

## Antifungal and Plant Growth Promotion Activities of Recombinant Defensin Proteins from the Seed of Korean Radish (*Raphanus sativus* L.)

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**Abstract:** In the present study, we analyzed the defensin protein deduced from Korean radish (*Raphanus sativus* L.) seeds. To express the genes in *E. coli*, we constructed a recombinant expression vector with a defensin gene, named *rKRs-AFP* gene isolated from Korean radish seeds. Over expressed rKRs-AFP proteins was separated by SDS-PAGE to determine the purity, and protein concentration was determined by the Bradford method. Antifungal activity was assessed by disk assay method against the tested fungi. As a result, when 500 mL of cell culture were disrupted by sonicator, 32.5 mg total proteins were obtained. The purified protein showed a single band on SDS-PAGE with estimated molecular weight about 6 KDa, consistent with the molecular mass calculated from the deduced amino acid sequence. The purified rKRs-AFP protein showed remarkable antifungal activities against several fungi including *Aspergillus niger*, *Botrytis cinerea* causing the gray mold disease, and *Candida albicans*. In field tests using the purified rKRs-AFP protein, the protein showed the reducing activity of disease spot and the mitigating effect of spreading of disease like agrichemicals. The immuno-assay of rKRs-AFP protein showed that the purified protein entirely accumulated at *B. cinerea* cytoplasm through the hyphal septa shown by fluorescence imaging. There was no fluorescence inside the cell, when the hypha was incubated without the protein. These all results indicate that the recombinant rKRs-AFP proteins can be utilized as a potential antifungal drug to control harmful plant fungal pathogens.

**Key Words:** anti-fungal protein, gray mold disease, *Raphanus sativus* L.

### INTRODUCTION

In the world, many fruit, vegetable and plants are damaged by plant fungal pathogens and one of them is *Botrytis cinerea* which is a casual agent of gray mold. To overcome plant fungal pathogens' problems, chemical cultivation has been performed in many countries for a long time. This practice causes not only environmental pollution but also harmful effects to human or animal. Due to the undesirable side-effect of synthetic agro-chemicals many biologists have been intensively studying biological control of plant fungal pathogens<sup>1-9</sup>.

Antimicrobial proteins such as radish antifungal protein in radish seeds, RIP (ribosome inactivating protein) and zeamatin in many plant seeds have been reported in the previous studies<sup>1,10-14</sup>. Several types of AMPs have been isolated, and their structures have been fully or partially characterized. Until now, defensin, only one conserved class of AMPs was found among plants, invertebrates, and vertebrates<sup>15</sup>. One type of these AMPs is small (3-5 KDa), basic, and cysteine-rich. Insect defensin and mammalian defensin are the most extensively studied Cys-rich AMPs<sup>16</sup>.

Plant defensins (45-54aa, 4 disulfide bridges) have been identified as a novel family of Cys-rich peptides<sup>17</sup>, which implicate in first-line host defense against fungal pathogens. Despite of their structural similarity<sup>18-20</sup>, plant defensins are highly varied in their primary amino

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acid sequences, with exception found in 8 structure-stabilizing Cys residues in common<sup>21</sup>). The variation in their primary sequences may account for the different biological activities reported for plant defensin, including antifungal activity<sup>22</sup>, antibacterial activity<sup>23</sup>. Mutational analysis of the radish (*Raphanus sativus*) Rs-AFP2 has revealed that the amino acid residues important for antifungal activity are clustered into two adjacent sites. The first site is around the type VI  $\beta$ -turn connecting  $\beta$ -strands 2 and 3, and the second sites is formed by residues on the loop connecting  $\beta$ -strand 1 and the  $\alpha$ -helix and the contiguous residues on the  $\alpha$ -helix and  $\beta$ -strand 3<sup>24</sup>). When fungal hyphae are treated with Rs-AFP2 or the *Dahlia merckii* defensin Dm-AMP1, there is a rapid influx of  $\text{Ca}^{2+}$ , efflux of  $\text{K}^+$ , and alkalization of the growth medium<sup>25</sup>). For these reasons, Plant defensin is considered as a hopeful agent of biological control against plant pathogens.

In this study, we described the cloning and purification of recombinant antifungal protein (rKRs-AFP) derived from Korean radish seeds, and estimated the antifungal efficacy of rKRs-AFP against *B. cinerea* using immuno-staining analysis and a field study.

## MATERIALS AND METHODS

### Sample, chemicals, microorganism and media

Korean radish seeds (*Raphanus sativus* L. cv. *Beakwoon*, Nongwoo Bio Co. Suwon, Korea) were used for the preparation of native antifungal protein<sup>1</sup>) and total RNA isolation. The total RNA of Korean radish seeds was isolated using easy-BLUE<sup>TM</sup> total RNA extraction kit (iNtRON, Seoul, Korea) and the cDNA of Korean radish seeds was synthesized using Power cDNA synthesis kit (iNtRON, Seoul, Korea). *Botrytis cinerea* (KACC 40574), *Aspergillus flavus* (KACC 40250), *Fusarium oxysporum* (KACC 40053), *Aspergillus niger* (KACC 40280), and *Candida albicans* (KACC30050) were used as the test pathogens. The fungal strains were grown in potato dextrose (PD) agar (Difco. Co. Detroit, USA), and spores were harvested and stored as previously described<sup>26</sup>). *C. albicans* was cultured overnight in YPD broth/agar (Difco. Co. Detroit, USA) at 30°C, respectively. *E. coli* Rosetta (DE3) *plac* (Novagen, UK.) was cultivated in LB broth (Difco. Co. Detroit, USA) containing appropriate concentration of antibiotics. Then it was used as a host for the expression of target protein.

### Construction of recombinant antifungal protein genes (rKRs-AFP genes)

From the amino acid sequence<sup>1,27</sup>), we deduced the genetic sequence of the rKRs-AFP genes. In order to amplify the consensus region of coding region form of KRs-AFP genes, the degenerated PCR primer was synthesized described as followings: The sequence of forward primer (mKRs-AFPf-Eco) is 5'-CCG GAA TTC CCA GAA GTT GTG YCA RAG G-3' which contained *EcoRI* restriction site. The sequence of reverse primer (mKRs-AFPr-Xba-His) is 5'-CCG GTC TAG ATT AGT GGT GGT GGT GGT GAC AWG GRA ART ARC AG-3', which contained *XbaI* restriction site and 6x His tag sequence. The PCR product amplified by a PCR Pfu DNA polymerase, cDNA of Korean radish seeds as the templates, and the described primer pairs was digested with *EcoRI/XbaI* and cloned into pHCEIb vector (Bioleaders Co. Seoul, Korea). The recombinant plasmid (rKRs-AFP genes) was sequenced and introduced into *E. coli* Rosetta (DE3) *plac*. The transformed *E. coli* was grown in 500 mL of LB broth (containing 50 ug/mL of ampicillin and 30 ug/mL of chloramphenicol) at 37°C with shaking for overnight.

### Purification of rKRs-AFP

The fully grown recombinant bacterial cells were harvested by centrifugation at 7,000g for 10 min at 4°C and the pellet was re-suspended in 50 mL of 20 mM Tris-HCl (pH 8.0). The cell suspension was sonicated for a total 20 min with a sonicator (Branson Model 250, Branson Ultrasonic, USA). The cracked bacterial cells were centrifuged at 10,000g for 15 min at 4°C. The pellet was re-suspended in 25 mL of 2 M Tris-HCl (pH 12) and 2 M urea and dissolved for 2h at 4°C. The fluid was centrifuged and filtered through a 0.22  $\mu\text{m}$  Acrodisc<sup>®</sup> syringe filter (Pall Co. USA) to remove insoluble materials<sup>28</sup>).

A Sephadex G-100 column (16×800 mm) was equilibrated with 1.5 column volumes of 20 mM Tris-HCl (pH 8.0) with a flow rate of 1 mL/min. The solubilized inclusion body was loaded onto a column and the eluted fractions were monitored at 280 nm. Pooled protein fractions were applied onto a metal chelating affinity chromatography column packed with 5 mL Ni-NTA resin (Qiagen Co. Germany) that was pre-charged with 50 mM  $\text{NiSO}_4$ . Before purification, the resin was equilibrated with 20 mM Tris-HCl (pH 8.0),

10 mM imidazole, and 0.5 M NaCl. The protein samples were loaded onto a column and washed with 10 volumes of 20 mM Tris-HCl (pH 8.0), 50 mM imidazole, and 0.5 M NaCl. Finally, the rPhlA protein was eluted with 20 mM Tris-HCl (pH 8.0), 500 mM imidazole, and 0.5 M NaCl, and the final elutant was dialyzed in 20 mM Tris-HCl (pH 8.0), containing 10% glycerol at 4 °C for 12 h. The purification steps were monitored by SDS-PAGE to determine the purity and the concentration of proteins was determined by Bradford method<sup>29</sup>).

#### Evaluation of antifungal activity

Antifungal activity was evaluated by disk assay method<sup>23</sup>. Firstly, *B. cinerea* KACC49574 was incubated on PDA plate at 25°C for 1 week. After spore formation, it was diluted in sterile water, centrifuged at 1,000 rpm for 1 min. In the supernatant, hyphae were removed and only spores were collected. Then the spores were added to sterile water, stored at 20°C and used as required. The obtained spores (100 µl) were mixed on 0.5% soft agar and was added onto a PDA plate. Afterwards, a sterile 5 mm paper disk (Whatman Ltd. Maidstone, UK) was placed on the PDA plate and samples were put on the disk. After cultivation at 25°C, the antifungal level of sample was determined. One antifungal unit was defined a minimal inhibitory dose against fungal growth.

#### Field study of rKRs-AFP

In order to estimate the consequence of rKRs-AFP onto plant infected gray mold rot disease under natural condition, the infected leeks were medicated with 50 mg/L of rKRs-AFP every week for 2 weeks. One 1 g/L solution of chemical drug Smirex (Dongbang Agro. Seoul, Korea) and distilled water (DW) were applied onto infected plants as a positive control and a negative control, respectively. After application, observations were made to notify the changes in disease points, number of leaves and leaf length for every single plant.

#### Immunization and immuno-fluorescence microscopy

A group of five 6 - 8 week old male Balb/c mice were immunized subcutaneously with 100 µl volumes containing 50 - 60 µg of purified native KRs-AFP in the presence of Freund's incomplete adjuvant. Two

immunizations were performed with a 2-week interval. A booster dose was given without adjuvant 1 month after the initial administration of the immunogen. The mice were sacrificed for preparative recovery of serum 2 weeks after the booster. The 2 mL of serum was applied to a column of 5 mL protein A Sepharose<sup>®</sup> CL-4B (Pharmacia, Sweden). The eluted IgG fraction was evaluated for its quality by SDS-polyacrylamide gel electrophoresis to reveal the presence of heavy and light chains. After dialysis against PBS, the materials was passed through a 0.22 µm syringe filter, quantified by Bradford assay, and then stored frozen in 0.5 mL aliquots at -70°C.

Further, in order to determinate the localization of rKRs-AFP, *B. cinerea* was incubated on the slide glass. First, 1 mL of PD agar was onto slide and covered with cover slip to be parallel between medium and slide. After being harden the medium, 1-2×10<sup>3</sup> numbers of spores were suspended with 100 µl of 0.5% soft agar then overlaid on slide. Every slide was incubated at 30°C until the spores which were embedded on slide were germinated sufficiently to observe the elongation of hyphae. 500 µl of 1 mg/mL rKRs-AFP was applied onto slide and incubated at 30°C for 6 h then the slides were fixed with 2.5% glutaraldehyde/1% paraformaldehyde mixture at 4°C for 4h. After fixation, the slides were washed with 0.1% of NaBH<sub>4</sub> for 1min to reduce auto fluorescence and then blocked with 2% goat serum / 0.1% Triton X-100 PBS for 1 h. The slide finished blocking step were treated with 200µl of mouse anti-KRs-AFP IgG diluted with 1% goat serum / 0.1% Triton X-100 PBS and incubated overnight at 4°C. In order to detect the binding of primary antibody, 500 × diluted Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes<sup>™</sup> USA) with 0.1% Triton X-100 PBS for 1hr in dark. Every interval of single step, the slide was washed with phosphate buffered saline (PBS) 3 times for 10 min.

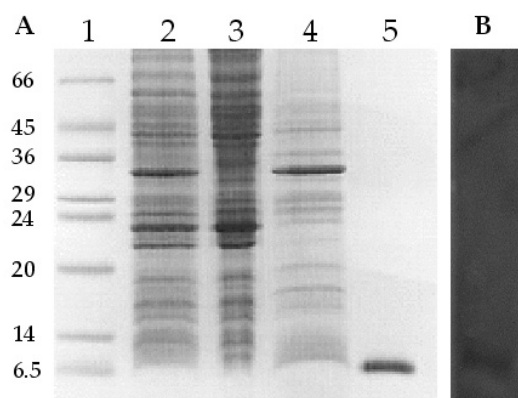
## RESULTS AND DISCUSSION

#### Purification of rKRs-AFP

Cells from 500 mL of culture were disrupted by sonicator and 32.5 mg total protein was obtained. After inclusion body was refolded, the specific activity increased by 5.3 fold while recovery was 77%. Finally, the rKRs-AFP was purified by metal affinity

**Table 1** Purification steps of rKRs-AFP from *E. coli*

	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification fold
Cell homogenate	32.5	1.25	40.63	100	1.0
Sephadex G-100	4.7	6.68	31.40	77	5.3
Ni-NTA affinity chromatography	1.9	12.5	23.75	58	10.0

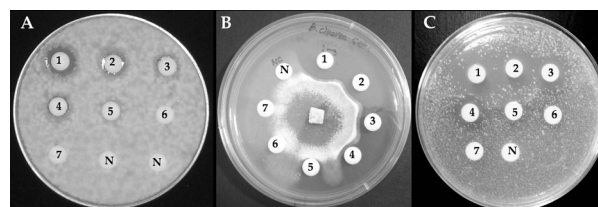


**Fig. 1.** SDS-PAGE (A) and immunoblotting (B) of recombinant Korean radish antifungal protein from *E. coli* Rosetta (DE3) *plac*. Lane 1, protein molecular weight markers (kDa); lane 2, crude cell extract; lane 3, cytosolic soluble fraction; lane 4, cytosolic insoluble fraction; lane 5, purified recombinant Korean radish antifungal protein from insoluble fraction.

chromatography and 1.9 mg of protein was obtained. The specific activity of purified rKRs-AFP was 12.5 U/mg (Table 1). The band was detected with mouse anti-rKRs-AFP IgG on immunoblotting analysis. The purified protein showed a single band on SDS-PAGE and the size was estimated about 6 kDa (Fig. 1). This value was in good agreement with the molecular mass calculated from the deduced amino acid sequence<sup>1,27</sup>.

#### Antifungal activity of rKRs-AFP

Previously we reported the antifungal efficacy of Korean radish against gray mold rot on Chinese chive caused by fungal pathogen *B. cinerea*<sup>30</sup>. In the present study, the antifungal activity of rKRs-AFP towards *B. cinerea*, *A. flavus*, *F. oxysporum*, *A. niger*, and *C. albicans* was observed. The antifungal activity was weakest against *A. niger* and the strongest against *B. cinerea*, respectively (Fig. 2). The rKRs-AFP revealed moderate antifungal activity against *C. albicans*. The strength of antifungal activity was positively correlated to the dose of rKRs-AFP. The minimal inhibitory doses of



**Fig. 2.** Determination of antifungal activities of recombinant Korean radish antifungal protein against pathogenic fungi and yeast by paper disk diffusion assay. 50  $\mu$ l of diluted samples were applied on each of paper disks. A, *Aspergillus niger*; B, *Botrytis cinerea*; C, *Candida albicans*: 1, 300  $\mu$ g; 2, 150  $\mu$ g; 3, 100  $\mu$ g; 4, 80  $\mu$ g; 5, 60  $\mu$ g; 6, 40  $\mu$ g; 7, 20  $\mu$ g/disc.

the antifungal activity of rKRs-AFP towards *B. cinerea*, *A. flavus*, *F. oxysporum*, *A. niger*, and *C. albicans* were 80, 120, 100, 150, and 100  $\mu$ g/disc, respectively. *In vitro*, the specificity of plant defensins activity was according to the ionic strength of growth medium<sup>26</sup> and in high strength growth medium, the spectrum of the activity was narrowed. We thought the difference of minimal inhibitory dose due to high ionic strength PDA growth medium and these purified recombinants rKRs-AFP can be usage for field experiment against gray mold rot.

#### Leek field study

To investigate the potential of rKRs-AFP as an effective antifungal bio-drug, the infected leeks were medicated with 50 mg/L of rKRs-AFP. The protein reduced the disease spot and consequently, mitigated spreading of disease like agrichemicals (Fig. 3). Moreover, the protein showed not only therapeutic efficacy but promotive effect on growth of leek plants. At two weeks after the treatment, the blades were averagely grown from 8.3 cm to 14 cm, while the plants treated with chemical drug were grown from 8 cm to 10.5 cm, and the numbers of leaves were increased from 1.5 to 2 (Fig. 4). In addition, the width of leaf was broader than positive control. Although the difference on the growth of plants was apparent, further studies

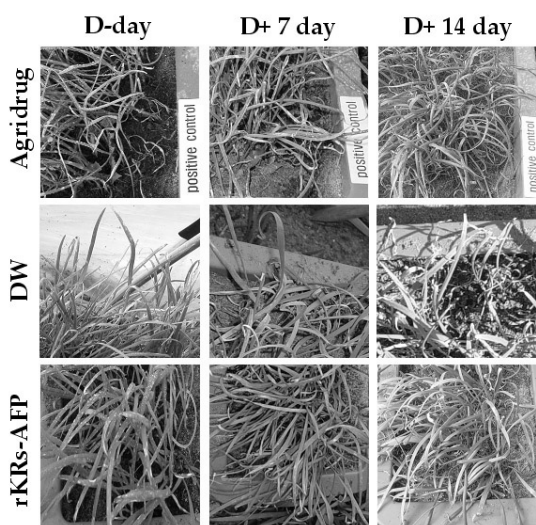


Fig. 3. Antifungal activity of rKRs-AFP against leek (*Allium tuberosum* Rottler) infected by gray mold rot disease. Every single pot was treated with 200 mL of 50 µg/ mL rRs-AFP, 1 mg/ mL agrichemicals or DW.

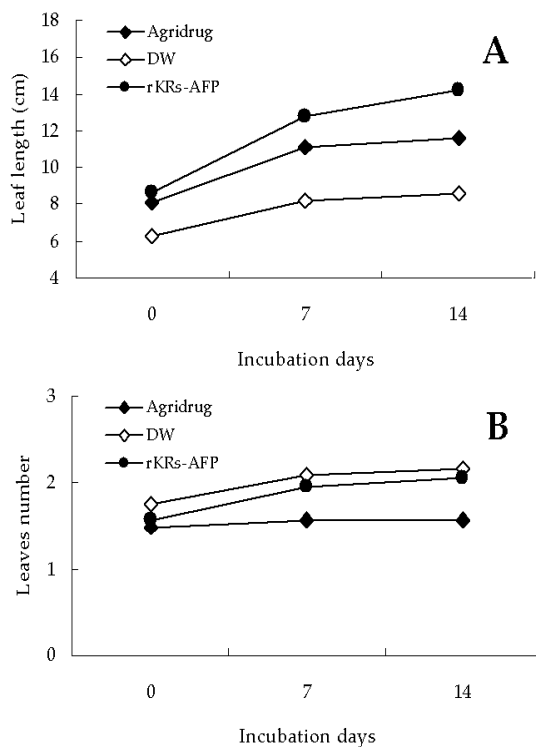


Fig. 4. The promotive effect of rKRs-AFP on growth of leek (*Allium tuberosum* Rottler) A : The leaf length was estimated for every single plant. B : The numbers of leaves was counted for every single plant. The amounts of proteins was used with 40 mg/ L.

are required to confirm growth stimulating effects.

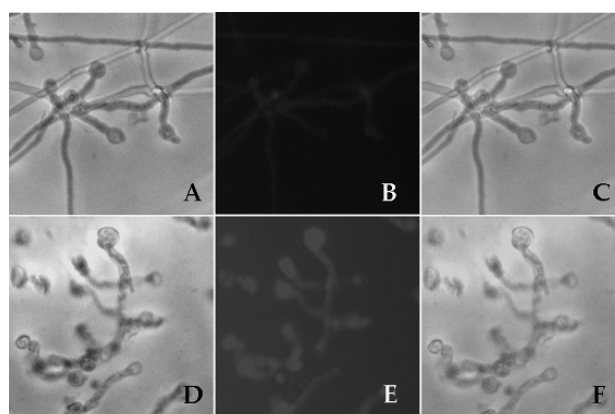


Fig. 5. Immunofluorescence microscope images to show the localization of rKRs-AFP in *B. cinerea* cells. (A) Normal image of *B. cinerea* cells untreated with rKRs-AFP. (B) rKRs-AFP Untreated cells are immunostained by mouse anti-rKRs-AFP IgG and GFP-labeled goat anti-mouse IgG. There was no detectable fluorescence signal. (C) Merged image of (A) and (B). (D) Normal image of disrupted cells (*B. cinerea*) by rKRs-AFP. (E) The disrupted cells were positively immunostained by mouse anti-rKRs-AFP IgG and GFP-labeled goat anti-mouse IgG (light green). (F) Merged image of (D) and (E).

#### Localization of rKRs-AFP in *B. cinerea*

We have reported that rKRs-AFP belongs to A group (A1, A2, A3 and A4) of defensins families based on its sequence<sup>27)</sup> and we proposed that rKRs-AFP is included in subfamilies (A3 and A4). To track the movement of antifungal proteins after application to the pathogen, rKRs-AFP was labeled with GFP. The rKRs-AFP which was treated to *B. cinerea* was easily detected with GFP-labeled antibody by fluorescence imaging. The results showed (Fig. 5) that the purified protein entirely accumulated at the cytoplasm through the hyphal septa with affecting pathogenic fungal morphology, but in hyphae incubated without the protein, no binding of primary antibody was observed, resulting in no fluorescence imaging inside the cell.

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