한국식물병리학회지 14(3) : 203-208(1998)
Korean J. Plant Pathol. 14(3) : 203-208(1998)

Purification and Characterization of an Antifungal Peptide from the Seeds of *Phytolacca americana*

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미국자리공 종실로부터 항균성 펩타이드의 분리 및 특성 연구

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ABSTRACT: We isolated and characterized an antifungal peptide from the seeds of *Phytolacca americana*. Growth inhibition assay with *Botrytis cinerea* was used to screen inhibitory proteins from 60 different plant species. A 4 kDa antifungal peptide (Pa-AFP) inhibitory to hyphal growth of *B. cinerea* was found in the seeds of *P. americana*. The peptide Pa-AFP was purified to homogeneity by chromatographies of Sephadex G-50, DEAE-Sepharose, Sephacryl S-300, and C_{18} reverse-phase HPLC. Western blot analysis showed that a polyclonal antibody raised against the purified peptide cross-reacted with a 4 kDa protein in seeds but not in root and leaf tissues of *P. americana*. Pa-AFP inhibited the hyphal growth of *Botrytis cinerea, Rhizoctonia solani, Fusarium oxysporum*, and *Magnaporthe grisea*. Pa-AFP exhibited growth inhibition of *Saccharomyces cerevisiae* strain BWG7a, which was sensitive to osmotin.

***Key words*: small peptide, antifungal activity, *Phytolacca americana, Botrytis cinerea*.**

Plants defend themselves against pathogens using various defense mechanisms. These mechanisms include cell wall lignification (3), synthesis of toxic phytoalexins (10), and accumulation of several defense-related proteins (4). Among them, pathogenesis-related (PR) proteins and seed PR-like proteins are already well known. Although the role of some PR proteins in the defense response of plants is not clearly elucidated, some PR proteins have been reported to have antifungal activity *in vitro* (20, 25, 26).

Different seed proteins with antifungal or antimicrobial activity are chitinases (12, 22), β-1,3-glucanases (15, 19), ribosome-inactivating proteins (15, 22), chitin-binding lectins (21), plant defensins (6), and hevein-type peptides such as the Pn-AMPs from *Pharbitis nil.* (14). The smallest antifungal peptides that consist of only 20 amino acids were isolated from the seeds of *Impatiens balsamina* (23). Basic proteins from seeds of cotton were found to have selective growth inhibitory activity against filamentous fungi such as *Botrytis cinerea, Alternaria brassicola, Chalara elegans*, and *Fusarium oxysporum* (8).

Fungal diseases caused severe limitation in production of major crops. To prevent plants from being destroyed by fungal pathogens several attempts were made. One of the alternative approaches is to produce transgenic plants resistant to diseases. Several transgenic plants which overproduce PR proteins were reported to enhance resistance to fungal diseases (2, 5, 13, 16, 17, 18, 24, 28). A preliminary and important part of the strategies in obtaining transgenic plants is the discovery and characterization of antifungal proteins from different plant species and the isolation of their encoding genes.

The aim of the present work is to isolate and characterize antifungal peptides from different plant species, eventually to produce transgenic plants with antifungal

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properties against *Botrytis cenerea*. Among 60 different plant species and seeds tested, we isolated and characterized a 4 kDa peptide with antifungal activity from the seeds of *Phytolacca americana*.

MATERIALS AND METHODS

Screening of antifungal proteins. Crude extracts were prepared by grinding 1 g of plant tissues in a mortar in 3 ml of 25 mM sodium phosphate buffer, pH 7.0. After filtration through four layers of gauze, the filtrate was centrifuged at 10,000*g* for 10 min. The supernatant was assayed by hyphal extension-inhibition of *Botrytis cenerea*.

Purification of antifungal peptide from *Phytolacca americana* seeds. *Step* 1: *Preparation of protein extracts*. One hundred gram of *P. americana* seeds was ground in a blender and the resulting meal was extracted for 4 h at 4°C with 500 ml of 25 mM sodium phosphate buffer, pH 7.0. The homogenate was squeezed through four layers of gauze and clarified by centrifugation at 10,000*g* for 20 min.

Step 2: Ammonium sulfate fractionation. Solid ammonium sulfate was added to the supernatant to obtain 30% relative saturation and the precipitate formed after standing 1 h on ice was removed by centrifugation at 10,000*g* for 20 min. The supernatant was adjusted to 70% relative ammonium sulfate saturation and the precipitate formed after standing 1 h on ice collected by centrifugation at 10,000*g* for 20 min.

Step 3: Sephadex G-50 gel filtration chromatography. The pellet from ammonium sulfate fractionation was suspended in 50 ml of the buffer and the solution was applied to the Sephadex G-50 column chromatography. Active fractions were pooled and then desalted by dialysis against the buffer.

Step 4: DEAE-Sepharose ion exchange chromatography. The desalted protein fraction was applied to a DEAE-Sepharose column previously equilibrated with the same buffer.

Step 5: Sephacryl S-300 gel filtration chromatography. The fractions containing antifungal activity from step 4 were pooled and the protein was precipitated with 70% saturated ammonium sulfate. The protein solution was then loaded onto a Sephacryl S-300 column equilibrated with the buffer.

Step 6: Reverse-phase HPLC. The active fractions from the Sephacryl S-300 column were combined and dialyzed against 0.1% trifluoroacetic acid (TFA) and subjected to C_{18} column connected to DIONEX HPLC system. Antifungal peptide was eluted with a linear gradient of 0~70% acetonitrile containing 0.1% TFA.

Preparation of antibody. The final preparation of antifungal peptide was subjected to 17% (w/v) SDS-PAGE. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250, the band corresponding to Pa-AFP excised, and the peptide eluted electrophoretically and dialyzed against 0.9% NaCl. One hundred microgram of the purified protein was emulsified with an equal volume of Freund's complete adjuvant and injected into a rabbit. After one month, 100 μg of the protein, emulsified with an equal volume of Freund's incomplete adjuvant, was injected subcutaneously into the rabbit at weekly intervals.

Western blot analysis. Proteins separated by 17% SDS-polyacrylamide were transferred electrophoretically to PVDF membrane using a semi-dry transfer kit (Hoefer Scientific Instrument) for 1 h at 0.8 mA/cm^2. The membrane was incubated with TTBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 6% non-fat dried milk for 4 h and treated for 1 h with antibody raised against Pa-AFP (dilution 1:2000). After incubation the membrane was washed three times with TTBS and then incubated with an anti-rabbit IgG-peroxidase conjugated (Amersham) as a secondary antibody (dilution 1:2000) for 1 h. The membrane was finally washed with TTBS. The protein-antibody complex was detected using the ECL chemiluminescence system (Amersham).

Assay of antifungal activity. Antifungal activity was assayed using a hyphal extension-inhibition assay as described previously (22). Fungal mycelia were harvested from the actively growing fungal plates and were placed on the center of petri dishes containing the potato dextrose agar (PDA). After incubation of the plates for 48 h at 18°C, several wells were made around the mycelium center and the aliquots of crude extracts or purified peptide were dropped to the well. The plates were then further incubated for 20 h at 18°C. Activity was determined by the appearance of crescents due to growth retardation of mycelia around the wells.

Measurement of yeast sensitivity to Pa-AFP. Sensitivity to Pa-AFP was determined in 0.5-ml liquid cultures containing various concentrations (0~8 μg/ml) of purified Pa-AFP. Overnight cultures were inoculated to an $OD_{600\ nm}$ of 0.02 in test tubes and incubated for 14~16 hrs at 30°C with shaking, and the growth of yeast was read at $OD_{600\ nm}$ at appropriate dilutions. The amount

of Pa-AFP that reduced growth of yeast by 50% is denoted as IC_{50}.

RESULTS AND DISCUSSION

Screening of antifungal peptides against *Botrytis cinerea*. To isolate strong antifungal proteins, we tested 60 different plant species using growth inhibition assay against *B. cinerea*. The strongest antifungal activity among the samples tested was found in seeds of *Phytolacca americana* and it was selected for further purification.

Purification of antifungal peptide from seeds of *Phytolacca americana*. To purify an antifungal peptide the cell homogenate obtained from seeds of *P. americana* was fractionated using various protein purification steps such as ammonium sulfate fractionation, DEAE-sepharose ion exchange chromatography, Sephacryl S-300 gel filtration, and C_{18} reverse-phase HPLC. Fractions from each purification step were examined for inhibitory effect of hyphal elongation of *B. cinerea*. The antifungal protein fraction which precipitated between 30 and 70% relative saturation with ammonium sulfate was subjected to anion exchange chromatography on DEAE-Sepharose (Fig. 1. A). The fractions showing antifungal activity were eluted in the pass-through fraction. The pass-through fraction was subjected to CM-Sepharose cation exchange column chromatography. Interestingly, the activity was again found in the pass-through fraction. To date most of the known antifungal peptides are highly basic and have a cysteine/glycine-rich domain (14, 23). Therefore, these antifungal peptides bind to cation resin. However, the antifungal peptide (designated as Pa-AFP) from *P. americana* was found in pass-through fraction in both cation and anion columns, suggesting that Pa-AFP may be highly hydrophobic. The antifungal-active fractions from the cation exchange chromatography were pooled and the protein was precipitated with 70% saturated ammonium sulfate. The pellet was dissolved in sodium phosphate buffer and then loaded onto a Sephacryl S-300 column (Fig. 1 B). Two protein peaks appeared and both peak fractions were subjected to inhibition bioassay against *B. cinerea*. The antifungal activity was observed only in the fractions of the first peak (Fig. 2). To further purify the antifungal peptide, fractions 27 to 30 from the Sephacryl S-300 column were combined and applied to C_{18} reverse phase HPLC. The antifungal peptide was eluted at the position of approximately 35% acetonitrile (in 0.1% TFA).

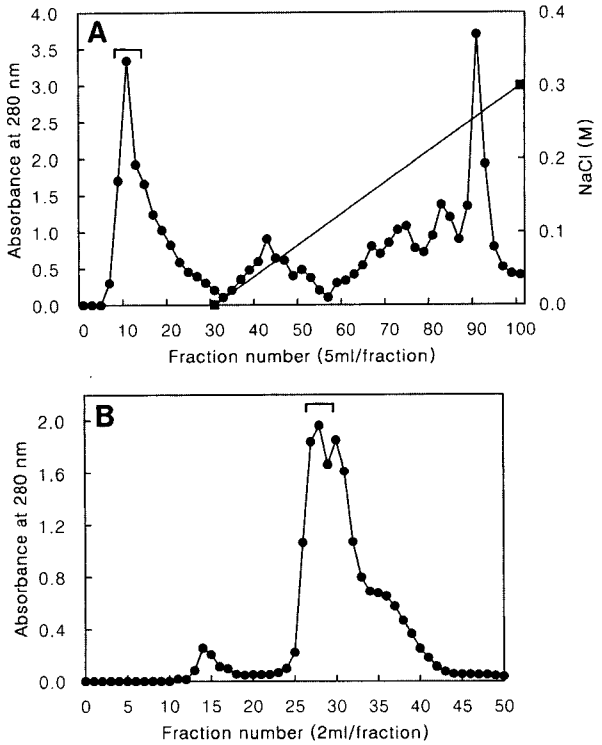

Fig. 1. Purification procedures of antifungal peptide from *Phytolacca americana* seeds. (A) Protein from 30~70% ammonium sulfate saturated fraction of 100 g seeds was desalted and applied to a DEAE-Sepharose column. Antifungal peptide was eluted in the unbound fraction (fractions 9 to 13). (B) The active fractions from the DEAE-Sepharose anion exchange chromatography were pooled and the protein was precipitated with 70% saturated ammonium sulfate. The pellet was dissolved in 25 mM sodium phosphate buffer, pH 7.0 and then loaded onto a Sephacryl S-300 column. The antifungal activity was observed in fractions from 27 to 30. The bar indicates the fractions showing antifungal activity.

Characterization of antifungal peptide from seeds of *Phytolacca americana*. The chromatographic steps of purification yielded approximately 1.8 mg of antifungal peptide from 100 g seed of *P. americana*. The molecular size of the purified peptide was approximately 4 kDa on an SDS-polyacrylamide gel (Fig. 3A).

To examine the presence of the antifungal peptide in other tissues, Western blot analysis was carried out using a polyclonal anti Pa-AFP antibody. A Western blot prepared from protein extracts obtained from seeds, root and leaf tissues was probed with the anti-Pa-AFP antibody. As shown in Fig. 3B, a single band was detected in seeds but not in the root and leaf tissues, suggesting that the Pa-AFP is specific to seeds of *P. americana*. Small peptides with a molecular size of ap-

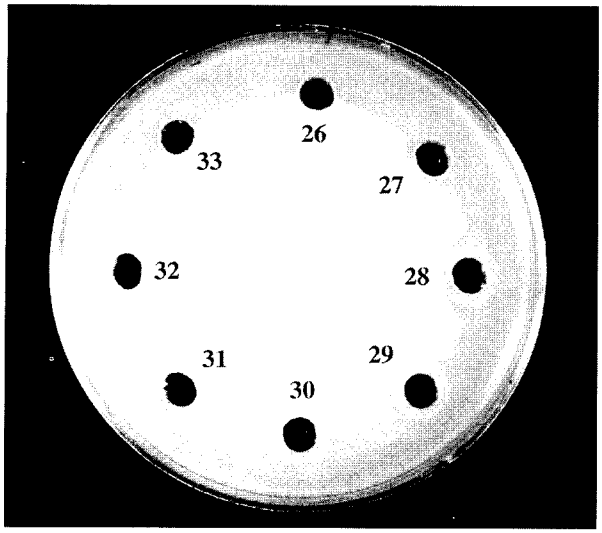

Fig. 2. Antifungal activity of each of Sephacryl S-300 gel filtration fractions against *Botrytis cinerea*. Fractions (50 μl per well) were placed near to *B. cinerea* grown on a PDA for 2 days, followed by further incubation at 18°C for 20 h. Numbers on the plate indicate the fractions shown in Fig. 1. (B).

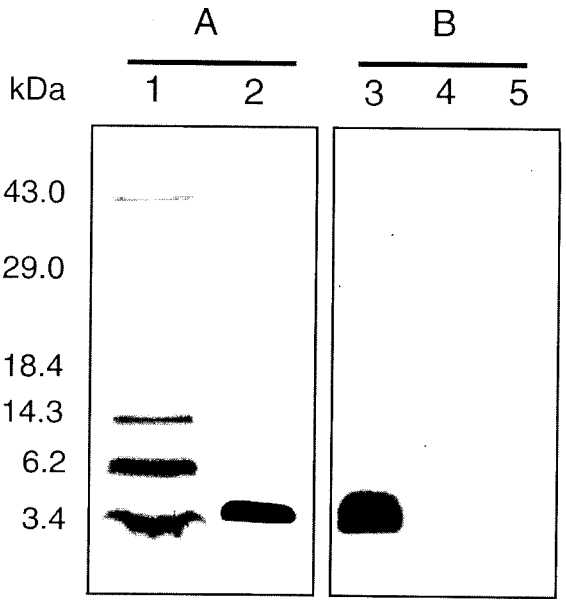

Fig. 3. SDS-PAGE (A) and Western blot analysis (B) of the antifungal peptide Pa-AFP. Electrophoresis of 1 μg of the reduced Pa-AFP was performed on 17% (w/v) SDS-PAGE and stained with Coomassie brilliant blue R-250 (lane 2). Five micrograms of extracts from seeds (lane 3), root (lane 4) and leaf tissues (lane 5) were separated on a 17% (W/V) SDS-PAGE, electroblotted to a nitrocellulose membrane and probed with the anti-Pa-AFP antiserum raised against the purified Pa-AFP. A single band of 4 kDa was detected in seeds. Size markers are shown in lane 1.

proximately 3~4 kDa, such as hevein-type peptides of Pn-AMPs from *Pharbitis nil.* (14), balsamin from the seeds of *Impatiens balsamina* (23), and Mj-AMPs from *Mirabilis jalapa* (7) have been shown to have strong antifungal or antimicrobial activities. Search through public protein data bases using Blastp program failed to identify any protein with significant amino acid sequence homology to Pa-AFP (Son, unpublished data). To further characterize the biochemical property of Pa-AFP, the active fractions obtained from HPLC was subjected to heat treatment. The Pa-AFP remained to be active after 15 min at 80°C, suggesting that the antifungal peptide is heat stable.

Antifungal activity against plant pathogens and yeast. To examine the specificity of antifungal activity of Pa-AFP, the partially purified Pa-AFP was assayed for antifungal activity *in vitro* against plant pathogenic fungi *Rhizoctonia solani, Magnaporthe grisea,* and *Fusarium oxisporum.* Strong inhibition against these pathogenic fungi was found at the concentration of 100 μg/ml of Pa-AFP (active fraction after Sephacryl S-300 gel filtration chromatography). Basic antifungal peptide from maize kernels was also found to have inhibitory activity against plant pathogenic fungi such as *Sclerotinia sclerotiorum, Alternaria longipes,* and *Fusarium moniliforme* (9).

The mechanism of the antifungal small peptides has been studied using various antifungal peptides. In the case of Pn-AMPs from *Pharbitis nil.* (14), fungal growth inhibition was due to burst of hyphal tips by rapid penetration of peptides that resulted in disruption of the fungal membrane and leakage of the cytoplasmic materials. Pa-AFP from *P. americana* has a similar molecular size and similar spectra of fungal inhibition. It is possible that Pa-AFP may also act as a mechanism similar to that of Pn-AMP to inhibit fungal growth. As an initial step to elucidate the mechanism of growth inhibition by Pa-AFP, we employed an alternative approach using *Saccharomyces cerevisiae* as a model system. Recently, Yun et al. (27) have shown that yeast can be a model system to investigate the mechanism of antifungal activity. Using this yeast system, we found that the yeast strains *S. cerevisiae*, BWG7a and GRF 167, differed in their sensitivity to Pa-AFP (Fig. 4). The value of IC_{50} was 1.5 μg/ml for the strain BWG7a and greater than 6 μg/ml for the strain GRF167. These results suggest that there is strict specificity between the antifungal protein, Pa-AFP, and its target cells. These results also strongly imply that the approach using the yeast model system could be valuable for the

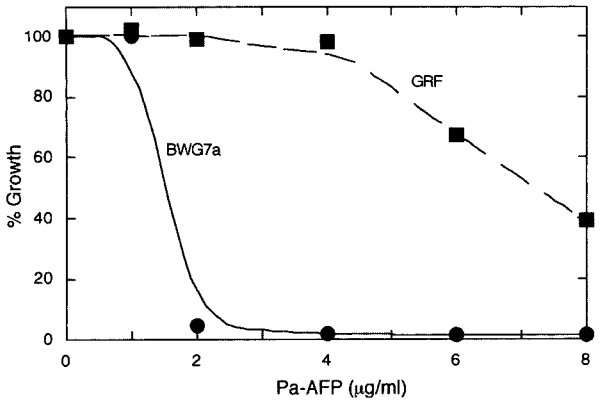

Fig. 4. Differential sensitivity of yeast strains to the cytotoxic effect of antifungal peptide Pa-AFP. The sensitivity of strains GRF167 (MATa ura3 his3) and BWG7a (MATa ade1 his4 leu2 ura3) to Pa-AFP was evaluated in liquid cultures. Values of the average of three independent experiments are the percentage of the $OD_{600\ nm}$ of control cultures without Pa-AFP used as a control.

elucidation of detailed mechanism of Pa-AFP. We are currently exploiting different approaches to characterize genes involved in Pa-AFP sensitivity/resistance, including complementation of the Pa-AFP sensitive strain BWG7a with a genomic DNA library from the resistant yeast strain GRF167 in a high copy plasmid. Many filamentous fungi in the class *Ascomycetes* are important phytopathogens (1) and even *S. cerevisiae* exhibits pathogen-like invasive growth allowing colonization of fruits such as grapes. Therefore, results obtained from model system of resistance/sensitivity of Pa-AFP using *S. cerevisiae* is expected to be readily applicable to economically important phytopathogenic fungi (11). Pa-AFP gene may be a useful candidate for genetic engineering of plants for increased tolerance against fungal or yeast infection.

요 약

본 연구에서는 미국자리공(*Phytolacca americana*) 종실로부터 항균성 펩타이드를 분리하여 그 특성을 조사하였다. 60여종의 식물 조추출액으로 잿빛곰팡이 균사의 생장 억제력을 조사한 결과, 미국자리공 종실의 조추출액이 잿빛곰팡이 균사의 성장을 강하게 저해하였다. 미국자리공 종실로부터 얻은 조추출액을 ammonium sulfate fractionation과 Sephadex G-25, DEAE-Sepharose ion exchange chromatography, Sephacryl S-300 gel filtration chromatography하였으며, C_{18} reverse-phase HPLC의 단계를 거쳐 순수 분리된 항균성 펩타이드(Pa-AFP)의 크기는 약 4 kDa이었다. 정제된 펩타이드로 조제한 항체를 가지고 Western blot analysis한 결과, 이 항균성 펩타이드는 종실에서만 특이적으로 발현되는 것을 알 수 있었다. Pa-AFP는 *Botrytis cinerea, Rhizoctonia solani, Fusarium oxysporum, Magnaporthe grisea* 및 효모의 성장도 저해하였다. 이들 결과는 순수 분리한 펩타이드가 식물병원진균에 내성을 갖는 형질전환식물의 창출을 위한 새로운 재료로 사용될 수 있음을 시사하고 있다.

ACKNOWLEDGMENT

We are grateful to Dr. I. Hwang (PMBBRC, Gyeongsang National University) for critical reading of this manuscript. This paper was supported by NON DIRECTED RESEARCH FUND, Korea Research Foundation, 1996.

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(Received April 28, 1998)