J. Microbiol. Biotechnol. (2000), **10**(6), 805–810

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Purification and Characterization of a Novel Antifungal Protein from *Paenibacillus macerans* PM1 Antagonistic to Rice Blast Fungus, *Pyricularia oryzae*

BAE, DONG-WON[1], YEON-SIK KAWK[1], JOON-TAEK LEE[1,2], DAE-YOUNG SON[2,3], SUNG-SIK CHUN[4], AND HEE-KYU KIM[1,2]*

[1]*Department of Agricultural Biology,* [2]*Genetic Engineering Institute*
[3]*Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Chinju 660-701, Korea*
[4]*Department of Food and Nutrition College Chinju, Chinju 660-759, Korea*

Received: July 24, 2000
Accepted: November 1, 2000

Abstract An antifungal protein antagonistic to the rice blast fungus, *Pyricularia oryzae* was purified from *Paenibacillus macerans* PM-1 by ammonium sulfate fractionation, Q Sepharose Fast Flow column chromatography, Phenyl Sepharose CL-4B column chromatography and Superose 12 gel filtration. An apparent molecular mass of the purified antifungal protein was determined as 8 kDa by SDS-PAGE and 9 kDa by analytical gel filtration, respectively, suggesting that the purified protein is a monomer. The antifungal protein was stable at pH range from 7–12 and up to 100°C. The protein was also stable at 0.1–1% Tween 20 and Triton X-100. The N-terminal amino acid sequence of the antifungal protein was Thr-Glu-Leu-Pro-Leu-Gly-Ile-Val-Met-Asp-Lys-Tyr-Thr-Asp-Ala-Phe-Lys-Phe-Asp-Met-Phe. Comparison of the determined sequence with other peptide and DNA sequences did not reveal homology at all. Therefore, the purified antifungal protein was speculated to be a novel protein. The conidial germination *in vitro* of *P. oryzae* KJ 301: 93–39 by the purified protein (5.9 μg/ml) was limited to 9±3.2% only, compared with 69±2.4% of the control. Ungerminated conidia were swollen at basal and mid cell by the purified protein. *In vivo* bioassay for inhibition of conidial germination of *P. oryzae* KJ 301, one of the most predominating races in Korea, the purified protein (5.9 μg/ml) strongly inhibited the conidial germination. The conidia, even though germinated, could not develop any further to produce appressoria efficiently.

Key words: *Pyricularia oryzae*, *Paenibacillus macerans*, antifungal activity, antifungal protein

*Corresponding author
Phone: 82-55-751-5443; Fax: 82-55-751-5199;
E-mail: heekkim@nongae.gsnu.ac.kr

The problems of fungicide resistance of phytopathogens and side effects which are unfavorable to environment have been caused by careless use of synthetic fungicides for the control of plant diseases [7, 8]. For instance, *P. oryzae* is the causal agent of rice blast that is the most destructive disease of rice throughout the rice-growing areas of the world. The frequent appearance of new races has reduced the effectiveness of resistant cultivars in the field [21]. Furthermore, environmental regulations have also restricted the use of chemical fungicides. These problems stimulated researchers to undertake screening for safe and potential compounds from microorganisms. Thus blastidin S [23] and kasugamycin [25] were discovered and introduced into the agricultural field for biocontrol of the rice blast caused by *P. oryzae*. These antibiotics of microbial origin have few side effects to the environment, little toxicity to plants, selective activity against plant pathogens, rapid decomposition after use and no residual toxicity in soils [19]. Recently, an antagonistic microorganisms for the biocontrol of plant diseases were isolated from *Trichoderma* spp., *Streptomyces* spp., *Agrobacterium* spp., *Pseudomonas* spp. and *Bacillus* spp. [11]. Most of the studies conducted on the antifungal proteins for the biocontrol of plant diseases were mostly pathogenesis-related (PR) and stress-responsed proteins [4, 6, 9, 20]. A novel antifungal antibiotic was reported to be produced by *Pseudomonas cepacia* [14]. Plant antifungal proteins from *Raphanus sativus* L. seeds [24], thermostable antifungal peptide (1,044 Da) from *B. amyloliquefaciens* [26] and antifungal metabolite from *Bacillus* spp. [10] were isolated and reported. Also, antifungal activity of aliphatic alkenals on *Saccharomyces cerevisiae* has been screened [16]. An antifungal protein BI from *B. subtilis* TG 26 antagonistic to *P. oryzae* was purified and characterized [17].

We previously reported antifungal substances antagonistic to *P. oryzae* from *Paenibacillus macerans* PM-1 [2]. During the course of the experiments, we found that the substances were produced extracellularly as well as intracellularly by the strain. In this paper, we described purification and characterization of the novel antifungal protein secreted from the strain.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Paenibacillus macerans PM-1 was cultivated in potato dextrose broth (Difco, pH 10.0) at 20°C for 24 h at 160 rpm. The cells were harvested by centrifugation at 8,000 rpm for 30 min.

Antifungal Protein Assay

The culture supernatant and protein solution were filtered with cellulose acetate membrane filter (pore size, 0.22 μm). The antifungal activity was measured by the method of hyphal extension-inhibition assay [22].

Protein Measurement

Protein concentration was measured by following the Bradford method using bovine serum albumin (BSA) as a standard [5].

Purification of Antifungal Protein

The culture supernatant was brought to 50% saturation with solid ammonium sulfate and centrifuged at 8,000 rpm for 30 min. Solid ammonium sulfate was added to the supernatant to reach 90% saturation. After centrifugation, the pellet was dissolved in a minimal volume of 50 mM Tris-HCl buffer (pH 7.5). The protein solution was dialyzed against the same buffer. Sodium chloride was added to the dialyzate to 0.2 M and the solution was centrifuged at 8,000 rpm for 30 min. The supernatant was applied to a Q Sepharose Fast Flow column (2.5×15 cm, Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl. After washing with the same buffer, the column was eluted at a flow rate of 2 ml min^{-1} (fraction size: 10 ml) with a linear gradient of 0.2–1 M NaCl. The active fractions were pooled and solid ammonium sulfate was added to 1 M. The supernatant that was recovered by centrifugation was put on a Phenyl Sepharose CL-4B column (2.5×15 cm, Pharmacia) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 1 M ammonium sulfate. After washing with the same buffer, elution was performed at a flow rate of 2 ml min^{-1} (fraction size: 10 ml) with a reverse gradient of 1–0 M ammonium sulfate. The active fractions were pooled and dialyzed with 50 mM sodium phosphate buffer (pH 7.0). Sodium chloride was added to the dialyzate to reach 0.15 M. The solution was centrifuged and concentrated to a small volume by ultrafiltration. The concentrate was put on a Superose 12 HR 10/30 column (1×30 cm, Pharmacia) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl, and eluted with the same buffer. The active fractions were then collected and stored at -20°C.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed on a 15% gel as described by the method of Laemmli [13]. Molecular mass markers (Pharmacia) were phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa).

N-Terminal Amino Acid Sequencing

Amino-terminal amino acid sequence analysis was carried out by automated Edman degradation in a 476A Protein sequencer (Applied Biosystems Inc., Foster City, CA) [18].

pH Stability

The purified protein solution was added to 0.1 M citrate (pH 4.0, 5.0), sodium phosphate (pH 6.0, 7.0), Tris-HCl (pH 8.0, 9.0), glycine-NaOH (pH 10.0), sodium phosphate-NaOH (pH 11.0), and KCl-NaOH (pH 12.0, 13.0) buffers. After incubation for 1 h at room temperature, the residual activity was measured.

Thermostability

Protein stability was studied over the range of 20–100°C. Pure protein was incubated for 30 min, aliquots were withdrawn, and assayed.

Surfactant Stability

The purified protein solution was incubated with Tween 20 and Triton X-100 at a concentration of 0.1% and 1% at room temperature for 1 h, and the residual activity was measured.

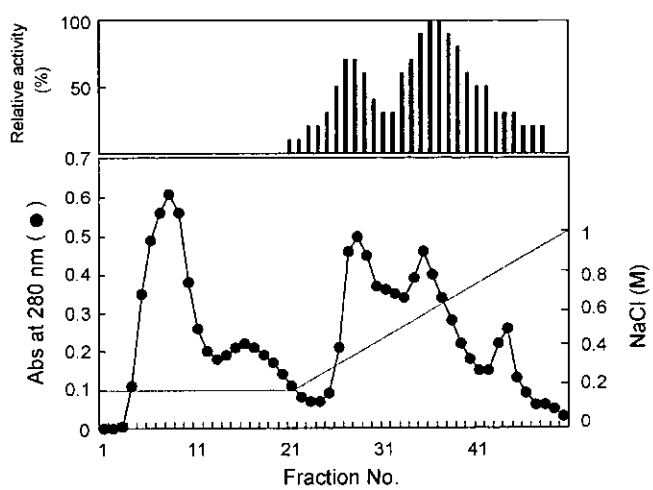

Fig. 1. Q Sepharose Fast Flow column chromatogram of *P. macerans* PM-1 crude protein.

The protein was eluted with a linear gradient of NaCl in 50 mM Tris-HCl buffer (pH 7.5) at 2 ml/min. The volume of each fraction was 10 ml.

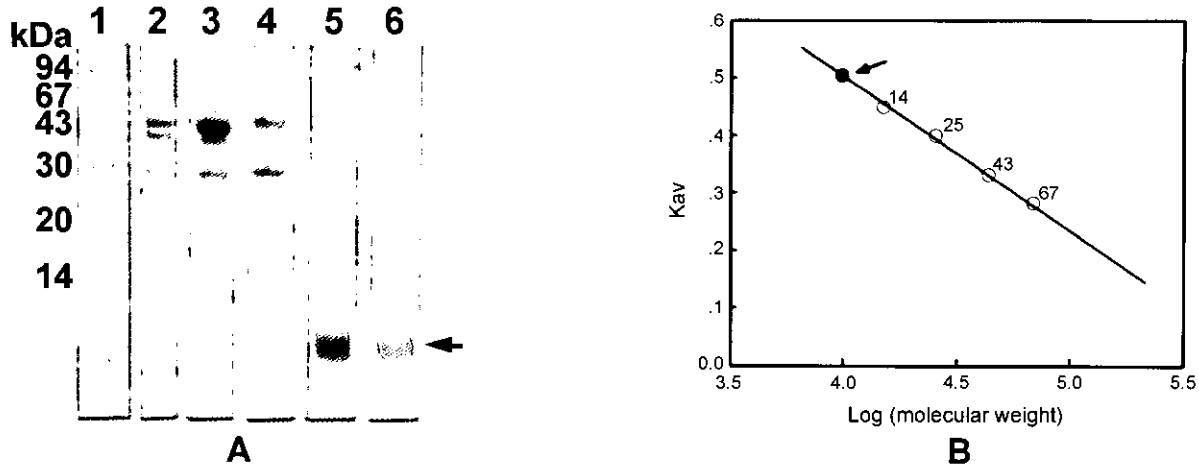

Fig. 2. SDS-PAGE of purified antifungal protein in *P. macerans* PM-1 at various steps (A) and molecular weight determination by gel permeation chromatography (B).

A: Lane 1, size marker; lane 2, culture supernatant; lane 3, after ammonium sulfate precipitation; lane 4, after Q Sepharose Fast Flow column chromatography; lane 5, after Phenyl Sepharose CL-4B column chromatography; lane 6, after Superose 12 gel filtration. B: The arrow indicates the position of the antifungal protein (●). Standard marker protein (○) used are as follow; 14 kDa, ribonuclease A; 25 kDa chymotrysinogen; 43 kDa, ovalbumin; 67 kDa, BSA.

Inhibition of Conidial Germination

The purified antifungal protein (5.9 μg/ml) was mixed with the conidial suspension (2.5×10^6/ml) of *P. oryzae* KJ 301: 93-39 (1:3, v/v), and the mixtures were placed on the hole slide glasses. The slide glasses were placed into the glass plates equipped with two glass rods on the moistened filter paper. After incubation of the plates at 28°C for 24 h, conidial germination was measured under a light microscope (Carl Zeiss SF, ×100). The ratio of conidial germination (%) was calculated as follows:

$$\text{Ratio of conidial germination (\%)} = \frac{\text{germinated conidia}}{\text{total conidia}} \times 100$$

In vivo Bioassay

A rice cultivar Nagdongbyeo susceptible to *P. oryzae* KJ 301 race was grown for 5 weeks in the greenhouse. Both 10 μl of conidial suspension (2.5×10^6 conidia of KJ 301: 93-39/ml) in Tween 20 and 40 μl of either culture supernatant or purified protein were mixed and applied to the rice leaf blades (about 5 cm), which were then placed in the humidity chamber at 28°C for 2 days. The germination and mycelial growth were examined under a light microscope (Carl Zeiss, Axiovert 135M, ×200).

RESULTS AND DISCUSSION

Purification of Antifungal Protein

Antifungal proteins were eluted from the Q Sepharose column at about 0.3-0.4 M and 0.5-0.8 M NaCl (Fig. 1). The eluate of the latter showing main antifungal activity was absorbed onto the Phenyl Sepharose column. The active fractions were eluted at about 0 M $(NH_4)_2SO_4$. After following the gel permeation chromatography on Superose 12, an antifungal protein was finally purified. The purified antifungal protein was homogeneous as seen by

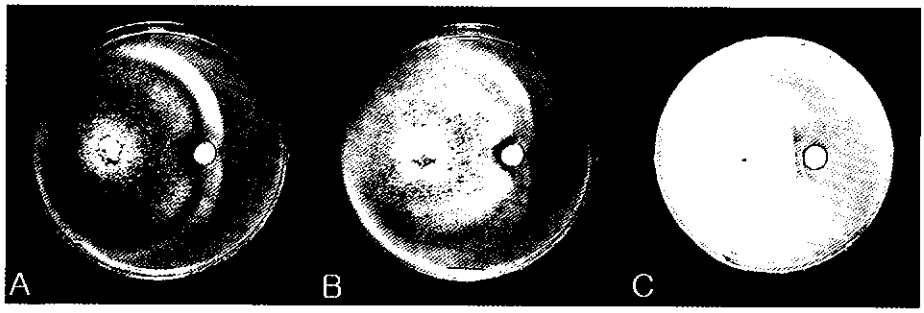

Fig. 3. Inhibition of hyphal growth of *P. oryzae* by purified protein.

A, KJ 101: 5-21, B, KJ 201: 88-62, C, KJ 301: 93-39. The PDA plates were inoculated with isolates of rice blast fungus. The inoculated plates were incubated at 28°C for 2 days prior to applying 50 μl (5.9 μg/ml) of purified protein per paper disc. The results were obtained by further incubation of the plates for 7 days.

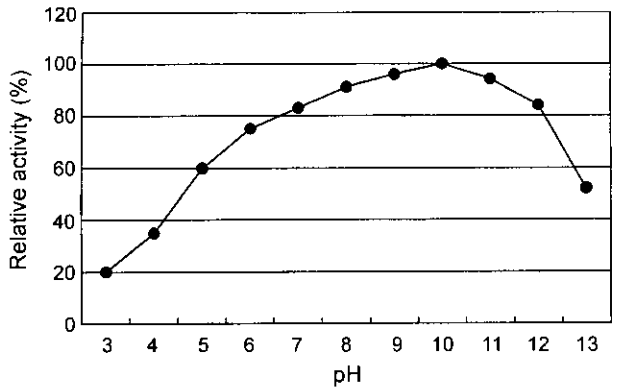

Fig. 4. Effect of pH on the stability of *P. macerans* PM-1 antifungal protein.
Purified antifungal protein was bioassayed after incubation of the reaction mixture at various pH values for 1 h.

SDS-PAGE (Fig. 2A). An apparent molecular mass of the protein by SDS-PAGE was 8 kDa. Gel filtration of the Superose 12 10/30 column indicated that the protein has an apparent molecular mass of 9 kDa (Fig. 2B). These results suggest that the purified protein has a monomeric structure. Recently, new approaches have been reported to develop antibiotic peptides that has powerful antifungal activity with little or no hemolytic activity [15].

N-Terminal Amino Acid Sequence

The N-terminal amino acid sequence of the purified protein was determined as Thr-Glu-Leu-Pro-Leu-Gly-Ile-Val-Met-Asp-Lys-Tyr-Thr-Asp-Ala-Phe-Lys-Phe-Asp-Met-Phe. A data base search for comparable peptides and corresponding nucleotide sequences was performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service [1]. The sequence of the purified protein showed no homology with any other proteins as well as with antifungal proteins and this suggests that the purified protein is a novel.

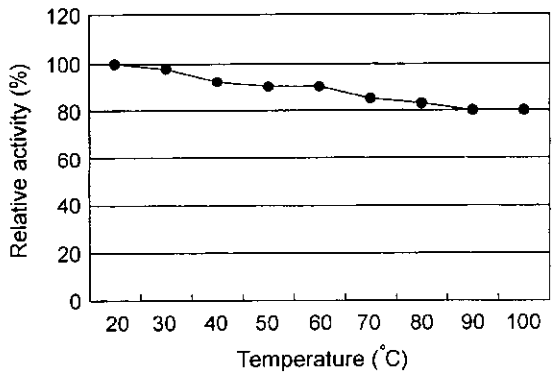

Fig. 5. Effect of temperature on the stability of *P. macerans* PM-1 antifungal protein.
Purified antifungal protein was bioassayed after incubation reaction mixture at various temperature for 30 min.

Table 1. Inhibitory activity of the purified antifungal protein from *P. macerans* PM-1 on conidial germination of *P. oryzae*.

Isolates	Treatment	Germination (% ±SD)
KJ 301: 93-39	Purified protein	9±2.4
	Control (distilled water)	69±3.2

Purified protein (5.9 μg/ml) was filtered through 0.22 μm membrane filter and the aliquots of 40 μl of purified protein were mixed with 10 μl of conidial suspension of 2.5×10^6 per ml. From this mixture aliquots of 30 μl were transferred to the hole slide glass in triplicates on the moisted filter paper supported with two glass rods in a petri-dish. The petri-dish was incubated at 28°C for 24 h. Conidial germination was examined under a light microscope at ×100 magnification.

Antifungal Activity

The antifungal activity of the purified protein was investigated by the inhibition of hyphal extension against three races of *P. oryzae* (KI 101: 5-21, KJ 201: 88-62, KJ 301: 93-39) on potato dextrose agar media. As shown in Fig. 3, this protein showed antifungal activity against all races of *P. oryzae*. Especially *P. oryzae* KJ 301: 93-39 was shown to be very susceptible to protein.

Physiological Properties

The purified antifungal protein (5.9 μg/ml) was stable at pH between 7.0– 12.0 but it was drastically inactivated at pH 13.0 (Fig. 4). The protein was significantly stable up to 60°C and showed about 80% of residual activity at 100°C (Fig. 5). These results suggest that the purified protein is an alkaline and very thermostable form. In fact, antifungal substances from other microorganisms had shown similar

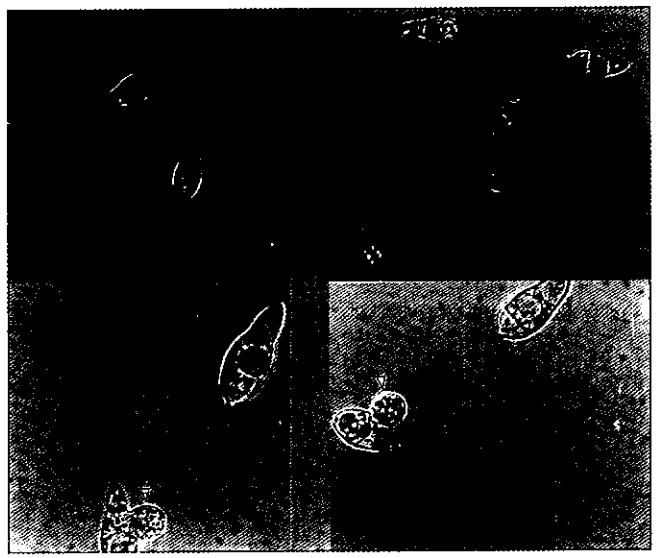

Fig. 6. Ungerminated and swollen conidia of *P. oryzae* race KJ 301: 93-39 in the presence of antifungal protein purified from *P. macerans* PM-1.
Hole slide glasses carrying 30 μl of conidial suspension of 2.5×10^6 per ml were incubated at 28°C for 10 h followed by a light microscopy at ×400 or ×630 magnification.

properties. Antifungal antibiotics from *B. subtilis* YB-70 showed high stability in a broad range of pH and temperature [12]. In a case of antifungal protein BI from *B. subtilis* TG 26, antifungal activity was not diminished up to 100°C, while 75% remained at 140°C [17].

In surfactant stability studies, protein was incubated with Tween 20 and Triton X-100 (0.1%, 1%) that was used in manufacturing biocontrol agents. The most antifungal activity remained in all surfactants and concentrations (data not shown). Moreover, the activity of this protein at 1% Triton X-100 was increased about 1.2-fold compared to control. When the protein was incubated with proteinase K (50 μg/ml) at 40°C for 30 min, about 80% of the activity remained (data not shown).

Inhibition of Conidial Germination

The purified antifungal protein (5.9 μg/ml) was mixed with the conidial suspension (2.5×10^6/ml) of *P. oryzae* KJ 301: 93-39 and the inhibitory activity of the protein on the conidial germination was investigated (Table 1). The germination was inhibited more about 7.7-fold than that of the control (sterilized water). The conidial morphology was also examined under a light microscope (Carl Zeiss, Axiovert 135 M, ×400, ×630). As shown in Fig. 6, the protein inhibited the conidial germination, and ungerminated conidia were swollen at basal and mid cell in the protein treatment.

In vivo Bioassay

The suppression of culture supernatant and purified protein on the conidial germination and mycelial growth of *P. oryzae* KJ 301: 93-39 was investigated with the leaves of Nagdongbyeo. The rice leaf treated with only water (control) was normally grown and extended with the formation of appressoria (Figs. 7A and 7B). The rice leaf treated with culture supernatant was substantially inhibited in the conidial germination (Fig. 7C). Although the germination occurred, an abnormal growth was closely observed. The conidial germination of pure protein-treated rice leaf was inhibited

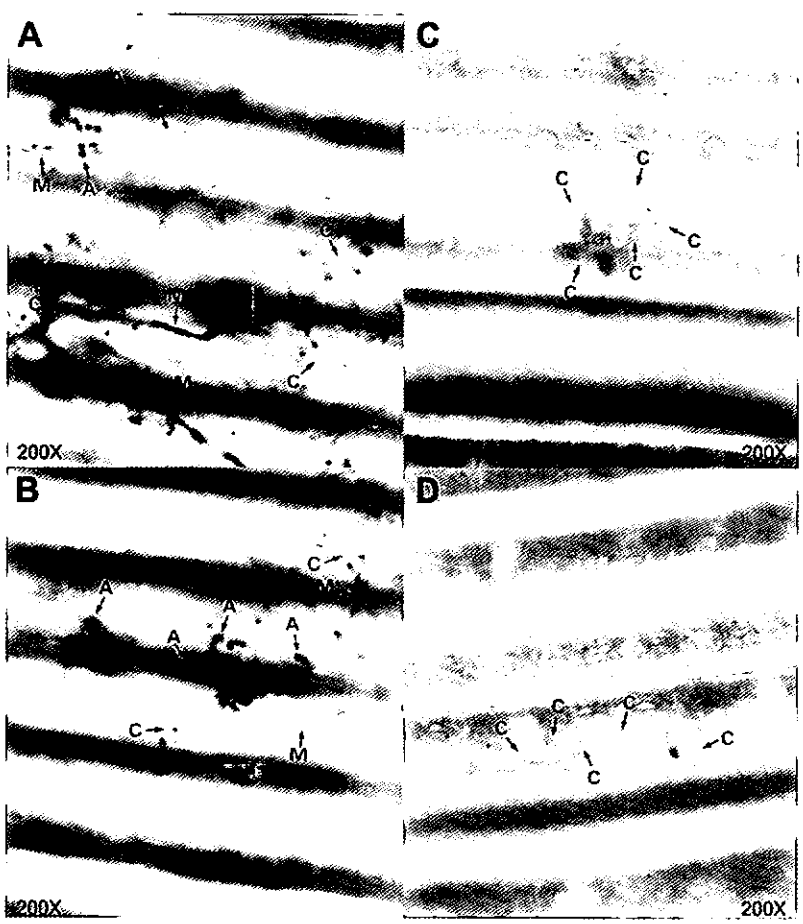

Fig. 7. Suppressive effect of the culture supernatant (C) and purified protein (D) compared to untreated control (A, B) on the conidial germination and mycelial growth *P. oryzae* KJ 301: 93-39 on the leaves of rice plant.

Both of 10 μl conidia suspension with 0.02% Tween 20 and 40 μl of either culture supernatant or purified protein was mixed and applied to the rice leaf blades of Nagdongbyeo, which were placed in the humidity chamber at 28°C for 2 days and examined microscopically for germination and mycelial growth. C: conidia, M: mycelium, A: appressorium.

more and the mycelial extension was very weak (Fig. 7D). These results are similar to the mechanism of inhibition observed in the formation of appressorium of *P. oryzae* by *Saccaromyces cerevisiae* α-factor [3].

Acknowledgments

This research was supported by a grant from the Korea Research Foundation, 1998.

REFERENCES

1. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215:** 403-410.
2. Bae, D. W. 1999. Purification and characterization of an antifungal protein *Paenibacillus macerans* PM-1 antagonistic to rice blast fungus, *Pyricularia oryzae* Cavara. Ph. D. thesis, Gyeongsang National University, Korea.
3. Beckerman, J. L., F. Naider, and D. J. Ebbole. 1997. Inhibition of pathogenicity of the rice blast fungus by *Saccharomyces cerevisiae* α-factor. *Science* **276:** 1116-1119.
4. Bol, J. F. and J. M. Linthorst. 1990. Plant pathogenesis-related proteins induced by virus infection. *Annu. Rev. Phytopathol.* **28:** 113-138.
5. Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
6. Cheong, N. E., Y. O. Choi, W. Y. Kim, I. S. Bae, M. J. Choi, I. H. Hwang, J. W. Kim, and S. Y. Lee. 1997. Purification and characterization of an antifungal PR-5 protein from pumpkin leaves. *Mol. Cells* **7:** 214-219.
7. Cohen, Y. and M. D. Coffey. 1986. Systemic fungicides and the control of oomycetes. *Annu. Rev. Phytopathol.* **24:** 311-338.
8. Gees, R. and M. D. Coffey. 1989. Evaluation of a strain of *Myrothecium rorodum* as a potential biological agent against *Phytophthora cinnamomi*. *Phytopathology* **79:** 1079-1084.
9. Kauffmann, S., M. Legrand, and B. Fritig. 1990. Isolation and characterization of six pathogenesis-related (PR) proteins of samsun NN tobacco. *Plant Mol. Biol.* **14:** 381-390.
10. Kharbanda, P. D., J. Yang, P. Beatty, S. Jensen, and J. P. Tewari. 1997. Potential of a *Bacillus* sp., to control blackleg and other diseases of canola (Abstracts). *Phytopathology* **87:** 51.
11. Kim, B. S., J. W. Ahn, and K. Y. Cho. 1998. Isolation and identification of myxobacteria KR025 and searching bioactive compounds. *Kor. J. Plant Pathol.* **14:** 345-349.
12. Kim, Y. S., J. K. Son, D. C. Moon, and S. D. Kim. 1997. Isolation and structure determination of antifungal antibiotics from *Bacillus subtilis* YB-70, a powerful biocontrol agent. *Kor. J. Appl. Microbiol. Biotechnol.* **25:** 62-67.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
14. Lee, C.-H., J. W. Suh, and Y. H. Cho. 1999. Immunosuppressive activity of cepacidine A, a novel antifungal antibiotic produced by *Pseudomonas cepacia*. *J. Microbiol. Biotechnol.* **9:** 672-674.
15. Lee, D. G., Z. Z. Jin, S. Y. Shin, J. H. Kang, K. S. Hahm, and K. L. Kim. 1998. Structure-antifungal activity relationships of cecropin a-magainin 2 and cecropin a-melittin hybrid peptides on pathogenic fungal cells. *J. Microbiol. Biotechnol.* **8:** 595-600.
16. Lee, J. R., S. H. Lee, I. Kubo, and S. D. Hong. 1998. Antifungal activity medium-chain (C_6-C_{13}) alkenals against, and their inhibitory effect on the plasma membrane H^+-ATPase of *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* **8:** 197-202.
17. Liu, Y. Q., Y. P. Wang, N. S. Pan, and Z. L. Chen. 1994. Identification of antagonistic strain TG 26 and purification of its antifungal protein BI. *Acta Botanica Sinica* **36:** 197-203.
18. Matsudaira, P. 1987. Sequence from picmole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **26:** 10035-10038.
19. Mirsa, A. K. 1986. Antibiotics as crop protectants. pp. 50-60. *In* Moats, W. A. (ed.), *Agricultural uses of antibiotics*, ACS symposium series 320, American Chemical Society, Washington, DC, U.S.A.
20. Niderman, T., I. Genetet, T. Bruyere, R. Gees, A. Stintzi, M. Legrand, B. Fritig, and E. Mosinger. 1995. Pathogenesis-related PR-1 protein are antifungal. *Plant Physiol.* **108:** 17-27.
21. Ou, S. H. 1985. *Rice Diseases*. 2nd ed. Commonwealth Mycological Institute, Kew, England.
22. Roberts, W. K. and C. P. Selitrennikoff. 1986. Isolation and partial characterization of two antifungal proteins from barley. *Biochem. Biophys. Acta* **880:** 161-170.
23. Takeuchi, S., K. Hirayama, K. Ueda, H. Sakai, and H. Yonehara. 1958. Blastidin S, a new antibiotic. *J. Antibiotics.* **11:** 1-5.
24. Terras, F. R. G., H. M. E. Schoofs, M. F. C. de Bolle, F. van Leuven, S. B. Rees, J. Vander leyden, B. P. A. Cammue, and W. F. Broekaert. 1992. Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J. Biol. Chem.* **267:** 15301-15309.
25. Umezawa, H., Y. Okami, T. Hashimoto, Y. Suhara, M. Hamada, and T. Takeuchi. 1965. A new antibiotic, kasugamycin. *J. Antibiotics.* **31:** 662-666.
26. Yu, G. Y. and J. B. Sinclair. 1997. Purification and identification of an antifungal protein produced by a potential biocontrol agent *Bacillus amyloliquefaciens* B94 (Abstracts). *Phytopathology* **87:** 107.