The presence of \textit{Drosophila melanogaster} sex peptide-like immunoreactivity in the accessory glands of male \textit{Helicoverpa armigera}

Vidya K. Nagalakshmi\textsuperscript{a,b}, Shalom W. Applebaum\textsuperscript{a}, Eric Kubli\textsuperscript{c}, Yves Choffat\textsuperscript{c}, Ada Rafaeli\textsuperscript{b,*}

\textsuperscript{a} Department of Entomology, The Hebrew University, P.O. Box 12, Rehovot 76100, Israel
\textsuperscript{b} Institute for Technology and Storage of Agricultural Products, ARO, Volcani Center, P.O. Box 6, 50250 Bet Dagan, Israel
\textsuperscript{c} Zoological Institute, University of Zurich-Irchel, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Received 14 July 2003; received in revised form 15 December 2003; accepted 22 December 2003

Abstract

In this study a highly specific polyclonal antibody to Drm\textsuperscript{SP} was produced and used to develop and standardize a sensitive direct ELISA. Structure–activity studies revealed that the antiserum is specific to the N-terminal of Drm\textsuperscript{SP}. This ELISA was used for the detection of Drm\textsuperscript{SP}-like immunoreactivity in the reproductive tissues of male \textit{Helicoverpa armigera} moths at femtomole levels. Two positive immunoreactive peaks were found in HPLC purified extracts of male accessory glands. The immunoreactive peak, which contained a higher amount of immunoreactivity, was also found to be pheromonostatic in PBAN-injected decapitated females as well as in intact female moths during their peak pheromone production. Lower levels of Drm\textsuperscript{SP}-like immunoreactivity were found in younger males (1–2 day-old) when compared to older males (3–7 day-old).

Keywords: Lepidoptera; \textit{Helicoverpa armigera}; PBAN; \textit{Drosophila melanogaster} sex peptide; ELISA; Pheromonostatic peptide

1. Introduction

In many insect species mating leads to a permanent or transient loss of female sexual receptivity, which is sometimes associated with a cessation of calling behaviour and initiation of oviposition. These changes have been attributed to the action of male-derived factors transferred to the female genital tract during copulation. In \textit{Drosophila melanogaster}, post-mating responses in the female were shown to be induced by several components derived from the male reproductive system. Amongst these components is a 36 amino acid peptide, \textit{D. melanogaster} sex peptide (Drm\textsuperscript{SP}), derived from the male accessory gland and induces female non-receptivity and ovulation (Chen et al., 1988; Kubli, in press). In addition, Drm\textsuperscript{SP} was shown to enhance the production of juvenile hormone (JH) by the adult \textit{D. melanogaster} corpora allata (CA) in vitro (Moshitzky et al., 1996). Drm\textsuperscript{SP} also activates uptake of vitellogenin by mature oocytes in vivo (Soller et al., 1999).

In Lepidoptera, female attraction is as a result of the release of sex pheromone, the biosynthesis of which, in many species is controlled by pheromone-biosynthesis-activating-neuropeptide (PBAN) (Rafaeli, 2002). Mating induced termination of sex pheromone production has been reported in a number of moth species (\textit{Helicoverpa zea}, Raina, 1989; \textit{Heliothis virescens}, Ramaswamy et al., 1996; \textit{Lymnantria dispar}, Giebultowicz et al., 1991; \textit{Argyrotaenia velutiana}, Jurenka et al., 1993; \textit{Epiphyas postvittana}, Foster, 1993; \textit{Bombyx mori}, Ando et al., 1996; \textit{Plodia interpunctella}, Rafaeli and Gileadi, 1999; \textit{Choristoneura fumiferana} and \textit{C. rosaceana}, Delisle and Simard, 2002). However, the identification and characterization of a male-derived protein responsible for pheromonostasis has only been established in \textit{H. zea},

* Corresponding author. Tel.: +972-3-9683729; fax: +972-3-9604428.
E-mail address: vtada@volcani.agri.gov.il (A. Rafaeli).

0022-1910/$ - see front matter $2003 Elsevier Ltd. All rights reserved.
where the pheromonostatic action after mating was attributed to pheromone suppression peptide (HezPSP) (KINGAN et al., 1993, 1995; ELIYAHU et al., 2003). No sequence homology exists between the moth derived HezPSP peptide and the dipteran derived DrmSP peptide except for the presence of a disulphide bridge separated by an equal number but dissimilar nature of amino acid sequence. The importance of a disulphide bridge for pheromonostatic activity has been questioned by our previous study, since partial sequences of HezPSP showed pheromonostatic activity in the absence of the disulphide bridge (ELIYAHU et al., 2003). The effect of synthetic DrmSP on both the activities of CA and pheromone glands of the moth Helicoverpa armigera was studied by us (FAN et al., 1999, 2000). DrmSP was found to stimulate JH biosynthesis by H. armigera CA in vitro in a similar fashion to its action on D. melanogaster CA. In addition, DrmSP inhibited pheromone biosynthesis in isolated pheromone glands treated with PBAN, as well as in decapitated females injected with PBAN and in intact female moths during peak pheromone production in the scotophase. Structure–activity studies determined that the N-terminus of DrmSP is chiefly responsible for CA activation (allatotropic), with no activation occurring with exclusively C-terminal containing partial sequences. For pheromonostasis the C-terminus was largely responsible (FAN et al., 2000) although lower levels of activity were also obtained with fragments lacking the C-terminus. These results indicated that DrmSP exhibits cross-reactivity in H. armigera, where it significantly suppressed pheromone production and activated JH biosynthesis. This strongly suggests that these peptides may resemble, in sequence or function, endogenous moth DrmSP-like compounds that may play an important role in eliciting post-mating behaviour.

We herein report results suggesting that endogenous DrmSP-like proteins are present in male reproductive organs of H. armigera and may be implicated in the inhibition of sex pheromone production by the female moths.

2. Materials and methods

2.1. Insect culture

H. armigera were raised on an artificial diet (Heliothis Premix, Stonefly Industries, Inc. Bryan, TX, USA) at a constant temperature of 26 °C and 14:10 (light: dark) photoperiod as reported previously (RAFAELI et al., 2003). The genetic pool of the culture is replenished yearly with field caught specimens. Pupae were sexed and males and females were allowed to emerge separately as adults.

2.2. Male accessory gland extraction and purification

The male accessory gland complex (MAG) including duplex and simplex were dissected during the photophase and the tissues were frozen using liquid nitrogen. They were stored as batches of 10 MAG per batch at −80 °C until ready for extraction. The MAG were homogenized in batches using a homogenizer (Ultra-Turrax T25) in a 20-fold excess (v/w) of Bennet’s buffer (Bennet, 1986) as reported by Kingan et al. (1995). The homogenate was centrifuged at 12,000×g for 15 min at 4 °C (Sorvall RC-5B). The supernatant was filtered through four layers of cheese cloth to remove excess fatty material. The pellet was washed twice using 10-fold excess of Bennet’s buffer and the supernatants were pooled. The pooled supernatant was then purified using two C₁₈ Sep-Pak cartridges in tandem. The acids and salts were rinsed through with 10% acetonitrile (AcN) containing 10 mmol l⁻¹ ammonium acetate (AmAc), pH 5.0, and proteinaceous material was eluted with 40% AcN containing 20 mmol l⁻¹ AmAc, pH 5.0. The partially purified extract was then fractionated by reverse-phase high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) using a wide pore C₄ column (Vydac, 4.6 mm × 25 cm) using an AcN gradient from 10% to 100% in 0.1% trifluoroacetic acid (TFA) increasing at 0.5% min⁻¹. One milliliter fractions were collected and stored at −80 °C until ready for immunoassay.

2.3. Detection of SP-like immunoreactivity

2.3.1. Reagents

The full sequence of DrmSP₁₃₆, partial sequence peptides (DrmSP₈₋₃₆, DrmSP₉₋₃₆, DrmSP₂₀₋₃₆, DrmSP₂₁), Dup99B and the partial sequence of HezPSP (HezPSP₃₅₋₅₇) were synthesised in the facilities of the University of Zurich-Irchel and kept at −20 °C. The anti-DrmSP, used as a primary antibody, was custom prepared in rabbits against the purified synthetic peptide in the Rehovot facilities of Sigma Chemical Co. (St. Louis, MO, USA). Alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO, USA) was used as a secondary antibody and this was detected using p-nitrophenyl phosphate (pNPP) tablets (Sigma Chemical Co., St. Louis, MO, USA) as the substrate. Costar 96 well (Easy wash-high binding) microtitre plates (Corning, NY, USA) were used for the immunoassays.

2.3.2. Buffers

DrmSP was coated on microtitre plates in 0.01 M carbonate–bicarbonate buffer, pH 9.6. Intermittent washings were performed using 0.01 M phosphate buffer saline (PBS) containing 0.05% of Tween-20 (PBS T-20), pH 7.4. One percent bovine serum albumin...
(BSA) (Sigma Chemical Co., St. Louis, MO, USA) in PBS T-20 was used as a blocking buffer and for the primary and secondary antibody incubations. Diethanol amine buffer 1 M, pH 9.8 was used for incubation with the substrate.

2.3.3. Immunoassay

Determination of DrmSP-like immunoreactivity was performed using a direct enzyme-linked immunosorbant assay (ELISA), where an antigen is adsorbed on the plate and quantified by its capacity to bind with its specific antiserum. DrmSP standards were prepared in the range of 10–400 fmole/µl in 30% AcN + 0.1% TFA and lyophilized directly on the microtitre plates (50 µl/well). HPLC purified extracts of *H. armigera* MAG complexes were dispensed on the microtitre plates at a concentration of 5 male equivalents per well and lyophilized. Carbonate–bicarbonate buffer was added (50 µl/well), the plates were sealed with Parafilm (Chicago, IL, USA) incubated for 30 min at room temperature in an orbital shaker incubator (TU-400 orbital shaker, Digisystem, Kyoto, Japan) at 50 rpm. This ensured a uniform coating of the wells. The plates were then incubated for 16 h at 4 °C. Subsequently, the plates were rinsed once with PBS T-20 and blocked with 1% BSA in PBS T-20 for 3 h, after which they were washed once and the primary antibody (anti-DrmSP) solution was added at 1:1000 dilution in PBS T-20 (50 µl/well). The plates were subsequently sealed, briefly incubated at room temperature in an orbital shaker incubator at 50 rpm for 30 min and thereafter incubated overnight at 4 °C. The plates were then washed twice and incubated with secondary antibody diluted 1:1000 (70 µl/well) in 30% AcN. Blocking was performed with 5% non-fat dry milk in TBS on an orbital shaker (60 rpm) at room temperature for 40 min. This was repeated twice with new blocking solution. The membrane was subsequently washed briefly with TBS containing 0.1% Tween-20 (TBS T-20) and incubated with DrmSP-antiserum at 1:500 dilution at 4 °C overnight. After washing with TBS T-20, three times for 30 min each secondary antibody (goat anti-rabbit IgG HRP, Pierce, Rockford, USA) was added at 1:4000 and incubated for 1.5 h at room temperature in an orbital shaker at 60 rpm. Subsequently, the membrane was washed with TBS T-20 four times for 20 min each. Chemiluminescence was detected after applying substrate (Supersignal West Dura, Pierce, Rockford, USA) using a CDC Imager (ChemiImager Alpha Innotech Co., San Leandro, CA, USA).

2.3.6. Dot-blot analysis

DrmSP and HPLC purified fractions were transferred onto a nitrocellulose membrane dampened with TBS (50 mM Tris buffer, 150 mM NaCl, pH 7.6). Blocking was performed with 5% non-fat dry milk in TBS on an orbital shaker (60 rpm) at room temperature for 40 min. This was repeated twice with new blocking solution. The membrane was subsequently washed briefly with TBS containing 0.1% Tween-20 (TBS T-20) and incubated with DrmSP-antiserum at 1:500 dilution at 4 °C overnight. After washing with TBS T-20, three times for 30 min each secondary antibody (goat anti-rabbit IgG HRP, Pierce, Rockford, USA) was added at 1:4000 and incubated for 1.5 h at room temperature in an orbital shaker at 60 rpm. Subsequently, the membrane was washed with TBS T-20 four times for 20 min each. Chemiluminescence was detected after applying substrate (Supersignal West Dura, Pierce, Rockford, USA) using a CDC Imager (ChemiImager Alpha Innotech Co., San Leandro, CA, USA).

2.3.7. Bioassay for pheromonostasis

Sex pheromone production was determined in 2 day-old female moths using an in vivo bioassay. The females were decapitated during the photophase of day 1 and subsequently maintained for an additional 24 h, after which they were injected with either physiological saline (control) or saline containing 10 pmol/female *Hez*PBAN (Bachem, Bubendorf, Switzerland) and in the additional presence or absence of HPLC purified pheromonostatic factors. In some experiments the effect of pheromonostatic peptides on endogenous pheromone production was determined using 3 day-old virgin female moths injected with the test compounds during the 5th h of the scotophase. Ovipositor tips (containing pheromone glands) of either decapitated or intact females were removed 3 h after injection and extracted for 10 min in hexane, which contained 25 ng tridecanyl acetate (Sigma Chemical Co., St. Louis,
MO, USA) used as an internal standard. The hexane extract was concentrated to 2–3 μl final volume under a slow stream of nitrogen and injected to a 30 m SE-54 fused silica capillary column (internal diameter 0.25 mm) (Alltech, Deerfield, IL, USA) for separation using a gas chromatographic system (Shimadzu, Kyoto, Japan). A temperature gradient from 120 to 270 °C at 10 °C/min was performed, and thereafter the oven temperature was maintained at the final temperature for 15 min. The detector temperature was held at 280 °C and the column inlet at 300 °C. Helium was used as a carrier at a flow pressure of 22 psi. Z11-hexadecenal was quantified using the internal standard quantification method as described previously (Rafaeli and Soroker, 1989).

2.3.8. Statistics
Statistical analysis was performed using a Statview 4.01 package on a Macintosh computer using analysis of variance.

3. Results
3.1. Determination of sensitivity and specificity of the DrmSP antiserum
The sensitivity of the antiserum was tested using different concentrations of DrmSP. Specificity was determined by cross-reactivity studies using the peptides: HezPBAN, HezPSP35-57, vasopressin and insulin. Using DrmSP antiserum at 1:1000 dilution, DrmSP levels in the range of 10–20 fmol/μl could be detected (Fig. 1). Cross-reactivity studies revealed that DrmSP antiserum was specific only to DrmSP and not to the other peptides tested, even at their highest concentrations; in addition, no cross-reactivity was obtained using HezPSP35-57 (Fig. 1). The specificity of the antiserum facilitates its usage in the detection of DrmSP-like factors in the male accessory glands of H. armigera.

3.2. Structure–activity studies
Experiments using partial N-terminal and C-terminal sequences of DrmSP revealed that the antiserum is highly specific to the N-terminal. Of all the fragments tested, only DrmSP1-36 containing the N-terminal end of DrmSP was immunoreactive (Fig. 2). DrmSP8-36 was slightly immunoreactive whereas the truncated fragments DrmSP6-21 and DrmSP20-36 did not respond to the antiserum. Moreover, Dup99B, which is highly homologous with the C-terminal region of DrmSP was not immunoreactive (Fig. 2). This implies that the presence of N-terminal amino acids is essential for detection by the antiserum.

3.3. Quantification of DrmSP-like peptides in the male accessory glands of H. armigera
Using the direct ELISA to quantify DrmSP-like immunoreactivity from HPLC extracts of MAG, two positive immunoreactive peaks were found (Fig. 3) at elution time 19–21 min (peak SP-IRa) and 62–66 min (peak SP-IRb). The elution time of both immunoreactive peaks differed from synthetic DrmSP1-36 (Fig. 3) which eluted at 45–47 min. The immunoreactivity of peak SP-IRb proved to be of higher DrmSP-like concentration than peak SP-IRa. In addition, the response of SP-IRa was not consistent in the various HPLC separations. When several HPLC separations were pooled and tested for immunoreactivity SP-IRa showed a loss in immunoreactivity. SP-IRb amounted to approximately 2.6 pmole-equivalents of DrmSP-like immunoreactivity in the accessory gland extract of one male equivalent (Table 1). The present assay is sensitive to a minimum of 0.5 male equivalents for SP-IRb.

Recovery studies were performed to verify the accuracy of the direct ELISA for quantification of...
DrmSP-like immunoreactivity in HPLC fractions by spiking both immunoreactive and non-immunoreactive fractions with 5000 fmole (100 fmole/μl) DrmSP. The results show a mean recovery of 87.19 ± 7.83% (Table 1).

### Table 1
Recovery of spiked DrmSP (100 fmole/μl) in HPLC-eluted samples

<table>
<thead>
<tr>
<th>Sample tested</th>
<th>DrmSP immunoreactivity (fmole)</th>
<th>Recovery (fmole)</th>
<th>% Recovery</th>
</tr>
</thead>
</table>
| Spiked DrmSP 5000 fmole/well
Elution time (5 male equivalents/well) | DrmSP present | DrmSP absent | |
| 5 min | 5012 ± 95 | 5012 0 5012 | 100 |
| 15 min | 2625 ± 88 | 0 ± 0 | 2625 53 |
| 25 min | 2750 ± 177 | 0 ± 0 | 2750 55 |
| 35 min | 5125 ± 88 | 0 ± 0 | 5125 103 |
| 35 min | 5875 ± 256 | 0 ± 0 | 5875 118 |
| 45 min | 7000 ± 345 | 0 ± 0 | 7000 140 |
| 55 min | 3500 ± 177 | 0 ± 0 | 3500 70 |
| 75 min | 3050 ± 141 | 0 ± 0 | 3050 60 |
| Peak SP-IRa (male equivalents/well) (pooled 18–23 min)
1 | 4250 ± 71 | 0 ± 0 | 4250 85 |
| Peak SP-IRb (male equivalents/well) (pooled 61–70 min)
0.25 | 4125 ± 106 | 0 ± 0 | 4125 83 |
| 0.5 | 5300 ± 424 | 1000 ± 0 | 4300 86 |
| 1 | 7300 ± 495 | 2600 ± 71 | 4700 94 |
| % Recovery (Mean ± SEM) | | | 87.19 ± 7.83 |

### 3.4. Dot-blot analysis

A dot-blot analysis revealed positive immunoreactivity of DrmSP with DrmSP antiserum (Fig. 4). In addition, immunoreactivity of the HPLC fractions (SP-IRa and b) was observed and confirmed the results obtained using the direct ELISA.

### 3.5. Evaluation of DrmSP-like immunoreactivity in MAGs of different age groups

Using C18 Sep-Pak purified accessory gland extracts, males of various ages were tested for DrmSP-like immunoreactivity. It was observed that 1 and 2 day-old males contain significantly lower levels of immunoreactivity compared to 3–7 day-old males (Table 2).

### 3.6. Biological activity of peak SP-IRb

Although immunoreactivity in peak SP-IRa was detected in individual HPLC runs it was unstable after pooling of several runs. Therefore the pooled peak was not tested for pheromonostatic activity. Peak SP-IRb at 5 male equivalents/female was active on decapitated females injected with PBAN (10 pmol/female) and reached a maximum of 60% inhibition of pheromone biosynthesis (Fig. 5). When tested at the latter concentration, it also showed 58% pheromonostatic activity on females at their peak pheromone production (during the 8th h of the scotophase) (Fig. 5 inset).

### 4. Discussion

Our study reveals the presence of DrmSP-like immunoreactivity in HPLC purified MAG extracts...
of *H. armigera*. Using the Drm specific antiserum we were able to quantify the Drm-like content in *H. armigera* MAG extracts. The sensitivity of the antiserum was tested using ELISA and immunoblot and its specificity was tested through its cross-reactivity with other non-target peptides. Results show that the antiserum used in our study is highly specific to Drm, detecting 10–20 fmole/l. Of the two immunoreactive peaks observed SP-IRA and b, SP-IRb was consistent and stable with a Drm-like concentration of 2.6 pmole/male equivalent of *H. armigera*. Moreover, the absence of immunoreactivity throughout the HPLC elution profile, other than SP-IRb, emphasizes the specificity and accuracy of the ELISA. A difference in the HPLC elution times of DrmSP and the positive immunoreactive peaks of *H. armigera* MAG extracts suggest, that these peptides may differ in their degree of hydrophobicity.

A study of the DrmSP-like content in MAG from various age groups of male moths showed an increase in DrmSP-like immunoreactivity in the MAG up to the third day after emergence, synchronizing with their active reproductive age (Rafaeli & Nagalakshmi, unpublished observations of male mating behaviour during the scotophase). Levels of DrmSP-like immunoreactivity in older males remained constant.

Studies using partial DrmSP sequences and Dup99B, which shares C-terminal homology with DrmSP, showed that this antiserum is highly N-terminal specific since none of the C-terminal peptides were immunoreactive. This aspect has an added advantage in elucidating the structure–function relationship of the DrmSP-like immunoreactive fraction from MAG extracts of *H. armigera*. We predict that the *H. armigera* DrmSP-like peptide (peak SP-IRb) will resemble DrmSP at the N-terminal to some extent, due to the antiserum specificity, as well as at the C-terminal, due to its pheromonostatic activity resembling that of DrmSP. Peptide sequence analysis of this DrmSP-like fraction in progress, will clarify this point.

In our study, when PBAN-treated decapitated females and females at their peak pheromone production period were challenged with 5 male equivalents of the HPLC purified DrmSP-like fraction, a maximum pheromonostatic effect of ~60% was obtained. Synthetic DrmSP at similar concentrations also caused
maximum inhibitory levels of 50–60% in vivo (Fan et al., 2000).

Mated females show a strong pheromonostatic effect reaching over 80% (data not shown). It is contemplated that the partial suppression obtained with DrmSP and SP-IRb might be because the primary role of N-terminal specific DrmSP is related to other post-mating activities. Indeed, the major effect of N-terminal specific DrmSP on JH synthesis was shown in H. armigera (Fan et al., 2000) and D. melanogaster (Moschitzky et al., 1996). The role of the DrmSP C-terminal sequence in the post-mating responses, namely non-receptivity and oviposition, was first demonstrated in D. melanogaster (Aigaki et al., 1991; Schmidt et al., 1993). Other post-mating responses reported for HPLC extracts of H. armigera MAG include egg maturation and oviposition (Jin and Gong, 2001). In H. armigera female moths, a HecPSP partial sequence was also shown to be pheromonostatic (Eliyahu et al., 2003).

Thus, several factors seem to be involved in coordinating post-mating responses and termination of receptivity. It is possible that efficient pheromone suppression after the mating process might be due to the collective action of endogenous DrmSP-like and HecPSP-like factors transferred by males to females during mating, in addition to their role on other post-mating effects. Hence, in the present system, since the antiserum is highly N-terminal specific, we may be delineating a DrmSP-like immunoreactive fraction in H. armigera whose primary role may be to stimulate JH production and/or oviposition. Our future studies on the influence of purified DrmSP-like fraction on the behaviour and physiology of female H. armigera will clarify the possible mode of action of this peptide in eliciting post-mating responses.

Acknowledgements

This work was supported in part by an Israel National Academy of Science and Humanities Grant (No. 539/02) to AR and SWA, and a Swiss National Foundation Grant No. 31-52, 440.97 to EK. This is contribution No. 412/03 from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. We would like to thank the anonymous reviewers for constructive comments.

References
