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# ODORANT-BINDING PROTEINS FROM A PRIMITIVE TERMITE

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**Abstract**—Hitherto, odorant-binding proteins (OBPs) have been identified from insects belonging to more highly evolved insect orders (Lepidoptera, Coleoptera, Diptera, Hymenoptera, and Hemiptera), whereas only chemosensory proteins have been identified from more primitive species, such as orthopteran and phasmid species. Here, we report for the first time the isolation and cloning of odorant-binding proteins from a primitive termite species, the dampwood termite, *Zootermopsis nevadensis nevadensis* (Isoptera: Termopsidae). A major antennae-specific protein was detected by native PAGE along with four other minor proteins, which were also absent in the extract from control tissues (hindlegs). Multiple cDNA cloning led to the full characterization of the major antennae-specific protein (ZnevOBP1) and to the identification of two other antennae-specific cDNAs, encoding putative odorant-binding proteins (ZnevOBP2 and ZnevOBP3). N-terminal amino acid sequencing of the minor antennal bands and cDNA cloning showed that olfaction in *Z. n. nevadensis* may involve multiple odorant-binding proteins. Database searches suggest that the OBPs from this primitive termite are homologues of the pheromone-binding proteins from scarab beetles and antennal-binding proteins from moths.

**Key Words**—Odorant-binding protein, chemosensory protein, *Zootermopsis nevadensis nevadensis*, Isoptera, Termopsidae, olfaction, dampwood termite.

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## INTRODUCTION

Insects perceive the world through small molecules that carry essential information about nestmates, food sources, intruders, and many other environmental cues. These hydrophobic semiochemicals are solubilized by odorant-binding proteins (OBPs) and transported through an aqueous environment (sensillar lymph) to the olfactory receptors, where the signal transduction starts (Kaissling, 2001). OBPs have been identified and/or cloned from various insect orders, such as Lepidoptera, Coleoptera, Diptera, Hymenoptera, and Hemiptera (Vogt et al., 2002). These proteins, subdivided into pheromone-binding proteins (PBPs) and general odorant-binding proteins (GOBPs), all have a hallmark of six conserved cysteine residues forming three disulfide bridges (Leal et al., 1999; Scaloni et al., 1999), which are essential for the rigidity of their three-dimensional structures (Horst et al., 2001; Sandler et al., 2000). A third class of small proteins has been identified in several sensorial organs from a number of insect orders (Lartigue et al., 2002). Although they may function as carrier proteins (Lartigue et al., 2002 and references therein), they have been separated into a group of chemosensory proteins (CSP), characterized by four cysteine residues and with low sequence similarity to OBPs. Interestingly, a thorough search for olfactory proteins in insect orders other than Lepidoptera (Marchese et al., 2000 and references therein) revealed that species from primitive insect orders, such as Orthoptera and Phasmatodea, possess CSPs, but not OBPs. This study was aimed at the identification of olfactory proteins from the primitive Dictyoptera, which includes the orders Mantodea (mantids), Blattodea (cockroaches) and Isoptera (termites). Here, we report the identification and cloning of OBP homologues in the dampwood termite, *Zootermopsis nevadensis nevadensis* HAGEN (Isoptera: Termopsidae).

## METHODS AND MATERIALS

*Protein Analyses*. Termite colonies were collected from ponderosa pine (*Pinus ponderosa* DOUGL. EX LAWS) stumps on the Eldorado National Forest near Placerville, California. Colonies were maintained in culture in 41 plastic containers (Rubbermaid Inc., Wooster, Ohio) for 5–6 months. Workers were anesthetized on ice and their antennae and hindlegs were collected with forceps under a microscope. After homogenizing in 10 mM Tris-HCl, pH 8 with an ice-cold Dounce tissue grinder (Wheaton), homogenates were centrifuged twice at 13,500 $\times$ g for 10 min at 4°C. The supernatants were concentrated by centrifugation under vacuum, analyzed by 15% native polyacrylamide gel electrophoresis (15% PAGE); bands were visualized with Coomassie blue R-250. For N-terminal amino acid microsequencing, proteins were separated by PAGE, transferred to polyvinyl difluoride (PVDF) membranes using electroblotting, identified bands were cut out and analyzed on a Procise Protein Sequencing System (Applied Biosystems).

*cDNA Cloning and RT-PCR.* Total RNA was extracted by TRIzol (Invitrogen) including 200 ng of glycogen (Invitrogen) from 130 antennae of workers. First strand cDNA was synthesized by using SuperScript II (Invitrogen) as transcriptase and SMART RACE cDNA Amplification Kit (Clontech). Degenerate primer,

5′-GC(A/C/G/T)AA(A/G)GA(A/G)GT(A/C/G/T)GA(C/T)GA(A/G)AA-3′

was designed on the basis of amino acid sequence of N-terminal region (AKEVDEK) of isolated antennae-specific protein. GCGTTTTTTTTTTTTTT (GCGT15) primer was used as a reverse primer. *Taq* DNA Polymerase in Storage Buffer B (Promega) was used as *Taq* polymerase. PCR were performed as follow: 70 cycles of 94°C for 1 min, 40°C for 2 min and 72°C for 3 min. PCR product was ligated into pBluescript SK (+) (Stratagene) and sequenced at an automated DNA sequencing facility (Davis Sequencing). More than 4 independent clones were sequenced to eliminate possible PCR mutations. For 5′-RACE a gene specific primer (Z1-r),

5′-CGATGGCCCTCTTCCACGATCTCTGGCGGT-3′,

was designed, and PCR was carried out as in the instruction manual of SMART RACE cDNA Amplification Kit. For cloning two other cDNAs, we used a N-terminal degenerate primer, GCGT15 primer and 4 gene-specific primers,

5′-CTCAACATGACTGGCAGTTTCCCCGATGAG-3′ (Z2-f),

5′-CCGAACGCCTCCTTCAACTTAGCGGCGACC-3′ (Z2-r),

5′-AATGAAGTGCTCGATCACCGGGACAACAGC-3′ (Z2-r2)

and

5′-GGAAACCCTTGCAGCTTCTCCACCACTGTC-3′ (Z3-r)

and performed PCR-cloning described above. Molecular mass, isoelectric point, BLAST database search, and ClustalW alignment of amino acid sequences were performed by MacVector (Accelrys). Signal peptide was predicted by SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP/). For RT-PCR, cDNAs were synthesized from total RNA extracted from antennae and legs as described above. The following combinations of gene-specific primers were used to detect the transcript of the cloned odorant-binding proteins:

Z1-f, 5′-GTGTCATGGTCGAACTCATGGCTCTCAACG-3′

and Z1-r (for ZnevOBP1), Z2-f and Z2-r (for ZnevOBP2), and

Z3-f, 5′-ACTTCGGCAGTTTCCCGGATGAGTCCGACC-3′,

and Z3-r (for ZnevOBP3). For detection of actin transcript (control), two degenerate primers were used: Actin-1,

5′;-AA(C/T)TGGGA(C/T)GA(C/T)ATGGA(A/G)AA-3′

and Actin-2,

5′-GCCAT(C/T)TC(C/T)TG(C/T)TC(A/G)AA(A/G)TC-3′.

To amplify OBP cDNAs 25 cycles of a stepwise program (94°C for 5 sec; 55°C for 10 sec; 72°C for 3 min) were carried out. Actin cDNA was amplified by 45 cycles of a stepwise program (94°C for 1 min; 45°C for 2 min; 72°C for 3 min). PCR products were separated by 1% agarose gel.

## RESULTS AND DISCUSSION

Our protein-based approach leads us to the identification of olfactory proteins by comparing the profile of proteins isolated from antennae and control tissues. A major antennae-specific protein was identified by native PAGE analyses of proteins from workers of *Z. n. nevadensis* (Fig. 1A).

The putative olfactory protein migrated faster than our reference protein, the pheromone-binding protein from *Bombyx mori*, BmPBP, and showed an N-terminal amino acid sequence (LTGRAFERAKEVDEK-RS; -indicates no signal) with 55% sequence identity (70% similarity) to the previously identified olfactory proteins (Pam6m and Pam6f) from the American cockroach, *Periplaneta americana* (Picimbon and Leal, 1999). In order to obtain further sequence information, the cDNA encoding this protein was cloned. A single-banded cDNA (550 bp) was amplified with a degenerate primer (based on the N-terminal amino acid sequence) and an GCGT15 primer. With this partial sequence available, we designed a gene specific primer, determined the 5′-region, and obtained the full length of cDNA, which encoded a protein with 151 amino acid residues (Fig. 1B). The predicted cleavage site of the signal peptide was in agreement with the N-terminal amino acid sequence of the isolated protein. Calculated molecular mass and the isoelectric point for the mature protein were 14,849 Da and pI 4.84, respectively. BLAST database search indicated considerable sequence similarity with previously identified odorant-binding proteins, with the highest scores (30–33% identity) to odorant-binding proteins from the scarab beetles *Exomala orientalis*, *Anomala osakana*, *Popillia japonica*, *Anomala cuprea*, and *A. octiescostata*, *Phyllopertha diversa*, *Heptophylla picea* and *Holotrichia parallela* (Deyu and Leal, 2002 and references therein). The sequence of a cockroach OBP, *Leucophaea maderae*, in the database (accession number AY116618) showed only 12% identity to ZnevOBP1. In addition to significant sequence similarity, the antennae-specific protein from *Z. n. nevadensis* possesses the hallmark of odorant-binding protein, six cysteine residues (at positions 16, 46, 50, 88, 97, and 106) (Fig. 1B). Hence,

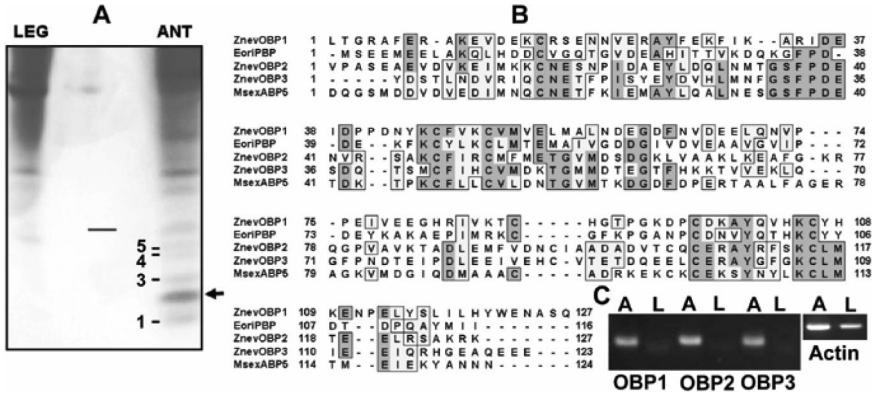


FIG. 1. Gel electrophoresis analysis of proteins extracted from *Z. n. nevadensis*, sequence alignment of deduced amino acids from the cloned OBP cDNAs, and RT-PCR analysis of transcription. (A) Soluble proteins analyzed by a 15% native PAGE and stained with Coomassie blue. LEG, control tissue; 100 midlegs-equivalent. ANT, 400 antennae-equivalent. Arrow indicates the major antennae-specific binding protein. Numbers are given to other minor antennae-specific proteins by their order of mobility. Migration of a reference protein, the pheromone-binding protein from *B. mori* (BmPBP), is denoted by a bar in the empty lane. A band with mobility similar to BmPBP was not antennae-specific. (B) Alignment of ZnevOBP1 with the protein in the database with the highest sequence identity, namely, the pheromone-binding protein from the Oriental beetle, *E. orientalis* (EoriPBP; AB040144). Alignment of ZnevOBP2 and ZnevOBP3 with the best fitting protein, i.e., the antennal-binding protein of *M.* sexta (MsexABP5; AF393498). For clarity, the putative signal peptide and the first 15 amino acid residues of MsexABP5 were omitted. The cDNA sequences for ZnevOBP1, ZnevOBP2, and ZnevOBP3 have been submitted to the GenBank database with accession numbers AY135381, AY135382, and AY135383, respectively. (C) RT-PCR data. cDNA fragments (ca. 100 bp) were amplified with gene-specific primers and antennal template (A), but not with template derived from legs (L). cDNA fragments (450 bp) of actin (control) were amplified using templates from both legs (L) and antennae (A).

we suggest that the isolated protein is an odorant-binding protein and named it ZnevOBP1.

While sequencing multiple clones for ZnevOBP1, we were able to identify two other cDNAs encoding putative odorant-binding proteins, which we named ZnevOBP2 and ZnevOBP3. One cDNA (ZnevOBP2) encoded a protein with 148 amino acid residues. The mature protein (127 amino acid residues) gave a calculated molecular mass and isoelectric point of 14,173 Da and pI 4.95, respectively. Interestingly, as the pheromone-binding protein from *Manduca sexta* (MsexPBP1) (Vogt et al., 2002), this protein has seven cysteine residues (Cys-17, 47, 51, 95, 104, 106, and 115) (Fig. 1B). The other cDNA (ZnevOBP3) encoded a

protein with 143 amino acid residues, the first 20 residue being the putative signal peptide. The calculated molecular mass and isoelectric point for the mature protein were 14,272 Da and pI 4.07, respectively. This protein possesses 6 cysteine residues in a pattern similar to that of ZnevOBP1, i.e., Cys-12, 42, 46, 88, 98, and 107. BLAST search indicated that ZnevOBP2 and ZnevOBP3 shared significant sequence similarity with several insect odorant-binding proteins, the highest scores being the antennal binding protein cloned from *M*. sexta MsexABP5 (AF393498), which showed 41 and 35% identity to ZnevOBP2 and ZnevOBP3, respectively (Fig. 1B).

In order to test whether the cloned cDNAs were related to the minor antennae-specific proteins from *Z. n. nevadensis* (see Fig. 1A), we attempted to determine the N-terminal amino acid sequences of bands 1, 3, 4, and 5. Given the scanty amounts of these proteins in the antennae, the obtained sequences were short, and the readings of the first amino acid residues were not reliable. However, the partial sequences showed that these minor antennae-specific bands are not encoded by the cloned cDNAs. Bands 1 and 3 gave partial sequences Ser-Pro-Leu/Phe-Asp and Gln-Met-Glu-Glu starting from the second and third residues, respectively. On the other hand, bands 4 and 5 gave sequences Val-Lys-Gln and Met-Leu-Asn, starting from the fourth residue. None of these residues is identical to those at the same positions in ZnevOBP2 and ZnevOBP3.

Two lines of evidence suggest that termites have multiple olfactory proteins. First, the minor bands 1, 3, 4, and 5 are specific to the antennae and show mobility similar to most acidic odorant-binding proteins identified to date; they are probably OBPs. Secondly, RT-PCR showed that ZnevOBP2 and ZnevOBP3 are also specific to antennae (Fig. 1C). In conclusion, the evidence from *Z. n. nevadensis* suggests that olfaction in termites (Isoptera) is mediated by multiple odorant-binding proteins.

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