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Research Article

AN EVIDENCE OF A SECRETORY PROTEIN TRANSFERRED TO FEMALE DURING MATING FROM THE MALE ACCESSORY GLANDS OF *SPODOPTERA LITURA* (F.)

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ABSTRACT

Comparison of the Tricine SDS-PAGE gels of male accessory gland secretions of *Spodoptera litura* virgin and mated males showed the occurrence of a protein <14KDa in virgin males from the time of eclosion while the level reached its peak at 24 hrs, remaining constant up to 48 hrs. The concentration of this protein drastically reduced just before the end of mating indicating the transfer of the protein to females during mating. During next 24 hrs following mating the concentration was recovered probably an indication that the male is ready for second round of mating. This study, a first of its kind in *Spodoptera litura*, demonstrating the possible transfer of protein from male to female, suggests that the protein identified from male accessory gland secretions may have an important influence on behavior, survival and reproduction in female moths.

INTRODUCTION

A well-known strategy evolved in most male insects is to use the accessory gland secretions to exert wide-ranging effects on female post-mating reproductive behavior. Male Accessory Gland (MAG) secretions are transferred to females, along with the sperms (Gillott, 2003) and these secretions play a significant role in the sperm maintenance, release and storage, stimulation of ovarian development, formation of mating plug, triggers oviposition and inhibits receptivity (Avila *et al*., 2011). The secretory products of MAG can be proteins, carbohydrates and lipids. It may contain a variety of biologically active components such as prostaglandins, juvenile hormone and sometimes toxic compounds that have been suggested to serve as egg protectants (Gillott, 2003).

During the last three decades, studies on various insects have demonstrated the occurrence of a number of peptides/proteins, which regulate reproduction, in the MAG secretions (Holman *et al*., 1990). These peptides/proteins are synthesized, activated and/or released at appropriate periods. One of the well characterized peptide which is 36 amino acids (SP; ACP70A) has been found to increase oviposition and inhibit receptivity in *Drosophila melanogaster* (Chen *et al*., 1988; Wolfner, 1997). Yi and Gillott, (1999) attributed the oviposition stimulating activity to a protein with molecular weight of 30 kDa in *Melanoplus sanguinipes*. A 13 kDa protein with similar activity was reported in *Locusta migratoria* (Lange and Loughton, 1985) and 60 kDa protein α-matrone in *Aedes aegypti* (Hiss and Fuchs, 1972). A preliminary work by Sridevi *et al*., 1987, reported that the MAG extracts of *Spodoptera litura* have an oviposition stimulating factor which induced oviposition in virgin female moths. Later, Divakara *et al* (2013) and Jin *et al* (2014) also demonstrated that MAG secretions modulate female post-mating behavior in *Spodoptera litura*.

Very few researchers have attempted to profile the MAG proteins in insects by electrophoresis. South *et al*., (2011) identified 14 distinct proteins through mass spectrometry present in the male accessory glands that get transferred to mated female, but absent in the virgin female reproductive tracts of *Tribolium castaneum*. In *Aedes aegypti*, Laura *et al*., (2009) reported 21 proteins in the reproductive tract of the mated females and suggested that they are transferred from males to females during mating using 15% SDS PAGE, but they could visualize only few proteins on the gel. Izadi and Subrahmanyam, (2005) analyzed MAG and their secretions in *Spodoptera litura* by electrophoresis and reported the presence of 23 proteins in the MAG and 14 in the secretions, but their study was limited to virgin males. As there are only couple of

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studies on *Spodoptera litura* about the MAG proteins transferred from male moths to female during mating the present investigation was undertaken to track these protein(s) by Tricine SDS-PAGE. This study will be of significance in the wake of emerging strategy of employing behaviour modifying proteins such as accessory gland proteins to contain the pest population such as tobacco caterpillar *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae), a polyphagous devastating pest of numerous wild and cultivated plants throughout the world. It has been reported to attack more than 120 species of agricultural crops distributed in 44 families worldwide (Qin *et al*., 2004) and over 60 cultivated plant species have been reported from India (Garad *et al.*, 1984).

MATERIALS AND METHODS

Insect Rearing

The parental stock of *Spodoptera litura* larvae (National Accession No. NBAII-MP-NOC-02) was obtained from National Bureau of Agricultural Insect Resources, (NBAIR, erstwhile PDBC, ICAR) Bellary Road, Bengaluru. The insects were reared on an artificial diet (Divakara *et al*., 2011) under controlled conditions in the laboratory at 25± 2ºC with 45±5% relative humidity and 12:12 (L:D) periods. The pupae collected were sexed and maintained separately according to their peripheral characters. Cotton dipped in 10% honey solution was provided as food to the adult moths soon after emergence.

Collection of tissue

The (MAG) tissues from virgin males were collected 24hrs after emergence and the MAG tissues from mated males were collected immediately after mating, since these insects exhibit mating behaviour on subsequent night of emergence. Both the virgin and mated males were dissected in ice-cold saline (Matsumoto *et al*., 2003) and were served as controls.

Further, to track the proteins, following experiments were designed to collect the MAG tissues.

1. MAG from virgin males: The MAG tissue was collected from virgin males from the time of emergence i.e., 0 hrs to 48hrs at an interval of 6hrs.
2. MAG from mated males: Male moths were provided in the ratio of 1:2 to females and were allowed to mate. The MAG from mated males was collected at every 6hrs up to 48 hrs.
3. MAG from males with interrupted mating: As mating duration lasts for a period of 40 minutes the tissues were collected by interrupting mating at 10, 20, 30 and 40 minutes (males separated by itself). The mating insects were frozen immediately after interruption to arrest the changes in protein profile and were soon dissected to collect the tissue.

The tissue was collected by sacrificing five insects at different intervals of time in every experiment.

Sample preparation and electrophoresis

The tissue was homogenized using a micro pestle with 100µl ice cold distilled water and centrifuged at 15,000rpm for 15 mins at 4ºC. 10µl of supernatant for each experiment as described above and 5µl of low range protein marker (SRL, India) was used for each gel, to which sample loading buffer was added. The samples were then heated at 70ºC for 5mins and were separated on 16% Tricine SDS-PAGE as described by Schagger H., (2006) at 100V for 7 hours, followed by fixation for an hour. The gel was then stained with Coomassie Brilliant Blue R250 for visualization of proteins and was documented using a gel documentation system (Syngene).

RESULTS

The work was initiated to locate the protein(s) transferred during mating from MAG to female by comparing the PAGE profile between virgin and mated males of *Spodoptera litura*. The presence of a protein <14 KDa in virgin males but absence of the same protein in mated males was observed suggesting the possibility of it being secretory and transferred to female during mating (Fig. 1). Further experiments were conducted to find the time at which the protein was released/synthesized in virgin males, the time of transfer of the protein during mating and resynthesis of protein after mating. The results revealed the synthesis of this protein in virgin males between 0-48hrs, with a gradual increase in the concentration between 0-24 hrs and remained almost constant thereafter till 48hrs (Fig. 2). Mating interruption experiment revealed the transfer of protein just before the end of mating (Fig. 3). However, the protein depleted after mating reappeared at 12hrs with a gradual increase in the concentration of protein reaching maximum at 48th hour. This suggests the resynthesis of protein and readiness of male for second mating (Fig. 4).

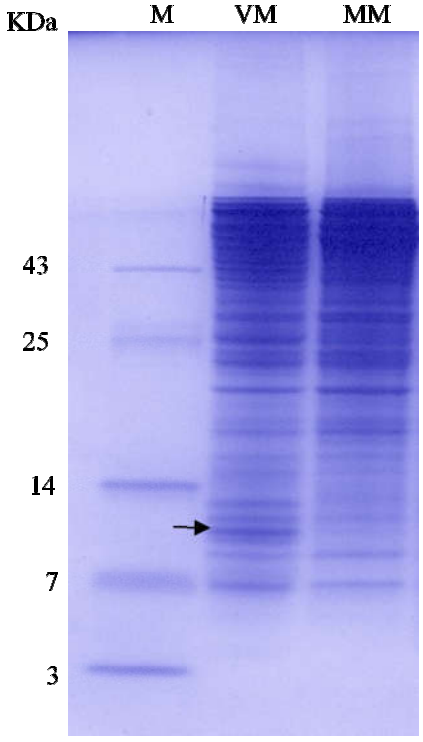

Figure 1 Protein profile comparison of Virgin and Mated Male Accessory Gland extracts. M- Protein marker, VM- Virgin male, MM- Mated male. Arrow indicates the presence of a band <14 KDa in virgin males and absence of the same in mated males.

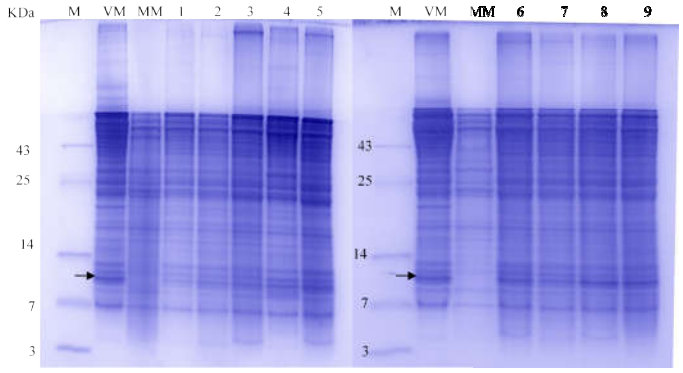

Figure 2 Determination of time of release/synthesis of protein. M- Protein marker,

VM- Virgin male, MM- Mated male, Lanes 1-9 represents the protein profile of virgin MAG extracts from the time of emergence at an interval of 6 hrs i.e. 0, 6, 12, 18, 24, 30, 36, 42 and 48hrs. The arrow indicates the presence of the protein in all the lanes with a gradual increase in the concentration between 0-24hrs and almost constant thereafter till 48hrs.

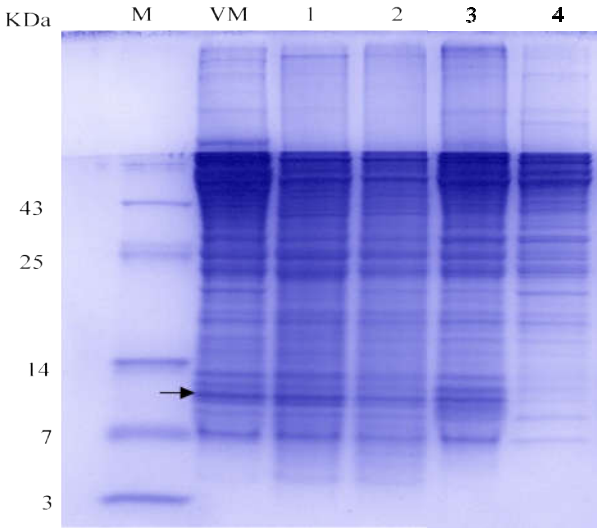

Figure 3 Determination of the time of transfer of protein during mating. M- Protein marker, VM- Virgin male, Lanes 1- 4 represents accessory gland protein profile of males mated for different durations of 10, 20, 30 and 40 minutes. The arrow shows the presence of protein in lane1-3 and absence in lane-4.

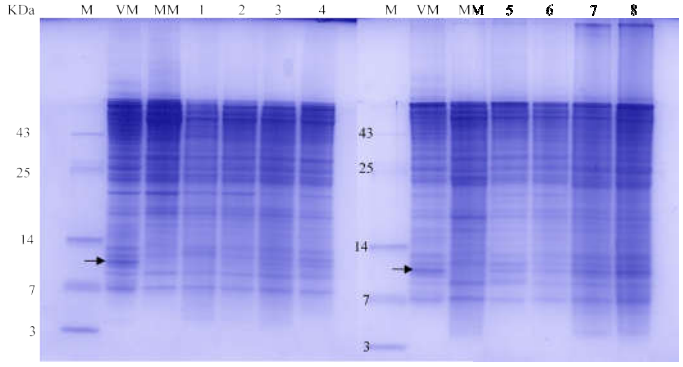

Figure 4 Determination of resynthesis/recovery of protein after mating. M- Protein marker, VM- Virgin male, MM- Mated male, Lanes 1-8 represents protein profile of accessory glands from mated moths, at an interval of 6hrs after mating i.e. 6, 12, 18, 24, 30, 36, 42 and 48 hrs. The arrow shows the reappearance of protein in lane-2 onwards with a gradual increase in the concentration of protein reaching maximum at 48th hour (lane-8).

DISCUSSION

The accessory gland proteins otherwise known as seminal fluid proteins (SFPs) produced in the reproductive tissue of male insects and transferred to females during mating induce numerous physiological and behavioural post-mating changes in females. These changes include decreasing receptivity to remating, affecting sperm storage parameters, increasing egg production, modulating sperm competition, feeding behaviours, and mating plug formation.

In the present study, we used Tricine SDS-PAGE to analyze the protein(s) transferred from male accessory glands during mating and we have found a protein of <14KDa to be present in virgin males but absent in mated moths, suggesting the possibility of it being a secretory protein transferred to females during mating and may be responsible for the post-mated behavioural changes in females. Several experiments were conducted to study the protein and the results revealed the appearance of the protein in virgin males which gradually increased reaching its maximum concentration at 24hrs after eclosion and thereby the concentration remained constant, suggesting the correlation of protein concentration reaching a peak at 24hrs to the males' readiness to mate, where earlier studies on this insect also reveal that males mate, the first time, on the subsequent night after emergence (Li *et al*., 2014).

The accessory gland protein profiles from males interrupted during mating showed the transfer of protein just before the end of mating where the mating duration in these insects was ~40 minutes (Li *et al* 2012) and we imply that the protein may be transferred to females along with the sperms during mating as earlier studies on *Drosophila melanogaster* have also suggested that MAG proteins are bound to the sperm tails and are transported to the female spermatheca during mating, where they are released gradually thereby maintaining the post-mating response in females (Peng *et al*., 2005).

The amount of protein in the male accessory glands is significantly diminished after mating and was recovered by 24hrs after mating signifying the resynthesis of protein and the chances of the male to remate. Resynthesis of this protein appears to be crucial in males which exhibit multiple mating as the case in *Spodoptera litura.* This type of regulation was also observed in *Drosophila melanogaster* wherein accumulation of the secreted protein Acp76A was seen 1 day after eclosion and upon mating the amount of protein dropped considerably and was recovered 24hrs after mating (Coleman *et al*., 1994). This accessory gland protein in *Drosophila melanogaster* was identified to be a member of the serpin superfamily of proteins (serine protease inhibitors) and was reported to be transferred to females during mating Using proteomics, transcript sequencing and bioinformatics tools, researchers have been able to identify a number of secretory proteins transferred to females during mating in several other insects. The proteins identified by these methods include the protease inhibitors, odorant binding proteins, chaperons, CRISPS (cysteine-rich secreted proteins), defensins, lectins, lipases, oxidoreductases and so on. These classes of proteins are relatively conserved across a wide range of insects (Walker *et al*, 2006; Baldini *et al*, 2012; Sirot *et al*, 2008; Scolari *et al*, 2012; Rafaeli and Hanin, 2013; Simmons *et al*, 2013; Baer *et al*, 2009) and hence in this study we suggest the probability of the protein to be

conserved falling into the same protein classes or may be a protein functionally unknown. Therefore, identification and exploration of the protein would provide new insight on its effect on the female reproductive behaviour. Efforts are on to sequence the protein and to study its fate in the mated female.

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