Improving Trapping and Mating Disruption of the Navel Orangeworm (NOW)

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 major long-term goals of this project are the development of female attractants and chemically stable, alternative sex pheromone blends for monitoring and controlling populations of the navel orangeworm by mating disruption. Throughout this project we focus our efforts on a molecular-based strategy for minimizing the number of blend constituents and developing chemically stable parapheromones for synthetic lures and on a chemical ecology-based approach for isolation of kairomones from natural sources for gravid female traps. This year we have focused our efforts on investigating the molecular and structural basis of pheromone reception to explore the use of a pheromone-binding protein, AtraPBP1, as a molecular target for the development of parapheromones. We have identified pheromone analogs that bind to AtraPBP1 with higher affinity than the natural pheromone. We have determined structural elements by examining the 3D structures of AtraPBP1 unbound as well as bound to pheromone constituents. Simultaneously, we are investigating by sensory physiology and behavioral studies if minor constituents of industrial batches of the synthetic pheromone can be eliminated to improve attraction and reduce pheromone degradation.

Materials and Methods:

Expression of non-labeled AtraPBP1 was performed in LB medium with transformed BL21(DE3) cells. Proteins in the periplasmic fraction were extracted with 10 mM Tris-HCl, pH 8 by using three cycles of freeze-and-thaw and centrifuging at 16,000 ×*g* to remove debris. The supernatant was loaded on a Hiprep™ DEAE 16/10 column (GE Healthcare, Piscataway, NJ). Separations by ion-exchange chromatography were done with a linear gradient of 0–500 mM NaCl in 10 mM Tris-HCl, pH 8. Fractions containing the target protein were further purified on a Q-Sepharose Hiprep™ 16/10 column (GE Healthcare) and, subsequently, on a Mono-Q HR 10/10 column (GE Healthcare). PBP fractions were concentrated by using Centriprep-10 (Millipore, Billerica, MA) and loaded on a Superdex-75 26/60 gel-filtration column (GE Healthcare) pre-equilibrated with 150 mM NaCl and 20 mM Tris·HCl, pH 8. Highly purified protein fractions were concentrated by Centricon-10, desalted on four 5-ml HiTrap desalting columns (GE Healthcare) in tandem and by using water as mobile phase, analyzed by LC-ESI/MS, lyophilized, and stored at -80ºC until use. The concentrations of the recombinant proteins were measured by UV radiation at 280 nm in 20 mM sodium phosphate, pH 6.5 and 6 M guanidine HCl by using the theoretical extinction coefficients calculated with EXPASY software (http://us.expasy.org/tools/protparam.html). LC-ESI-MS was performed with a LCMS-2010 (Shimadzu, Kyoto, Japan). HPLC separations were done on a ZorbaxCB C8 column (150 × 2.1 mm; 5 μm; Agilent Technologies, Palo Alto, CA) with a gradient of water and acetonitrile plus 2% acetic acid as a modifier. The detector was operated with the nebulizer gas flow at 1.0 l/min and the curved desolvation line and heat block at 250°C. ^{15}N-labeled AtraPBP1 was prepared as previously described for BmorPBP1. CD spectra were recorded by using a J-810 spectropolarimeter (Jasco, Easton, MD) with 0.2 mg/ml AtraPBP1 in either 20 mM ammonium acetate, pH 7 or 20 mM sodium acetate, pH 5. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorometer with 10 mg/ml of AtraPBP1 in 20 mM of one of the following buffers: sodium acetate, pH 4, 5 and ammonium acetate, pH 5.5-7. The protein solution was excited at 280 nm and the emission spectra were recorded between 285 and 420 nm. Excitation and emission slits were set at 1.5 and 10 nm, respectively. NMR was obtained on a Bruker Avance 600 MHz spectrometer equipped with a four-channel interface and triple-resonance cryogenic probe. The 15N–1H HSQC spectra were obtained with ^{15}N-labeled 0.5 mM AtraPBP1 in 95% H_20 and 5% ^2H_2O, with pH adjusted to 4.5 (20 mM sodium acetate), 5.5 (20 mM sodium acetate), and 7.4 (20 mM sodium phosphate).

Binding was measured by incubating AtraPBP1 with test ligands, separating unbound and bound protein, extracting pheromone from the latter sample, and analyzing by gas chromatography, according to a previously reported protocol. After lowering pH to release ligand, bound protein fractions were extracted and analyzed by gas chromatography (GC) for quantification and by GC-mass spectrometry (GC-MS) for confirmation of ligand identity. GC and GC-MS were done on a 6890 series GC and a 5973 Network Mass Selective Detector (Agilent Technologies, Palo Alto, CA), respectively. Both instruments were equipped with the same type of capillary column (HP-5MS, 25 m × 0.25 mm; 0.25 μm; Agilent Technologies) operated under the same temperature program (150°C for 1 min, increased to 250°C at a rate of 10°C/min, and held at the final temperature for 10 min). Pure pheromone samples, including isomers of 11,13-hexadecadienal, were supplied by Bedoukian Research Inc. Each compound was tested at least five times. Test compounds were incubated with AtraPBP1 in the ratio of

10:1, ligand:protein. For competitive binding assays, all ligands were added to a protein solution at the same concentration.

GC-EAD (Electroantennographic Detection) was done with a gas chromatograph (HP 5890, Agilent Technologies, Palo Alto, CA) equipped with transfer line and temperature control units (Syntech, Kirchzarten, Germany). The effluent from the capillary column was split into EAD and flame ionization detector (FID) in a 3:1 ratio. Antennae were placed in EAG probes of an AM-01 amplifier (Syntech) and held in place with Spectra 360 electrode gel (Parker Laboratories, Orange, NJ). The analog signal was fed into an A/D 35900E interface (Agilent Technologies) and acquired simultaneously with FID signal on an Agilent Chemstation. GC-MS was obtained on a 5973 Network Mass Selective Detector (Agilent Technologies). GC-MS and GC-EAD were equipped with capillary columns (HP-5MS, 30 m x 0.25 mm; 0.25 μm; Agilent Technologies) and (HP-INNOWAX, 30 m x 0.25 mm; 0.25 μm; Agilent Technologies), respectively. The temperature program started at 50°C for 1 min, increased at a rate of 10°C/min to 250°C, and held at this final temperature for 10 min. Both GCs were operated under splitless mode with the injection port at 230°C and purge time 2 min.

Results and Discussion:

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). 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.

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 the NMR structure of AtraPBP1 at low pH. In addition, 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.

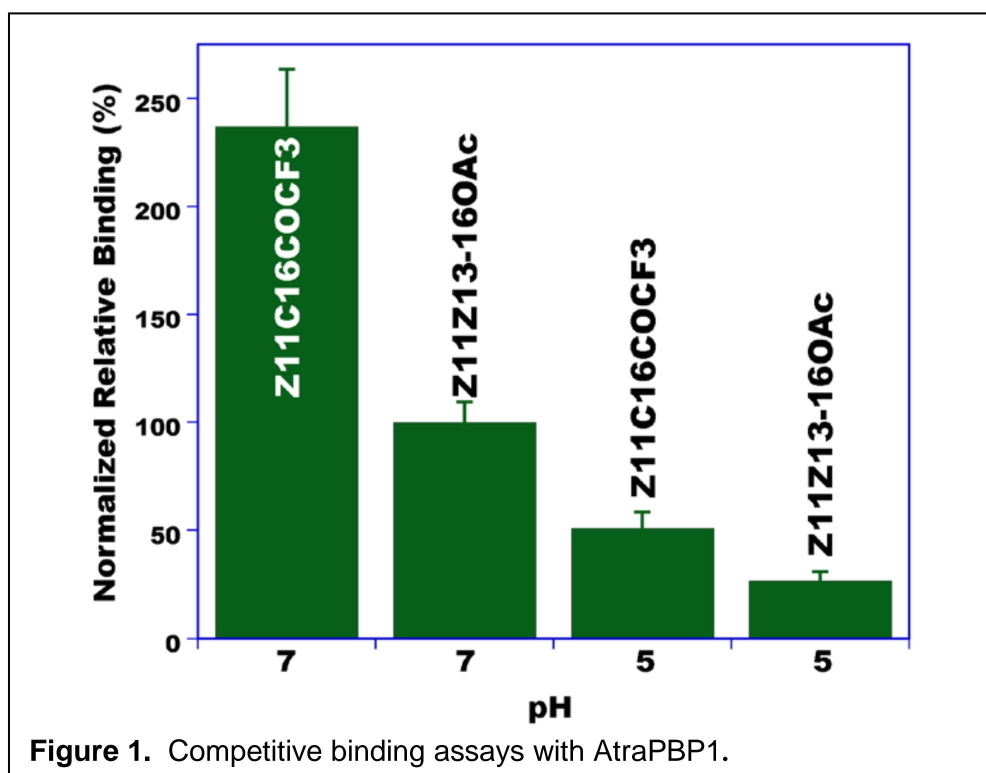

Figure 1. Competitive binding assays with AtraPBP1.

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 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 (**Figure 2**), which may be inhibitory. These samples are currently being analyzed by Dr. Ring Cardé (UC Riverside, Department of Entomology, see Project No.09-ENTO9-Cardé). Later, we will compare physiological and behavioural data in attempt to identify and eliminate industrial inhibitors and, consequently, improve sex pheromone formulations.

In conclusion, we have demonstrated that a pheromone-binding protein, AtraPBP1, previously isolated from male antennae of the navel orangeworm, can be used as a molecular target for the development of parapheromones. Although the natural sex pheromone system has already been identified, some of the constituents are unstable, particularly for use in monitoring. While results of applications of synthetic pheromone in mating disruption are promising, the synthetic pheromone is not reliable as a monitoring tool as it loses activity after 1-day in the field. To shed more light on the development of more stable pheromone substitutes, we are studying the three dimensional structures of AtraPBP1 unbound as well as bound to constituents of the sex pheromone. In addition to develop more stable attractants, we are carefully examining by GC-EAD and behavioral assays possible inhibitory effects of minor constituents of the synthetic pheromone. Our ultimate goal is the development of stable formulations for monitoring navel orangeworm populations, which in an essential step for evaluation of mating disruption effect on populations.

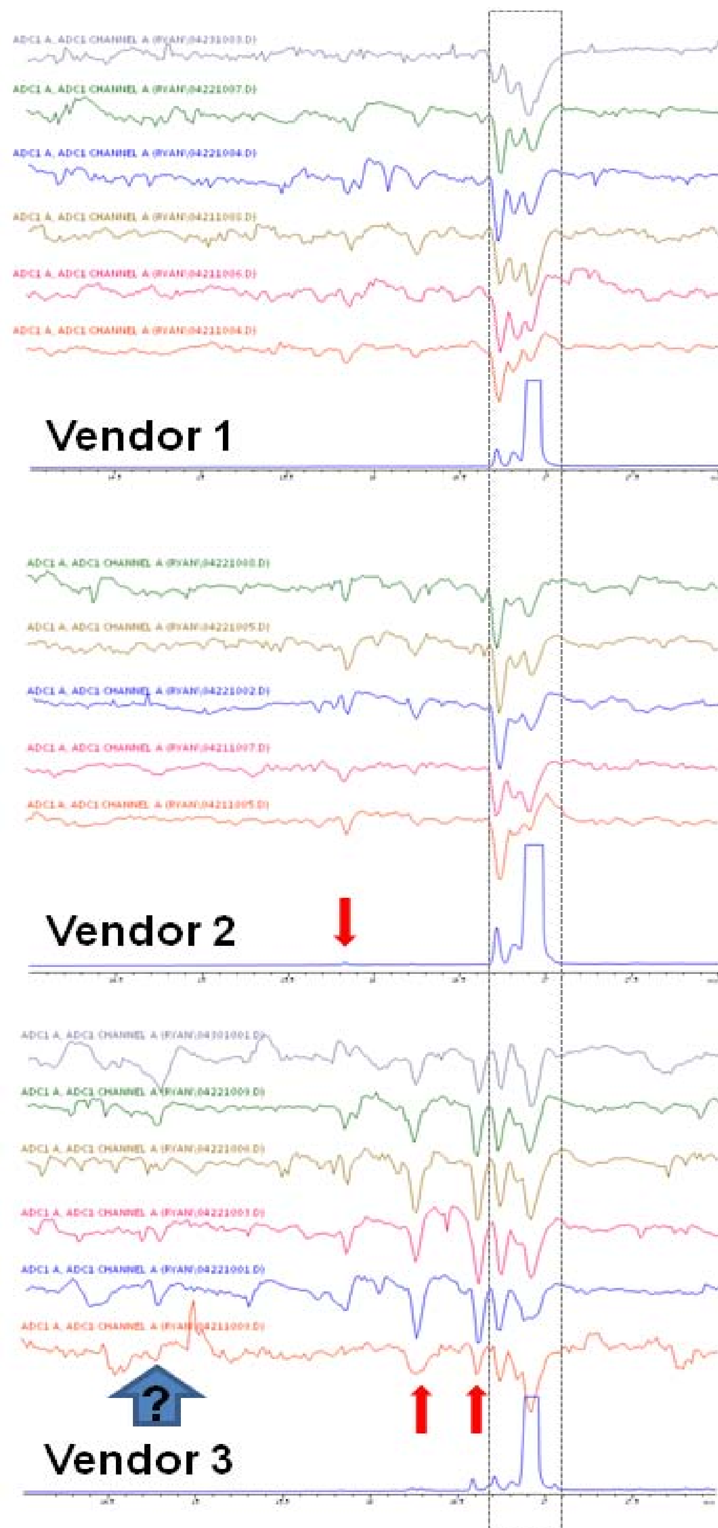

Figure 2. Comparison of synthetic pheromone samples from 3 different sources. At least two, possibly three, additional EAD-active peak have been identified in sample 3. These impurities may be inhibitory. The dashed box indicates the EAD peaks elicited by the four isomers of the pheromone. Arrows indicate possibly inhibitors.

Research Effort Recent Publications:

Leal WS, Ishida Y, Pelletier J, Xu W, Rayo J, Xu X, Ames JB (2009) Olfactory proteins mediating chemical communication in the navel orangeworm, *Amyelois transitella*. PLoS ONE 4: e7235

Xu X, Xu W, Rayo J, Ishida Y, Leal WS, Ames JB (2010) NMR structure of navel orangeworm moth pheromone-binding protein (AtraPBP1): Implications for pH-sensitive pheromone detection. Biochemistry 49: 1469-1476

Liu Z, Vidal DM, Syed Z, Ishida Y, Leal WS (2010) Pheromone binding to general odorant-binding protein from the navel orangeworm. J Chem Ecol 36: 787--794

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