Antifungal Activities of *Streptomyces blastmyceticus* Strain 12-6 Against Plant Pathogenic Fungi

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ABSTRACT

*Streptomyces blastmyceticus* strain 12-6 was isolated from a forest soil sample of Cheonan area on the basis of strong antifungal activities against plant pathogenic fungi. Butanol extracts of the cultural filtrates were active against *C. acutatum*, *C. coccodes*, *C. gloeosporioides*, *F. oxysporum*, and *T. roseum*. Active fractions were prepared by thin layer chromatography using silica gel plate; 12-6-2 (Rf 0.36), 12-6-3 (Rf 0.44). Scanning electron microscopy showed that the active fractions caused a change in surface texture of fungal spores from smooth surface to wrinkled surface. The lethal effect on the spores of the active fractions varied from 56% to 100%. It was shown that the spores of *C. acutatum* were more sensitive to the antifungal fractions than the spores of *F. oxysporum*. Fluorescence staining using TOTO-1 indicated that the antifungal fractions could make the spores more sensitive to the fluorescence dye. Thus, it was suggested that antifungal agents prepared in this study exhibited the antifungal activity by damaging the plasma membrane of both fungal spores and hyphae. Identification of antifungal agents in the active fraction using GC-MS analysis revealed the presence of cyclo-(Leu-Pro) and 9-octadecenamide as major components that have already been known as antifungal substances.

1. Introduction

Around 20% of economically important crops are lost worldwide due to plant diseases caused by plant pathogens and 85% of them are known as fungi [1]. The pathogens, for example, known for anthracnose are *Colletotrichum* species; for gray mold rot are *Botrytis allii* or *B. cinerea*; for fusarium wilt, *Fusarium oxysporum*; for rice blast disease, *Magnaporthe grisea*; for phytophthora blight are *Phytophthora capsici*, *P. infestans*, *P. aeruginosa*, or *P. cactorum*; for blight, *Rhizoctonia solani*; for sclerotial rot, *Sclerotinia sclerotiorum*, and so on. Chemical control methods using pesticides have been the most effective for the control of plant pathogens [2]. However, overuse of chemical pesticides has resulted in many unexpected side effects. Examples of adverse reactions include the appearance of resistant pathogens, soil acidification, groundwater contamination, destruction of ecosystems [3], and toxicity to humans and livestock [4]. To reduce the use of chemical pesticides, a variety of biological control options using antagonistic microorganisms or microbial pesticides have been developed as effective alternatives for their reduced toxicity and good biodegradability in natural environment [5,6], and thus are recognized as eco-friendly control methods [7–9]. Therefore, the needs for the development of biological antifungal agents are ever growing. In this paper, we isolated an actinomycete strain having excellent antifungal activity from forest soil and identified the antifungal fractions produced by the strain.

2. Materials and methods

2.1. Microorganisms

*Streptomyces blastmyceticus* strain 12–6 was originally isolated from a soil sample collected from a forest region of Cheonan city, South Korea. It was grown at 28°C on yeast extract-malt extract (YEME) agar medium containing 2 g/L dextrose, 4 g/L yeast extract, and 10 g/L malt extract. For liquid culture, agar was omitted from YEME medium and shaken at 200 rpm at 28°C. Plant pathogenic fungi and yeasts tested in this study are listed in Table 1. Test microorganisms were grown on potato dextrose agar (PDA; BD, Sparks, MD) medium at 25°C. Submerged culture was carried out at 25°C with the agitation rate of 200 rpm in a potato dextrose broth (PDB; BD).
2.2. Extraction of culture filtrate

Strain 12-6 was grown in liquid YEME at 200 rpm at 28°C for 7 days after which the mycelia were removed by filtration using Whatman No. 2 filter paper. The antifungal fraction was extracted by vigorous mixing of the same volume of n-butanol and the culture filtrate, and the solvent layer was separated using a separation funnel and concentrated under reduced pressure at 50°C in a vacuum rotary evaporator (R-114; Büchi, New Castle, DE). The residue on the evaporation flask was dissolved in a small amount of methanol.

2.3. Disk diffusion assay

The antifungal activities of active fractions from culture filtrate of the strain 12-6 were determined by disk diffusion assay. PDA media were first inoculated with test microorganisms, and then paper disk (φ 8mm) soaked with 10μL of each active fraction was placed on the PDA. After incubation at 25°C for 7 days, the size of inhibition zone was measured for determination of antifungal activities.

2.4. Thin layer chromatography (TLC)

TLC was performed to resolve the antifungal fractions in n-butanol extract. A total of 10μL n-butanol extract was applied to a TLC glass plate (Silica gel 60 F254; Merck, Kenilworth, NJ), and n-butanol-acetic acid-water (12:3:5, v/v/v) was used as a developing solvent. After development, samples were sprayed with 0.3% ninhydrin and heated at 100°C for 5 min. The resulting color spots were named according to their respective Rf values [10].

To identify spots with antifungal activity, semipreparative TLC was performed starting with 200μL extract on the starting line of a TLC plate. Then individual spots were scraped off and the antifungal fraction was eluted with methanol. Finally, 10μL of the eluent was absorbed onto a filter paper disk, and the antifungal activity was confirmed via disk diffusion assay.

2.5. Minimal inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of the antifungal fractions was measured using spot-eluents diluted in methanol to concentrations ranging from 2500–156μg/mL. Antifungal activity at each concentration was examined using the paper disk method on PDA medium inoculated with about 5 × 10⁴ spores of each test strain.

2.6. Microscopy of fungi

The spores of test fungi (5 × 10⁵/mL) were suspended in 1mL of PDB. The antifungal extract (0.4mg/mL for C. acutatum, or 0.8mg/mL for F. oxysporum) was added into the spore suspension and incubated at 25°C, 200 rpm for 12 h, and then observed with an optical microscope (Axioskop 40; Carl Zeiss, Oberkochen, Germany) or a scanning electron microscope. For scanning electron microscopy, the spores were fixed with 4% glutaraldehyde for 2 h and secondly fixed with OsO₄ for 1 h. The fixed samples were serially dehydrated in 50%, 70%, 90%, 95%, and 100% ethanol for 20min, respectively, and then dried with isopropyl alcohol. They were subsequently subjected to platinum coating using an ion sputter (Hitachi E-1030; Hitachi, Tokyo, Japan) and observed with a scanning electron microscope (Hitachi S-4300; Hitachi) operating at 15kV.

For optical microscopy of fungal hyphae, spores were incubated in PDB for 12 h at 25°C to develop submerged mycelia. Then, 0.4mg/mL (for C. acutatum) or 0.8mg/mL (for F. oxysporum) of the antifungal fraction was added into the liquid media, and the mycelia were further incubated for 12 h under same conditions. Microscopic observations were performed following the same procedures described for spore observation.

2.7. Determination of lethal effect of the antifungal fractions

Fungal spores that have been treated with antifungal fractions as described above were collected from the
culture solution by centrifugation, washed three times, and plated on PDA plates, to count the number of colonies formed after 48 h incubation at 25°C. As control experiment, the colony counting was performed for spores without antifungal treatment following the same procedure on PDA plate. The mortality rate was expressed as % of the number of killed spores to the number of spores of the control experiment. The experiment was repeated three times.

2.8. Fluorescence microscopy
Fluorescence staining using TOTO-1 (514/533; Invitrogen, Carlsbad, CA) was performed to confirm the damage to spore membranes caused by the antifungal substance. The spores were collected as described above and resuspended in 300 µL diluted TOTO-1 (1/5000 with TE buffer) for 3 min. Then, the spores were washed three times in TE buffer and observed with a fluorescence microscope (Nikon Eclipse TE2000-U; Nikon, Tokyo, Japan). Dead spores appeared green while normal spores were not stained [11]. As a control, spores not treated with the antifungal fraction were observed under an optical microscope (Axioskop 40; Carl Zeiss), and the ratio of fluorescent spores to total spores under optical microscope was determined as the mortality rate.

2.9. GC/MS
GC/MS analyses were carried out at the Seoul Center at the Korea Basic Science Institute. The antifungal fractions from semi-preparative TLC were placed in an Agilent GC/MS system equipped with a J&W DB-5ms capillary column. Analysis conditions were as follows: injector, split ratio (2:1); injection temperature, 250°C; injection volume, 1 mL; carrier gas, He (1.0 mL/min); column, DB5-MS J&W Scientific (Folsom, CA); oven temperature, from 60°C (2 min) to 320°C at intervals of 10°C/min; interface temperature, 280°C; ion source, EI, 230°C; analyzer, quadrupole, 150°C; mass range, 40–800 m/z.

2.10. Data analysis
The data were expressed as the average ± standard deviation (SD) of triplicate determinations. Significant differences were analyzed using one-way analysis of variance (ANOVA). Differences at \( p < 0.05 \) were considered significant. Statistical analysis was performed using Microsoft Office Excel 2016 software.

3. Results

3.1. Antifungal activity of culture extract
Strain 12-6 was cultured in YEME liquid medium for 7 days, and the resulting culture filtrate was extracted with n-butanol and concentrated to confirm its antifungal activities. The n-butanol extract was most effective against \( T. \) roseum, \( C. \) coccodes, \( C. \) gloeosporioides, and \( F. \) oxysporum f sp lycopersici.

3.2. Antifungal activity and minimal inhibitory concentration of TLC fraction
The n-butanol extract was developed on a glass TLC plate and stained through a ninhydrin reaction, yielding four distinct spots. Each spot was scraped and dissolved in methanol to elute the antifungal compounds. Of these four eluates, two spots designated 12-6-2 (\( R_f \) 0.36) and 12-6-3 (\( R_f \) 0.44) exhibited antifungal activities. The purity of each eluent was confirmed using TLC (Figure 1).

Spot 12-6-2 had an MIC of 310 µg/mL for \( C. \) acutatum, \( C. \) coccodes, and \( C. \) gloeosporioides, and >500 µg/mL for \( B. \) cinerea, \( F. \) oxysporum, and \( T. \) roseum. The MICs of 12-6-3 were 310 µg/mL for both \( C. \) acutatum and \( F. \) oxysporum, and >500 µg/mL for all other test strains (Table 2). It was approximately consistent with the antifungal activity determined by the size of the inhibition zone described above, except for \( T. \) roseum which, contrary to expectations, had relatively high MIC values in disk diffusion assays.
3.3. Spore killing by antifungal fractions

For quantitative analysis of the antifungal activity, the lethality of spores of *C. acutatum* and *F. oxysporum* in the presence of each antifungal fraction were determined. As shown in Table 3, at 0.4 mg/mL, fraction 12-6-2 showed 96.67% lethality against *C. acutatum* spores while fraction 12-6-3 showed only 56.67%. At 0.8 mg/mL, both fractions exhibited 100.00% lethality. Against *F. oxysporum* spores, similar level of lethality was shown at 0.4 mg/mL; 76.33% by 12-6-2 and 79.33% by 12-6-3. But, again, at 0.8 mg/mL, both of fractions killed the spores completely (Table 3).

Overall, at lower concentration, fraction 12-6-2 was more active against *C. acutatum* while at higher concentration, both fractions were equally effective to both fungi. These results suggest that spore germination and mycelial growth could not occur due to the fungicidal effects of each fraction.

Next, TOTO-1 fluorescent staining was used to confirm the fungicidal effects of each fraction. Normal spores were observed with a light microscope but showed no fluorescence upon staining with TOTO-1. However, when spore permeability was increased due to membrane damage, TOTO-1 was able to penetrate into cells, resulting in detectable fluorescence. Virtually all spores of both *C. acutatum* and *F. oxysporum* became fluorescent after antifungal treatment, with no fluorescence evident in the absence of antifungal treatment (Figures 2 and 3).

These results suggest that the cell membrane of spores may be sufficiently damaged by the antifungal fractions extracted from *S. blastmyceticus* strain 12-6.

### Table 2. MICs of the antifungal fractions against various plant pathogenic fungi.

| Test microorganism       | 12-6-2 | 12-6-3 |
|--------------------------|--------|--------|
| *Botrytis cinerea*       | >500   | >500   |
| *Colletotrichum acutatum*| 310    | 310    |
| *Colletotrichum cocodes* | >500   | >500   |
| *Colletotrichum gloeosporioides* | 310 | >500   |
| *Fusarium oxysporum*     | >500   | 310    |
| *Trichotheicum roseum*   | >500   | >500   |
| *Candida albicans*       | >500   | >500   |
| *Saccharomyces cerevisiae* | >500   | >500   |
| *Trichosporon asahii*    | >500   | >500   |

Antifungal fractions were diluted with methanol to concentrations ranging from 2500–156 μg/mL, and the antifungal activity at each concentration was examined via disk diffusion assay on PDA medium inoculated with ~5 x 10^5 spores of each test strain.

### Table 3. Inhibition (%) of spore germination by the antifungal fractions.

| Fraction | μg/ml | *C. acutatum* | *F. oxysporum* |
|----------|-------|---------------|----------------|
| 12-6-2   | 0.4   | 96.67 ± 0.58  | 76.33 ± 1.53   |
| 12-6-2   | 0.8   | 100.00 ± 0.00 | 100.00 ± 0.00  |
| 12-6-3   | 0.4   | 56.67 ± 2.08  | 79.33 ± 0.58   |
| 12-6-3   | 0.8   | 100.00 ± 0.00 | 100.00 ± 0.00  |

Each value is the average ± standard deviation of triplicate determinations.

Fungal spores that have been treated with antifungal fractions were washed three times, plated on PDA plates, and incubated for 48 h at 25°C to assess colony counts. In control experiments, spores without antifungal treatment were plated on PDA. Fungicidal activity is expressed as a percentage of spores killed relative to the number of spores observed in the control experiment.

3.4. Abnormal morphology caused by antifungal fractions

To investigate the effects of antifungal substances on the morphology of fungi, *C. acutatum* spores were cultured for 12 h in liquid potato dextrose medium supplemented with 0.4 mg/mL fraction 12-6-2, and the growth was observed under optical and electron microscopy.
microscopes. While the control spores germinated normally and produced viable mycelia, germination and growth were not observed in the presence of the antifungal fractions. Electron micrographs revealed shrunken, wrinkled spores and hyphae following treatment with the antifungal extracts, compared to the smooth surface of control spores and fraction 12-6-3 also exhibited similar structural damage to the spores and hyphae of *F. oxysporum* (Figure 3). Combined with the data above, these data suggest that the killing effects of the antifungal fractions are directly related to the structural damage to both spores and mycelium.

GC-MS analysis of the antifungal fractions revealed that propyl hexanoate, levoglucosan, 9-octadecenamide, and dipeptide, Leu-Pro were the major components (data not shown). Among them, antifungal activity and antibacterial activity of Leu-Pro, in the form of cyclo-(Leu-Pro), have been reported most frequently [12]. 9-Octadecenamide has also been reported to have antimicrobial activity [13]. Antifungal activities of other substances have not been known. Therefore, antifungal activity of *S. blastmyceticus* strain 12-6 is thought to be due to synergistic effect of two compounds, cyclo-(Leu-Pro), and 9-octadecenamide.

4. Discussion

The isolated fungicides acted on both spores and hyphae of *C. acutatum* and *F. oxysporum*, completely inhibiting spore germination and mycelial growth. These antifungal activities appeared comparable to that of natamycin against *F. oxysporum* and *V. fungi-cola* spores (39% and 82%, respectively) [11]. Natamycin has an MIC < 10 ppm with superior activity against *Aspergillus* sp. and *Fusarium corneal* [14]. The electron micrographs showed that the antifungal agents of the strain 12-6 induced the abnormal crumpled surface structure of the spores and hyphae. This strongly suggested that the action mechanism of the antifungal agents be related to the membrane structure. Fluorescence staining with TOTO-1 also indicated increased membrane permeability. Taken together, these data suggest that the antifungal agents described here are similar to natamycin, an amphoteric macrolide of *S. natalensis* that binds to ergosterol in the cell membranes of fungi [14].

GC/MS analysis of the components of each TLC spot showed that 9-octadecenamide was most abundant. 9-Octadecenamide has been found in...
cerebrospinal fluid, and its physiological functions to induce sleep in animals and potential utilization as a treatment for cannabinoid-regulated depression have been known. Occasionally, 9-octadecenamide is also present as a major component in bacteria and has been reported to have antimicrobial activity [15]. Meanwhile, the more comprehensively studied antimicrobial agents found in bacteria was the cyclic dipeptides. It has been known as one of the cyclic dipeptides with antimicrobial activity [12]. For example, cyclo(Leu-Pro) of Streptomyces sp. KH-614 has been reported to be active against pathogenic fungi such as Pyricularia oryzae and Trichophyton rubrum as well as vancomycin-resistant enterococci [16,17]. Cyclo(Leu-Pro), which was also found in the culture of Lactobacillus casei, showed antifungal activity against Penicillium sp. [18]. Cyclo(Leu-Pro) purified from the culture medium of Lactobacillus plantarum was found to inhibit the growth of Ganoderma boninense and Candida albicans [19]. It has been reported that cyclo(Leu-Pro) exists in the culture medium of Leuconostoc mesenteroides or in kimchi prepared with this bacterium [20]. According to recent reports, cyclic peptides can penetrate the lipid bilayer layer of the cell membrane, destroying the membrane structure and changing the membrane permeability to exhibit antimicrobial activity [21]. Therefore, S. blastmyceticus strain 12-6 used in this study produced 9-octadecenamide and cyclo(Leu-Pro), and their cooperative activities were thought to be the cause of antifungal activity.

Disclosure statement

No potential conflict of interest was reported by the authors.

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