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Antifungal Mechanism of a Novel Antifungal Protein from Pumpkin Rinds against Various Fungal Pathogens

Seong-Cheol Park,[†] Jin-Young Kim,[†] Jong-Kook Lee,[†] Indeok Hwang,[‡] Hyeonsook Cheong,[‡] Jae-Woon Nah,[∥] Kyung-Soo Hahm,*[,†,§] and Yoonkyung Park*[,†,‡]

[†]Research Center for Proteineous Materials (RCPM) and [‡]Biotechnology and BK21 Research Team for Protein Activity Control and [§]Department of Cellular Molecular Medicine, School of Medicine, Chosun University, Kwangju, 501-759, Korea, and [∥]Department of Polymer Science and Engineering, Sunchon National University, Jeonnam, 540-742, Korea

A novel antifungal protein (Pr-2) was identified from pumpkin rinds using water-soluble extraction, ultrafiltration, cation exchange chromatography, and reverse-phase high-performance liquid chromatography. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry indicated that the protein had a molecular mass of 14865.57 Da. Automated Edman degradation showed that the N-terminal sequence of Pr-2 was QGIGVGDNDGKRGKR−. The Pr-2 protein strongly inhibited in vitro growth of *Botrytis cinerea*, *Colletotrichum coccodes*, *Fusarium solani*, *Fusarium oxysporum*, and *Trichoderma harzianum* at 10−20 μM. The results of confocal laser scanning microscopy and SYTOX Green uptake demonstrated that its effective region was the membrane of the fungal cell surface. In addition, this protein was found to be noncytotoxic and heat-stable. Taken together, the results of this study indicate that Pr-2 is a good candidate for use as a natural antifungal agent.

KEYWORDS: Pr-2; pumpkin rinds; antifungal protein; *Fusarium oxysporum*

INTRODUCTION

A number of studies have recently been conducted to identify novel and potent antimicrobial proteins because these so-called "natural antibiotics" have the potential to overcome antimicrobial resistance. The emergence of clinical bacterial strains exhibiting resistance against conventional antibiotics has urged the search for novel antibiotic agents. Plants serve as a major source of protein and carbohydrates for humans and livestock. Many functional classes of protein have been purified and characterized from plant food.

Plant responses to pests and pathogens include de novo synthesis of proteins and peptides with antifungal activity. Antifungal proteins and peptides exert a protective activity against fungal invasion and play an important role in defending crops against fungal attacks (*1*). Over the past decade, many studies have focused on screening antifungal proteins from various sources because they have economical value due to their ability to protect important crops from fungal pathogens. In addition, it has been reported that transgenic plants expressing antifungal proteins have increased resistance to fungal diseases (*2*, *3*).

To date, antifungal proteins have been identified from a large number of leguminous species (*1*). In addition to other physiological or mechanical functions, these proteins may act as protective barriers or growth inhibitors against various pathogens. Antifungal proteins can be categorized into the following subfamilies: chitinases, β-1,3-glucanases, thaumatin-like proteins, proteinase inhibitors, endoproteinases, peroxidases, plant defensins, ribosome-inactivating proteins, and immunophilin proteins with other biological properties (*3*−*10*). Novel antifungal proteins have the potential for applications in medicine, food safety, and agriculture.

Pumpkin has been traditionally used for its medicinal value in many countries, including China, Yugoslavia, Argentina, India, Mexico, and Korea (*11*, *12*). Its popular medicinal uses have been the focus of many studies over the last few decades, and the antibiotic effects of pumpkin have been analyzed, along with its antidiabetic, antihypertension, antitumor (*13*), antibacterial (*14*, *15*), anti-inflammatory, and antimutagenic effects (*16*). It has been reported that some proteins isolated from pumpkin have toxic effects on phytopathogens. This study was conducted to identify pumpkin proteins with antimicrobial activity and to analyze their protective roles.

An antifungal PR-5 protein with a molecular mass of 28 kDa and an amino acid sequence that was highly homologous to that of thaumatin was previously isolated from pumpkin leaves (*17*). This protein was also found to exert a synergistic effect against the growth of *C. albicans* when administered with nikkomycin, a chitin synthase inhibitor. In addition, a ribosome-inactivating protein purified from *Cucurbita moschata* was found to inhibit the growth of phytopathogenic bacteria including *Phytophthora infestans*, *Erwinia amylovora*, and *Pseudomonas solanacearum* (*18*). Cucurmoschin, an antifungal peptide abundant in arginine, glutamate, and glycine residues, was identified in black pumpkin seeds (*14*). Cucurmoschin was found to inhibit the mycelial growth

*To whom correspondence should be addressed. (K.-S.H.) Fax: +82-62-227-8345. E-mail: kshahm@chosun.ac.kr. (Y.P.) Fax: +82-62-230-6854. E-mail: y_k_park@chosun.ac.kr.

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of the fungi *Botrytis cinerea*, *Fusarium oxysporum*, and *Mycosphaerella oxysporum*. MAP2 (MW, 2249 Da), MAP4 (MW, 4650 Da), and MAP11 (MW, 11696 Da), which are isolated from pumpkin seeds, were found to inhibit the growth of yeast cells, with MAP11 being the most effective inhibitor (*13*). In the present study, we isolated a novel antifungal protein with a broad spectrum of activity against pathogenic fungi and reported the mechanism of its antifungal action.

MATERIALS AND METHODS

Biological Materials. Pumpkin (*C. moschata*) was purchased from a local market. For the quantitative antifungal assay, the following fungal strains were obtained from either the Korea Collection for Type Cultures (KCTC) or the Korea Agricultural Culture Collection (KACC): *Aspergillus fumigates* (KCTC 6145), *Aspergillus parasiticus* (KCTC 6598), *Penicillium verrucosum* var. *verrucosum* (KCTC 6265), *F. oxysporum* (KCTC 16909), *Fusarium solani* (KCTC 6326), *Trichoderma harzianum* (KCTC 6043), *Trichoderma viride* (KCTC 6047), *B. cinerea* (KACC 40573), *Colletotrichum coccodes* (KACC 40803), and *Didymella bryoniae* (KACC 40669). Fungal cells were grown on PDA (potato dextrose agar) plate and subcultured at 2−3 weeks.

Chemicals. Standard marker proteins for the determination of molecular mass and all chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN) and water [high-performance liquid chromatography (HPLC) grade] were obtained from Burdick & Jackson Inc. (Muskegon, MI), and trifluoroacetic acid (TFA) was obtained from Merck.

Protein Extraction and Purification. Pumpkin rinds were homogenized in extraction buffer [25 mM Tris-HCl, 1.5 M LiCl, and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4]. The homogenate was then centrifuged, after which the soluble extracts were heated at 70 °C for 20 min to isolate heat-stable proteins. The antifungal protein was then purified using ultrafiltration, cation exchange chromatography on CM-sepharose, and C_{18} reversed-phase (RP)-HPLC. Briefly, the heat-soluble extracts were ultrafiltered on a cutoff membrane with a molecular mass of 30 kDa. The ultrafiltered samples were dialyzed with 20 mM phosphate buffer (pH 6.0) containing 1 mM EDTA and 10 mM NaCl, and they were then applied directly to a CM-sepharose Fast Flow cation-exchange column (4.6 cm × 10 cm, Amersham Pharmacia Biotech). A stepwise gradient procedure was used for fractionation. After the unadsorbed material was washed out, the fractions exhibiting antifungal activity were eluted with 0.1 M NaCl. The fraction was then dialyzed with 10 mM ammonium acetate buffer (pH 5.0) and lyophilized. The dried samples were suspended with water containing 0.1% (v/v) TFA, and the substances were then injected onto a C_{18} column (4.6 mm × 250 mm; Vydac, Hesperia, CA) that was previously equilibrated with 10% ACN containing 0.1% (v/v) TFA. Linear gradient elutions were employed using 10−95% ACN over 60 min after washing with 10% ACN for 10 min. The flow rate was maintained at 1 mL/min at 40 °C, and the absorbance was monitored at 214 nm. Throughout the purification of Pr-2, the antifungal activities of the eluted fractions were analyzed by measuring its inhibition against *F. solani* and *C. albicans*.

Tricine-Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis was conducted according to the method described by Schagger and Jagow (*19*) to separate peptides with a low molecular mass. After electrophoresis, the gel was stained with Coomassie Brilliant Blue G. The molecular mass marker for tricine-SDS-PAGE consisted of triosephosphate isomerase from rabbit muscle (26.6 kDa), myoglobin from horse heart (17 kDa), α-lactalbumin from bovine milk (14.2 kDa), aprotinin from bovine lung (6.5 kDa), insulin chain B, oxidized, bovine (3.5 kDa), and bradykinin (1 kDa).

Proteolytic Digestion. After extraction, dialyzed crude extracts were digested with 0.25 mg/mL trypsin at 37 °C for 4 h to determine if the antimicrobial compounds were proteinaceous. Before and after protease treatment, the antifungal activity of the extracts toward *C. albicans* was evaluated by antifungal assay (*20*).

Protein Quantification and N-Terminal Sequencing. The protein concentration was determined using the BCA protein assay method (*21*) using bovine serum albumin as a standard. All protein assays were conducted in triplicate. To determine the sequence of Pr-2, after homogeneous Pr-2 was subjected to tricine-SDS-PAGE, blotting was performed at 200 mA for 2 h using a polyvinylidene fluoride (PVDF) membrane with a pore size of 0.2 μM. The Pr-2 band used for sequencing was cut out and air-dried. The N-terminal amino acid sequence was determined by Edman degradation (*22*) on an amino acid sequencer (Applied Biosystems Inc., model 473A). The amino acid sequence was compared with other sequences in the NCBI database using the BLAST program.

Antifungal Assay. A radial growth inhibition assay was performed to examine the antifungal activity of the fractions during the purification steps. Fungal fragments, preculutred in mycelial growth, were placed in the center of PDA plates, and then, the cultures were incubated for 60 h at 28 °C in the dark. After incubation, sterilized blank paper disks were placed around and at an appropriate distance away from the fungal fragment, and an aliquot of protein in 25 mM Hepes buffer (pH 7.2) was then introduced to the disk. Next, the plates were incubated for 72 h at 28 °C until mycelial growth from the central disk enveloped the periphery. To determine the EC_{50} value of the purified protein against various fungal pathogens, fungal spores from 10 day old cultures grown on a PDA plate at 28 °C were collected using 0.08% Triton X-100. The spores were diluted to a concentration of 2×10^4 /mL in half-strength potato dextrose broth (12 g/L PDB, Difco) supplemented with 50 mM MES buffer (pH 6.0). Next, 50 μL of each spore suspension was placed in flat-bottomed polystyrene 96-well plates (Nunc, Roskilde, Denmark), and 50 μL of protein that had been serially diluted by 2-fold in 50 mM MES buffer was added. After 36−48 h of incubation at 28 °C, the germination and hyphal growth of spores were microscopically evaluated using an inverted light microscope. The absorbance was also measured at 580 nm using a VersaMax microtiter reader (Molecular Devices, CA) (*23*).

Antibacterial Assay. The antibacterial activity of Pr-2 toward *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) was assessed using a microdilution assay performed in a 96-well plate according to a previously described method (*24*).

Hemolysis against hRBCs (Human Red Blood Cells). The hemolytic activities of Pr-2 were assessed using hRBCs collected from healthy donors on heparin. The fresh hRBCs were rinsed three times in phosphate-buffered saline (PBS) (1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 135 mM NaCl, pH 7.4). The protein dissolved in PBS was then added to 100 μL of the stock hRBCs suspended in PBS (final RBC concentration, 8% v/v). The samples were then incubated with agitation for 60 min at 37 °C, after which they were centrifuged for 10 min at 800*g*. Next, the absorbance of the supernatants was assessed at 414 nm. hRBCs in PBS (A_{blank}) and in 0.1% Triton X-100 (A_{triton}) was used as negative and positive controls, respectively. The percent hemolysis was calculated according to the following equation:

$$\% \text{ hemolysis} = [(A_{sample} - A_{blank})/(A_{triton} - A_{blank})] \times 100$$

Each measurement was conducted in triplicate.

Rhodamine Labeling of Protein. Three hundred micrograms of purified Pr-2 protein was suspended with 50 mM sodium borate buffer (pH 8.5) and mixed with the 5(6)-carboxytetramethylrhodamine N-succimidyl ester solution dissolved in dimethyl sulfoxide (DMSO), and the mixture was then incubated for 2 h at 37 °C in the dark. Rhodamine-labeled Pr-2 was purified on C_{18} RP-HPLC system as the above purification method.

Confocal Laser Scanning Microscopy. Confocal laser scanning microscopy was used to analyze the cellular distribution of the protein. Fungal cell suspensions (10^4 conidia/mL) were poured on poly-L-lysine-coated glass slides, which were subsequently incubated at room temperature (RT) for 45 min to allow the cells to adhere to the slides. Next, after which the slides were washed with PBS, rhodamine-labeled Pr-2 was added. The samples were incubated for 30 min at RT, and the slides were then rinsed several times with PBS. The cells were observed using a confocal laser scanning microscope (CLSM, 510META, Zeiss, Gottingen, Germany) (*24*).

SYTOX Green Uptake Assay. *C. albicans* was grown in YPD at 28 °C and then suspended (2×10^4 conidia/mL) in PBS and incubated with 1 μM SYTOX Green for 15 min in the dark. After the protein was then added, the increase in fluorescence was monitored at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The

absorbance of the samples was then evaluated for 100% uptake with 0.1% Triton X-100. In addition, the effects of protein against *F. oxysporum* were observed by fluorescence microscopy.

RESULTS AND DISCUSSION

Purification of the Antifungal Protein, Pr-2. In this study, homogeneous Pr-2 was successfully purified using a three-step procedure. An antifungal protein obtained from pumpkin rinds was purified using ultrafiltration, cation exchange chromatography on CM-sepharose, and C_{18} RP-HPLC. In the first isolation step, ultrafiltration through a 30 kDa molecular mass cutoff membrane yielded two components: one with molecular masses >30 kDa and one with molecular masses <30 kDa.

For the initial screening of crude extracts from pumpkin rinds, we examined the antimicrobial activity of the protein against both pathogenic bacteria and yeast. The crude extracts showed a potent antifungal activity, but they had no effect against bacterial strains (data not shown). To determine if the extracts were proteinaceous, the susceptibility of their antifungal activity toward *C. albicans* was determined after treatment with trypsin for 4 h. Tricine-SDS-PAGE showed that the proteins were almost completely digested (**Figure 1A**) and that the digested extract lost all of its antifungal activity (**Figure 1B**). These results suggest that the extract contains antifungal proteins.

Heat-soluble compounds, which are believed to have thermostable characteristics, were applied to cation exchange chromatography on a CM-sepharose column because antimicrobial peptides and protein have a basic character. The elution profile resulted in the unadsorbed (**Figure 2A**, fraction I) and absorbed fractions (**Figure 2A**, fractions II−IV). Inhibition of the growth for the test yeast, *C. albicans*, was then assayed with the four eluates. The active fraction, eluted by 0.1 M NaCl (**Figure 2A**, fraction II), was then collected. To further purify the protein, the active substance was injected onto a RP C_{18} column using the HPLC system, and one main peak was obtained (**Figure 2B**). This substance was then assayed for its antifungal activity and subjected to tricine-SDS-PAGE to determine its homogeneity. As shown in **Figure 3**, this substance produced a single band upon tricine-SDS-PAGE analysis (**Figure 3A**) and inhibited the germination of *F. solani* (data not shown). These findings demonstrate that a pure antifungal protein was successfully purified. As summarized in **Table 1**, the yield of the Pr-2 protein purified from mature pumpkin was 1.3 mg of pure Pr-2 per 200 g of pumpkin powder.

Protein Identification. To determine the precise molecular mass of the Pr-2 that was represented by the single band shown in

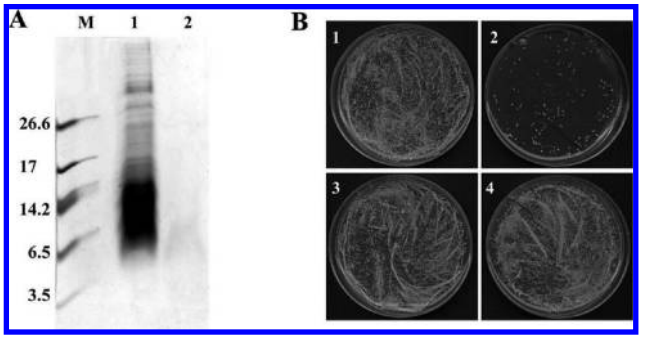

Figure 1. Proteolytic digestion of crude extracts with trypsin. (**A**) Crude extracts were digested with 0.25 mg/mL trypsin at 37 °C for 4 h, and then, the sample was subjected to SDS-PAGE. Lane 1, without trypsin; and lane 2, with trypsin. (**B**) Digested crude extracts were assayed toward *C. albicans*. Panels: 1, control (no extracts and trypsin); 2, only extracts (no trypsin); 3, digested extracts (with trypsin); and 4, trypsin only (no extracts).

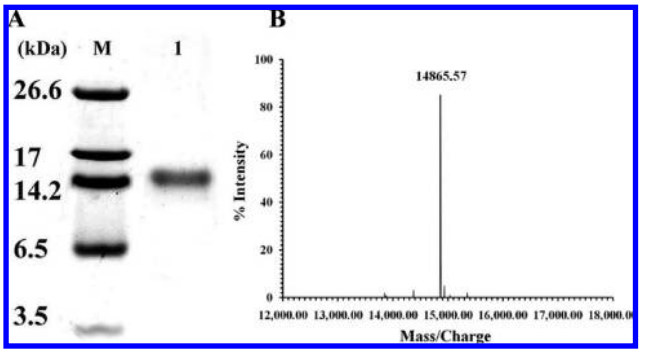

Figure 3. Tricine-SDS-PAGE and molecular mass of purified Pr-2. (**A**) Pr-2 purified by RP-HPLC was subjected on tricine-SDS-PAGE with 16.5% separating gel, and the gel was stained with Coomassie brilliant blue G-250. M, molecular mass marker proteins; 1, the Pr-2 protein was purified by RP-HPLC. (**B**) The molecular mass of the purified Pr-2 was examined using MALDI-TOF/MS.

Table 1. Summary of the Purification of Pr-2 from Pumpkin Rind Powders

step	amount of proteins (mg/200 g powder)	recovery (%)
1. extraction	246.32	100
2. heat	53.90	21.88
3. ultrafiltration	12.62	5.12
4. CM-sepharose	2.94	1.19
5. RP-HPLC	1.3	0.53

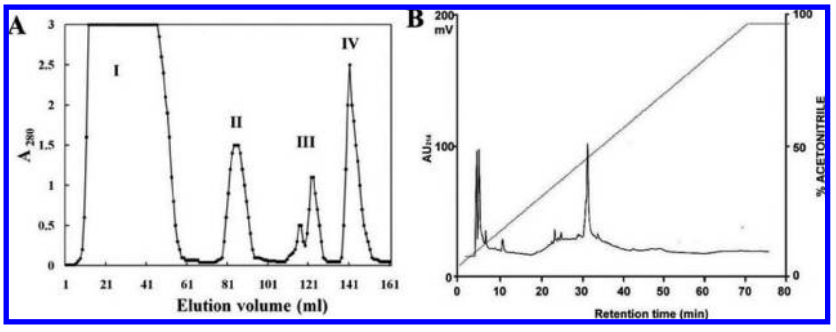

Figure 2. Chromatographical purification of antifungal proteins (Pr-2). (**A** and **B**) Pr-2, antifungal protein, was purified using cation exchange chromatography on CM-Sepharose (**A**) and reverse-phase HPLC on a C_{18} column (**B**). Extraction of the soluble proteins and chromatographic procedures are described in the Materials and Methods section. Fractions: I, unadsorbed fraction; II−IV, fraction eluted with 0.1 (II), 0.3 (III), and 1 M (IV) NaCl. Fraction II containing antifungal activity was collected and purified on a C_{18} column (**B**).

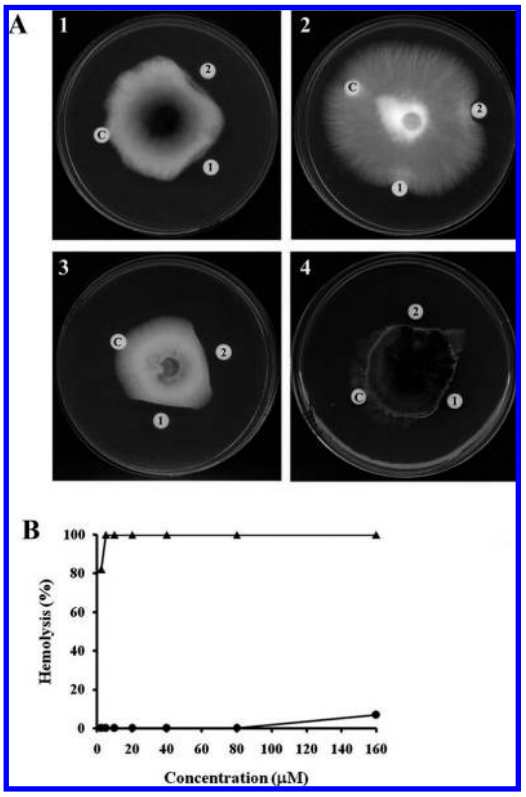

Figure 4. Inhibitory activity of purified Pr-2 for fungal growth (**A**) and its noncytotoxic effect for hRBCs (**B**). (**A**) Purified Pr-2 was subjected to radial growth inhibition tests using *F. solani* (1), *B. cinerea* (2), *T. harzianum* (3), and *C. coccodes* (4). The amounts of 150 and 300 μg of Pr-2 protein were loaded onto paper disk-1 and -2, respectively. Paper disk-C was loaded with 25 mM Hepes buffer, pH 7.2, as a negative control. (**B**) The dose response of hemolysis toward 8% hRBCs with Pr-2 (●) and melittin (▲) was evaluated.

Figure 3A, we evaluated the protein by matrix-assisted laser desorption/ionization time-of-flight/mass spectrometry (MALDI-TOF/MS). The results revealed that the molecular mass of the protein was 14865.57 Da (**Figure 3B**). Additionally, the N-terminal amino acid sequence of the protein was determined to be QGIGVGDNDGKRGKR by Edman degradation. This N-terminal amino acid sequence was not identical to that of any other proteins in the database, although the N-terminal amino acid sequence contained only 15 amino acids (approximately 11% of the full sequence). These results indicate that Pr-2 was a novel antifungal protein.

Antifungal and Nonhemolytic Effects of Pr-2. The purified Pr-2 protein was tested for its antimicrobial and hemolytic effects. As shown in **Figure 4A**, the protein exhibited potent antifungal activity against four phytopathogenic fungi. The ED_{50} of Pr-2 against fungal and bacterial cells is summarized in **Table 2**. Investigation of the inhibition spectrum revealed that Pr-2 showed good inhibition activity toward fungal cells but that it had no antibacterial effects. Pr-2 also had a low hemolytic activity against hRBCs at a concentration of 160 μM, whereas melittin, which was used as a control, showed 100% hemolysis at 5 μM (**Figure 4B**). Furthermore, Pr-2 was found to exhibit noncytotoxic activity for the HaCaT cell, which is a human keratinocyte cell line (data not shown).

Target Region of Pr-2 in Fungal Cells. To examine the target sites and better understand the antifungal activity of Pr-2, the organisms were observed by confocal laser scanning microscopy after which rhodamine-labeled Pr-2 was added to cultures of

Table 2. In Vitro Activity of the Antifungal Protein, Pr-2, against Fungal and Bacterial Cells

pathogens	ED_{50}[a] (μM)
fungi	
A. fumigates	40
A. parasiticus	80
B. cinerea	20
C. coccodes	10
D. bryoniae	40
P. verrucosum var. *verrucosum*	NA
F. oxysporum	10
F. solani	10
T. harzianum	20
T. viride	20
C. albicans	20
bacteria	
E. coli	NA
S. aureus	NA

[a] ED_{50}, effective dose for 50% inhibition; NA, not active at a concentration of 160 μM.

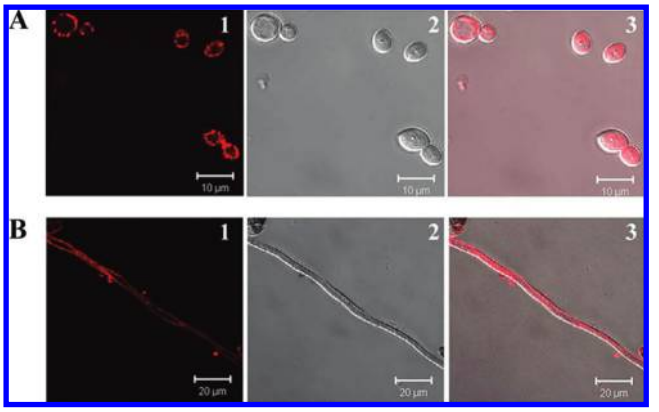

Figure 5. Localization of rhodamine-labeled Pr-2 in fungal cells. Fungal cells with rhodamine-labeled Pr-2 were visualized on confocal laser scanning microscopy. Conidia of *C. albicans* (**A**) and hyphae of *F. oxysporum* (**B**) were incubated with 20 μM rhodamine-labeled Pr-2 for 30 min. A representative fluorescence image of fungal cells with the protein is shown in panel 1, and a bright field image of the same cells is presented in panel 2. The merged image of the two panels is shown in panel 3.

C. albicans and *F. solani* (**Figure 5**). The results revealed that rhodamine-labeled Pr-1 accumulated in the cell wall or membrane of *C. albicans* (**Figure 5A**) and *F. solani* (**Figure 5B**). Furthermore, we measured the influx of SYTOX Green dye into *C. albicans* fluorimetrically to identify the effective site of Pr-2. SYTOX Green cannot enter the cytoplasm by itself without damaging the cell wall and membrane, and its fluorescence increases when it is bound to intracellular nucleic acids. A dose-dependent increase in fluorescence was displayed after the addition of Pr-2, while fluorescence did not increase in samples that were treated with bovine serum albumin as a negative control (**Figure 6A**). We also confirmed the entrance of dye into the cells by fluorescence microscopy (**Figure 6B**,**C**). Taken together, the results indicate that the fungal activity of Pr-2 occurred due to damage to the membrane of fungal cells.

A major mechanism of antimicrobial peptides was known that they kill the microorganism via destabilizing or pore formation of the cytoplasmic membrane by extracellular distribution and

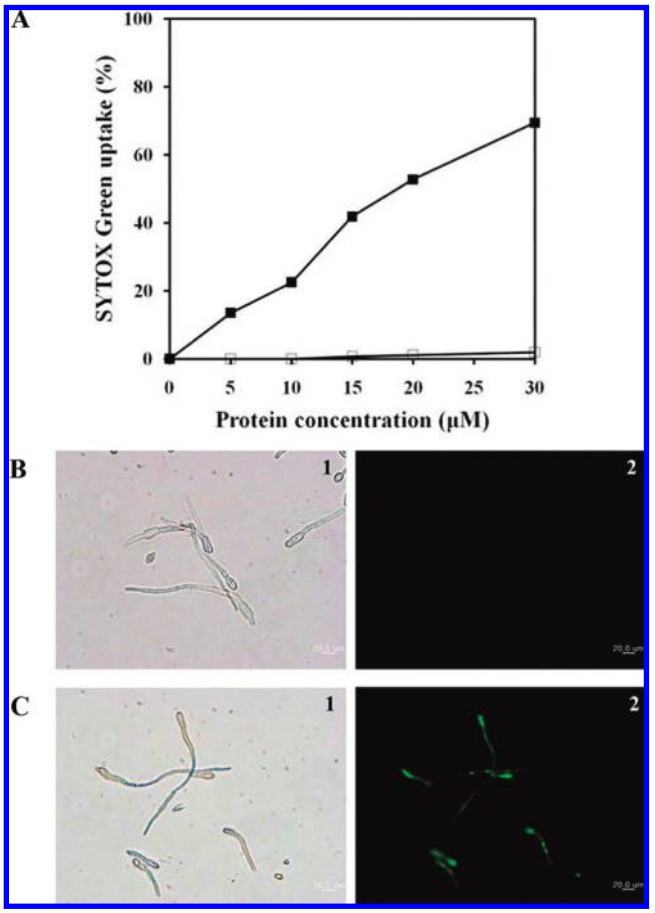

Figure 6. Permeable activity of Pr-2 in fungal cells. (**A**) SYTOX Green uptake into *C. albicans* was measured at an excitation of 484 nm and an emission of 520 nm after the addition of Pr-2 (■) and bovine serum albumin (BSA) (□). The fluorescence obtained by 0.1% triton X-100 was taken as 100% uptake. (**B** and **C**) Visualization of SYTOX Green uptake by *F. oxysporum* treated with BSA (**B**) or Pr-2 (**C**) at a concentration of 20 μM was observed using fluorescence microscopy. Panels 1 and 2 show the bright field image and fluorescence image, respectively. BSA was used as a negative control.

inhibit the growth of microorganism via inhibition of cell division or macromolecular synthesis by intracellular penetration (*25*, *26*). Similar to most antimicrobial peptides, Pr-2 may permeate the membrane by destabilizing or disrupting the plasma membrane of the microorganism.

In summary, Pr-2, a novel antifungal protein, appears as a single band after SDS-PAGE and has a heat-stable character. Additionally, Pr-2 exhibits growth inhibition against 10 species of harmful pathogenic fungi. Although further efforts are needed to determine another possible biological role of Pr-2, we predict that Pr-2 could be developed as an antifungal agent and for use in the development of phytopathogen-resistant transgenic plants.

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