Chem. Senses 26: 529–531, 2001

# Detection and Removal of an Artefact Fatty Acid from the Binding Site of Recombinant *Bombyx mori* Pheromone-binding Protein

**Neil J. Oldham, Jürgen Krieger[1], Heinz Breer[1] and Aleš Svatoš[2]**

Max-Planck-Institute for Chemical Ecology, Carl-Zeiss-Promenade 10, D-07745 Jena, [1]Institute of Physiology, University of Stuttgart-Hohenheim, Garbenstrasse 30, D-70593 Stuttgart, Germany and [2]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, Prague 6-Dejvice, CZ-166 10, Czech Republic

*Correspondence to be sent to: Neil Oldham, Max-Planck-Institute for Chemical Ecology, Carl-Zeiss-Promenade 10, D-07745 Jena, Germany. e-mail: oldham@ice.mpg.de*

## Abstract

Recombinant *Bombyx mori* pheromone-binding protein (PBP), purified from an *Escherichia coli* expression system, has been found to contain (11*Z*)-octadecenoic acid (*cis*-vaccenic acid) as an artefact ligand. An efficient delipidation procedure is described to overcome what would appear to be a general problem with recombinant lepidopteran PBPs.

## Introduction

Odorant-binding proteins (OBPs) are a group of small globular proteins found in the sensillum lymph of insects (Vogt and Riddiford, 1981) and the nasal mucus of mammals (Bignetti *et al.*, 1985). Although OBPs have been the subject of detailed study, their exact role in the olfactory process is yet to be elucidated. In the most widely accepted model, OBPs are responsible for binding hydrophobic odour molecules and transporting them through the aqueous sensillum lymph, or nasal mucus, to membrane-bound olfactory receptors (Steinbrecht, 1998). Among the known OBPs from insects there exists a well-characterized group with selective expression in pheromone sensitive sensilla (Vogt and Riddiford, 1981; Steinbrecht *et al.*, 1995). It is proposed that these pheromone-binding proteins (PBPs) may preferentially interact with certain odorant molecules, i.e. pheromone components, thus contributing to the remarkable specificity of pheromone reception (Krieger and Breer, 1999; Steinbrecht, 1998). This theory is, in part, supported by a number of ligand binding studies (Du and Prestwich, 1995; Oldham *et al.*, 2000; Plettner *et al.*, 2000), but the degree of specificity exhibited by PBPs appears to be lower than that of the entire receptor neuron.

Recently, we reported an electrospray–mass spectrometry (ESI-MS) based method for studying complexes of the *Bombyx mori* PBP (BmPBP) with a number of ligands, including bombykol (Oldham *et al.*, 2000). During this work, we discovered the presence of an unknown lipid in the binding site of recombinant BmPBP, which appeared to originate from the *Escherichia coli* expression system. Here we describe the phenomenon in more detail, identify the lipid and present an effective delipidation procedure.

## Materials and methods

Recombinant *B. mori* pheromone-binding protein (BmPBP) was produced and purified as previously described (Wojtasek and Leal, 1999; Oldham *et al.*, 2000). Delipidation of BmPBP was achieved in three steps. First, the protein was transferred into 25 mM ammonium acetate buffer, pH 4.5 (buffer A), using a Vivaspin ultrafiltration concentrator (capacity 500 µl, molecular weight cut-off (MWCO) 5 kDa, Vivascience Ltd, Stonehouse, Gloucestershire, UK). [Note: the use of a volatile buffer is only necessary for MS applications.] The BmPBP solution was centrifuged (10 000 r.p.m., 4°C) until the retentate volume was <50 µl (~7–9 min). Following addition of buffer A (500 µl) to the retentate, the solution was re-centrifuged. This washing step was repeated once, before a final dilution with buffer A (350 µl) to give a BmPBP solution in $NH_4OAc$ buffer at pH 4.5. In the second step of the delipidation procedure, BmPBP was incubated with Lipidex™-1000 (a lipophilic, hydrophobic gel derived from Sephadex LH-20; Canberra-Packard, Dreieich, Germany). Lipidex™-1000/methanol suspension (500 µl) was pipetted into a MC-Ultrafree centrifuge filter cup (capacity 500 µl, pore size 0.22 µm, Millipore, Bedford, MA). Following centrifugation at 10 000 r.p.m. for 1 min, the filtrate was discarded and a further aliquot of Lipidex™-1000 suspension added to the filter. The methanol was once more spun through and discarded. After washing the Lipidex™-1000 twice, by

thoroughly mixing with buffer A (2 × 500 µl) and centrifuging at 10 000 r.p.m. for 1 min, BmPBP solution (pH 4.5) was added. Following thorough mixing, the resulting suspension was incubated, in the filter cup, at 37°C for 1 h with gentle shaking. In the third step of the delipidation protocol the Lipidex™-1000 was removed by centrifuge filtration, washed once with buffer A (500 µl), and the combined filtrate concentrated and adjusted to pH 7 with $NH_4OAc$ buffer (2.5 mM, pH 7, buffer B), using a Vivaspin concentrator (see above). The final volume of buffer was adjusted to give a protein concentration of 5 mg/ml. For detection of binding, excess bombykol (20-fold) was added as a 0.6 M solution in ethanol. ESI-MS was performed as previously described (Oldham *et al.*, 2000). To identify the lipid contaminant, a sample of Lipidex™-1000, used for delipidation, was extracted with methanol (500 µl), the extract methylated with diazomethane (Blau and Darbre, 1993) and analysed by gas chromatography/mass spectrometry (Oldham and Svatoš, 1999). A 3 ml *E. coli* BL21 (DE3) pET22b (+)/BmorPBP stem culture was extracted, as previously described (Hohoff *et al*., 1999), and analysed by GC/MS as above.

## Results

Figure 1a shows the deconvoluted native ESI-MS of BmPBP after purification from the *E. coli* expression system. The peak at 15 878 Da corresponds to the predicted molecular mass of BmPBP, as deduced from its amino acid sequence. A major adduct peak, at 16 160 Da (M+282), was only present under native ESI-MS conditions, and was attributed to a non-covalent complex between the protein and an unknown lipid. In an attempt to remove this contaminant, a procedure described for the delipidation of fatty acid-binding proteins was employed. However, incubating BmPBP with Lipidex™-1000, as outlined (Hohoff *et al*., 1999), did not remove the lipid. In order to overcome this problem, we took advantage of the recent finding that BmPBP appears to release ligands at low pH (Wojtasek and Leal, 1999; Oldham *et al*., 2000), and incubated the protein with Lipidex™-1000 at pH 4.5. A delipidation protocol was established using ultrafilter concentrators (MWCO 5 kDa), to adjust the pH of the BmPBP solution, and a centrifuge filter (pore size 0.22 µm), to remove the Lipidex™-1000 (see Materials and methods section). Following delipidation with this method, the mass spectrum of BmPBP showed no evidence of lipid contamination (Figure 1b). Furthermore, treatment with bombykol produced a clear BmPBP–pheromone complex (Figure 1c), indicating that the low pH conditions did not irreversibly denature the protein. Following BmPBP delipidation, analysis of a methanol wash of the Lipidex™-1000, by gas chromatography–mass spectrometry (Oldham and Svatoš, 1999), identified the contaminant lipid as essentially pure (11*Z*)-octadecenoic acid (*cis*-vaccenic acid). Extraction and analysis of the total

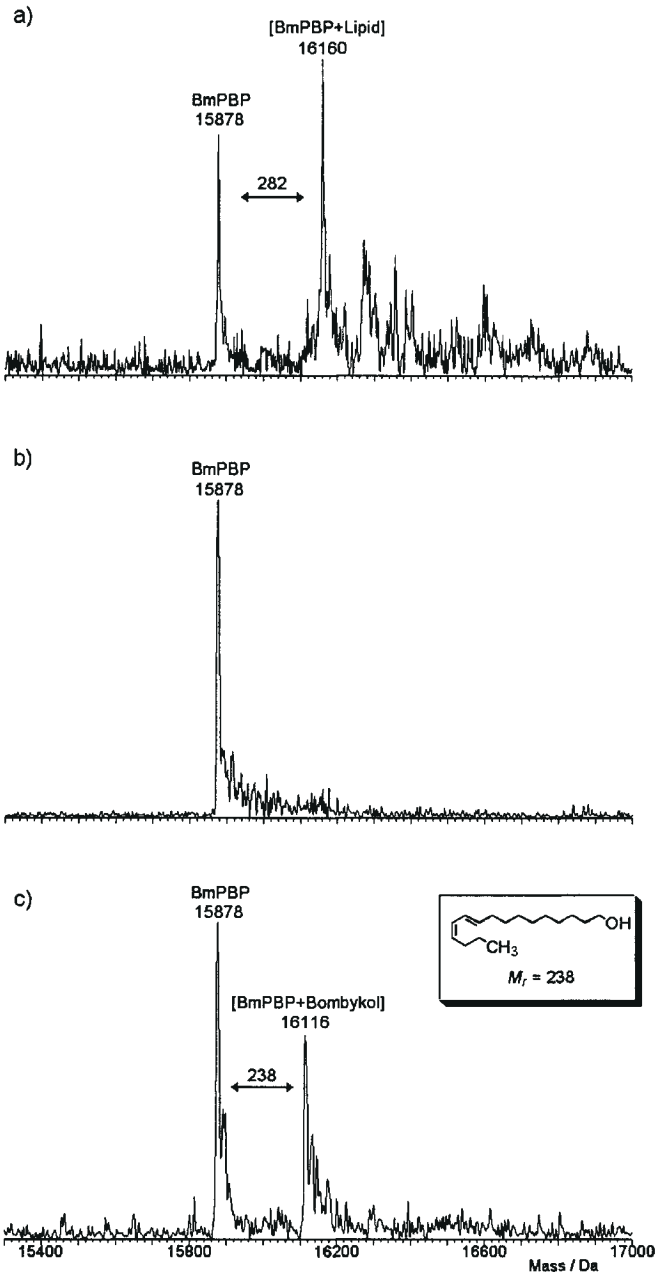


**Figure 1** Deconvoluted native electrospray-mass spectra of recombinant BmPBP **(a)** before delipidation, **(b)** after delipidation and **(c)** after exposure to bombykol.

lipids from *E. coli* [strain BL21 (DE3)—containing the BmPBP expression plasmid], however, demonstrated the presence of four major fatty acids (Table 1). Thus, in the presence of these compounds, BmPBP appeared to bind only the Δ11-unsaturated $C_{18}$ fatty acid.

## Conclusions

Recombinant *B. mori* pheromone-binding protein contains (11*Z*)-octadecenoic acid as an artefact lipid from the expression system. The fatty acid can be removed by

**Table 1** Composition of major fatty acids extracted from *E. coli* expression strain BL21 (DE3) pET22b(+)/BmorPBP

| Fatty acid | mol % |
|---|---|
| *cis*-Palmitoleic acid | 10.5 |
| Palmitic acid | 44.2 |
| *cis*-Vaccenic acid | 27.6 |
| Stearic acid | 17.7 |

incubation with Lipidex™-1000 at pH 4.5 without permanent loss of protein binding activity. BmPBP binds vaccenic acid ($C_{18}$) selectively, despite the fact that the lipid molecule is two carbons longer than bombykol ($C_{16}$). This is particularly remarkable given that palmitoleic acid ($C_{16}$) is present in *E. coli*. Assuming that the protein has access to both vaccenic and palmitoleic acids, it would appear that the intramolecular distance from C1 to the double bond, in the fatty acid, is important for binding. Preliminary studies on the recombinant PBPs from *Mamestra brassicae* and *Antheraea polyphemus*, by native ESI-MS, show that they too appear to form PBP–fatty acid complexes. Thus, it would seem that the presence of *E. coli*-derived lipids in the binding site of recombinant lepidopteran PBPs is a general phenomenon.

## Acknowledgements

The authors are grateful to Prof. C. Cambillau for a sample of *M. brassicae* PBP.

## References

**Bignetti, E., Cavaggioni, A., Pelosi, P., Persaud, K.C., Sorbi, R.T.** and **Tirindelli, R.** (1985) *Purification and characterization of an odorant-binding protein from cow nasal tissue*. Eur. J. Biochem., 149, 227–231.

**Blau, K.** and **Dabre, A.** (1993) *Esterification*. In Blau, K. and Dabre, A. (eds), Handbook of Derivatives for Chromatography. Wiley, Chichester, pp. 11–30.

**Du, G.** and **Prestwich, G.D.** (1995) *Protein structure encodes the ligand binding specificity in pheromone binding proteins*. Biochemistry, 34, 8726–8732.

**Hohoff, C., Börchers, T., Rüstow, B., Spener, F.** and **van Tilbeurgh, H.** (1999) *Expression, purification, and crystal structure determination of recombinant human epidermal-type fatty acid binding protein*. Biochemistry, 38, 12229–12239.

**Krieger, J.** and **Breer, H.** (1999) *Olfactory reception in invertebrates*. Science, 286, 720–723.

**Oldham, N.J., Krieger, J., Breer, H., Fischedick, A., Hoskovec, M.** and **Svatoš, A.** (2000) *Analysis of the silkworm moth pheromone binding protein-pheromone complex by electrospray ionization-mass spectrometry*. Angew. Chem. Int. Ed. Engl., 39, 4341–4343.

**Oldham, N.J.** and **Svatoš, A.** (1999) *Determination of the double bond position in functionalized monoenes by chemical ionization ion-trap mass spectrometry using acetonitrile as a reagent gas*. Rapid Commun. Mass Spectrom., 13, 331–336.

**Plettner, E., Lazar, J., Prestwich, E.G.** and **Prestwich, G.D.** (2000) *Discrimination of pheromone enantiomers by two pheromone binding proteins from the gypsy moth* Lymantria dispar. Biochemistry, 39, 8953–8962.

**Steinbrecht, R.A.** (1998) *Odorant-binding proteins: expression and function*. Ann. N.Y. Acad. Sci., 855, 323–332.

**Steinbrecht, R.A., Laue, M.** and **Ziegelberger, G.** (1995) *Immunolocalisation of pheromone-binding protein and general odorant-binding protein in olfactory sensilla of the silk moths* Antheraea *and* Bombyx. Cell Tissue Res., 282, 203–217.

**Vogt, R.G.** and **Riddiford, L.M.** (1981) *Pheromone binding and inactivation by moth antennae*. Nature, 293, 161–163.

**Wojtasek, H.** and **Leal, W.S.** (1999) *Conformational change in the pheromone binding protein from* Bombyx mori *induced by pH and by interaction with membranes*. J. Biol. Chem., 274, 30950–30956.

*Accepted February 20, 2001*