Journal of Asia-Pacific Entomology 19 (2016) 59–64

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Molecular cloning and antifungal activity of an inhibitor cysteine knot peptide from the bumblebee *Bombus ignitus*

Hee Geun Park [a], Yijie Deng [a], Kwang Sik Lee [a], Bo Yeon Kim [a], Hyung Joo Yoon [b], Kyeong Yong Lee [b], Byung Rae Jin [a,*]

[a] College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Republic of Korea
[b] Department of Agricultural Biology, National Academy of Agricultural Science, Wanju 565-850, Republic of Korea

ARTICLE INFO

Article history:
Received 24 August 2015
Revised 26 October 2015
Accepted 28 October 2015
Available online 25 November 2015

Keywords:
Bombus ignitus
Bumblebee
Inhibitor cysteine knot peptide
Innate immunity
Antifungal peptide

ABSTRACT

The honeybee inhibitor cysteine knot (ICK) peptide acts as an antifungal peptide and insecticidal venom toxin. However, the ICK peptide from bumblebees has not been characterized. Here, we report the molecular cloning and antifungal activity of a bumblebee (*Bombus ignitus*) ICK peptide (BiICK). We identified a BiICK that contains an ICK fold. The *BiICK* was expressed in the epidermis, fat body, and venom gland of *B. ignitus* worker bees. A 6.7-kDa recombinant BiICK peptide was expressed in baculovirus-infected insect cells. Recombinant BiICK peptides directly bound to *Beauveria bassiana*, *Ascosphaera apis*, and *Fusarium graminearum*, but they did not bind to *Escherichia coli*, *Paenibacillus larvae*, or *Bacillus thuringiensis*. Consistent with this finding, BiICK exhibited antifungal activity against fungi. These results demonstrate that BiICK acts as an antifungal peptide.

Introduction

Inhibitor cysteine knot (ICK) peptides have been identified in a wide variety of plants species, invertebrates, and vertebrates (Osaki et al., 1999; Fujitani et al., 2002; Barbault et al., 2003; Escoubas and Rash, 2004; Tian et al., 2010; Gilly et al., 2011; Horita et al., 2011; Smith et al., 2011; Zhang and Zhu, 2012; Chan et al., 2013; Gao et al., 2013; He et al., 2013; Smith et al., 2013; Park et al., 2014). The ICK fold is a common structural motif exhibiting small and triple-stranded β-sheets that contain three disulfide bridges (Zhu et al., 2003; Tian et al., 2010; Zhang and Zhu, 2012). ICK peptides are known to possess various biological actions. Based on their functional roles, ICK peptides are divided into two groups: venom toxins and antimicrobial peptides (Tian et al., 2010; Zhang and Zhu, 2012). Previous studies have shown that the ICK peptides from various organisms have multifunctional roles that include ion channel-blocking activity (Escoubas and Rash, 2004; Deng et al., 2013; Gao et al., 2013), insecticidal activity (Horita et al., 2011; Smith et al., 2011), and antimicrobial activity (Osaki et al., 1999; Liu et al., 2000; Fujitani et al., 2002; Barbault et al., 2003; Tian et al., 2010; Zhang and Zhu, 2012; Park et al., 2014).

Additionally, insect ICK peptides may be involved in innate immunity (Tian et al., 2010). Our previous study showed that the ICK peptide from the honeybee *Apis cerana* acts as an antifungal peptide and an insecticidal venom toxin, indicating that the honeybee ICK peptide has dual functions, with different mechanisms of action in both the body and venom (Park et al., 2014). In comparison to the biological and toxicological actions of ICK peptides from scorpions and spiders, the roles of ICK peptides from bees have remained relatively unexplored. Moreover, a recent study has identified twenty-six ICK peptides from seven ant species (Zhang and Zhu, 2012). Until now, ICK peptides from bumblebees have not been characterized. In the present study, we report the molecular cloning and antifungal activity of an ICK peptide from the bumblebee *Bombus ignitus* (BiICK). We cloned the *BiICK* cDNA and expressed recombinant BiICK in baculovirus-infected insect cells. Using recombinant BiICK, we assayed for microbial binding and antimicrobial activity. Finally, we found that BiICK exhibits antiproliferative activity against fungi but not against bacteria.

Materials and methods

cDNA cloning and sequence analysis

The cDNA encoding BiICK was cloned from a set of expressed sequence tags (ESTs) from a *B. ignitus* cDNA library (Choo et al., 2010). Plasmid DNA extraction was performed using a Wizard Mini-Preparation Kit (Promega, Madison, WI, USA) according to the manufacturer's protocols. The cDNA sequence was analyzed using a BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). A signal sequence was predicted using the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP). Pairwise sequence

* Corresponding author. Tel./fax: +82 51 200 7594.
E-mail address: brjin@dau.ac.kr (B.R. Jin).

http://dx.doi.org/10.1016/j.aspen.2015.10.012
1226-8615/

comparisons were performed using the DNASIS and BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST). MacVector (ver. 6.5, Oxford Molecular Ltd., Oxford, UK) was used to align the ICK amino acid sequences. A phylogenetic analysis of the hymenopteran ICK amino acid sequences was performed using the bootstrap 75% majority-rule consensus tree with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford, 2000). The hymenopteran ICK amino acid sequences retrieved from database searches for the alignment and phylogenetic analyses are summarized in Table 1.

RNA extraction and northern blot analysis

The bumblebee *B. ignitus* was supplied by the Department of Agricultural Biology, National Academy of Agricultural Science, Republic of Korea. Ten *B. ignitus* worker bees were dissected on ice, and tissue samples were collected from the epidermis, fat body, gut, muscle, and venom gland. The total RNA from these tissue samples was prepared using a Total RNA Extraction Kit (Promega). The total RNA (5 μg/lane) was separated using a 1.0% formaldehyde agarose gel, transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany), and hybridized at 42 °C with a *BiICK* cDNA probe labeled with [α-^{32}P] dCTP (Amersham Biosciences, Piscataway, NJ, USA) using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocols. The hybridization conditions and the exposure used in this study were previously described elsewhere (Lee et al., 2014; Park et al., 2014).

Recombinant protein expression and purification

Recombinant BiICK was produced using a baculovirus expression system (Je et al., 2001). The *BiICK* cDNA sequence was PCR-amplified from *pBluescript-BiICK* using the forward primer (74-94) 5′-GGATCC ATGTCCAAATTTATGCTATCC-3′ and the reverse primer (284-301) 5′-TCTAGATTAATGATGATGATGATGATGATAATCCTTCCCTTT-3′; the reverse primer included a His-tag sequence (underlined). The PCR cycling conditions were 3 min for denaturation at 94 °C, followed by 30 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min) and a final 5 min extension at 72 °C. The resulting PCR products were sequenced as described above. The *BiICK* fragment was inserted into the *pBacPAK8* transfer vector (Clontech, Palo Alto, CA, USA) under the control of the *Autographa californica* nucleopolyhedrovirus (AcNPV) polyhedrin promoter. Co-transfection with 500 ng of the expression vector (*pBacPAK8-BiICK*) and 100 ng of AcNPV viral DNA (Je et al., 2001) was performed as previously described (Lee et al., 2014; Park et al., 2014). Recombinant viruses were cultured in *Spodoptera frugiperda* (Sf9) insect cells in TC100 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco BRL) at 27 °C. Recombinant proteins were purified using the MagneHis™ Protein Purification System (Promega) according to the manufacturer's protocols. The recombinant proteins were identified using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analysis with anti-His antibody (BETHYL Laboratories, TX, USA). The western blot analysis was performed using an enhanced chemiluminescence western blotting system (Amersham Biosciences, Piscataway, NJ, USA) with the anti-His antibody and horseradish peroxidase-conjugated anti-mouse IgG diluted 1:5000 (v/v) as the secondary antibody. The protein amounts were quantified using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Microbial binding assay

Escherichia coli DH5α (Park et al., 2014) and *Bacillus thuringiensis* strain 656-3 (Choi et al., 2004; Park et al., 2014) were cultured in Luria–Bertani (LB) medium, and *Paenibacillus larvae* (Choi et al., 2013b) was cultured in Brain–Heart Infusion (BHI) medium. *Beauveria bassiana* SFB-205 (Kim and Je, 2010; Park et al., 2014), *Ascosphaera apis* (Choi et al., 2013a), and *Fusarium graminearum* wild-type strain GZ3639 (Bowden and Leslie, 1999; Park et al., 2014) were grown in potato dextrose broth. The bacterial or fungal culture was harvested and washed with 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). The microorganism samples were incubated with 0.7 μg of recombinant BiICK (suspended in 40 μl of PBS) at room temperature for 10 min. After centrifugation at 4000 rpm for 5 min, the pellets were washed and then resuspended in 40 μl of PBS. The pellet and supernatant samples were resolved by 15% SDS–PAGE and identified using western blot analysis, as described above with an anti-His antibody.

Confocal microscopy

E. coli, *B. thuringiensis*, *P. larvae*, *B. bassiana*, *A. apis*, or *F. graminearum* was cultured as described above. The obtained microorganisms were washed three times with PBS and incubated with 0.7 μg of recombinant BiICK (suspended in 40 μl of PBS) at room temperature for 10 min. The microorganism samples were fixed in acetone (−20 °C) for 2 min and air-dried. The samples were washed three times in PBS and then incubated with 2% BSA (suspended in PBS) at room temperature for 20 min. After the incubation, the samples were washed once with PBS and incubated for 1 h with anti-His antibody [diluted 1:500 (v/v)]. For secondary staining, the samples were washed twice with PBS for 10 min and subsequently incubated with fluorescein-conjugated goat anti-mouse antibody [diluted 1:400 (v/v); Santa Cruz Biotech., Inc.,

Table 1
Hymenopteran ICK amino acid sequences retrieved from database searches.

Family	Genus species	Common name	GenBank no.	Definition
Apidae	*Bombus ignitus*	Bumblebee	KT374284	Inhibitor cysteine knot peptide
	Bombus terrestris	Bumblebee	XM_003395714.2	Omega-conotoxin-like protein 1
	Apis cerana	Aisatic honeybee	KJ530970.1	Inhibitor cysteine knot peptide
	Apis mellifera	Honey bee	XM_006560014.1	Uncharacterized protein
	Apis dorsata	Giant honeybee	XM_006617301.1	Uncharacterized protein
	Apis florea	Little honeybee	XM_003691529.2	Omega-conotoxin-like protein 1
Megachilidae	*Megachile rotundata*	Alfalfa leafcutting bee	XM_003700188.2	Omega-conotoxin-like protein 1
Formicidae	*Linepithema humile*	Argentine ant	ADOQ01003051.1	Uncharacterized protein
	Pognomyrmex barbatus	Red harvester ant	XM_011646163.1	Omega-conotoxin-like protein 1
	Wasmannia auropunctata	Little fire ant	XM_011708498.1	Omega-conotoxin-like protein 1
	Monomorium pharaonis	Pharaoh ant	XM_012676372.1	Omega-conotoxin-like protein 1
	Harpegnathos saltator	Jerdon's jumping ant	XM_011156654.1	Omega-conotoxin-like protein 1
	Camponotus floridanus	Florida carpenter ant	XM_011259206.1	Omega-conotoxin-like protein 1
	Solenopsis invicta	Red fire ant	XM_011161167.1	Omega-conotoxin-like protein 1
	Acromyrmex echinatior	Panamanian leafcutter ant	XM_011053427.1	Omega-conotoxin-like protein 1
	Vollenhovia emeryi	Japanese ant	XM_012022800.1	Omega-conotoxin-like protein 1