Synthesis and Field Evaluation of the Sex Pheromone from the Ten-Lined June Beetle

Project No.: **09-ENTO5-Leal**

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Objectives:

- Identification and synthesis of the sex pheromone of the ten-lined June beetle (TLJB), *Pollyphyla sobrinia*
- Formulation of synthetic sex pheromome
- Developing the use of sex pheromones for detection, monitoring, and risk assessment
- Exploring the use of sex pheromones for mass trapping and/or mating disruption
- Reverse chemical ecology approach for the development of scarab beetle attractants

Interpretive Summary:

Sex pheromones and other semiochemicals are invaluable tools in insect control programs. These green chemicals can be employed in integrated pest management (IPM) programs for monitoring established populations and to reduce and optimize insecticide sprays as well as for detection and survey programs for exotic species. To implement an effective IPM approach, it is critical to know which pest species are present (detection), and whether or not their population densities warrant control (monitoring and risk assessment). Detection of the presence or absence of a pest species is often a concern in quarantine when an invasive species is expanding its range, and area-wide action can be taken to limit or prevent that spread when early colonizers are first detected. Sex pheromones are also used for direct control of insect populations in mass trapping, mating disruption, and attract and kill. The objective of mass trapping is to reduce pest density without other intervention. If the trapped stage is the damaging stage of the insect, the effectiveness of mass trapping could be proportional to the number of insects removed. The cornerstone of successful IPM programs is the identification and synthesis of sex pheromones of insect pests. Elucidation of the chemical

structure of the sex pheromone from the Ten-Lined June Beetle (TLJB) has been challenging because of its novelty, the amounts of pheromone produced, the number of beetles available to extract the pheromone, and the chemical stability of the pheromone structure. Using gas choramatography and the beetle antennae as the sensing element (the so-called GC-EAD, or– Gas Chromotography-Electroantennographic Detection) and gas chromatography-mass spectrometry we gathered limited structural information that led us to tentative structures of the pheromone. In addition, we expressed the protein that transport this pheromone in the beetle's antennae and will obtain additional structural features based on test compounds affinity to the pheromone carrier protein. We will synthetize putative compounds and identify which of them is the sex pheromone by conducting future trapping experiments.

Materials and Methods:

To collect female beetles for pheromone extraction, professors, postdoctoral scholars, staff research associates, graduate and undergraduate students took multiple trips to the fields in Manteca during the flight season from June to August. Collected betteles were transported to the lab and kept at room temperature until the following night for extraction during the calling period. In addition, a local student and a collaborator (DJR) collected beetles daily and send to the lab by overnight carrier. First, we pooled the satellite fractions (3% and 5%), which were leftovers from previous years and kept at -80^{o}C. Then, we combined with new extracts from female beetles captured this year, and re-isolated the pheromone using GC-MS to identify the active peak. With this newly isolated sample, we obtained additional insights on the molecular structure of the sex pheromone.

To apply a reverse chemical ecology approach based on a pheromone-binding protein (PsobPBP) that transports pheromone to the pheromone receptors, we have constructed an expression cassette, pET22b●PsobPBP, and the protein was over-expressed in LB medium using transformed BL21(DE3) cells. Proteins in the periplasmic fraction were extracted by freeze-and-thaw and purified by ion-exchange chromatography (DEAE and MonoQ) and gel filtration (Sepharose) to generate pure samples.

Results and Discussion:

Structure elucidation of the TLJB sex pheromone has been challenging because the amounts of pheromone produced per individual is probably the smallest known in pheromone communication, the number of beetles available for pheromone extraction is limited to females we can collect during the flight season, and the chemical structure is novel and refractory to most chemical derivations. We serendptiously observed, however, that proton exchange took place when the isolated pheromone sample was dissolved in deuterated methanol, CD_3OD. The increase in mass-to-charge ratios (m/z) of various fragments strongly suggests that the pheromone molecule has one free hydroxy group. Comparison with the original MS indicates shifts in m/z 283→284, 241→242, and 171→172 (base peak). Although this new information alone is not enough for structure elucidation, it adds new key pieces to the puzzle, the most important being the occurrence of a free OH group. The polarity of this hydroxy group might be stabilized by intramolecular hydrogen bonding, as indicated by the low polarity of the compound (difference in Kotatz indexes, Δpolar - non-polar, 295). This low polarity is

consistent with elution from silica gel column with 4% ether in hexane. Unfortunately, we could not collect enough beetles to generate the amount required for structure elucidation.

As a contingency plan we explored a “reverse chemical ecology” approach which we previously devised to be used in combination with conventional chemical ecology approaches to tackle challenging problems, such as the identification of unusual moth pheromones [1] and effective mosquito oviposition attractants [2]. In the reverse chemical ecology approach olfactory proteins are isolated, cloned, expressed, and employed in binding assays to screen for potential attractants and generate “lead” compounds. Simply put, structure elucidation of sex pheromones and other naturally-occurring chemicals is a puzzle composed of pieces of information derived mostly from spectral data and chemical derivatizations. The reverse chemical ecology approach generates additional pieces of molecular-based information, i.e., affinity of related compounds to an olfactory protein. To employ this reverse chemical ecology approach in the case of the ten-lined June beetle we isolated, cloned, and expressed a pheromone-binding protein expressed specifically in the antennae of this species, which we named PsobPBP. Recombinant samples of PsobPBP were prepared by bacterial expression and isolated by a combination of ion-exchange chromatography and gel filtration to yield pure samples as indicated by high performance liquid chromatography (HPLC) and gel electrophoresis with denatured proteins (SDS-PAGE) (**Figure 1**)

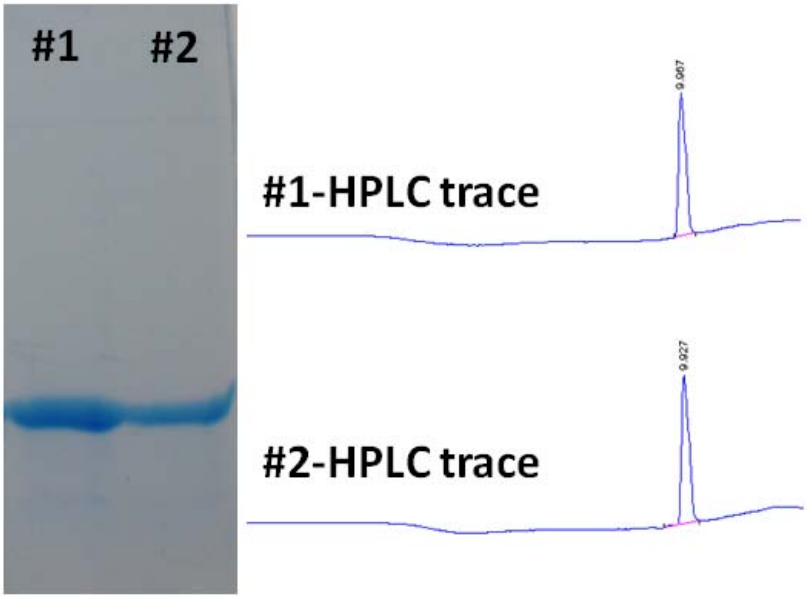

Figure 1. Pure recPsobPBP samples as indicated by SDS-PAGE (left) and HPLC (right).

To confirm that the recombinant protein was identical to the native PBP we compared the recombinant sample by native gel electrophoresis (Native-PAGE), protein sequencing, and mass spectrometry. Migration of native PsobPBP and recPsobPBP in native PAGE (**Figure 2**), the N-terminal sequence of recPsobPBP, obtained by Edman degradation (MSEEME), and molecular mass, obtained by LC-ESI/MS (**Figure 3**), confirmed that recPsobPBP is identical to the native PBP. We examined the secondary structure of recPsobPBP by circular dichroism

(CD). As indicated by the maximum at 192 nm and the two minima at 210 and 219 nm (**Figure 4**), PsobPBP is an □ helix-rich protein. This type of secondary structure is a common feature of pheromone-binding proteins. We will use this recombinant protein as a molecular target in binding assays-oriented identification of lead compound(s), which will also be tested as possible attractants.

As for pheromone identification, we will synthesize possible candidates. Once we have putative synthetic pheromone compounds we will return to the Almond Board of California for possible financial support to test these compopunds and to subsequently implement strategies for controlling and/or monitoring TLJB populations.

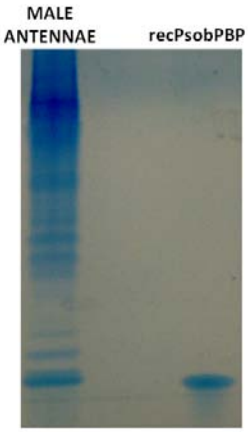

Figure 2. Native-PAGE (15%) analysis of native and recombinant proteins.

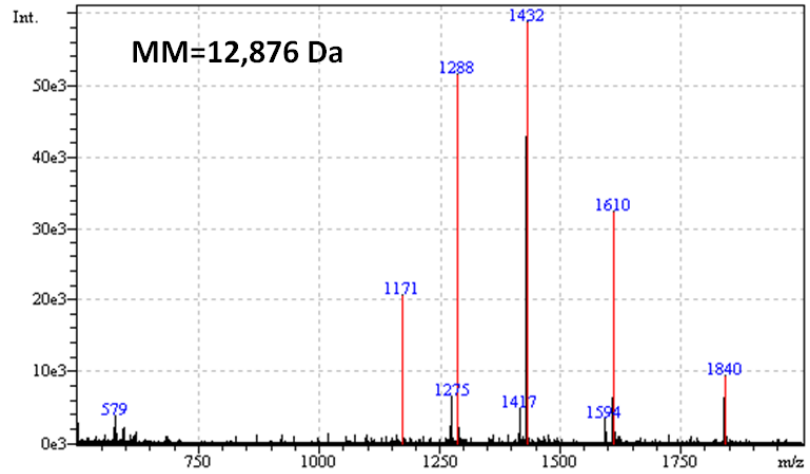

Figure 3. LC-ESI/MS data indicating that observed (12,876 Da) and calculated (12,875 Da) molecular masses are in agreement.

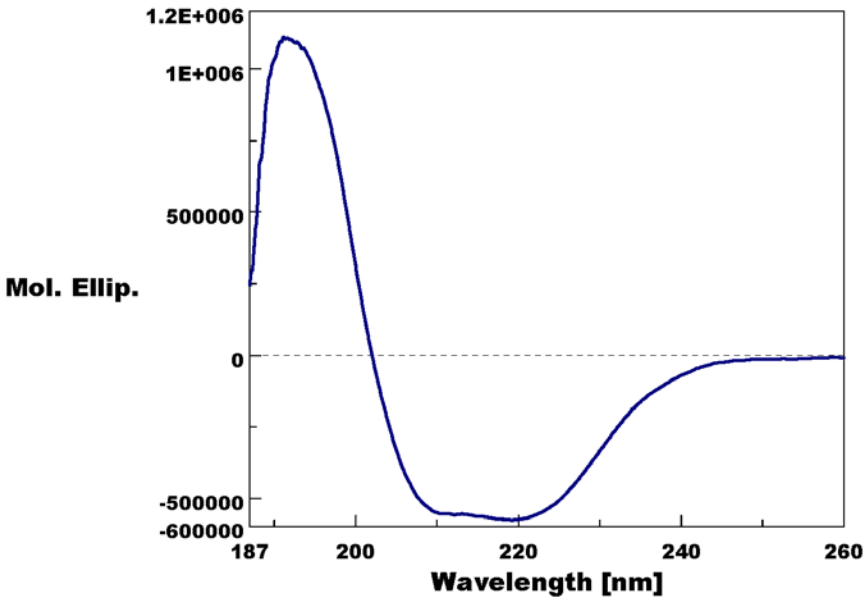

Figure 4. Far-UV CD spectrum of PsobPBP at pH 7

Research Effort Recent Publications:

No publications

References Cited:

Leal WS, Parra-Pedrazzoli AL, Kaissling KE, Morgan TI, Zalom FG, et al. (2005) Unusual pheromone chemistry in the navel orangeworm: novel sex attractants and a behavioral antagonist. Naturwissenschaften 92: 139-146.

Leal WS, Barbosa RM, Xu W, Ishida Y, Syed Z, et al. (2008) Reverse and conventional chemical ecology approaches for the development of oviposition attractants for Culex mosquitoes. PLoS ONE 3: e3045.