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Molecular cloning and characterization of two peptide toxins from the spider *Araneus ventricosus*

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ABSTRACT

Spider toxins have great potential in the development of biopesticides. Here, we report the molecular cloning and characterization of two peptide toxins from the spider *Araneus ventricosus*. Two cDNAs encoding peptide toxins were cloned from *A. ventricosus*. Analysis of the cDNA sequence shows that the mature peptides of AvT-39 and AvT-48 consist of 39-amino acid residues and 48-amino acid residues, respectively. Both of the mature peptides include six conserved cysteine residues and a principal structural motif typical of spider toxins. The *AvT-39* and *AvT-48* cDNAs, which encode the mature peptide, were expressed in baculovirus-infected insect cells. AvT-39 and AvT-48 expression in insect cells significantly decreased cell viability. Additionally, the median lethal time (LT_{50}) of *Spodoptera exigua* larvae inoculated with recombinant AcNPV expressing AvT-48 was approximately 1 day shorter than that of larvae expressing wild-type AcNPV, demonstrating that the recombinant virus reduced LT_{50} by approximately 25%. Taken together, our findings describe the molecular characterization of two peptide toxins from *A. ventricosus* and demonstrate the potential for these toxins to be used as biopesticides.

Introduction

Spiders produce toxins to paralyze their prey or to defend themselves against predators. The majority of toxins produced by spiders are small peptides with molecular masses ranging from 3 to 7 kDa, which are stabilized by 3 to 5 disulfide bonds (Liang, 2004; Kozlov et al., 2005; Yuan et al., 2008). Peptide toxins are involved in various physiological processes such as ion channel blocking, insecticidal activity, blood coagulation, and fibrinolysis (Liang, 2004; Yuan et al., 2008). Thus, these spider toxins appear to have a strong potential to be developed into pharmaceuticals and insecticides (Rash and Hodgson, 2002; Zhang et al., 2003; Liang, 2004; Chong et al., 2007; Whetstone and Hammock, 2007; Corzo et al., 2008).

In baculoviral insecticides, several genes have been introduced into the baculovirus genome through genetic manipulation to increase the lethality of baculoviruses (Whetstone and Hammock, 2007). Among these attempts, scorpion venom-derived insect-specific toxins expressed by recombinant baculoviruses showed excellent efficacy in inducing the death of pests (Stewart et al., 1991; Zlotkin et al., 2000). In addition to scorpion peptide toxins, insect-selective and highly potent toxins have been identified from several other organisms including spiders (Hughes et al., 1997; Johnson et al., 1998; Zhang et al., 2003).

Among the spider species, tarantula toxins are the best studied because tarantulas are among the most venomous spiders (Liang, 2004; Yuan et al., 2008). Many effective biotoxins have been identified and characterized from tarantula venom. Although the cloning and expression of cDNAs encoding spider silk proteins from *Araneus ventricosus* have been reported (Lee et al., 2007, 2012), the molecular characterization of *A. ventricosus* spider toxins remains relatively unexplored. The objective of this study is to illustrate the gene structure and toxicity of the *A. ventricosus* peptide toxins. In this study, we describe the molecular cloning and characterization of two peptide toxins from *A. ventricosus* by cloning and sequencing cDNAs encoding *A. ventricosus* peptide toxins. We also expressed recombinant *A. ventricosus* peptide toxins in baculovirus-infected insect cells. Additionally, we characterized the insecticidal activity of a recombinant baculovirus expressing *A. ventricosus* peptide toxins.

Materials and methods

cDNA cloning and sequence analysis

A cDNA library was constructed using whole bodies of *A. ventricosus* as previously described (Lee et al., 2003). Clones containing the correct spider toxin cDNA inserts were selected from the expressed sequence tags (ESTs) that were generated from an *A. ventricosus* cDNA library. Plasmid DNA was extracted using the Wizard Mini-Preparation kit

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(Promega, Madison, WI, USA). cDNA sequences were analyzed using an ABI310 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Sequenced cDNAs were compared using the DNASIS and BLAST programs published online by NCBI (http://www.ncbi.nlm.nih.gov/BLAST). MacVector (ver. 6.5, Oxford Molecular Ltd., Oxford, UK) was used to align the deduced amino acid sequences of the spider toxin genes.

Cell culture and virus

Spodoptera frugiperda (Sf9) cells were maintained at 27 °C in TC100 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) as previously described (Je et al., 2001). Wild-type *Autographa californica* nucleopolyhedrovirus (AcNPV) and recombinant AcNPV were propagated in Sf9 cells.

Construction of recombinant virus expressing spider toxin

A baculovirus expression system (Je et al., 2001) using AcNPV and the Sf9 insect cell line was employed to construct recombinant virus expressing *A. ventricosus* toxin. The *AvT-39* cDNA, which encodes 39 amino acids as a mature peptide, was PCR-amplified from *pBluescript-AvT-39* using the primer set indicated in Table 1. The *AvT-48* cDNA, which encodes 48 amino acids as a mature peptide, was PCR-amplified from *pBluescript-AvT-48* using the primer set indicated in Table 1. Reverse primers for the amplification of *AvT-39* and *AvT-48* were engineered to include His-tag sequences. PCR cycling conditions were as follows: 94 °C for 3 min, 30 cycles of amplification (94 °C for 30 sec; 55 °C for 30 sec; 72 °C for 1 min), and 72 °C for 5 min. PCR products were sequenced using the BigDye terminator cycle sequencing kit and an automated DNA sequencer (Perkin-Elmer Applied Biosystems). The *AvT-39* and *AvT-48* fragments were inserted into the *pBacPAK8* vector (Clontech, Palo Alto, CA, USA) to generate an expression vector under the control of AcNPV polyhedrin promoter. The honeybee melittin signal peptide (Tessier et al., 1991) was used as a signal sequence for the secretion of AvT-39 and AvT-48 (Table 1). For expression experiments, 500 ng of the construct (*pBAC1-AvT-39* or *pBAC1-AvT-48*) and 100 ng of the AcNPV viral DNA (Je et al., 2001) were co-transfected into $1.0–1.5\times10^6$ Sf9 cells for 5 h using the Lipofectin transfection reagent (Gibco BRL). Transfected cells were cultured in TC100 medium (Gibco BRL) at 27 °C for 5 days. Recombinant baculoviruses were propagated in Sf9 cells cultured in TC100 medium at 27 °C.

Western blot analysis

Western blot analysis was performed using an enhanced chemiluminescence (ECL) Western blotting system (Amersham Biosciences, Piscataway, NJ, USA). Protein samples were mixed with sample buffer [0.0625 M Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, and 0.125% bromophenol blue], boiled for 5 min, and separated using 14% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and then the membrane was blocked in 1% bovine serum albumin (BSA). Then, the membrane was incubated with anti-His antibody at room temperature for 1 h and washed in tris-buffered saline spiked with Tween-20 (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 washes with TBST, the membrane was incubated with ECL detection reagents (Amersham Biosciences) and exposed to autoradiography film.

Table 1
Primer sequences used for PCR amplification.

Primer	Sequence
AvT-39-F	5′-GACCAAACATGTAGGAAA-3′
AvT-39-R	5′-GGTACCTTAATGATGATGATGATGATGAAAGAGTGGTTCTTGGCA-3′
AvT-48-F	5′-GACCAAGAATGCAGGAAA-3′
AvT-48-R	5′-GGTACCTTAATGATGATGATGATGATGGTTATTGGGTTTCCT-3′
Melittin-F	5′-GGATCCATGAAATTCTTAGTCAAC-3′
Melittin-R	5′-GGCCGCATAGATGTAAGA-3′

Cell immunofluorescence staining

Sf9 insect cells infected with the recombinant virus expressing spider toxin or wild-type AcNPV were harvested at 48 h, 60 h, or 72 h post-infection (p.i.) and washed three times in phosphate-buffered saline (PBS: 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Sf9 cells were fixed in acetone (−20 °C) for two minutes and air-dried. The cells were washed three times in PBS and then pre-incubated in PBS containing 2% BSA at room temperature for 20 min. After washing once with PBS, the cells were incubated with mouse anti-His antibody diluted 1:500 (v/v) in PBS containing 1% BSA for 1 h, washed twice in PBS for 10 min each, and then incubated in goat anti-mouse fluorescein-conjugated antibody diluted 1:400 (v/v) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in PBS containing 1% BSA for 1 h. Cells were washed five times in PBS for 5 min each, and then the cells were wet mounted. Visualization and localization of spider toxins within Sf9 cells were performed by laser scanning confocal microscopy (Carl Zeiss LSM 510, Zeiss, Jena, Germany).

RNA extraction and Northern blot analysis

Total RNA was isolated from the epidermis, fat body, silk gland, and venom gland using a Total RNA Extraction Kit (Promega). The total harvested RNA (5 mg/lane) was separated using a 1.0% formaldehyde agarose gel, transferred onto a nylon blotting membrane (Schleicher & Schuell), and hybridized at 42 °C with the appropriate probe diluted in hybridization buffer containing 5× SSC (1× SSC is 0.15 M sodium chloride and 0.15 M sodium citrate), 5× Denhardt's solution (0.1% each of BSA, Ficoll, and polyvinylpyrrolidone), 0.5% SDS, and 100 mg/ml denatured salmon sperm DNA. *AvT-39* or *AvT-48* cDNA was labeled with [α-^{32}P] dCTP (Amersham Biosciences) using the Prime-It II Random Primer Labeling kit (Stratagene, La Jolla, CA, USA), and labeled cDNA was used as a probe for hybridization. After hybridization, the membrane filter was washed three times for 30 minutes each in 0.1% SDS and 0.2× SSC at 65 °C and then was exposed to autoradiography film.

Bioassay

Larvae of the beet armyworm *Spodoptera exigua* were reared on an artificial diet as previously described (Kwon et al., 2006; Wan et al., 2012). Day 1 third instar *S. exigua* larvae were inoculated with 1×10^5 polyhedra/larva of wild-type AcNPV or recombinant AcNPV expressing AvT-48. Accumulated mortality was measured for 144 h post-infection (p.i.). The median lethal time (LT_{50}) was calculated using the Probit method and SoftTOX software, version 1.1 (WindowChem Inc., USA). The 95% confidence intervals (CI) were also calculated for the LT_{50} values.

Statistical analysis

Data are expressed as the mean ± SD of experiments performed in triplicate and were analyzed for statistical significance using one-way analysis of variance (ANOVA). Differences with a *P*-value less than 0.05 were considered statistically significant for all treatments.