobilized and

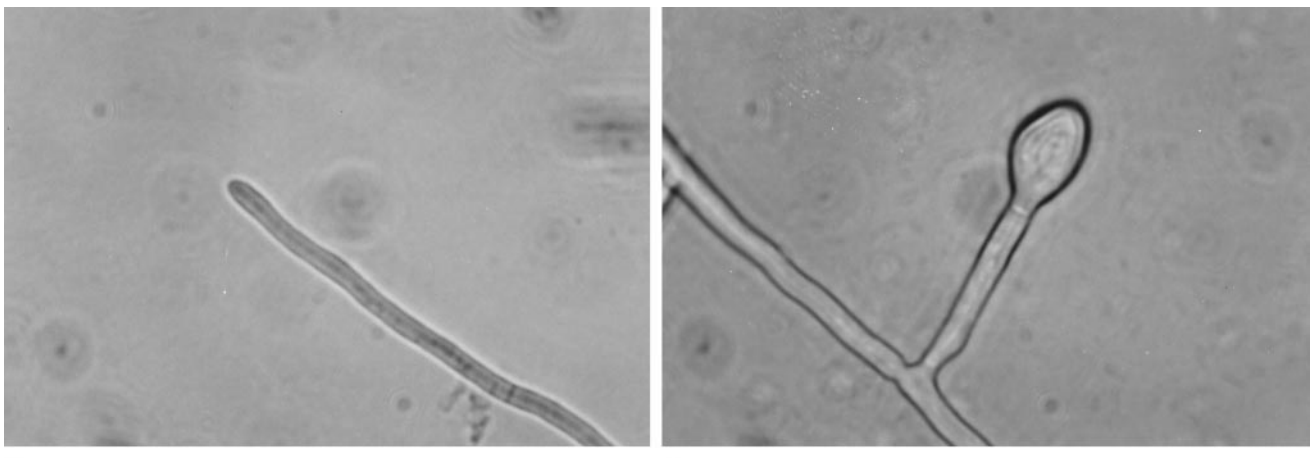

Fig. 4. Effect of purified pafungin from *P. aeruginosa* K-187 on morphology of *F. oxysporum*. (A) Normal mycelia of *F. oxysporum*; (B) swelling of *F. oxysporum* hyphae grown in presence of 0.83 mg/ml of purified pafungin.

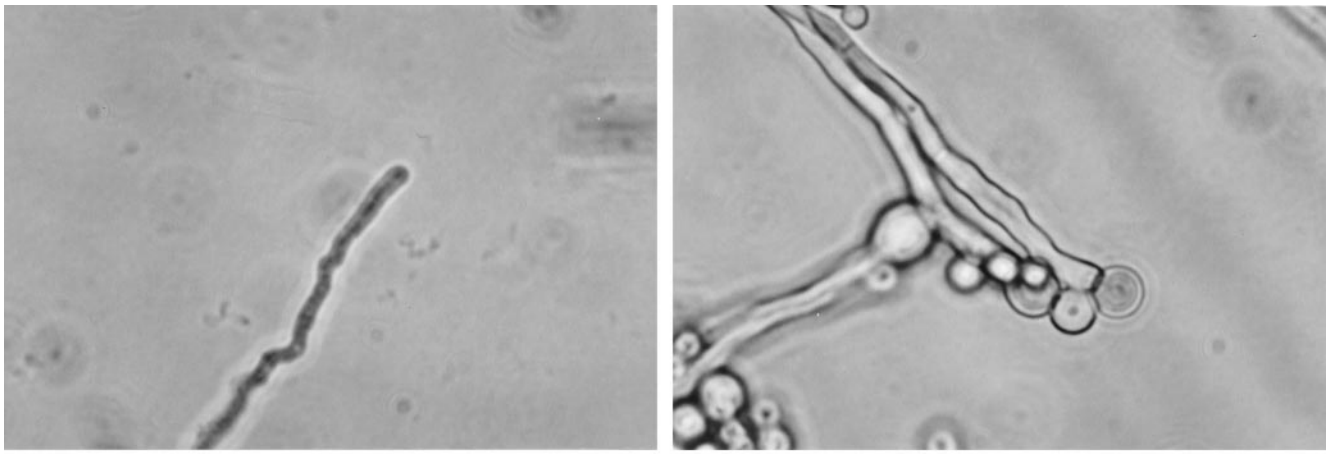

Fig. 5. Effect of purified pafungin from *P. aeruginosa* K-187 on morphology of *A. fumigatus*. (A) Normal mycelia of *A. fumigatus*; (B) swelling of *A. fumigatus* hyphae grown in presence of 0.83 mg/ml of purified pafungin.

free pafungin displayed the same antifungal activities. When examined under the light microscope, it was also found that immobilized pafungin caused hyphae swelling of *F. oxysporum* and *A. fumigatus*. The phenomena were similar to those caused by the free pafungin.

4. Discussion

In a recent report [15] we showed that culture broth of *P. aeruginosa* K-187 grown aerobically in a SCSP medium displayed antifungal activity, and inhibited the growth of many pathogenic fungi. In this paper, we carried out the isolation and characterization of an antifungal compound named pafungin from culture broth of *P. aeruginosa* K-187. Pafungin was characterized to be a complex carbohydrate with molecular weight of 66 000, which contains 17% of amino acids, and 62% of carbohydrate. When the inhibitory activity to *F. oxysporum* was measured, the optimum pHs for this compound were found to be pH 5–7, and it was extremely thermostable and remained intact after extensive dialysis in high salt solution. The thermostability of pafungin is remarkable, it is stable up to 100°C and maintains around 40% of its activity even at high temperature for 50 min. This stability is markedly higher than the thermal stabilities observed for antifungal proteins of other origins [3,23,24]. To our knowledge, this is the first report of a high-molecular-weight complex carbohydrate that have antifungal activity, and it is effective for all 39 strains of fungi tested. It will be interesting to determine whether this compound produced by *P. aeruginosa* K-187 resembles known antifungal agents. Many known antifungal compounds were small molecules and most are peptides [1] or proteins [6,7, 23,25] in nature. Many of these antifungal peptides had chitin-binding abilities [26], whereas no such binding activity of pafungin was found (data not shown). Most of the antifungal proteins were shown to have chitinase activities; however, pafungin showed no activity of chitinase and/or lysozyme. Therefore, our results suggested pafungin was not the bifunctional chitinase/lysozyme produced by this microorganism in SCSP as previously reported by our group [14], neither was it one of these previously described antifungal agents. Our previous observation [15] that bifunctional chitinase/lysozyme thus produced showed no antifungal activity further confirmed this conclusion. The yield of pafungin produced by *P. aeruginosa* K-187 is extremely high (197.6 mg/l) in comparison to other antifungal compounds produced by other microorganisms. For example, it was 20 times more than the antifungal protein (10 mg/l) produced by *Gliocladium virens* [27].

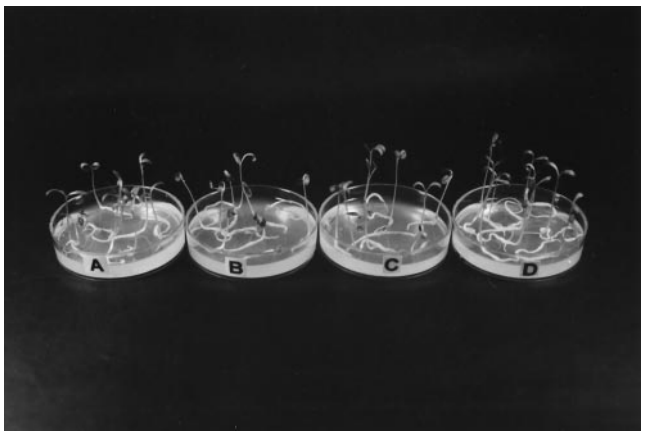

Fig. 6. Plate assay for disease of alfalfa seedlings after treatment of: (A) sterilized water; (B) *F. oxysporum*; (C) mixture of *F. oxysporum* spores suspension and purified pafungin (1:1); (D) purified pafungin.

Several apparent or plausible mechanisms, by which biocontrol organisms or antifungal compounds may become involved in the antagonism of pathogenic fungi have been proposed previously. These included interference with spore germination or germ tube elongation [4,6,28–30],

inhibition by abnormal hyphae swelling [1,2], lysis and complete degradation of hyphal tip [2,7,31–33], and suppression by competition for nutrients [25,34]. We have previously shown that crude extract from the culture broth grown aerobically in a SCSP medium inhibited the growth of *F. oxysporum*, *F. moniliforme*, *P. italicum*, and *A. flavus* by blocking spore germination as well as causing hyphae swelling [15]. In this study, the purified pafungin produced by *P. aeruginosa* K-187 was shown causing the same effects on both *F. oxysporum* and *A. fumigatus*. Similar phenomena were seen when *Trichoderma viride* was treated with chitinase purified from pea tissue [9], or pathogenic *Botrytis cinerea* was treated with endochitinase produced by *Trichoderma harzianum* [3,29]. We have also shown that the crude extract from the culture broth of *P. aeruginosa* K-187 even caused lysis or degradation of hyphae tips of the tested fungi [15], similar to the phenomena observed, when Iturin produced by *Bacillus subtilis* was used for *F. oxysporum* [10], or endochitinase from *T. harzianum* was used for pathogenic *B. cinerea* [3,29]. However, the purified pafungin did not display such activity. It is reasonable to suggest that some other agents in the crude extract were needed to act together with pure pafungin to swell and lyse the hyphae. This is in accord with the knowledge that chitinase and β-1,3-glucanase purified from pea pods acted synergistically in the degradation of fungal cell walls. Purified chitinase and β-1,3-glucanase, tested individually, did not inhibit growth of most of the test fungi. However, combinations of both effectively inhibited all fungi tested. [9]. Similar studies showed the chitinase and β-1,3-glucanase produced by *Pseudomonas stutzeri* YPL-1 acted together to completely degrade the hyphae of *Fusarium solani* [2], and these two enzymes produced by *T. harzianum* must act simultaneously to decompose hyphal walls of *Schizophyllium commune* [7,35]. The synergistic factor required to act together with pafungin is yet to be explained.

A simple, rapid assay was developed by Handelsman et al. [21] to screen bacteria for the ability to reduce the mortality of Iroquois alfalfa seedlings that were inoculated with *Phytophthora megasperma* f. sp. *medicaginis* zoospores. By the use of this method with slight modification in which alfalfa seedlings were inoculated with *F. oxysporum* to result in 100% mortality of control seedlings, the crude extract from the culture broth of *P. aeruginosa* K-187 was shown to reduce seedling mortality profoundly [15]. The purified pafungin was as effective as the crude extract in protecting seedlings from damping off. The mechanism by which crude extract or pafungin suppress damping off remains to be determined. However, in view of the effects on the hyphal morphology of *F. oxysporum* induced by both crude extract and purified pafungin, it is reasonable to suggest that hyphae swelling and lysis play a major part in suppression of seedling damping off.

Although many questions remain to be addressed before its application in the field, pafungin appears to have potential as a biocontrol agent for alfalfa damping off. One particularly attractive characteristic that may further enhance its potential as a biocontrol agent is its immobilizability. When covalently immobilized on an enteric coating polymer (hydroxypropyl methylcellulose acetate succinate), which has reversible soluble-insoluble characteristics with pH change, the pafungin retained its antifungal activity completely. Immobilizing enzyme to reversible soluble-insoluble carrier, in contrast to conventional water-insoluble support, has been suggested as a means to solve the diffusional problems encountered in a heterogeneous reaction system and to aid in product separation [36]. Immobilization of chitinase on such carrier for chitin hydrolysis has been shown before [22,37]. We have attempted to immobilize pafungin and studied the initial ammonium sulfate precipitate of culture broth of *P. aeruginosa* K-187 grown in a SCSP medium, which is known to contain chitinase [37]. Accidently, we found such preparation displayed antifungal activity. Since chitinase from *P. aeruginosa* had no antifungal activity [17], it was speculated that pafungin was immobilized. This was confirmed by immobilization of purified pafungin to such a carrier. Pafungin, with its wide range of antifungal activity, extreme thermostability, and immobilizability, should find great potential in plant pathogen control. The mode of antagonism and the site of interaction between pafungin and the pathogen are currently under investigation with scanning electron microscopic techniques.

Acknowledgments

This report was supported by Grant NSC987-B-212-003 from the National Science Council, Republic of China.

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