Improving Trapping and Mating Disruption of the Navel Orangeworm

Project No.: **09-ENTO2-Leal**

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Objectives:

- Elucidation of the three-dimensional structures of the major pheromone-binding protein from the navel orangeworm, AtraPBP1
- Oviposition preference studies
- Developing dispenser for slow release of pheromones
- Test possible use of kairomones as pheromone replacement
- Examine pheromone batches to identify possible inhibitors

Interpretive Summary:

The navel orangeworm (NOW), *Amyelois transitella* Walker (Lepidoptera: Pyralidae), is the most serious insect pest of almonds and pistachios in California, and a major pest of a number of other crops, including walnuts and figs. The navel orangeworm is primarily controlled during the growing season with pyrethroids and insect growth regulators, but alternative methods of control are sorely needed. Sex pheromones offer an environmentally-friendly alternative to control insect populations by mating disruption or other strategies in integrated pest management. Typically, sex pheromones and other attractants (aka semiochemicals) are identified by a bioassay-guided isolation of natural products.

Alternatively, olfactory proteins may be used in a reverse chemical ecology approach for screening potential attractants on the basis of their affinity to odorant-binding proteins. These proteins are part of a large family of carrier proteins, for which we coined the term encapsulins , but those directly involved in semiochemical reception are grouped

into pheromone-binding proteins (PBPs) and general odorant-binding proteins (GOBPs) based on their transport of pheromones or other semiochemicals. We have now isolated, cloned and expressed olfactory proteins from the navel orangeworm and set the stage to use them in reverse chemical ecology.

Although the sex pheromone system of the navel orangeworm has already been identified, some of the constituents are unstable. Reverse chemical ecology in this case can be used for the development of alternative compounds (parapheromones). Thus, this report is focused on a male antennae-specific PBP, which we named AtraPBP1. We just published a detailed report on the identification of other olfactory proteins.

Having previously observed that PBPs from the silkworm moth, *Bombyx mori,* and the wild silkworm moth, *Antheraea polyphemus*, undergo pH-dependent conformational changes that lead to lack of binding at low pH we assessed the effect of pH on the conformation of AtraPBP1. We prepared samples of recombinant AtraPBP1 by using a recombinant pET vector without His6-Tag that generates PBPs with identical conformation and disulfide bridge formation as the native protein. Samples were highly purified by a combination of ion-exchange chromatography (DEAE), high-resolution ion-exchange chromatography (Mono Q), and gel filtration, with the purity confirmed by SDS-PAGE and LC-ESI/MS (>99.5%). We prepared samples for circular dichroism (CD) and fluorescence analysis by taking aliquots of the same sample and diluting with buffers of the desired pH. Far-UV-CD spectrum of AtraPBP1 at pH 7 with a maximum at 193 nm and two minima at 208 and 223 nm demonstrated that this PBP is α-helical rich like BmorPBP1 and ApolPBP1. At lower pH, the intensity of the second minimum at 223 nm was clearly reduced and thus indicated that there is unwinding of helical secondary structure. Similar changes have been observed with CD spectra of BmorPBP1 and ApolPBP1. Apparently, the formation of a C-terminal helix does not offset the unwinding of the N-terminal α-helix thus causing a reduction in the overall content of this secondary structure. pH-Titration by intrinsic fluorescence showed a dramatic transition between pH values of 5 and 6.5 thus suggesting that AtraPBP1 exists in two distinct conformations, one at the pH of the sensillar lymph and the other at low pH as in the vicinity of dendritic membranes.

Nuclear Magnetic Resonance (NMR) analysis revealed very striking spectral changes upon changing the pH from 4.5 to 7.4. The ^{15}N-^{1}H heteronuclear single quantum coherence spectrum at pH 4.5 exhibited the expected number of sharp and well-resolved main-chain amide resonances (142 peaks), indicating the protein forms a uniform, stable, and monomeric tertiary structure at low pH. At pH 5.5, the number of NMR peaks increased almost two-fold (284 peaks), indicative of an equal mixture of protonated and deprotonated forms of the protein at this intermediate pH. The NMR resonances at pH 7.4 appear broadened with chemical shift heterogeneity (185 peaks), suggesting a heterogeneous mixture of protein structures at neutral or slightly acidic pH. Such heterogeneity may be stabilized with a ligand. We are, therefore, pursuing the three-dimensional structures of AtraPBP1 at low and neutral pH by NMR and X-ray crystallography, respectively. We have already determined NMR backbone assignments for AtraPBP1 at low pH and a full structure determination is currently underway. On the other hand, we were able to co-crystallize AtraPBP1 with pheromone constituents and

obtain crystals that diffract to atomic resolution thus allowing determination of structures of AtraPBP1-pheromone complexes.

To assess affinity of AtraPBP1 for pheromone constituents, we used a previously developed binding assay, which is based on the separation of bound and unbound ligand by a centrifugal device. After the free ligand is removed by filtration, the PBP-bound ligand is released from the protein by lowering the pH, extracted with organic solvent and analyzed by gas chromatography (GC) for quantification and gas chromatography-mass spectrometry (GC-MS) for identification of the bound ligand. The major constituent of the sex pheromone system, (*Z,Z*)-11,13-hexadecadienal, hereafter referred to as Z11Z13-16Ald, bound to AtraPBP1 with apparent high affinity at neutral pH and low or no binding affinity at low pH. This pH-dependent binding affinity may be explained by the formation of a C-terminal α-helix, which competes with the ligand for the binding cavity at low pH. Although only one of the four isomers of 11,13-hexadecadienal is known to be behaviorally active, pheromone-detecting sensilla in male antennae are sensitive to the four isomers of this compound, namely, Z11Z13-16Ald, Z11E13-16Ald, E11E13-16Ald, and E11Z13-16Ald. We compared binding of Z11Z13-16Ald and E11E13-16Ald and found no difference (data not shown) thus suggesting that AtraPBP1 alone cannot discriminate stereoisomers of the major constituent of the sex pheromone.

Next, we tested binding affinity of other constituents of the navel orangeworm sex pheromone. Female-produced sex pheromones in moths are normally complex mixtures of straight chain acetates, alcohols and aldehydes, with 10-18 carbon atoms and up to three unsaturations, the so-called Type I pheromones. Type II sex pheromone is comprised of polyunsaturated hydrocarbons and epoxy derivatives with long straight chains. The navel orangeworm is unusual in that its sex pheromone system in composed of a complex mixture that includes constituents of both types: Z11Z13-16Ald, Z11Z13-16OH, Z11Z13-16OAc (behavioral antagonist), (*Z*,*Z*,*Z*,*Z*,*Z*)-3,6,9,12,15-tricosapentaene and (*Z*,*Z*,*Z*,*Z*,*Z*)-3,6,9,12,15-pentacosapentaene, and other minor constituents. As opposed to Type I pheromones that give very low background indicating negligible non-specific binding, it was difficult to assess binding of the pentaene compounds because their hydrophobicity led to high background levels. On the other hand, the secondary constituent, Z11Z13-16OH bound to AtraPBP1 with affinity comparable to that of the major constituent, but showed no affinity at low pH. Interestingly, the behavioral antagonist, Z11Z13-16OAc showed the highest affinity to AtraPBP1 of all tested ligands. Next, we performed competitive binding studies with AtraPBP1 incubated with the three ligands at the same concentration. These competitive binding assays mirrored what was observed with non-competitive binding assays, AtraPBP1 was bound with the highest affinity to Z11Z13-16OAc, whereas the aldehyde and alcohol showed similar affinity. These results suggest that a single PBP may be involved in the reception of multiple constituents of sex pheromones.

To further explore the potential use of AtraPBP1 for the development of parapheromones, we tested binding of a pheromone analog, (*Z*)-1, 1,1-trifluoro-13-octadecen-2-one (hereafter referred to as Z11C16COCF3). Trifluoromethyl ketones (TFMK) are compounds which inhibit a variety of hydrolytic enzymes, such as

acetylcholinesterase, chymotrypsin, trypsin, juvenile hormone esterase, human liver microsomal CEs, and pheromone degrading esterases in male olfactory tissues. They have been demonstrated to interrupt insect chemical communication and to bind to pheromone-binding proteins, but their mode of action is still a matter of debate. We compared by competitive binding the affinity of Z11C16COCF3 and Z11Z13-16OAc to AtraPBP1. Surprisingly, Z11C16COCF3 binds to AtraPBP1 with much higher affinity than the behavioural antagonist Z11Z13-16OAc (**Figure 1**). Although binding activity decreased dramatically at low pH, this TFMK showed binding affinity at low pH almost half of that of the best natural ligand (Z11Z13-16OAc) at neutral pH (**Figure 1**). We, therefore, concluded that AtraPBP1 may be employed for the development of an affinity-based approach for the development of parapheromones.

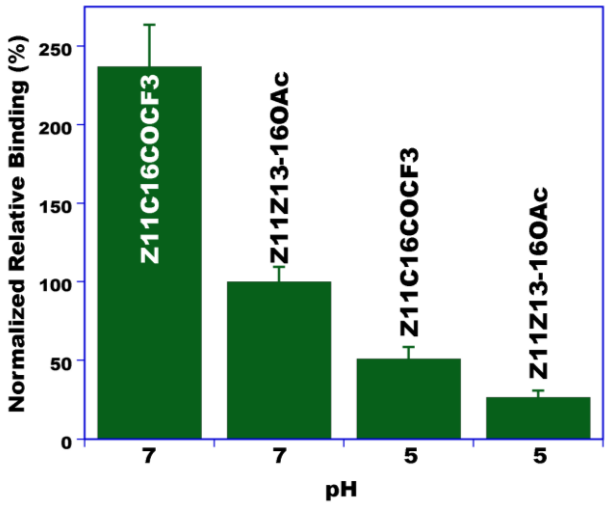

Figure 1. Competitive binding assays with AtraPBP1**.**

In addition to pheromones and parapheromones, we are prospecting for plant-derived attractants (kairomones). Previously, we have observed in the laboratory that gravid NOW females prefer to oviposit on pistachios than almonds. We have collected airborne volatiles from pistachios and analyzed the extracts by gas chromatographic-electroantennographic detection (GC-EAD) using female antenna as a sensing element. We have identified 5 EAD-active peaks and are now in the process of identifying these putative kairomones.

Lastly, we are analyzing industrial batches of synthetic constituents of the NOW sex pheromone to identify possible inhibitors. We have identified a number of EAD-active compounds, which may be inhibitory. These samples are currently being analyzed by Dr. Ring Cardé (Department of Entomology, UC Riverside). Later, we will compare physiological and behavioural data in attempt to identify and eliminate industrial inhibitors and, consequently, improve sex pheromone formulations.

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