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RESEARCH ARTICLE

Molecular cloning and characterization of venom peptides from two Honeybee species (*Apis cerana* and *Apis mellifera*)

Muttugoud Revataganv[a], N.Balakrishnan[b], M.R.Srinivasan[b], S.Mohankumar[a]

[a]Department of Plant Biotechonlogy, Centre for Plant Molecular Biotechnology, Tamil Nadu Agricultural University, Coimbatore-641003.
[b]Department of Agricultural Entomology, AC&RI, Tamil Nadu Agricultural University, Killikulam, Vallanadu-628252.

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ABSTRACT

Two honey bee species (*Apis cerana* and *Apis mellifera*) are domesticated for the economic products like honey, royal jelly and venom. Three components of honey bee venom peptide *viz.,* melittin, phospholipase A2 (PLA2), and secapin have proven effects in pharmaceutical and clinical applications and these components were cloned and characterized in the present study. Bee venom glands were dissected out for RNA isolation and cDNA was synthesized from the total RNA. PCR was carried out using gene-specific primers for melittin, PLA2, and secapin genes, using cDNA as template. PCR products were cloned in the TA-cloning vector and sequenced. Amino acid sequences were deduced from nucleic acid sequences and a phylogenetic tree was constructed to deduce the evolutionary relationship among the honey bee species. Crude bee venom was collected by electric milking method and dissolved in sterile water to separate the venom peptides by SDS-PAGE. Crude bee venom was tested against the rice pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* which causes bacterial blight disease of rice. Agar diffusion assay of bee venom was performed to confirm the antimicrobial properties of crude venom. Both *A. cerana* and *A. mellifera* venom showed antibacterial activity against *X. oryzae* pv. *oryzae*.

Keywords: *RT-PCR, Bee venom peptides, Melittin, Phospholipase A2, Secapin, antibacterial activity*

INTRODUCTION

Honeybee venom is composed of various biologically active substances like multidomain proteins, polypeptides, enzymes, amines, lipids, and amino acids. Enzymes like phospholipaseA2, phospholipase B, hyaluronidase, phosphatase, alpha-glucosidase and peptides like melittin, mast cell degranulation (MCD) peptide, secapin, adolapine, crdiopep (Vick *et al.,* 1974), tertiapin (Gauldie *et al.,* 1976) and melittin F. Amines like histamine (Nagamitu 1935), apamine which is a neurotoxin (Habermann and Cheng-Raude 1975) and minimine (Lowy *et al.,* 1971). Bee venom is produced from a long thin, convoluted exocrine gland present at the abdomen of the queen and worker bees. Honey bees use venom for protecting the colonies and themselves when they are exposed to predators (Peiren *et al.,* 2008). Venom is lethal for other insects and small vertebrates, even for larger animals also it is lethal if they stung by a greater number of stings (Piek *et al.,* 1984). Bee venom has got significant medicinal property in which it is effective against arthritis, multiple sclerosis and cancer diseases (Jo *et al.,* 2012). The main constituent of bee venom is melittin, which accounts for 50% of total bee venom (Habermann 1972) and possesses the lytic activity and anti-microbial action. Melittin gene was isolated from *A. mellifera* (Neumann *et al.,* 1952), *Vespula sp.*, and *Polistes sp.* (Li *et al.,* 2005). Cloning of PLA2, the utmost studied toxic enzyme, that exhibits inflammatory effect, was done in *A. mellifera* (Chunsheng *et al.,* 2014) and bumblebee, *Bombus ignites* (Xin *et al.,* 2009). Secapin is a serine protease inhibitor-like peptide that exhibits anti-fibrinolytic, anti-elastolytic, and anti-microbial activities (Lee *et al.,* 2016, Hou *et al.,* 2014). Cloning and characterization of these venom peptides from *A. cerana* and *A. mellifera* is done in the present study. Besides, as bee venom exhibits antibacterial activity, experiments were carried out to test the effect of bee venom extracts on plant pathogenic organisms like *X. oryzae* pv. *oryzae*.

MATERIAL AND METHODS

Venom collection

Honey bee venom is collected from two species of honey bees *viz., A. cerana* and *A. mellifera* maintained at Apiary, Department of Agricultural Entomology, Tamil Nadu Agricultural University Coimbatore. Venom collection was carried out by two

*Corresponding author's e-mail: smktnau@gmail.com

methods, one by electric milking (Dotimas and Hider, 1987) using honey bee venom collector instrument and the other was by a manual collection of venom by dissecting out venom reservoir from the abdomen of the honey bee and squeezing it mechanically (Piek 1986). The venom was dried, lyophilized and stored at -20ºC until use. Venom was mixed with sterile water and centrifuged at 13000rpm for 10 min to precipitate the impurities. The supernatant was filter sterilized using a 0.22 µm membrane filter and used as a venom sample for SDS-PAGE analysis. The concentration of 10µg/µL was obtained by dissolving 4 mg of venom in 400 µL of sterile water.

Cloning and characterization of venom peptides

RNA was isolated from honey bee venom glands using TRI-reagent. cDNA was synthesized from total RNA using Revert Aid First Strand cDNA synthesis kit (Thermo Fisher Scientific India). The polymerase chain reaction was carried out using peptide related gene-specific primers (Table.1) using cDNA as template. Amplified products were separated on 2 per cent agarose gel. PCR amplification was performed with a total volume of 25 µL reaction containing 2.5 µL reaction 10X buffer, 2.5 µL dNTP's (25mM), forward and reverse primer 2.5µL (10mM), 0.3 µL Takara Taq (1.5 units) DNA Polymerase and 14.6 µL of nuclease-free water. PCR temperature profile for melittin gene was as follows: 5 mins at 94˚C, followed by 35 cycles of 94˚C for 1 min, 51˚C for 30 sec and 72˚C for 40 sec with a final extension of 72˚C for 10 min. PCR temperature profile for escaping gene was as follows: 5 mins at 95˚C, followed by 35 cycles of 95˚C for 1 min, 55˚C for 30 sec and 72˚C for 40 sec with a final extension of 72˚C for 10 min. PCR temperature profile for PLA2 gene was as follows: 5 mins at 95˚C, followed by 35 cycles of 95˚C for 1 min, 53˚C for 30 sec and 72˚C for 40 sec with a final extension of 72˚C for 10 min.

PCR products were cloned into TA-cloning vector (pMD20-T) and were transformed into *E. coli* (DH5α) cells which were then plated on LB-agar medium containing ampicillin (50 mg/ml), X-gal (20 mg/ml), and IPTG (100 mM/µl). White colonies were selected and plasmid was isolated. Transformants were confirmed by subjecting plasmid to double digestion with *Eco*RI and *Hin*dIII restriction enzymes (Sambrook *et al*., 1989). Plasmids were sequenced by AgriGenome Labs Pvt Ltd. Cochin, India using BigDye Terminator Cycle Sequencing Kit.

Sequence analysis

Nucleotide sequences of melittin, secapin and PLA2 were deduced to amino acid sequences by using the ExPASy (Expert Protein Analysis System) translation tool. Sequences were aligned with the ClustalW programme using Bioedit tool. MEGA X software tool was used to construct the phylogenetic tree by neighbor-joining method. The amino acid sequences of out-group used in the phylogenetic analysis were obtained from the NCBI database.

The SDS-PAGE analysis of crude venom

Protein samples were mixed with protein sample buffer (0.0625M Tris–HCl, pH 6.8, 2 % SDS,10 % glycerol, 5 % β-mercaptoethanol, and 0.125 % bromophenol blue), boiled for 4 min. The protein samples were separated using 15% SDS-PAGE. Following electrophoresis, gels were fixed and stained with 0.1% Coomassie brilliant blue R-250 (Laemmli, 1970)

Anti-bacterial assay

Agar disc diffusion assay was carried out using nutrient agar (NA) medium for *X. oryzae pv. oryzae*. Bacterial strain *Xanthomonas oryzae* pv. *oryzae* was obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore and sub cultured on nutrient agar medium. Twenty-four hour fresh bacterial cultures were used as inoculum by spreading on NA medium. Crude venom extract (50µg and 30µg) from *A. cerana* and *A. mellifera* was pipetted on to the autoclaved Whatman's filter paper discs (7mm) and allowed to dry. Discs containing venom were placed on NA medium for the diffusion of venom components. Discs without venom extract and water alone were used as control (Hudzicki 2009). Zones of inhibition in bacterial cultures were measured after 24 h incubation period. The experiment was repeated 5 times.

RESULTS AND DISCUSSION

Cloning and characterization of venom peptides

The PCR result showed that amplified products were of the expected size, for melittin (263 bp; Fig.1A), secapin (257 bp; Fig.2A) and PLA2 (559

Table. 1. Primer sets used for the RT-PCR analysis (Hou *et al.,* 2013, Chunsheng *et al.,* 2014)

Gene	Primers	Sequences	Expected product size
Melittin	Forward	5'-GAAGCGATCGGAGAAATCAT-3'	263 bp
	Reverse	5'-GATAGGTCGTAAATCGGCAT-3'	
Secapin	Forward	5'-AGAAGAATTATGAAGAACT-3'	257 bp
	Reverse	5'-AAGTTCATACGAATTTAAG-3'	
PLA2	Forward	5'-TGTAACCTCCGCTTCCCTT-3'	559 bp
	Reverse	5'-TCCGCCCCGTGAATTTATC-3'	

bp; Fig.3A). for both *A. cerana* and *A. mellifera*. respectively. Recombinant clones were identified by *Eco*RI and *Hin*DIII restriction enzyme analysis. The size of digested gene fragments was increased compared to actual gene size because of extra flanking sequences of other restriction sites present in the cloning vector.

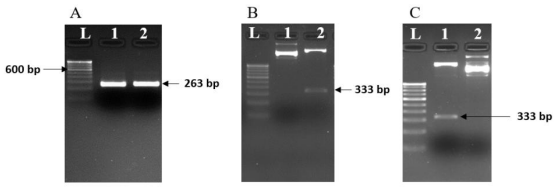

Figure 1. (A) Melittin gene PCR product (L: 100bp ladder, 1: *A. mellifera*. 2: *A. cerana* (B) Restriction digestion of recombinant plasmid with melittin gene of *A. cerana* with EcoRI and HinD III (L: 100bp ladder, 1: plasmid undigested, 2: plasmid digested) (C) Restriction digestion of recombinant plasmid with melittin gene of *A. mellifera* (L: 100bp ladder,1: plasmid digested, 2: plasmid undigested).

Sequence alignment and phylogenetic tree analysis

Multiple alignments of amino acid sequences of melittin and escaping from *A. cerana* and *A. mellifera*, showed more than 95% homology with the other species. where, *A. cerana* and *A. mellifera* differed by two positions in melittin (Fig.5) and three positions in secapin (Fig. 6). Amino acid sequences of PLA2 gene from *A. cerana* and *A. mellifera* showed 64% similarly with other species. But differed in many positions. (Fig.7).

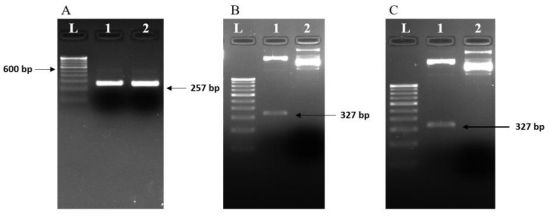

Figure 2. (A) secapin gene PCR product (L: 100bp ladder, 1: A. mellifera. 2: A. cerana (B) Restriction digestion of recombinant plasmid with secapin gene of A.cerana with EcoRI and HinD III (L: 100bp ladder, 1: plasmid digested, 2: plasmid undigested) (C) Restriction digestion of recombinant plasmid with secapin gene of A. mellifera (L: 100bp ladder,1: plasmid digested, 2: plasmid undigested).

Phylogenetic tree of melittin gene revealed that honey bee species *A. mellifera* of India and European species were closely related, whereas *A. cerana* of India and Republic of Korea species were closely related (Fig.8). Phylogenetic tree of secapin

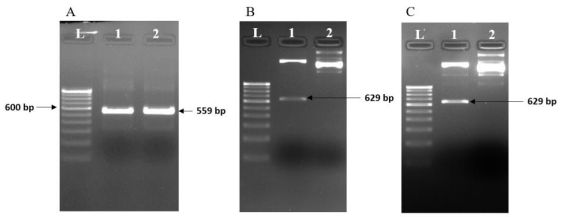

Figure 3. (A) PLA2 gene PCR product (L: 100bp ladder, 1: A. mellifera. 2: A. cerana (B) Restriction digestion of recombinant plasmid with PLA2 gene of A.cerana with EcoRI and HinD III (L: 100bp ladder, 1: plasmid digested, 2: plasmid undigested) (C) Restriction digestion of recombinant plasmid with PLA2 gene of A. mellifera (L: 100bp ladder,1: plasmid digested, 2: plasmid undigested).

gene revealed that *Apis mellifera* from America and Europe were closely related, whereas *Apis cerana* from Republic of Korea and *Apis mellifera* from India were closely related (Fig.9). Likewise, the phylogenetic tree of PLA2 revealed that *A. cerana* from India and China were closely related, whereas *Apis mellifera* from India and America were closely related (Fig.10).

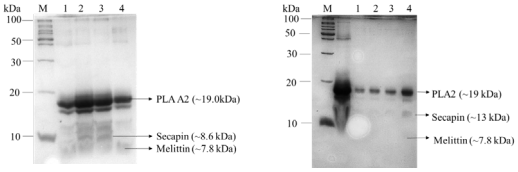

Figure 4. SDS-PAGE analysis of honeybee venom (A) Apis mellifera and (B) Apis cerana M: Protein marker, 1 to 4: 0.2 to 0.5 µl respectively.

Antibacterial activity bee venom against X. oryzae pv. oryzae.

Agar disc diffusion assay was conducted to check the antibacterial activity of the honey bee venom against the *X. oryzae pv. oryzae* (Fig.11). Results showed that crude bee venom of both *A. cerana* and *A. mellifera* had antibacterial activity against the pathogen. (Table.2)

Values given in table represents mean ± S.D. of (n=5) replicates. In antimicrobial activity assay *A. cerana* and *A.* mellifera crude venom showed strong inhibitory activity at 50µg with an inhibition zone of 15.00±0.32(mm) and 15.00±0.79 respectively. The same phenomenon was observed at 30µg in which *A. cerana* & *A.* mellifera crude venom showed 10.00±0.22 and 10.00±0.7(mm) zone of inhibition respectively.

The present study describes, cloning and characterization of three genes from honeybee venom gland *viz.,* melittin, PLA2, and escaping of two honeybee species *A. cerana* and *A. mellifera*. Melittin gene cDNA of 263 bp and secapin of 257 bp were found to be similar to the earlier reports (Hou *et al.,* 2013). PLA2 gene of 559bp was found

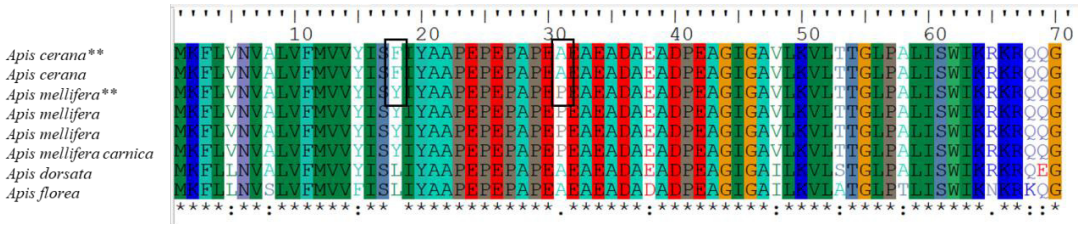

Figure 5. ClustalW multiple sequence alignment of melittin deduced amino acid sequences of Apis spp. represents species used for the study.**

to be similar to reports by Chunsheng *et al.,* (2014). The amino acid sequences of *A. cerana* melittin peptide deduced from the nucleotide sequences was 70 as similar to earlier reports (Vlasak *et al.,* 1983). PLA2 and secapin were 169, and 77 amino acids respectively. *A. cerana,* PLA2 amino acid sequences found to be 169 reported (Chunsheng *et al.,* 2014). ClustalW multiple sequence alignment of deduced amino acid showed that there was 95% homology with other species in case of melittin

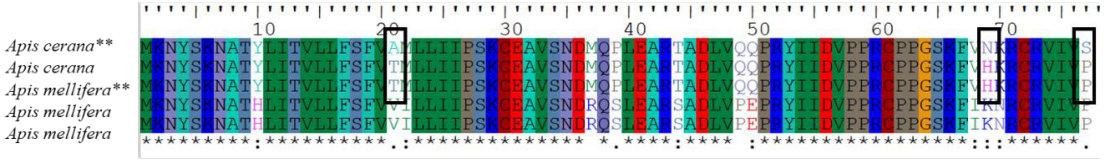

Figure 6. ClustalW multiple sequence alignment of secapin deduced amino acid sequences of Apis spp. represents species used for the study**

and secapin peptides and more conserved regions were found. In the case of PLA2, 64% of the amino acid regions were shared with other species of the honey bee. Although, some regions of amino acids were found to be semi-conserved. The phylogenetic tree was constructed based on deduced amino acid sequences among the related honey bee species to understand genetic roots and evolutionary relationship of melittin, secapin, and PLA2. Where all peptides of *A. cerana* and *A. mellifera* clustered into

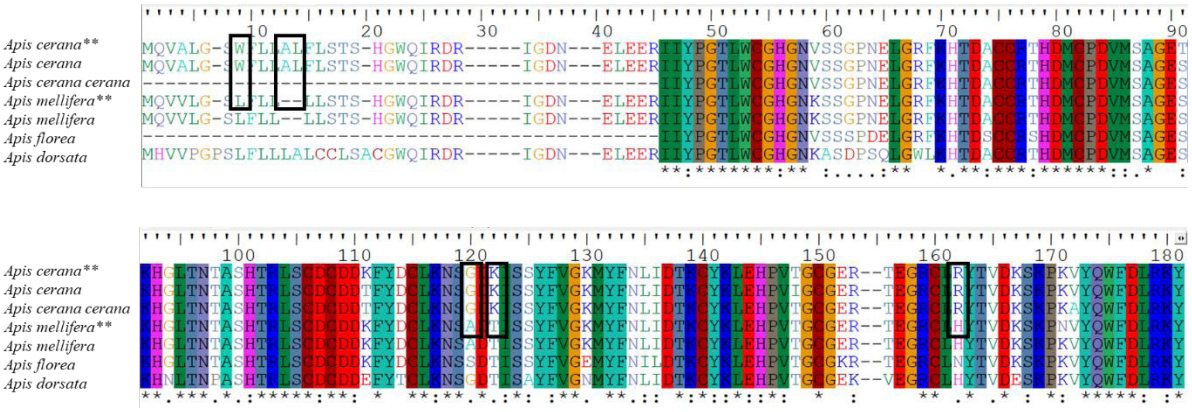

Figure 7. ClustalW multiple sequence alignment of PLA2 deduced amino acid sequences of Apis spp. represents species used for the study.**

the distinct group in terms of different geographical regions, demonstrating that they share geographical identities. In silico analysis of peptides showed that melittin peptide of *A. cerana* has the isoelectric point (pI) of 4.69 and contains 9 negatively charged residues and 6 positively charged residues.

Table 2. Antibacterial activity bee venom against *X. oryzae* pv. *oryzae*

	Zone of Inhibition (mean of 5 replications in mm)					
Pathogenic Organism	Water control	*A. cerana* 50 g	*A. cerana* 30 g	*A. mellifera* 50 g	*A. mellifera* 30 g	Blank disc
X. oryzae pv. *oryzae*	0.00	15.00 0.32	10.00 0.22	15.00 0.79	10.00 0.7	0.00

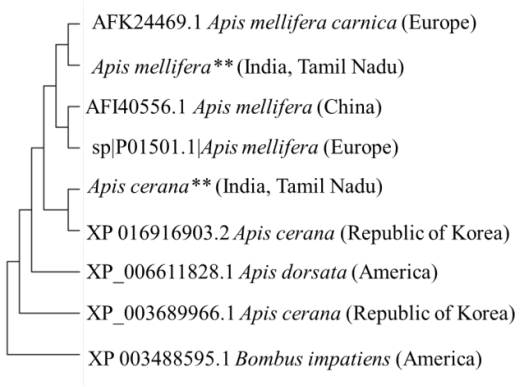

Fig.8 Phylogenetic relation of *A. cerana* and *A. mellifera* in melittin peptide with other honey bee species of different geographical regions.

Melittin has estimated the half-life of 30 hr in mammalian reticulocytes *in vitro*, more than 20 hr in yeast *in vivo* and more than 10 hr in *E. coli*. Aliphatic index of the peptide was 107.43 and has instability index of 46.7 which shows it was an unstable peptide.

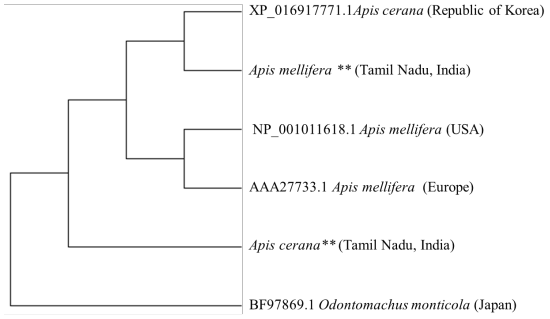

Fig.9 Phylogenetic relation of *A. cerana* and *A. mellifera* for secapin peptide with honey bee species of different geographical regions.

As compared to *A. cerana*, *A. mellifera* melittin shows almost same parameters in terms of molecular weight of 7.8 kDa, isoelectric point (pI) and amino acids charges except for aliphatic index of 106.00 and instability index of 51.70 which shows peptide was unstable. In the case of PLA2 of *A. cerana, the* peptide has a molecular weight of 19.9 kDa, and isoelectric point (pI) of 7.55. It consists of 21 negatively charged residues and 22 positively charged residues.

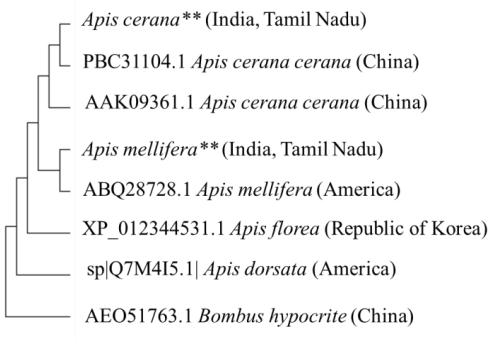

Fig.10 Phylogenetic relation of *A. cerana* and *A. mellifera* for PLA2 peptide with honey bee species of different geographical regions.

It has estimated the half-life of 30 hr in mammalian reticulocytes *in vitro*, more than 20 hr in yeast *in vivo* and more than 10 hr in *E. coli*. Aliphatic index of the peptide was 63.43 and has instability index of 23.33 which is a stable peptide. PLA2 of *A. mellifera* peptide has a molecular weight of 19.5 kDa and has the isoelectric point (pI) of 6.69. It consists of 21 negatively charged residues and 20 positively charged residues. Estimated half-life of PLA2 was 30 hr in mammalian reticulocytes *invitro*, more than 20 hr in yeast *in vivo* and more than 10 hr in *E. coli* systems. Aliphatic index of PLA2 peptide was 65.93 and has instability index of 28.11 which was a stable peptide. Whereas, secapin peptide of *A. mellifera* and *A. cerana* has an isoelectric point (pI) of 9.45 with 5 negatively charged residues and 10 positively charged residues. It has estimated the half-life of 30 hr in mammalian reticulocytes *in vitro*, more than 20 hr in yeast *in vivo* and more than 10 hr in *E. coli* systems. Aliphatic index of the peptide was 102.47and has instability index of 30.69 which was a stable peptide.

CONCLUSION

Although, many studies have been made on melittin and PLA2 peptides, still the secapin peptide has to be explored more. Antimicrobial properties of bee venom are less explored against plant pathogens compared to human pathogens.

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