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The expression and phylogenetics of the Inhibitor Cysteine Knot peptide OCLP1 in the honey bee *Apis mellifera*

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ABSTRACT

Small cysteine-rich peptides have diverse functions in insects including antimicrobial defense, phenoloxidase activity regulation, and toxic inhibition of ion channels of prey or predator. We combined bioinformatics and measurements of transcript abundance to start characterizing AmOCLP1, a recently discovered Inhibitor Cysteine Knot peptide in the honey bee *Apis mellifera*. We found that the genomes of ants, bees, and the wasp *Nasonia vitripennis* encode orthologous sequences indicating that OCLP1 is a conserved peptide and not unique to the honey bee. Search of available EST libraries and quantitative real time PCR analyses indicate that the transcript of *AmOCLP1* is ubiquitous with expression in life stages ranging from embryos to adults and in all tested tissues. In worker honey bees *AmOCLP1* expression was not associated with age or task and did not show clear enrichment in any of the tested tissues. There was however a consistent trend toward higher transcript levels in the abdomen of foragers relative to levels in the head or thorax, and compared to levels in the abdomen of younger worker bees. By contrast, in drones *AmOCLP1* transcript levels appeared higher in the head relative to the abdomen. Finer analyses of the head and abdomen indicated that the *AmOCLP1* transcript is not enriched in the stinger and the associated venom sac or in cephalic exocrine glands. The evolutionary conservation in the Hymenoptera, the ubiquitous expression, and the lack of enrichment in the venom gland, stinger, exocrine glands, and the brain are not consistent with the hypotheses that OCLP1 is a secreted honeybee toxin or an endotoxin acting in the central nervous system. Rather we hypothesize that OCLP1 is a conserved antimicrobial or phenoloxidase inhibitor peptide.

1. Introduction

Cysteine rich peptides are important for many vital processes in insects including defense, immune responses, wound healing, and neuromodulation. This group of peptides is extremely variable in terms of primary sequence and function. It includes ion channel inhibitors, antimicrobial peptides, phospholipases, protease inhibitors, disintegrins, defensins, and proteins with additional functions. There is no clear relationship between sequence and function, and peptides with a similar function may show profound sequence variability. For example, ion channel inhibitors, a functional group of animal toxin peptides (ATP), which inhibit the same target channels, often vary in sequence and structural folds (Billen et al., 2008; Mouhat et al., 2004). The high diversity in their sequence, structure, and function makes it challenging to detect and annotate these peptides with standard automatic classification methods. To contend with this challenge Kaplan et al. (2007) built a computational classifier for identifying animal toxins and toxin-like peptides (ATP and ATP-like) and applied it to 10,157 predicted protein sequences of the sequenced honey bee (*Apis mellifera*) genome (Weinstock et al., 2006). Using this tool 19 predicted honey bee ATP-like proteins were identified, eight of which were predicted to possess a signal peptide, as expected of ATPs. Two of the proteins are well-known bee venom toxins, Apamin and MCD (Mast Cell Degranulating Peptide). Two additional high scored sequences were termed *'Raalin'* and *'OCLP1'* (ω-conotoxin-like protein 1, later also termed Amickin-1; Tian et al., 2010) and studied with more detail (Kaplan et al., 2007; Tirosh et al., 2012). In the current report we describe expression and phylogenetic analyses for the honey bee *AmOCLP1* gene.

AmOCLP1 is a predicted 74 amino acid residue sequence that possesses a signal peptide followed by a cysteine-rich domain of ~30 amino acid residues and an unstructured tail. The six cysteines are linked by three bridges forming an *Inhibitor Cysteine Knot* (ICK) fold, an evolutionarily conserved structural motif shared by a large

Abbreviations: ATP, animal toxins and toxin-like peptides; HPG, hypopharingeal glands; ICK, Inhibitor Cysteine Knot; OCLP1, ω-conotoxin-like protein 1; PO, phenol oxidase; qRT-PCR, quantitative reverse transcription PCR.

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group of polypeptides with diverse sequences and bioactivities (Craik et al., 2001). For example, this fold characterizes toxins of venomous animals such as spiders, scorpions, and assassin bugs in which it is necessary for their tertiary structure and presumed toxicity toward ion channels (Billen et al., 2008; Corzo et al., 2001; Zhu et al., 2003). In other studies ICK peptides were shown to function in antimicrobial defense (Fujitani et al., 2002) or as phenoloxidase inhibitors (Daquinag et al., 1995; Lu and Jiang, 2007; Tsukamoto et al., 1992). Kaplan et al. (2007) showed that AmOCLP1 is similar to three saliva toxins of the assassin bug that appear to function as voltage-gated Ca^{2+} ion channel inhibitors, and which contain a fold similar to that of the snail ω-conotoxins (Bernard et al., 2004, 2001; Corzo et al., 2001). The premise that AmOCLP1 is a toxin is also consistent with the observation that injection of an expressed AmOCLP1 peptide into fish (but not fly maggot) caused a significant, reversible, short-term paralytic effect and compromised locomotion (Kaplan et al., 2007). However, preliminary mRNA analysis in the same study showed that the *AmOCLP1* transcript is expressed in the honey bee brain. Although there is some evidence that venom toxins may be additionally expressed in non-venomous tissues, including the brain (Ma et al., 2001; Miwa et al., 1999), these findings may point to the possibility that AmOCLP1 is not a constituent of the honey bee venom and its expression may not be brain specific. For example, mosquitoes and flies express small proteins that share profound similarities in the cysteine motifs and tertiary structure with AmOCLP1. These peptides are expressed throughout the body and were suggested to function as phenoloxidase inhibitors necessary for fine-tuning the melanization process and its associated release of cytotoxic reactive oxygen intermediates (Shi et al., 2006; Tsukamoto et al., 1992). As mentioned above, peptides with a similar ICK motif also function as antimicrobial peptides that may also have ubiquitous expression.

In order to study the expression pattern and possible functions of AmOCLP1 in the honey bee, we combined bioinformatics and qPCR analyses for different tissues and for bees differing in age, task, or gender. These analyses indicate that sequences similar to AmOCLP1 are encoded in the genomes of additional hymenopterans and suggest that AmOCLP1 has a ubiquitous expression in the honey bee body with no enrichment in the brain, stinger, or cephalic exocrine glands.

2. Materials and methods

2.1. Bees

Honey bee colonies were derived from a mixture of European races typical to Israel. We maintained the colonies in the field at the bee research facility in the Edmond Safra campus of the Hebrew University of Jerusalem, Israel. The colonies were maintained according to standard beekeeping practices. Each source colony was headed by a queen that was instrumentally inseminated with semen from a single drone, with the exception of colony H5 that was headed by a naturally mated queen. Given that queens typically mate with 10–20 drones, a single-drone insemination helps to reduce genetic variability between sister bees in source colonies used in the reported experiments. Foragers and nurses were identified according to established criteria (e.g., (Bloch and Robinson, 2001; Huang et al., 1991). Nurses were identified as 7-day-old worker bees inserting their heads into honeycomb cells containing larvae; foragers were identified as bees returning to their hive with loads of pollen in their pollen baskets. To obtain 1-day-old bees, we removed honeycomb frames containing pupae (sealed in cells) from source colonies in the field, removed all adult bees, and immediately transferred the frames to a light-proof container that we placed in a dark incubator (32 ± 2 °C, 50 ± 10% relative humidity).

2.2. AmOCLP1 expression in honey bee EST libraries

We used Blast to search the NCBI EST database by limiting the species to *A. mellifera*, and using as query the *AmOCLP1* mRNA sequence (accession number XM_001120252, GB19297, Kaplan et al., 2007). In addition, we used the BeeBase GBrowse to search for EST sequences similar to *AmOCLP1* (using the search term GB19297) in *A. mellifera* Genome Assembly 4.5 (http://hymenopteragenome.org/cgi-bin/gb2/gbrowse/bee_genome45).

2.3. OCLP1 like sequences in other insects

We first used the Blastp and tBlastn algorithms to search NCBI nr databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for sequences similar to the premature (including the signal peptide) or mature predicted AmOCLP1 protein sequence. We also used the BeeBase GBrowse to search the *A. mellifera* Genome Assembly 4.5 for sequences similar to AmOCLP1 in the genome sequences of 7 ant species (*Acromyrmex echinator, Atta cephalotes, Camponotus floridanus, Harpegnathos saltator, Linepithema humile, Pogonomyrmex barbatus, Solenopsis invicta*), the parasitic wasp *Nasonia vitripennis*, and the SwissPort Metazoa database (which is linked to BeeBase). Predicted mRNA sequences were translated to proteins, and used in a Blastx search for finding similar proteins in the NCBI nr protein database.

Multiple sequence alignment was performed using ClustalW (Thompson et al., 1994). To infer the evolutionary history we conducted a Neighbor-Joining analyses (Saitou and Nei, 1987) using the MEGA5 software package (Tamura et al., 2011). In addition to the sequences mentioned above, we also included in this analysis sequences of three assassin bug toxins (Ptu1, accession number P58606.1, (Bernard et al., 2001); Ado1, accession number P58608.1, (Bernard et al., 2004); Iob1, accession number P58609.1, (Corzo et al., 2001)), insect phenoloxidase inhibitors (POI) from *Manduca sexta* (accession number BE015616, (Lu and Jiang, 2007)) *Musca domestica* (accession number P81765.1, (Daquinag et al., 1995), and *Anopheles gambiae* (accession number AAX22219, (Shi et al., 2006)), and three *A. mellifera* toxins or toxin-like peptides (AmRaalin, BeeBase GB11222; AmApamin, accession number P01500.2; AmMCD [Mast Cell Degranulating Peptide], accession number P01499.2). The robustness of the obtained phylogenetic tree was assessed using bootstrap analysis with 1000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All ambiguous positions were removed for each sequence pair. There were a total of 668 positions in the final dataset.

2.4. Dissections

We performed all dissections on dry ice (with the exception of the finer expression analyses in the second experiment in which the tissue was dissected in ice-cold saline, see below) and maintained the tissue frozen throughout the procedure. We detached the heads and abdomens from the rest of the body and transferred each separately into a frozen 15 ml tube for RNA extraction (see below). To facilitate brain dissection, the heads were detached after a freeze-dry procedure (DNA Plus freeze dryer, Heto Drywinner, −55 °C, 100 mbar) as described (Hagai et al., 2007; Rubin et al., 2006; Tirosh et al., 2012). We removed the compound eyes, ocelli, hypopharyngeal glands, and any other glandular tissues during brain dissection and placed each brain separately in a 1.5 ml tube.