Insect Biochemistry and Molecular Biology 48 (2014) 51–62

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Identification of pheromone components and their binding affinity to the odorant binding protein CcapOBP83a-2 of the Mediterranean fruit fly, *Ceratitis capitata*

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ARTICLE INFO

Article history:
Received 30 July 2013
Received in revised form
17 February 2014
Accepted 18 February 2014

Keywords:
Medfly
Ceratitis capitata
Olfaction
Odorant binding protein
Pheromone binding protein
Pheromone
Binding studies
Protein expression
Electroantennography
GC–EAG
Fluorescence displacement

ABSTRACT

The Mediterranean fruit fly (or medfly), *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae), is a serious pest of agriculture worldwide, displaying a very wide larval host range with more than 250 different species of fruit and vegetables. Olfaction plays a key role in the invasive potential of this species. Unfortunately, the pheromone communication system of the medfly is complex and still not well established. In this study, we report the isolation of chemicals emitted by sexually mature individuals during the "calling" period and the electrophysiological responses that these compounds elicit on the antennae of male and female flies. Fifteen compounds with electrophysiological activity were isolated and identified in male emissions by gas chromatography coupled to electroantennography (GC–EAG). Within the group of 15 identified compounds, 11 elicited a response in antennae of both sexes, whilst 4 elicited a response only in female antennae. The binding affinity of these compounds, plus 4 additional compounds known to be behaviourally active from other studies, was measured using *C. capitata* OBP, CcapOBP83a-2. This OBP has a high homology to *Drosophila melanogaster* OBPs OS-E and OS-F, which are associated with trichoid sensilla and co-expressed with the well-studied *Drosophila* pheromone binding protein LUSH. The results provide evidence of involvement of CcapOBP83a-2 in the medfly's odorant perception and its wider specificity for (*E*,*E*)-α-farnesene, one of the five major compounds in medfly male pheromone emission. This represents the first step in the clarification of the *C. capitata* and pheromone reception pathway, and a starting point for further studies aimed towards the creation of new powerful attractants or repellents applicable in the actual control strategies.

1. Introduction

Insect pheromones play an important role in intra- and inter-species communication, inducing specific behavioural responses in terms of sexual attraction, mating aggregation and host-marking of oviposition sites. Odour perception is regulated by a fine molecular pathway that involves multigene families including odorant-binding proteins (OBPs), chemosensory proteins (CSPs) and odorant receptors (ORs). Within the OBP family, pheromone-binding proteins (PBPs) are proven to be involved in insect sexual communication, but the molecular basis underlying this process is still unknown for most Dipteran insects and is the target of several studies. The Mediterranean fruit fly or medfly, *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae) is a serious pest of agriculture worldwide (Arita and Kaneshiro, 1989; Maddison and Bartlett, 1989). This species shows a wide larval host range comprising more than 250 different species of fruits and vegetables (Joint-FAO/IAEA-Division, 1985; Malacrida et al., 2007), which is a major factor in its biological success. Olfaction plays a key role in the invasive potential of this species, regulating essential behaviours such as i) localisation of plant hosts, ii) detection of pheromones during recognition and location of mates for mating, iii) discrimination

Abbreviations: OBP, Odorant binding protein; PBP, Pheromone binding protein; CSP, Chemosensory protein; OR, Odorant receptor; EAG, Electroantennography; GC–EAG, Gas chromatography coupled to electroantennography; SIT, Sterile Insect Technique.

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http://dx.doi.org/10.1016/j.ibmb.2014.02.005

between suitable and already pierced hosts for oviposition. The pheromone communication system of the medfly is complex and still not fully understood. Medfly mating behaviour has been a topic of extensive research in the last few decades (Arita and Kaneshiro, 1989; Feron, 1959; Levinson et al., 1987; Prokopy and Hendrichs, 1979; Shelly et al., 1994; Whittier et al., 1992; Yuval and Hendrichs, 2000; for a review, see Eberhard, 2000). In this species the males attract females (Arita and Kaneshiro, 1989; Feron, 1959; Prokopy and Hendrichs, 1979) by emitting a mixture of pheromone compounds. Several decades ago Feron (1962) reported compounds released by *C. capitata* males. Since then, various studies have been conducted, with the aim of identifying the active components of the pheromone mixture (Alfaro et al., 2011; Feron, 1962; Goncalves et al., 2006; Jacobson et al., 1973; Jang et al., 1989; Ohinata et al., 1977). Jacobson and colleagues (Jacobson et al., 1973) described the sex pheromone as a mixture of 15 substances, including carboxylic acids and other compounds such as methyl (*E*)-6-nonenoate and (*E*)-6-nonen-1-ol. In 1977, Ohinata and colleagues reported that these mixtures were attractive to both sexes in laboratory tests, but only to males in an open field trial (Ohinata et al., 1977). Jang et al. (1989) detected 69 compounds from the male headspace, while the female headspace samples contained traces of only a few compounds, mainly short-chain aldehydes. Goncalves (Goncalves et al., 2006) published another list of compounds and reported the composition of aeration samples of calling males collected in Tenax tubes. More recently, Alfaro et al. (2011) reported the medfly volatile profiles at different physiological states and characterised groups of compounds according to their emission pattern. As the exact composition and function of the complex pheromone blend, and the molecular mechanisms by which it is sensed by both males and females, is not well defined, further studies on these topics may furnish powerful tools for the improvement of current pest control strategies, i.e. by developing specific synthetic attractants or repellents that can be used in Sterile Insect Technique (SIT) programmes.

The analysis of medfly EST libraries and the genome sequence resulted in the identification of a number of *obp* genes (Gomulski et al., 2012, 2008; Scolari et al., 2012; Siciliano et al., 2014). Further molecular characterisation and analysis of expression profiles of five identified putative *obp* genes (*CcapOBP69a*, *CcapOBP19d-1*, *CcapOBP83a-1*, *CcapOBP83a-2* and *CcapOBP28a*) underlined a possible implication of these genes in odorant perception and represented a first step in the elucidation of the molecular pathway regulating olfactory behaviours in the medfly (Siciliano et al., 2014). One of these five genes, *CcapOBP83a-2,* was found to be highly enriched in antennae with the highest expression in sexually mature individuals (Siciliano et al., 2014). In a phylogenetic analysis (Siciliano et al., 2014) it is clustered together with *Drosophila melanogaster* OBPs OS-E and OS-F also known as OBP83a and OBP83b that have been shown to associate with trichoid sensilla, which are strongly implicated in the detection of volatile pheromones. OS-E and OS-F were identified by McKenna and colleagues (McKenna et al., 1994). OS-F was also independently and simultaneously identified as PBPRP3 by Pikielny et al. (1994). OS-E and OS-F are known to co-express with a well-studied *Drosophila* OBP called LUSH in the pheromone sensitive sensilla (Shanbhag et al., 2001); while ligands are known for LUSH as 11-*cis*-vaccenyl acetate (cVA) (Ha and Smith, 2006), no ligands are known for OS-E and OS-F. Interestingly the analyses of medfly EST libraries and the genome sequence identified a LUSH-like OBP gene, *CcapOBP19a,* in the medfly genome (Siciliano et al., 2014). In the present study, we investigate the chemicals emitted by sexually mature medfly individuals during the "calling" period using coupled gas chromatography–electroantennography (GC–EAG) and coupled GC–mass spectrometry (GC–MS) in order to identify the physiologically active compounds. We then expressed the first *C. capitata* OBP CcapOBP83a-2 and used it to examine the relative binding ability of the newly identified pheromone components. We provide evidence for the possible involvement of CcapOBP83a-2 in olfaction processes (possibly in the pheromone communication) and its specificity for (*E*,*E*)-α-farnesene, one of the five major compounds in the medfly male pheromone emission.

2. Material and methods

2.1. Collection of volatiles

Medfly individuals were obtained from the well-established ISPRA strain and reared under standard conditions in the quarantine facility at the University of Pavia, Italy. Volatile collections were performed over 24 h (from 15:00 h to 15:00 h) using 4 day-old virgin flies (60 flies per vessel) from males and females separately. Each set of 60 individuals (2 replicates per sex) was placed inside an airtight 2-L glass vessel connected to the outlet of an air compressor which pumped air through a charcoal filter guaranteeing the use of ultrapure air (550 ml/min). Volatiles were collected on Porapak Q (0.05 g, 60/80 mesh; Supelco) in a glass tube (5 mm Ø) inserted into the collection port on top of the vessel. Another pump drew air through this tube. To ensure that no unfiltered air was drawn into the vessel from outside, the rates of airflow were set so that more purified air was pumped in than was drawn out. After 24 h, volatiles were eluted from the Porapak Q tube with 0.5 ml of redistilled diethyl ether, providing a solution that contained the isolated volatile compounds. Samples were then stored at −20 °C until used. Before use, all the equipment was rinsed with acetone, ethanol and distilled water and then dried in an oven at 180 °C for at least 2 h. Porapak Q tubes were cleaned by elution with redistilled diethyl ether and heated at 132 °C for 2 h under a stream of purified nitrogen to remove contaminants. Charcoal filters were conditioned before use by attaching to a constant stream of nitrogen in an oven at 150 °C for 2 h.

2.2. Gas chromatography (GC) analysis

GC analysis was performed by injecting 4 μl of volatile sample onto a nonpolar capillary column (HP-1, 50 m, 0.32 mm internal diameter, 0.52 μm film thickness) using an Agilent 6890 GC equipped with a cold on-column injector and flame ionization detector (FID). The oven was maintained at 30 °C for 2 min and then programmed for increments at 5 °C min^{-1} to 250 °C. Quantification was carried out by calculating and comparing peak areas with known amounts of authentic external standards.

2.3. Coupled gas chromatography–mass spectrometry (GC–MS) analysis

Attractive headspace samples were analysed on a capillary GC column (HP-1, 50 m, 0.32 mm i.d., 0.52 μm f.t.) directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments, Manchester, UK) equipped with a cold on-column injector. Ionization was initiated by electron impact (70 eV, 250 °C). The oven was maintained at 30 °C for 2 min and then programmed for increments at 5 °C min^{-1} to 250 °C. Tentative GC–MS identifications were confirmed by peak enhancement with authentic standards on two GC columns of different polarities (Ukeh et al., 2009).

2.4. Coupled gas chromatography–electroantennography

Electrical responses to chemical stimuli were recorded from the antennae of both sexes using 4 day-old virgin individuals.