Article Addendum

An oviposition stimulant binding protein in a butterfly

Immunohistochemical localization and electrophysiological responses to plant compounds

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Oviposition is evoked by plant compounds, which are recognized by chemoreceptive organs of insects. The swallowtail butterfly, *Atrophaneura alcinous*, oviposits its eggs on the host plant, *Aristolochia debilis*, in the presence of only two stimulating compounds: an alkaloid, aristolochic acid, and a monosaccharide, sequoyitol. In our previous study, a unique protein of 23 kDa [Oviposition stimulant(s) binding protein (OSBP)] was found in the forelegs of female, but not male *A. alcinous*. The electrophysiological response of *A. alcinous* to an extract of *A. debilis* was depressed by the presence of OSBP antiserum, suggesting that OSBP presumably binds to oviposition stimulant(s). We show here, using a highly sensitive fluorescence micro-binding assay that native OSBP binds to a main oviposition stimulant, aristolochic acid, from its host plant, *A. debilis*. Three-dimensional molecular modeling studies also gave a reasonable structure for the OSBP/aristolochic acid complex. This is the first report of a native chemoreceptive protein binding to an oviposition stimulant ligand in insects.

**An Oviposition Stimulant Binding Protein**

Sensory reception plays very important roles in survival, especially for insects,1-7 and butterflies have evolved a specialized chemoreception system for laying eggs. For adult female butterfly, oviposition is an essential behavior for generating progeny. Therefore, it is necessary for the female butterflies to recognize their specific host plants. Chemoreceptive proteins in taste organs are thought to have important roles for the precise recognition of host plant compounds.6-7 The swallowtail butterfly, *Atrophaneura alcinous*, oviposits its eggs on the host plant, *Aristolochia debilis*, in the presence of only two triggering compounds: an alkaloid, aristolochic acid and a monosaccharide, sequoyitol. The oviposition behavior of butterflies is induced by recognition of the plant compounds via receptors in the tarsus of the foreleg. Using scanning electron microscopy, we observed tarsal contact chemosensilla in the foreleg; the female of *A. alcinous* has a toothbrush-like dense cluster of sensilla, which is larger than that of the male (Fig. 1).

Insect odorant-binding proteins (OBPs) are small, water-soluble proteins that are widely found in the olfactory systems of various species.8-10 OBPs are involved in the first specific biochemical step of odor reception and are thought to carry lipophilic odorants to the olfactory receptor cells through hydrophilic surroundings.5-16 The molecular cloning of insect OBPs is ongoing; however, few structural studies correlating function to ligand-binding activities have been reported.17,18 We have isolated a soluble protein of 23 kDa from *A. alcinous*.19 Western blot analysis showed that this protein was expressed only in the female, and not in the male. Moreover, the protein is localized in the tarsi. We isolated this protein and determined the sequence of the N-terminal 23 amino acids. We then cloned its cDNA by RT-PCR and RACE. The deduced sequence of 212 amino acids is 38% homologous to a bilin-binding protein (BBP), of the cabbage butterfly, *Pieris brassicaceae*. Two consensus sequences from the lipocalin family of proteins were found in the sequence, suggesting that it is a binding protein for lipophilic ligands. This protein possibly plays an important role in the sensory process for oviposition, and is considered to be an oviposition stimulant(s) binding protein (OSBP).

Three-dimensional structure modeling of OSBP, based on the crystal structure of BBP, has suggested that aristolochic acid, an oviposition stimulant compound of *A. alcinous*, could bind OSBP.20 Indeed, a binding assay, measuring fluorophore conjugated to a ligand molecule under an internal reflection fluorescence microscope, showed that OSBP binds to aristolochic acid.21-24

**Immunohistochemistry**

Localization of OSBP in male and female tarsi was investigated by immunohistochemistry using an anti-OSBP antiserum. Sections were counter-stained with Evans blue to reduce auto-fluorescence.
shown in red-orange. Strong auto-fluorescence was observed in tarsi, especially at the cuticle (Fig. 2A). Green fluorescence in Figure 2A (without the anti-OSBP antiserum) is probably due to the non-specific binding of the secondary antibody. When sections were reacted with the anti-OSBP antiserum (Fig. 2B), strong green signals were observed at the sensilla. OSBP is, therefore, likely to be localized at the sensilla of female tarsi.

**Electrophysiological Responses of Chemosensilla**

An electrophysiological study showed that the female tarsus was stimulated by a methanolic extract from the host plant, *A. debilis* (Fig. 3A and B). We investigated the responses to two main compounds of *A. debilis*, hydrophilic sequoyitol and lipophilic aristolochic acid. When sensilla were treated with aristolochic acid, one or two kinds of impulses were observed (Fig. 3C). When sensilla were treated with sequoyitol, only one kind of impulse was observed (Fig. 3D). When sensilla were stimulated by a methanolic extract from *A. debilis*, two to three different trains of impulses with differently sized amplitudes were usually observed. The sensilla were then pretreated with the antiserum raised against OSBP for ten minutes and then stimulated with the stimulating solution. The response was partially suppressed by the pretreatment (Fig. 3E). The suppression by anti-OSBP antibody was removed by washing with water, suggesting that the binding of the antibody is reversible.19 The results of the electrophysiological experiments suggest that OSBP plays the role of a binding receptor in the chemosensory signal transduction system for oviposition, probably as a carrier protein of the stimulants.

OSBP is present only in females of *A. alcinous* and can bind to aristolochic acid, a major oviposition stimulant of the host plant, *A. debilis*.20 OSBP binds to aristolochic acid, suggesting that OSBP is involved in the chemosensory mechanism of *A. alcinous*. OSBP is, therefore, a candidate molecule for the transfer of aristolochic acid to receptors or for the activation of receptor molecules of the chemosensory neurons following ligand binding.

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