Journal of Insect Physiology 73 (2015) 37–46

Contents lists available at ScienceDirect

Journal of Insect Physiology

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

Characterization of *Melanoplus sanguinipes* oviposition stimulating protein expression and re-examination of its potential role in stimulating oviposition

E.A. Sieminska [a,b], J.A. Andres [a], C.D. Todd [a], M.A. Erlandson [a,b,*]

[a] Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[b] Agriculture and Agri-Food Canada, Saskatoon Research Centre, Saskatoon, Saskatchewan, Canada

ARTICLE INFO

Article history:
Received 27 May 2014
Received in revised form 7 January 2015
Accepted 12 January 2015
Available online 21 January 2015

Keywords:
Melanoplus sanguinipes
Oviposition
Oviposition stimulating protein
Reproduction

ABSTRACT

Melanoplus sanguinipes oviposition stimulating protein (MsOSP) was characterized and its role in stimulating oviposition in virgin females was examined. A 967 nt *MsOSP* mRNA sequence with homology to previously characterized N-terminal amino acid sequence data for MsOSP was identified in a RNAseq library generated from an mRNA pool from the long hyaline tubule (LHT) of the male accessory gland complex. This transcript contained a predicted 729 nt open reading frame encoding the 242 aa putative MsOSP protein and had the second highest read abundance in the library. The *MsOSP* transcript was detected exclusively in the LHT tissue of adult males and its abundance increased with time until 7 days post-eclosion. Western blot analysis using an anti-MsOSP antibody showed high levels of MsOSP protein in the LHT luminal secretions of virgin males and to a lesser degree was associated with the aedeagus and ejaculatory duct. MsOSP was shown to be a major protein component of the spermatophore packet transferred from the male to female during copulation. However, only minor amounts of MsOSP could be detected in the female bursa, spermatheca and oviduct. Intrahemocoelic injection of LHT luminal protein into mature virgin females stimulated oviposition in ~65% of females. A similar but non-significant trend was observed upon injection of purified recombinant MsOSP protein, and immunoprecipitation of LHT protein with anti-MsOSP antibody led to abrogation of oviposition stimulation upon injection of mature virgin females. Despite the demonstration of stimulation of oviposition upon intrahemocoelic injection of LHT-derived-MsOSP into mature virgin females, the potential mode of action of MsOSP in this process remains to be determined. MsOSP cannot be detected in the tissues other than the bursa, spermatheca and oviduct of female grasshoppers and relatively large quantities of MsOSP are required to stimulate oviposition upon injection.

1. Introduction

Any deviation from monogamy may result in sexual conflict because individuals' lifetime reproductive interests will not coincide (Rice 2000; Parker, 2006). In polyandrous insects, sperm storage and internal fertilization enhance sperm competition which may, in turn, lead to the evolution of characters that increase reproductive success in one sex but are costly to the other. Support for this type of sexual conflict comes from the negative relationship between male sperm competition and early female mortality (Civetta and Clark, 2000).

In insects, male accessory gland complexes produce secretions that influence sperm competitive success. These include a rich mixture of proteins (i.e. seminal fluid proteins, SFPs), some of which are complexed with carbohydrates and lipids (Gillott 2003; Herndon and Wolfner, 1995). Detailed biochemical, genetic and molecular studies in the model system *Drosophila melanogaster* have shown that these SFPs play key roles in a variety of reproductive parameters including the transfer, protection and storage of sperm (den Boer et al., 2009; Xue and Noll, 2000), sperm competition (Chapman et al., 2000; den Boer et al., 2010) as well as more direct impacts on females including fecundity (den Boer et al., 2009), ovulation (Peng et al., 2005; Ram and Wolfner, 2007), oviposition (Herndon and Wolfner, 1995; Jin and Gong, 2001; Lange and Loughton, 1985) and mating behavior (Yapici et al., 2008; Yang et al., 2009). In *Drosophila* positive selection is an important force driving the evolution of SFPs (e.g.

* Corresponding author at: Saskatoon Research Centre, Agriculture and Agri-Food Canada, Agriculture et Agroalimentaire Canada, 107 Science Place, Saskatoon, SK S7N 0X2, Canada. Tel.: +1 (306) 956 7276; fax: +1 (306) 956 7247.
E-mail address: Martin.Erlandson@AGR.GC.CA (M.A. Erlandson).

http://dx.doi.org/10.1016/j.jinsphys.2015.01.003
0022-1910/

Almeida and Desalle, 2009; Begun and Lindfors, 2005; Begun et al., 2006; Swanson et al., 2001). This selection pressure may arise primarily as the result of the underlying conflict between the reproductive interest of males and females (e.g. Arnqvist and Nilsson, 2000; Arnqvist and Rowe, 2005; Brommer et al., 2012; Fricke et al., 2009; Wigby and Chapman, 2005). Support for this type of sexual conflict comes from the negative relationship between male sperm competition and early female mortality (Civetta and Clark, 2000). However, this is not always the case. In field crickets, during copulation, males transfer seminal fluid products that increase female life span and fecundity (Wagner et al., 2001). Thus, in other insects, selection may not necessarily be driven by conflict but by male–male (sperm) competition and cryptic female choice (sensu Eberhard, 1996). Distinguishing the role of all these evolutionary forces in the rapid evolution of reproductive proteins (reviewed in Clark et al., 2006) requires a detailed knowledge of the molecular physiology of SFPs. However, although SFPs fall into biochemical conserved classes (Avila et al., 2011; Braswell et al., 2006; Mueller et al., 2004), very little is known about SFP-mediated effects in other species. As a result of this strong taxonomic bias, in most insect groups a crucial question remains: what post-mating responses in females are triggered by male SFPs?

In orthopterans SFPs are known to upregulate egg production in mated females (Lange and Loughton, 1985; Yi and Gillott, 1999). For example, in the migratory locust (*Locusta migratoria*) an oviposition-stimulating factor secreted by the male's opalescent accessory gland induces oviposition (Lange and Loughton, 1985; Paemen et al., 1991). Similarly, oviposition in field crickets (*Allonemobius*) seems to be controlled by a very different SFP known as "protein X" suggesting that male derived oviposition factors show functional convergence in this group (Fedorka and Zuk, 2005). In this paper we characterize an oviposition factor in the grasshopper *Melanoplus sanguinipes*, a close relative of the American and African locusts, that is one of the most important agricultural pests in central North America. In an early study, *M. sanguinipes* male accessory gland extracts were shown to stimulate oviposition in virgin females (Friedel and Gillott, 1976). An attempt to purify and characterize the oviposition-stimulating protein from the long hyaline tubule (LHT) using Sephadex G-100 column purified fractions indicated that a 60 kDa component caused stimulation of oviposition when injected into the hemolymph of virgin females (Yi and Gillott, 1999). This fraction was characterized as a dimer of a 30 kDa protein called oviposition-stimulating protein (OSP). A polyclonal antibody generated against a crude preparation of *M. sanguinipes* OSP (MsOSP) reacted with the viscous secretion associated with spermatophore transfer, the spermatheca and the egg-pod froth of mated females, confirming transfer of MsOSP from male to female during copulation (Yi and Gillott, 1999).

Here we extend the characterization of MsOSP, its expression profile in male grasshoppers and further investigate its possible role in stimulating oviposition in virgin females. This study is part of a larger transcriptomic and proteomic project characterizing the accessory reproductive glands of male *M. sanguinipes* (Bonilla et al., 2014). Based on the available N-terminal 21 aa sequence from MsOSP (Yi and Gillott, 1999) we were able to identify a full-length putative open reading frame for MsOSP and express it as a recombinant protein in the Bac-to-Bac baculovirus expression vector system. We also used RT-PCR and RT-qPCR to characterize the temporal expression profile of MsOSP in male grasshoppers and Western blots to examine its tissue localization and potential transfer to females during copulation.

2. Materials and methods

2.1. Insect cultures: staging adults and tissue dissections

The *M. sanguinipes* colony was reared as described by Ewen and Hinks (1986). Late 5th instar nymphs were selected from the colony, separated by sex, and the males and females reared separately. Each day, newly eclosed adults were isolated for the purposes of temporal staging. At prescribed developmental stages, grasshoppers were anesthetized with CO_2 and specific tissues were isolated by dissection or bioassays were carried out as described below.

2.2. RNAseq 454 pyrosequencing

The accessory glands were dissected from 8 mated and 8 virgin males (10 days old) and the LHT was isolated, excised and homogenized in Triazol reagent according to the manufacturer's protocol (Invitrogen). Total RNA was resuspended in 100 μL of sterile DEPC-treated H_2O and RNA quantified using a Nanodrop spectrophotometer (Fisher-Thermo). The mRNA was then purified from total RNA (400–500 μg) by passing the sample twice through a Poly(A) Purist kit (Ambion) purification protocol. The mRNA fractions were resuspended in 25 μL of RNA storage buffer (Ambion), quantified using Nanodrop and found to contain 7.0 and 7.7 μg mRNA per μL for mated and virgin samples, respectively. cDNA library construction and 454 pyrosequencing were conducted at the DNA Technology Laboratory, Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, SK. The samples were quantified and checked for mRNA quality using RNA 6000 Nano Kit and Bioanalyzer 2100 (Agilent). Subsequently, mRNA was fragmented using the high temperature Zn^{2+} method and fragments purified with selection for 300–1000 nt size fragments using a cDNA Rapid Library Prep Kit (Roche). The RNA fragments were used to prepare cDNA libraries for pyrosequencing according to manufacturer's instructions (Roche). The output from the GS FLX titanium sequencing runs was assembled into contigs, quantified as to the number of reads per contig and annotated via a *blastx* algorithm using the CLC Genomics Workbench software suite (CLC). The contig libraries generated for the mated and virgin LHT were searched using the N-terminal 21 amino acid sequence (ALPSFVPAXKKDDPKLDEEFF) obtained by Yi and Gillott (1999). This search returned a single contig in the mated library containing 13,909 reads and a single contig in the virgin library containing 11,129 reads.

2.3. MsOSP cloning

The coding sequence for the putative MsOSP was amplified from cDNA synthesized with InFusion SMARTer Directional cDNA Library Construction Kit (Clontech Laboratories) using the primers 5′-TCGTCTAGAATGTCTGCCCTGCAACTCTGC-3′ and 5′-GGGAAGCTTTTAGGCGTAGTCGGGCACGTCGTAGGGGTAAGGGAACAATTCATCGACTAC-3′ and HiFiTaq Polymerase (Invitrogen) according to manufacturer's protocol. The nucleotide sequence corresponding to the human influenza hemagglutinin (HA) epitope tag (underlined) was incorporated into the reverse primer. The PCR product was ligated into the TA cloning vector pGEM (Promega), propagated in DH10B cells and plasmid DNA isolated for Sanger sequencing. Sequences with 100% alignment to the *MsOSP* coding sequence were excised with *Xba*I and *Hin*dIII and cloned into the Bac-to-Bac baculovirus expression vector, pFastBac1, under the control of polyhedrin promoter. The pFastBac1-*MsOSP* construct was transformed into competent DH10Bac™ *Escherichia coli* cells (Invitrogen, Bac-to-Bac Baculovirus Expression System Manual) containing the bacmid