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Production of an antifungal protein for control of *Colletotrichum lagenarium* by *Bacillus amyloliquefaciens* MET0908

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Abstract

A plant pathogenic fungus, *Colletotrichum lagenarium*, causing watermelon anthracnose, was isolated from naturally infected leaves, stems, and fruits of watermelon. A bacterial strain, MET0908, showing a potent antifungal activity against *C. lagenarium*, was isolated from soil. An antifungal protein was purified by 30% ammonium sulfate saturation and concentrated using Centricon 10, DEAE–Sepharose™ Fast Flow column and Sephacryl S-100 gel filtration chromatography. The molecular weight of the purified protein was estimated as 40 kDa by SDS–PAGE. The purified protein was stable at 80 °C for 20 min and exhibited a broad spectrum of antifungal activity against various plant pathogenic fungi. Confocal microscopy image analysis and scanning electron microscopy showed that the protein acted on the cell wall of *C. lagenarium*. The purified antifungal protein exhibited β-1,3-glucanase activity. The N-terminal amino acid sequence of the purified protein was determined as Ser-Lys-Ile-x-Ile-Asn-Ile-Asn-Ile-x-Gln-Ala-Pro-Ala-Pro-x-Ala. A search of the sequence with NCBI BLAST showed no significant homology with any known proteins, suggesting that the purified protein may be novel.

Keywords: *Colletotrichum lagenarium*; Watermelon anthracnose; *Bacillus amyloliquefaciens*; Antifungal agent; β-Glucanase

1. Introduction

Watermelon is widely cultivated and consumed around the world. Anthracnose, which causes serious problems for the cultivation of watermelon, is caused by the fungal pathogen, *Colletotrichum lagenarium* [1]. To control it, agricultural chemicals have been used for a long time. Their excessive use has led to environmental degradation and pollution [2]. Such chemicals can be lethal to useful soil insects and beneficial microorganisms in the rhizosphere, and they may also enter the food chain [3]. Moreover, their efficiency is decreased due to the development of resistant pathogens [4]. Alternative control methods include the use of biological control microorganisms, and numerous studies have been attempted over the last 25–30 years [5–8]. The potential use of microorganisms to control soilborne plant pathogens, such as *C. lagenarium*, offers a non-polluting alternative. Biocontrol mechanisms are classified based on their mode of action, such as root colonization, degradative parasitism, antibiosis or competitive antagonism [9–11]. In controlling fungal plant pathogens, a variety of mechanisms contribute to the biocontrol activity of microbes. Cell-wall-degrading enzymes (CWDEs), such as β-1,3-glucanases, cellulases, proteases, and chitinases, are involved in the antagonistic activity of some biological control agents against phytopathogenic fungi [12–16]. In particular, numerous correlations between fungal antagonism and bacterial production of chitinases and/or β-1,3-glucanases have been noted [17].

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Chitin and β-1,3-glucans are major constituents of many fungal cell walls, and various workers have demonstrated in vitro lysis of fungal cell walls either by microbial chitinase or β-1,3-glucanase [17–19]. Understanding the mode of action of biocontrol agents is a prerequisite for: (1) developing rational procedures to select more effective antagonistic microbial strains, (2) developing appropriate production and formulation methods that enhance biocontrol activity, and (3) fulfilling some requirements of the toxicological and registration packages needed for commercial development. The objectives of this study were to isolate the fungal pathogen that causes watermelon anthracnose from naturally infected plants, and then screen antifungal bacteria from soil for use in biological control. Also, antifungal substances produced by the antagonists were purified, characterized, and finally applied to an agricultural system as natural biological agents against various plant pathogens.

2. Materials and methods

2.1. Microorganisms, culture and growth conditions

The phytopathogenic fungi, *C. lagenarium* KACC 40809, *Fusarium graminearum* KACC 40532, *Didymella bryoniae* KACC 40669, and *Colletotrichum gloeosporioides* KACC 40003 were obtained from Korean Agricultural Culture Collection, Suwon, Korea. *Pythium ultimum* PAT0918, *Phytophthora capsici* PAT0928, and *Monosporascus cannonballus* PAT0938 were obtained from the Laboratory of Phytopathology, Chonnam National University, Kwangju, Korea. The pathogenic fungi were incubated at 24–28 °C and maintained on potato dextrose agar (PDA, Difco). The bacterial antagonistic isolates were isolated from soil in a diseased watermelon field in Dongkang-Myun, Naju, Korea. Tenfold serial dilutions from soil suspensions in phosphate-buffered saline (PBS, 0.05 M, pH 7.4) were plated on Luria–Bertani (LB) agar. The plates were incubated at 37 °C and kept on LB broth at −80 °C as stocks.

2.2. Isolation and pathogenicity testing of C. lagenarium

Diseased watermelon leaves, stems, and fruits were collected from various fields in Dongkang-Myun, Naju, Korea. The samples were maintained in sterilized disposable tubes, then incubated on PDA at 24 °C for 5 days. The isolated pathogens were stored at 4 °C for further study. The morphological characteristics of the pathogenic fungus were examined, and compared with *C. lagenarium* KACC 40809 under a microscope, and then identified as *C. lagenarium* [20]. To test pathogenicity, isolated strains were inoculated on leaves and stems of watermelon plants and grown for 30 days in a greenhouse at 30 °C and 95% relative humidity. For controls, uninoculated plants were incubated under the same conditions.

2.3. Screening and identification of antagonistic bacteria

Antagonistic activity of bacterial isolates against *C. lagenarium* was measured according to a modified method of Lim et al. [17]. Soil samples (1 g) were suspended in 9 ml of PBS and then diluted serially. The diluted samples were incubated on LB agar at 37 °C for 1 day and the bacterial isolates were obtained. *C. lagenarium* was inoculated on both sides of PDA plates and then incubated at 28 °C for 2 days. The bacterial isolates then were streaked in the center, 60 mm away from the fungus. Inhibition of mycelial growth in millimeters was measured after further incubation for 5 days. Among the isolates, one strain showing the strongest activity was selected. To identify the bacterial strain, gram staining, a catalase test, morphology and carbon source utilization patterns were assayed. The selected antagonistic bacterial strain was identified according to the methods described in standard reference manuals [14,15,21,22] and the MicroLog™ System, Release 4.0 (INSUNG Chromatec Co., Korea).

2.4. Antifungal activity of co-culture filtrates

Antifungal activity of the isolates against *C. lagenarium* was assayed according to the method of Roberts et al. [23]. The fungus was grown in PD broth for 2 days at 30 °C, then the antifungal bacteria were inoculated and co-cultured. Their cell-free filtrates were collected by centrifugation and the supernatants were concentrated by Centricon 10 (Amicon Grace Co., MA, USA) followed by aseptic filtration (0.45 μm). A paper disk containing culture filtrates (40 μl) was placed in the center of a plate with the fungal pathogen, then the growth of the fungus was measured after 3 days of incubation at 30 °C.

2.5. Purification of antifungal agent

To purify the antifungal agent, solid ammonium sulfate was added to the co-culture supernatant to 30% saturation and stored overnight at 4 °C. The resulting precipitates were harvested by centrifugation at 42,000*g* for 40 min at 4 °C. The pellets were dissolved in 25 mM of Tris–HCl buffer (pH 8.1) and dialyzed with the same buffer for 24 h with three changes. The resulting protein solution was applied to a DEAE–Sepharose™ Fast Flow column equilibrated with 25 mM of Tris–HCl buffer (pH 8.1). After washing the column with the same buffer, the absorbed proteins were eluted with a linear

gradient formed from two buffer solutions containing 0 and 1.0 M NaCl. The active fractions were pooled and concentrated and then the antifungal activity was monitored as described above. The concentrated antifungal protein was applied to a Sephacryl S-100 column equilibrated with 25 mM of Tris–HCl buffer (pH 8.1) containing 0.15 M NaCl. Elution was carried out with the same buffer. The active-peak fractions were pooled, concentrated, and used as the purified antifungal protein source. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to check the protein purity and to determine the molecular weight of the protein as described by Laemmli [24].

2.6. Thermal stability and antifungal spectrum

The effect of temperature on antifungal activity was evaluated in the range of 30–100 °C for 20 min and the remaining antifungal activity was assayed by the previously described paper disk method. To examine the antifungal spectrum, phytopathogenic fungi, including *Fusarium graminearum* KACC 40532, *Didymella bryoniae* KACC 40669, and *Colletotrichum gloeosporioides* KACC 40003, *Pythium ultimum* PAT0918, *Phytophthora capsici* PAT0928, and *Monosporascus cannonballus* were used.

2.7. Confocal microscope image analysis and scanning electron microscopy

To analyze intracellular distribution of the purified protein, confocal laser scanning microscopy was performed as described by Kim et al. [9]. Fungal cells were incubated with FITC-labeled proteins for 6 h at 30 °C. After incubation, cells were harvested by centrifugation and washed with PBS. Visualization and localization of the labeled protein was performed using a Leica TCS 4D camera connected to a Leica DAS upright microscope (Leica Lasertech. GmbH, Heidelberg, Germany). Scanning electron microscopy was performed according to the method of Barbara et al. [25]. The subcultured fungal pathogen was incubated at 30 °C for 1–3 days with 120 mg/ml of the purified protein. The cells were fixed with an equal volume of 4% glutaraldehyde and 1% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2). After fixation at 30 °C for 3 h, the samples were centrifuged at 1500*g* and washed with the same buffer. The fungal cells were dehydrated in graded ethanol. After lyophilization and gold coating, the fungal cells were examined on a HITACHI S-2400 electron microscope (Tokyo, Japan).

2.8. Enzyme assay and N-terminal amino acid sequencing

To determine substrate specificity to cell wall materials, the purified protein was mixed with colloidal chitin, colloidal chitosan, cellulose, and laminarin (β-1,3-glucan). All enzyme assays were carried out at 50 °C for 15 min in 100 mM sodium acetate buffer (pH 5.0) in a reaction volume of 200 μl with a final substrate concentration of 0.66% (w/v). The hydrolysis of each substrate was quantified by measuring the reducing sugars released by the dinitrosalicylic acid method of Miller [26]. The concentration of protein was determined by the method of Lowry et al. [27] and the protein concentration of column fractions was measured by absorbance at 280 nm. Total activity is defined as the number of μmol of glucose released per ml of filtrate per hour, while specific activity is defined as the ratio of glucose released to the amount of protein in the culture filtrates. The N-terminal sequence of the antifungal protein was determined using automated Edman degradation with Perkinelmer Procise (USA) from the National Instrument Center for Environmental Management (NICEM), Seoul National University, Korea. The amino acid sequence was compared with others in the National Center for Biotechnology Information (NCBI) database by using the BLAST program.

3. Results

3.1. Screening and identification of antagonist

For the selection of a potential antagonist to inhibit *C. lagenarium*, over 250 bacteria were isolated from soil. Among these isolates, only strain MET0908 showed a powerful antagonistic effect (Fig. 1(A)), and the supernatant of a co-culture with *C. lagenarium* inhibited mycelial growth (Fig. 1(B)). However, antifungal activity was not observed when MET0908 was grown in pure culture. This was confirmed by using the supernatant of bacteria grown in the absence of fungal mycelium. MET0908 was gram-positive, catalase-positive, motile, endospore-forming, rod shaped and aerobic. Based on the MicroLog™ system, MET0908 utilized α-D-glucose, D-mannose, sucrose, turanose, pyruvate, glycerol, glycogen, alanyl-glycine, and alanine as carbon sources, and was identified as *Bacillus amyloliquefaciens* by this data base.

3.2. Purification and antifungal spectrum of protein

An antifungal protein was purified by ammonium sulfate fractionation, DEAE–Sepharose™ Fast Flow, and Sephacryl S-100 column chromatography. A Coomassie blue-stained SDS–PAGE gel revealed a single band with molecular weight of about 40 kDa (Fig. 2). The band showed antifungal activity against *C. lagenarium* and other plant pathogens, including *F. graminearum*, *D. bryoniae*, *C. gloeosporioides*, *P. ultimum*, *P. capsici*, and *M. cannonballus* (data not shown).

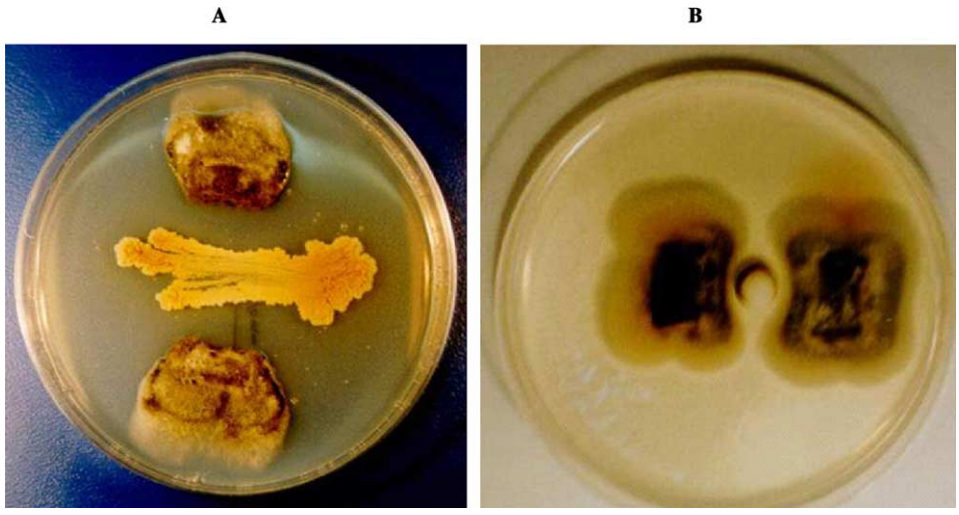

Fig. 1. Antifungal activity of antagonist (A) and co-culture filtrate (B) against *Colletotrichum lagenarium*.

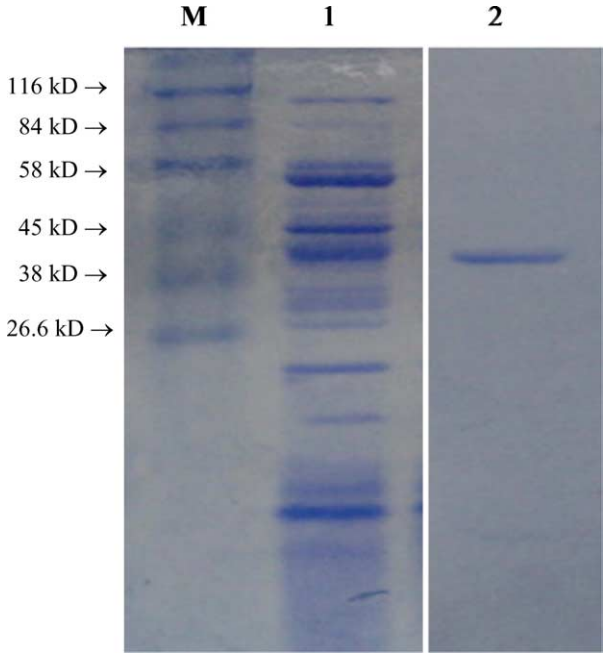

Fig. 2. SDS–PAGE of the purified protein from *Bacillus amyloliquefaciens* MET0908. Lane M, protein marker; lane 1, crude extract; lane 2, purified protein.

3.3. Mode of action of the antifungal protein

3.3.1. Confocal microscopy image analysis

To track the antifungal protein after application to the pathogen, it was labeled with FITC. FITC-labeled protein inside hyphae was easily detected by fluorescent imaging in confocal microscopy. The results showed that the purified protein accumulated at the hyphal septa (Fig. 3(B)), but in hyphae incubated without the protein, there was no detectable fluorescence inside the cells (Fig. 3(A)).

3.3.2. Scanning electron microscopy

Morphological changes of the pathogen were examined for 3 days after treatment with the protein. After 1 day of incubation, the hyphal septa were abnormally swollen (Fig. 4(B)). Most hyphae were degraded after 2 days (Fig. 4(C)) and then burst (Fig. 4(D)). Control cultures showed no morphological changes for 3 days (Fig. 4(A)). From these results, it was thought that the mode of action of the antifungal protein is disruption of the cell wall.

3.4. Enzyme assay and N-terminal amino acid sequence

The antifungal protein was examined for its ability to hydrolyze cell wall-related substrates. The purified protein exhibited no activity on colloidal chitin, colloidal chitosan, and cellulose under standard assay conditions. However, laminarin (β-1,3-glucan), which is a component of the fungal cell wall, was effectively hydrolyzed by the protein (data not shown). Crude extracts and fractions from the purification steps were collected and analyzed for enzyme activities and protein concentrations. The results of purification are summarized in Table 1. Antifungal activity of the protein was unaffected by temperatures of 30–70 °C. The remaining antifungal activity after 20 min at 80 and 90–100 °C was 70% and 50%, respectively (Table 2). The N-terminal amino acid sequence of the purified protein was determined as: Ser-Lys-Ile-x-Ile-Asn-Ile-Asn-Ile-x-Gln-Ala-Pro-Ala-Pro-x-Ala. (x means undetermined amino acids). A data base search for comparable proteins and corresponding amino acid sequences was performed at the (NCBI) using the BLAST network service. The sequence of the protein showed no homology with any other β-1,3-glucanases, including antifungal proteins, so these results suggest that the purified protein may be novel.

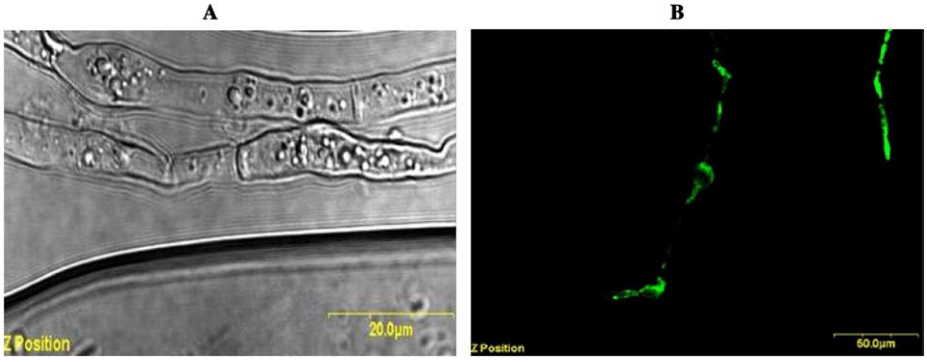

Fig. 3. Confocal microscopy of hyphae of *C. lagenarium* treated with antifungal protein. Hyphae were treated for 6 h at 30 °C with 80 mg/ml FITC-labeled antifungal protein (B) or untreated with labeled antifungal protein (A). The fungi were washed with PBS buffer (pH 7.4) and visualized by laser-scan microscopy. (A) is a optical image of the control, (B) is a fluorescence image of hyphae treated with FITC-labeled antifungal protein.

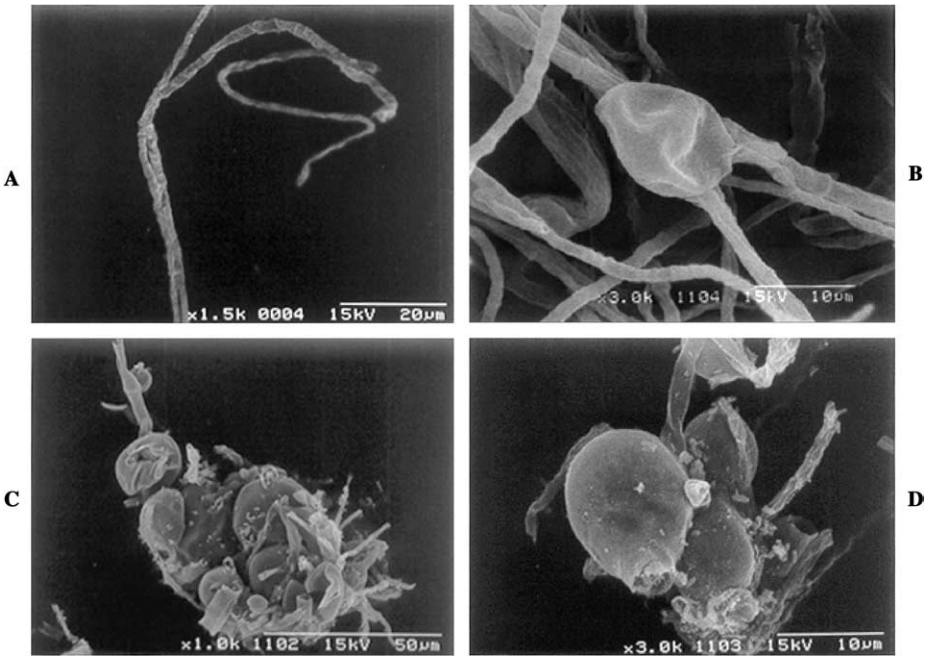

Fig. 4. Scanning electron microscopy of mycelium treated with the antifungal protein for 1–3 days at 30 °C. Fungal mycelia were treated with antifungal protein (120 mg/ml). After incubation for 1–3 days, morphological changes were investigated. (A) Control; (B) 1 day; (C) 2 days; (D) 3 days. The control was normal mycelium incubated at 30 °C for 3 days.

Table 1
Purification of the antifungal protein from *B. amyloliquefaciens* MET0908

Step	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold
Culture filtrate	189.2	15.2	12.44	100	1.0
$(NH_4)_2SO_4$	161.7	8.96	18.1	85.4	1.46
DEAE–Sepharose	96.9	1.48	65.5	59.9	3.62
Sephacryl S-100	66.9	0.156	428.8	69.0	6.55

4. Discussion

In most fungi, chitin and non-cellulosic β-glucans are the most abundant skeletal or microfibrillar components, while proteins and β-glucans are the main cementing components [28]. β-1,3-Glucanase (EC 3.2.1.39) and β-1,3-1,4-glucanase (EC 3.2.1.73) constitute the glycosyl hydrolase family 16 [29]. The former catalyzes the cleavage of β-1,3-linkages of β-1,3-glucan and shows antifungal activity, whereas the latter hydrolyzes

Table 2
Effect of temperature on stability and activity of the antifungal protein

Temperature (°C)	Inhibition distance (mm)	Antifungal activity (%)
4	15 ± 0.3	100
30	15 ± 0.3	100
40	15 ± 0.3	100
50	15 ± 0.3	100
60	15 ± 0.5	100
70	13.5 ± 0.5	90
80	10.5 ± 1.0	70
90	7.5 ± 1.0	50
100	7.5 ± 1.0	50

β-1,4-glucosidic bonds adjacent to β-1,3-linkages in β-1,3-1,4-glucans. In our study, *B. amyloliquefaciens* MET0908 secreted an extracellular β-1,3-glucanase, which is a key enzyme in the decomposition of fungal hyphal walls [17–19], when co-cultured with *C. lagenarium*. Confocal image analysis microscopy showed that the antifungal agent was embedded in the septa of the hyphal wall of the pathogen. Scanning electron microscopy revealed abnormal swelling, degradation, and burst by the excretion of lytic enzymes from the antagonist. Applications of β-glucanases, such as lichenase, in processing of animal feed, control of fungal pathogens, and release of intracellular materials from microbial cells have been reviewed [30]. β-1,3-Glucanase produced by *B. amyloliquefaciens* MET0908 showed strong activity against plant pathogens in a watermelon pot assay test and was stable at high temperatures. In our experiments we only used 120 mg/ml of protein and showed that it killed the fungal mycelium. It is possible that lower concentration which were not tested also would have been effective. From these results, we suggest that *B. amyloliquefaciens* MET0908 would seem to be a good biocontrol-candidate and a successful antagonist, although the question remains as to how effective this antagonistic bacterium would be under field conditions. The production of lytic enzymes during mycoparasitism may play a role in the biocontrol of fungal pathogens, including *C. lagenarium*. Also, this β-1,3-glucanase may be useful in the brewing and animal feedstuff industries because of its demonstrated thermostability, although further evidence is needed.

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