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Expression and Antibacterial Activity of a *Bombus ignitus* Apidaecin in Baculovirus-Infected Insect Cells

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The apidaecins are highly active against Gram-negative bacteria. Here, we show the expression and antibacterial activity of the bumblebee, *Bombus ignitus,* apidaecin. We PCR-amplified 51 bp of the active domain sequence of the *B. ignitus apidaecin* gene and expressed the recombinant *B. ignitus* apidaecin active domain in baculovirus-infected insect cells. The recombinant *B. ignitus* apidaecin active domain shows bactericidal activity against Gram-negative bacteria, including *Pseudomonas tolaasii,* a serious pathogen in cultivated mushrooms, but not Gram-positive bacteria. This result suggests that the active domain of the *B. ignitus* apidaecin is a potential antibacterial agent for the control of bacterial brown blotch diseases.

Key words: Antibacterial activity, Apidaecin, Bumblebee, Insect cells, Mushroom, *Pseudomonas tolaasii*

Introduction

Bacterial brown blotch diseases of the button (*Agaricus bisporus*) and oyster (*Pleurotus ostreatus*) mushrooms are caused by *Pseudomonas tolaasii* (Paine, 1919). *P. tolaasii* is a serious pathogen in cultivated mushrooms; it is endemic in mushroom farms and results in significant reductions in mushroom yield and quality (Prashanth *et al*., 2011). However, the control of bacterial diseases in cultivated mushrooms, such as *Agaricus* and *Pleurotus* spp., has been limited because suitable control measures and bactericides are lacking (Gill, 1995). Hence, novel and alternative control methods are needed. Currently, bacterial brown blotch diseases are controlled in mushroom cultivation houses by fumigation and by sterilizing water. A recent study has shown that the introduction of a *Bacillus thuringiensis* crystal protein into an antimicrobial *Bacillus brevis* strain has dual insecticidal and antibacterial activities, protecting against mosquito larvae and *P. tolaasii,* respectively (Roh *et al*., 2010).

Four genes encoding antibacterial peptides (*apidaecin*, *hymenoptaecin*, *abaecin*, and *defensin*) were cloned from the bumblebee *Bombus ignitus* (Choi *et al*., 2008). Among these antibacterial peptides, the apidaecins are highly active against Gram-negative bacteria (Casteels *et al*., 1989; Li *et al*., 2006). In this study, we evaluated the activity of the *B. ignitus* apidaecin against a Gram-negative bacterium, *P. tolaasii*, and report on the expression and antibacterial activity of the recombinant *B. ignitus* apidaecin. We PCR-amplified and expressed the active domain sequence of the *B. ignitus apidaecin* gene in baculovirus-infected insect cells. Additionally, we assayed the antibacterial activity of the recombinant *B. ignitus* apidaecin active domain against three Gram-negative bacteria and four Gram-positive bacteria.

Materials and Methods

Gene cloning and sequence analysis

The *B. ignitus apidaecin* active domain was PCR-amplified using a primer set designed from the *B. ignitus apidaecin* gene sequence (Choi *et al*., 2008): the forward primer (321-329), 5'-GATCCAGAAGAATTCATGGCCAACCGA-3' (*Eco*RI site was introduced) and the reverse primer (363-371), 5'-CAGCTTCGCCTCGAGAAGACGCGG-3' (*Xho* I site was introduced). PCR was performed according to a defined protocol: 94°C for 2 min, 30 cycles of amplification (94°C for 30 sec; 55°C for 30 sec; and 72°C for 30 sec) and 72°C for 5 min. The nucleotide sequences of PCR products were determined using a BigDye termina-

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tor cycle sequencing kit and an automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA).

Expression of the recombinant peptide

A baculovirus expression vector system, based on the *Autographa californica* nucleopolyhedrovirus (AcNPV) and the Sf9 insect cell line (Je *et al*., 2001), was used for the production of the recombinant *B. ignitus* apidaecin active domain. The 51 bp *B. ignitus apidaecin* active domain was inserted into the pBAC1 vector (Clontech, Palo Alto, CA) to generate an expression vector in which the expression of the recombinant protein is under the control of the AcNPV polyhedrin promoter. Recombinant baculoviruses were propagated in Sf9 cells cultured in TC100 medium (Gibco BRL, Gaithersburg, MD) at 27°C. Recombinant proteins were purified using a HisTrap column (Amersham Biosciences, Arlington Heights, IL). Protein concentrations were determined using the Bio-Rad Protein Assay Kit.

Antibody preparation and Western blot analysis

The purified recombinant *B. ignitus* apidaecin active domain (20 μg) was mixed with an equal volume (200 μl) of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO) and injected into BALB/c mice (Semtaco Bio Korea Co., Korea). Two subsequent injections were administered using antigens mixed with equal volumes of Freund's incomplete adjuvant (200 μl) at one-week intervals, beginning one week after the first injection. Blood was collected three days after the last injection (antigen only) and centrifuged at 10,000 × g for 10 min after clotting at 4°C overnight. The supernatant antibodies were stored at −70°C until use. Western blot analysis was performed using an enhanced chemiluminescence (ECL) western blotting analysis system (Amersham Biosciences, Piscataway, NJ). Protein samples were mixed with sample buffer, boiled for 5 min, and loaded on a 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the proteins were blotted onto a sheet of nitrocellulose transfer membrane (Schleicher & Schuell, Dassel, Germany). After blotting, the membrane was blocked by incubation in a 1% bovine serum albumin (BSA) solution, incubated with an antiserum solution (1:1,000 v/v) at room temperature for 1 hr, and washed in TBST (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% (w/v) Tween 20). The membrane was then incubated with horseradish peroxidase–conjugated anti-mouse IgG diluted 1:5,000 (v/v). After repeated washing, the membrane was incubated with ECL detection reagents (Amersham Biosciences) and exposed to film.

Antibacterial assay

The antibacterial activity of the recombinant *B. ignitus* apidaecin active domain was assayed, as described previously (Choo *et al*., 2010). Purified recombinant *B. ignitus* apidaecin active domain was tested for antibacterial activity against three Gram-negative bacteria (*Escherichia coli*, *P. tolaasii*, and *P. aeruginosa*) and four Gram-positive bacteria (*B. thuringiensis*, *B. megaterium*, *B. subtilis*, and *B. cereus*). Inocula (200 μl of a culture containing 1.5 × 10^6 cfu/ml) of *P. tolaasii* and *B. thuringiensis* 656-3 were spread onto sterile Luria-Bertani (LB) agar plates, and the surface of the plates was allowed to dry for approximately 5 min. A 20-μl aliquot of recombinant *B. ignitus* apidaecin active domain (40 mg) was then dropped onto LB agar plates that had been inoculated with bacteria. An equivalent volume (20 μl) of phosphate-buffered saline (PBS: 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4) was used as a control. The plates with recombinant *B. ignitus* apidaecin active domain were incubated at 30°C (Gram-negative bacteria) or 37°C (Gram-positive bacteria) for 18 hr, and the resulting activity was determined by measuring zones of inhibition. In addition, the antibacterial activity of the recombinant *B. ignitus* apidaecin active domain was monitored using a liquid growth inhibition assay. A total of 200 μl of the adjusted inoculum (1.5-2.0 × 10^5 cfu/ml) was added to each well of a 96-well plate containing serial dilutions of recombinant *B. ignitus* apidaecin active domain, and the same volume of PBS was, again, used as a control. LB broth was used as the bacterial culture medium used in this study. The 96-well plates were incubated at 30°C (Gram-negative bacteria) or 37°C (Gram-positive bacteria) for 18 hr with shaking at 230 rpm. The inhibition of bacterial growth was determined by measuring the absorbance at 560 nm. The growth inhibition results are expressed as the mean value of three independent replicates. The minimal inhibitory concentration (MIC) for the antibacterial assay is expressed as the lowest concentration that causes 50% inhibition of bacterial growth.

Results and Discussion

Expression of the recombinant *B. ignitus* apidaecin active domain

To assess the expression of the *B. ignitus apidaecin* active domain, the 51 bp active domain was PCR-amplified from *B. ignites* cDNA using a primer set designed from the *B. ignitus apidaecin* gene sequence (Choi *et al*., 2008). The resulting cDNA fragment was inserted into a baculovirus transfer vector (Fig. 1A) to generate a recombinant

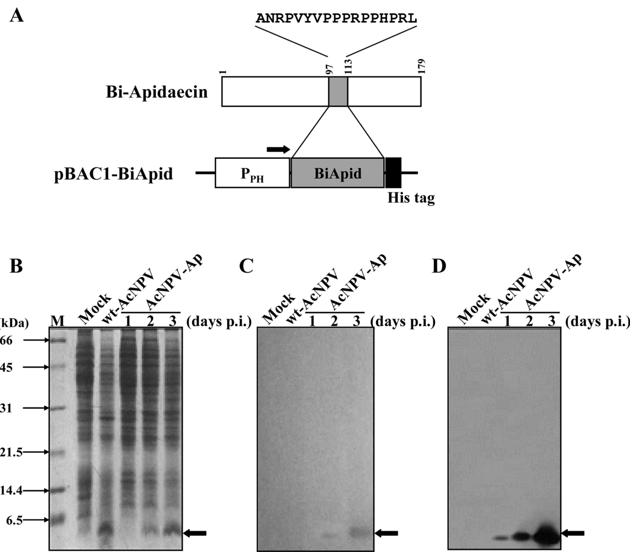

Fig. 1. Expression of the *B. ignitus* apidaecin active domain in baculovirus-infected insect cells. (A) The transfer vector pBAC1-BiApid for the production of recombinant AcNPV-Ap expressing the *B. ignitus* apidaecin active domain. The *B. ignitus apidaecin* active domain gene was placed under the control of the polyhedrin (P_{PH}) promoter. The arrow indicates the direction of transcription. (B-D) SDS-PAGE and Western blot analysis of the recombinant *B. ignitus* apidaecin active domain. Sf9 cells were mock-infected or infected with either the wild-type AcNPV or the recombinant AcNPV-Ap at an MOI of 5 pfu per cell. The cells were collected at 1, 2, or 3 days post-infection (dpi). Total cellular lysates were subjected to 15% SDS-PAGE (B), electroblotted, and incubated with either anti-*B. ignitus* apidaecin antibody (C) or anti-His-tag antibody (D). Molecular weight standards were used as size markers. The recombinant *B. ignitus* apidaecin active domain is indicated by arrows.

virus expressing the *B. ignitus* apidaecin active domain. A transfer vector (pBAC1-BiApid) was constructed by placing the *B. ignitus apidaecin* active domain cDNA under the control of an AcNPV polyhedrin promoter in pBAC1 (Fig. 1A). Recombinant AcNPV, which we have termed AcNPV-Ap, was produced in insect Sf9 cells by co-transfection with wild-type AcNPV DNA and the transfer vector.

The expression of the recombinant *B. ignitus* apidaecin active domain in Sf9 cells infected with the recombinant virus AcNPV-Ap was analyzed by SDS-PAGE (Fig. 1B). The recombinant *B. ignitus* apidaecin active domain was present as a single band of 2 kDa in cells infected with the recombinant virus but not in cells infected with the wild-type AcNPV or mock-infected cells.

To characterize the expression of the recombinant *B. ignitus* apidaecin active domain in baculovirus-infected insect cells, the recombinant *B. ignitus* apidaecin active

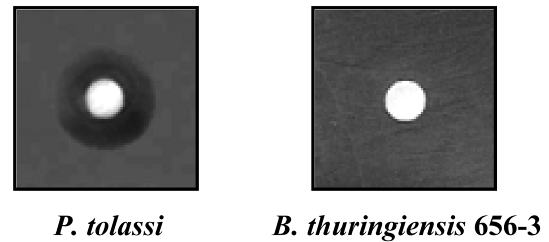

Fig. 2. Antibacterial activity of the *B. ignitus* apidaecin active domain. The inhibition of bacterial growth by the *B. ignitus* apidaecin active domain is shown using the disc diffusion assay. The Gram-negative bacterium *P. tolassi* (left) and the Gram-positive bacterium *B. thuringiensis* 656-3 (right) are shown.

domain, with a molecular mass of 2 kDa, was purified from the recombinant baculovirus (AcNPV-Ap)-infected Sf9 cell culture supernatants using a HisTrap column. The purified recombinant *B. ignitus* apidaecin active domain was injected into mice to produce polyclonal antibodies. The expression of the recombinant *B. ignitus* apidaecin active domain in baculovirus-infected insect cells was confirmed by Western blot analysis using either antibodies targeting the recombinant *B. ignitus* apidaecin active domain (Fig. 1C) or the His-tag (Fig. 1D)

Antibacterial activity of the recombinant *B. ignitus* apidaecin active domain

The Gram-negative bacterium *P. tolaasii* causes brown blotch disease in *A. bisporus* and *P. ostreatus* (Jo *et al*., 2011). The apidaecins are highly active against Gram-negative bacteria (Casteels *et al*., 1989; Li *et al*., 2006). Therefore, it seems likely that the apidaecin from *B. ignitus* may have antibacterial activity against *P. tolaasii*. Thus, we evaluated the antimicrobial activity of the recombinant *B. ignitus* apidaecin active domain. Our findings demonstrate that the recombinant *B. ignitus* apidaecin active domain shows a bactericidal activity against *P. tolaasii* but lacks activity against *B. thuringiensis* (Fig. 2), which shows insecticidal activity against mushroom flies (Choi *et al*., 2004).

To determine if the bactericidal activity of the recombinant *B. ignitus* apidaecin active domain is more widespread, we assessed its antibacterial activities against Gram-negative and Gram-positive bacteria using a liquid growth assay. The result shows that the inhibitory effect of the recombinant *B. ignitus* apidaecin active domain on bacterial growth is exclusive to Gram-negative bacteria (Table 1), with activity against *E. coli* (MIC, 20 μM), *P. tolaasii* (MIC, 125 μM), and *P. aeruginosa* (MIC, 149 μM) and no activity against Gram-positive bacteria, even at the highest concentration (300 μM) (Table 1).

Table 1. Antibacterial activity of the *B. ignitus* apidaecin active domain against Gram-negative and Gram-positive bacteria

Bacterial strain	MIC (μM)
Gram-negative	
E. coli	20
P. tolaasii	125
P. aeruginosa	149
Gram-positive	
B. thuringiensis 565-3	ND[a]
B. megaterium	ND
B. subtilis	ND
B. cereus	ND

[a] Not detected.

These results show that the *B. ignitus* apidaecin active domain is active against Gram-negative bacteria, including *P. tolaasii*.

In conclusion, these data demonstrate that the *B. ignitus* apidaecin is active against Gram-negative bacteria. The fact that the *B. ignitus* apidaecin active domain is active against *P. tolaasii* suggests that it is a potential antibacterial agent for the control of bacterial brown blotch diseases. Our study provides a basis for future studies focused on the development of bactericides for the control of bacterial pathogens in cultivated mushrooms.

Acknowledgments

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