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Identification of Accessory Gland Proteins from *Helicoverpa armigera*: a Comparative Study Between Virgin and Mated Male Moths

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A B S T R A C T

The accessory gland proteins (MAG) secreted in male moths are known to influence female reproductive behavior and physiology, when they are transferred along with the sperm during mating. Through, two-dimensional gel electrophoresis (2-DE) we have identified these MAG proteins in *Helicoverpa armigera*, a devastating polyphagous pest. The comparative analysis of virgin and mated male moths revealed a total of 139 and 154 protein spots respectively. The differentially expressed proteins following mating were, 25 over-expressed and 13 under-expressed. Further, 11 highly expressed protein spots unique to virgin males were analyzed using MALDI- MS and mascot search. The proteins detected were found to be involved in different functions such as, metabolism, spermatogenesis, oogenesis, olfaction, and protease inhibition.

Key words: Cotton bollworm, MAG proteins, 2DE, MALDI-MS, Peptide mass fingerprinting

The old-world bollworm *Helicoverpa armigera* also called cotton bollworm is one of the most dreadful polyphagous pests worldwide (Jones *et al*. 2019). The larvae of this pest have been reported to cause damage to vegetative and reproductive plant stages by feeding on major parts of the plants such as leaves, stems, and most preferably the buds, inflorescence, fruits and pods (Reed 1965). They feed on more than 180 crops, which include commercially important crops such as cotton, soybean, tomato, chickpea, sorghum, groundnut, sunflower, okra and maize (Ravi *et al*. 2005, Pomari *et al*. 2015). It is estimated that, about 5 billion US dollars spent annually across the globe to control this pest. In Brazil, alone 0.8 billion US dollars have been spent during 2012-2013 (de Freitas Bueno and Sosa Gomez,2014). In India the insecticide used against the pest annually was worth 28000 billion rupees on most of the crops and 50% was used on cotton alone (Honnakerappa and Udikeri 2018).

As there is an increased usage of chemical insecticide the pest is gaining resistance to most of these chemicals like pyrethroids, organophosphates, carbamates, oxadiazines, macrocyclic lactone spinosad, avermectins and paraffinic spray oils (Honnakerappa and Udikeri 2018). The alternative way of controlling the pest other than the use of chemical insecticide is a major research challenge. Hence, the use of biomolecules such as behavior-modifying proteins secreted from male accessory glands (MAG) of the pest moth can be a futuristic approach for pest control as MAG secretions especially the proteins are known to play a key role in the reproduction of insects. They induce several behavioral changes in females, when they are transported along with sperms to her system, such as termination of pheromone biosynthesis, declined receptivity, stimulation and acceleration of the oviposition, increased rate of egg development and also providing immunity (Gillott 2003, Avila 2011). Use of MAG proteins to alter the behavior of females so as to disable her to lay fertilized eggs either by inducing mating inhibition or early egg laying, is a new concept in pest control. For this approach the protein profiling of MAG is very important.

The 2-dimensional gel electrophoresis (2-DE) along with Mass spectrometry (MS) is the most effective and

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widely used technique in proteomics for analyzing the proteins in a complex mixture from a biological sample. This approach finds its application in the study of differentially expressed proteins between different groups for comparative proteomic analysis (Kumar *et al*. 2017). In several insects with 2-DE approach MAG proteins were studied, for instance, in *Callosobruchus maculatus* 98 proteins were identified, which were possibly transferred to the female during mating (Bayram *et al*. 2017). Twenty-one seminal fluid proteins were identified in *Teleogryllus oceanicus* and some of these proteins were found to be involved in post-mating behavioral changes in females as per previous reports in other species (Simmons *et al*. 2013). From the male reproductive organs (testis, seminal vesicle, accessory gland, ejaculatory duct and ejaculatory bulb) >400 proteins were detected in *Drosophila melanogaster*, and most of these proteins were associated with odorant/pheromone-binding, lipid metabolism, proteolysis and antioxidation and were expressed tissue specifically in the male reproductive system (Nobuaki and Yamamoto 2009). In *Cimex lectularius* 76 proteins were detected from seminal fluid among which 32 were found in the mated female but not in virgin females suggesting that these proteins might have been transferred from male to female during mating (Reinhardt *et al*. 2009). A total of 566 proteins were detected in the MAG secretion of *Spodoptera litura*, among these, 91 proteins were found to be differentially expressed in virgin and mated males (Mamtha *et al*. 2019). The seminal fluid proteins, in insects, belongs to a wide range of functional groups that are involved in metabolism, gametogenesis, odorant binding, nucleotide binding and other cellular processes (Wolfner 2002, Gillott 2003, Avila 2011).

The identification of male accessory gland proteins in *Helicoverpa armigera* will lead us to understand the functional aspects and their role played in influencing female reproductive behavior as well as the physiology in males. Hence the present work was conceptualized to identify accessory gland proteins in pre and post-mated conditions of the male moths through the 2-DE approach.

MATERIALS AND METHODS

Insect collection

Helicoverpa armigera culture (Accession no: NBAII-MP-NOC-01) were procured from the National Bureau of Agricultural Insect Resources (NBAIR), Bangalore. The Larvae were fed with artificial diet prepared as described by Shobha *et al*. (2009) with slight modification. After pupation, which happens after the 6th instar of the larval stage, the pupae were collected, sexed and kept separately until the emergence of moth.

Tissue collection and sample preparation

The two days old virgin male and Mated male were dissected in ice-cold Lepidopteran saline (Matsumoto *et al*. 2003) to collect Male accessory gland tissue. The MAG tissue was homogenized with 20µl Mili-Q water in cold condition, centrifuged at 12000 rpm at 4°C and the supernatant collected was stored at -40°C until further use.

Two-dimensional gel electrophoresis

The total protein content in the sample was estimated and accordingly, the MAG extract containing 300µg of protein was diluted in rehydration buffer (8M urea, 4% CHAPS, 40mM Dithiothreitol (DTT), 2% Bio-Lyte, pH 3-10; Bio-Rad) and was incubated at 25°C-28°C for 12 hrs. After incubation the samples were centrifuged at 10,000 rpm, for 15min. the supernatant was collected and loaded onto an 18cm Linear IPG (Immobilized pH gradient) strips of pH 3-10 and was kept in the Ettan IPGphor3 system (GE Healthcare) for Iso-Electric Focusing (IEF). The IEF was performed initially for 15min at 250V and then for 3 hours, 10000V was provided. On completion of IEF, the IPG strip was removed and equilibrated twice, initially with 1% DTT along with equilibration buffer (6M urea, 75mM Tris-HCL pH-8.8, 2% SDS, 29.3% Glycerol, 0.002% bromophenol blue) for 1hr and then with 2.5% iodoacetamide (IDA) along with equilibration buffer for 45mins. Subsequently, the second dimension was carried out for protein separation by using only resolving gel of 10% SDS PAGE (Laemmli 1970) which was stacked with IPG strip carefully and one lane was loaded with a standard protein marker of 10 - 250kDa (Bio-Rad). The electrophoresis was carried out overnight at 70V (Ettan Daltsix Gel Electrophoresis unit; GE Healthcare).

Silver staining

The 2D gel after electrophoresis was incubated overnight in fixing solution (40% methanol and 10% acetic acid) in a gel rocker at room temperature. The gel was washed thrice with distilled water for 20mins each. Further sensitization of the gel was done by incubating with 0.02% sodium thiosulphate for 1min and then washing step was performed, where the gel was washed three times with distilled water for 1min each and in the next step the gel was incubated with 0.2% silver nitrate solution for one hour in a dark chamber. Then the gel was again washed with distilled water thrice for 30s each. In the final step the developer solution (2% sodium carbonate and 35% formaldehyde in water) was added to the gel and by gentle rocking the gel was observed for the development until protein bands were visualized. Then immediately the stopper solution of 5% acetic acid was added to the gel to stop the reaction from further development (Dunn 1987, Shevchenko *et al*. 1996).

Image analysis

The stained gel was further documented using Epson Expression 11000XL Scanner (GE Healthcare). The gel image was analyzed using Image-Master 2D Platinum 7 software. Wherein, the software automatically detects and assigns the spot Id's and matches Id's to all the protein spots present in the gel. Hence, it provides the details about differentially expressed proteins in virgin MAG and mated MAG. Based on that, the protein spots present in one gel and not in the other were considered as unique spots. With detailed examination, some of the prominently expressed unique spots from virgin MAG gel were selected and were considered for peptide mass fingerprinting (PMF) using MALDI- TOF/TOF MS.

In-gel trypsin digestion

The stained gel spots were excised and processed individually. The gel pieces were minced into small pieces and transferred to sterile tubes. The gel pieces were then destained using a destaining solution (1:1 ratio of 15mM K3 [Fe (CN)6] and 50mM Na2S2O3) for 10 minutes intervals (3 - 4 times) until the gel pieces become translucent white. Once the gel pieces were destained completely then the gel was dehydrated using 100µl of 1:1 ratio 25mM ammonium bicarbonate and 100% acetonitrile, and the solution was kept in a thermomixer until complete dryness where the gel will be significantly smaller in size. The gel pieces were further rehydrated with 100µl of 10mM DTT for 1 hour at 56°C, after incubation DTT solution was removed using a pipette, subsequently the alkylation step was performed by adding 55mM IDA and incubated in dark at room temperature for 45min. The IDA solution was removed and the gel was incubated with 100µl of 25mM ammonium bicarbonate solution at room temperature for 10min. After incubation the solution was removed and the gel pieces were dehydrated with 100µl of 100% acetonitrile for 10 minutes at room temperature in a thermomixer until complete dryness. To the dried gel 10µl of Trypsin solution was added and incubated for 10min at 4°C. After incubation, 150µl of 25mM ammonium bicarbonate was added and kept for incubation overnight on thermomixer at 37°C. The digested solution was then transferred to a fresh sterile tube (eppendorff). The gel pieces were extracted thrice with extraction buffer (1:1 ratio 0.1% trifluoroacetic acid in MS Grade water and 100% acetonitrile) and the supernatant was collected each time into the same tube used earlier which contained the digested solution and the resultant solution was concentrated using speed vac.

MALDI – TOF/TOF MS

The Trypsin digested dried peptide mix was suspended in TA buffer (30:70 acetonitrile: 0.1% TFA). The peptides obtained were solubilized with HCCA (α-Cyano-4-hydroxycinnamic acid) matrix in 1:1 ratio and from this 2µl was spotted onto the MALDI plate (MTP 384 ground steel; Bruker Daltonics, Germany) and air dried. After air drying the sample was analyzed using Ultraflex III MALDI TOF/TOF-MS instrument. External calibration was done with standard peptide (PEPMIX Mixture) supplied by Bruker, with masses ranging from 1046 to 3147 Da. Further analysis was done by using FLEX ANALYSIS SOFTWARE (Version 3.3; Bruker Daltonics, Germany) in positive reflectron ion mode with an average of 500 laser shots at mass detection range between 500 to 5000 m/z with 50-400 ppm mass tolerance for obtaining the MS/MS spectra. The masses obtained were submitted for the Mascot search engine (Matrix Science) for similarity search against Lepidoptera taxonomy (675 sequences) with Swiss Prot database (SwissProt 2018_02 (556825 sequences; 199652254 residues). The search was performed for peptide mass fingerprinting using following search parameters, where, up to 2 missed cleavage was allowed with trypsin as cleavage enzyme, the peptide tolerance, and fragment tolerance was set to ±1.2Da and 0.6Da respectively, fixed modification of cysteine carbamidomethylation and variable modification of methionine oxidation were considered. The maximum protein hits were set to 20 and peptide charge as 1. The MOWSE scores > 41 were allowed and a significant threshold point of <0.05 was considered. The Proteins identified were further checked for the presence of signal peptide using SignalP 4.1 (http://cbs.dtu.dk/services/SignalP).

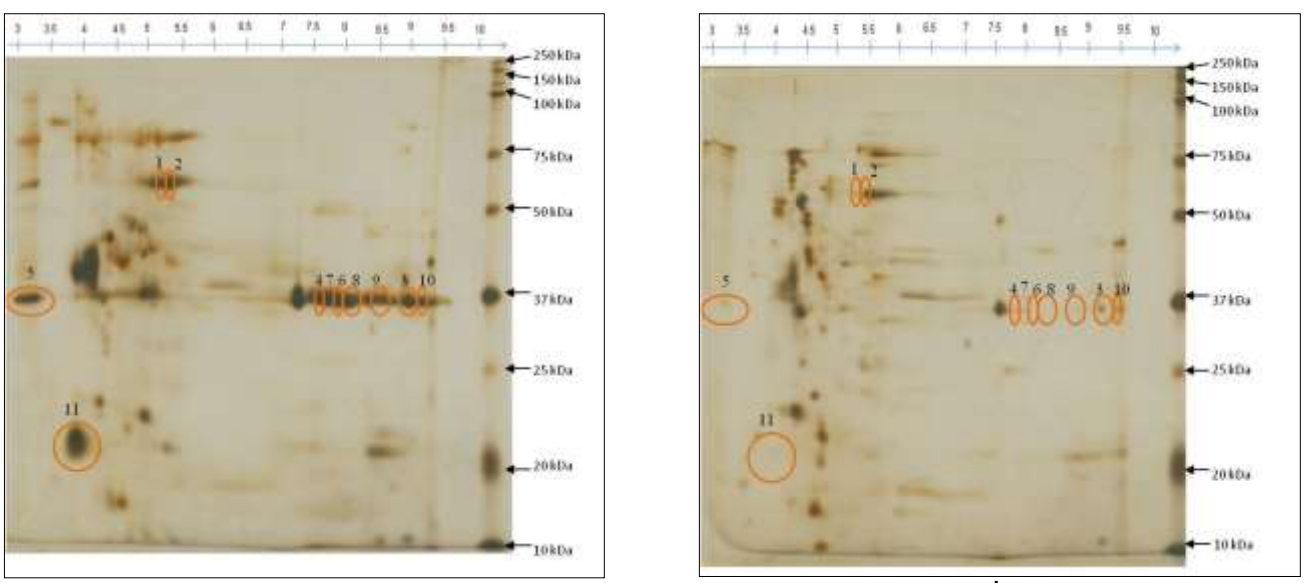

Fig 1 Differentially expressed proteins from silver stained 10% 2-DE gel of MAG from *H. armigera* (a) virgin moth (b) mated moth

RESULTS AND DISCUSSION

The 2DE gel image revealed that the protein pattern of virgin and mated MAG samples (Fig 1a-1b) was differentially expressed between two conditions. This revealed that 25 proteins were overexpressed and 13 proteins were under expressed in mated moth compared to the virgin, this was observed among the 60 proteins spots

common in both virgin and mated. However, the total number of protein spots detected in virgin and mated males was 139 and 154 respectively, within the molecular mass ranging from 10kDa - 250kDa. Among which, 79 protein were unique to virgin moth and 94 protein were unique to mated moth. The protein number in the mated moth is higher compared to virgins. Eleven protein spots highly expressed only in virgin but not in mated were selected for PMF analysis these protein spots ranged between 20kDa - 73kDa.

The MS/MS spectra of 11 spots obtained from MALDI-TOF were searched in the mascot search engine against the order Lepidoptera, based on the sequence similarities the proteins were identified. These proteins were further investigated for the presence of signal peptide and among 11 proteins 7 proteins confirmed the presence of signal peptide (Table 1). Usually, the presence of signal peptides is an indication that the protein is secretory in nature, and based on requirement these proteins are synthesized. The presence of these proteins before mating and depletion of the same after mating indicates the importance of these proteins in reproduction and are presumed to be transferred to the female. These proteins were predicted (based on literature) to be involved in different functions such as, metabolism, spermatogenesis, oogenesis, olfaction, and protease inhibition based on earlier reports in other insects (Table 2).

Table 1 List of MAG proteins identified in *H. armigera* through 2-DE and mass spectroscopy

Protein spot ID	Spot intensity (% vol)	Mol Wt	pI	Accession No.	Protein name	Organism name	Signal Peptide
2274	1.37439	73	5.14711	BXB12_BOMMO	Bombyxin B-12	*Bombyx mori*	Yes
2262	7.36448	20	3.7	BXRA_AGRCO	Bombyxin-related peptide A	*Agrius convolvuli*	Yes
2273	1.99985	72	5.33482	PBAN_BOMMO	PBAN-type neuropeptides	*Bombyx mori*	Yes
2217	0.917063	38	9.31564	OBP1_BOMMO	General odorant-binding protein 1	*Bombyx mori*	Yes
2269	4.51006	38	7.92374	PBAN_BOMMO	PBAN-type neuropeptides	*Bombyx mori*	Yes
2299	3.94273	37	3.13754	A1AT_BOMMO	Antitrypsin	*Bombyx mori*	Yes
2271	2.30944	37	8.55028	ARYB_MANSE	Arylphorin subunit beta	*Manduca sexta*	Yes
2268	2.57157	37	8.38827	EIF3H_BOMMO	Eukaryotic translation initiation factor 3 subunit H	*Bombyx mori*	No
2270	4.26854	37	8.18994	JHAMT_BOMMO	Juvenile hormone acid O-methyltransferase	*Bombyx mori*	No
2225	1.27427	37	9.13128	VATF_MANSE	V-type proton ATPase subunit F	*Manduca sexta*	No
2222	1.56607	37	9.60614	DHSO_BOMMO	Sorbitol dehydrogenase	*Bombyx mori*	No

In this study, two proteins Bombyxin B12 and Bombyxin related peptide A were detected in MAG tissue of *H. armigera*. These proteins belong to the insulin family (Kondo *et al*. 1996). Bombyxin was the first insulin like peptide isolated from insects (Ishizaki and Suzuki 1994, Mizoguchi and Okamoto 2013). Bombyxin was reported to be expressed predominantly in the brain of *Bombyx mori*. However, through RT-PCR Iwami *et al*. (1996) showed the bombyxin mRNA expressed at a lower level in tissues like epidermis, ganglia, testis, ovary, silk gland, fat body, malpighian tubule, midgut, and hindgut in fifth instar larvae of *Bombyx mori*. Bombyxin plays an important role in the stimulation of ROS mediated signaling in prothoracic glands which activates the synthesis of ecdysone (Gu and Chen 2020), responsible for molting and development. In brain removed pupae of Samia cynthia a 5 kDa peptide, currently referred to as bombyxin, is responsible for the adult development (Ishizaki and Suzuki 1994). Bombyxin is also speculated to play an important role in ovarian development (Orikasa *et al*. 1993, Fujinaga *et al*. 2019) and in the regulation of carbohydrate metabolism (Satake *et al*. 1997, Kawabe *et al*. 2019). Yet the function of bombyxin is still obscure for a tissue specific expression in male insects. Till date bombyxin has not been detected in MAG tissue of insects, however, our study identified the presence of these proteins in MAG tissue. The absence of these proteins in mated male moth indicates that these proteins might be transferred to females and also can be speculated to play an important role in the development of ovary in females.

Another protein, which belongs to a class of olfactory proteins, was detected and identified as general odorant binding protein 1 (GOBP1). Odorant binding proteins (OBPs) are a group of small, water soluble, low molecular weight proteins, present in both vertebrates and invertebrates. OBPs are reported to be present in the aqueous fluid, near the sensory dendrites; they serve as carrier molecules to transport odorants to the odorant receptors (Vogt *et al*. 1991). In vertebrates they belong to a superfamily of carrier proteins called lipocalins (Pelosi 1994). In invertebrates, especially insects, OBPs are divided into 3 subfamilies such as pheromone binding proteins (PBPs), general odorant binding proteins (GOBPs) and antennal binding protein (ABPs) (Gyorgyi *et al*. 1988, Zhou 2010). The expression of GOBPs was initially detected in antennae of both male and female lepidopteran insects, moreover, they showed sequence similarity to PBPs. GOBPs are further divided into subfamilies GOBP1 and GOBP2 (Vogt *et al*. 1991). The predominant expression of GOBPs

in antennae suggests playing a role in the detection of host volatiles (Vogt *et al*. 1999, Zhou 2010). A recent study showed that antennal GOBPs from *Agrotis ipsilon* (AipsGOBP1 and AipsGOBP2) not only bind to host volatiles but, also binds to sex pheromones (Huang *et al*. 2018) and can influence reproduction. Sun *et al*. (2012) cloned and expressed one strain of OBPs i.e. HarmOBP10 in the male reproductive tissues of *H. armigera*, and also confirmed the transfer of this protein to females as it was detected on the surface of fertilized eggs. After this observation HarmOBP10 was suggested to influence the physiology of female system. Previously, HarmGOBP1 and HarmGOBP2 were confirmed to be expressed only in antennae of *H. armigera* (Wang *et al*. 2001, Zhang *et al*. 2011) and were not detected in reproductive tissue. In this study we have detected GOBP1 from MAG tissue of *H. armigera*. Based on Sun *et al*. (2012) observation we can confirm that OBPs contribute in the reproduction process. Furthermore, the studies on MAG secretions in other insects, such as in *Tribolium castaneum* (South *et al*. 2011), *Drosophila melanogaster* (Takemori and Yamamoto 2009), *Bactrocera dorsalis* (Wei *et al*. 2015) and *Bombyx mori* (Dong *et al*. 2016) have shown the presence of GOBPS/OBPs. The OBPs, secreted and transferred to females during mating might regulate the reproductive physiology by possibly interacting with a receptor in the female's reproductive tract (Wei *et al*. 2015).

Pheromone biosynthesis activating neuropeptide (PBAN) is extensively studied in female moth of various lepidopteran species (Rafali *et al*. 2009). PBAN belongs to pyrokinin family and it is reported that the timely release of PBAN stimulates the biosynthesis process for pheromone production in females, by binding to the PBAN-receptor (PBAN-R) present in the pheromone gland (Tillman *et al*. 1999, Jurenka 2003, Rafaeli 2009). The initial isolation of PBAN was from *Helicoverpa zea*, named after its functional stimulation of pheromone biosynthesis in female adult moths (Raina *et al*. 1989). Furthermore, the PBAN-like peptides of pyrokinin family regulates different functions across the insecta other than pheromone biosynthesis such as, muscle contraction in the hindgut of *Leucophaea maderae* (Holman *et al*. 1986) melanization in Lepidoptera larvae (Matsumoto *et al*. 1990); embryonic diapause in *B. mori* (Imai *et al*. 1991); acceleration of puparium formation in flies (Zdarek *et al*. 1997, Verleyen *et al*. 2004); pupal diapause development in heliothine moths (Sun *et al*. 2003, Zhang *et al*. 2004) and ecdysone biosynthesis in prothoracic glands of *B. mori* (Watanabe *et al*. 2007). The presence of PBAN like peptides is less studied in males, wherein this study reports two PBAN like neuropeptide in MAGs of *H. armigera* which was not previously reported. The study on *Mamestra brassicae* reported the presence of PBAN like peptides in the brain of male moth (Jacquin *et al*. 1994). In the crustacean, *Paratya compressa* a study revealed that during rearing in the absence of males there was a delayed ovarian development in females whereas when the females were reared along with the male the ovarian development occurred normally suggesting that male shrimps secrete an ovary-stimulating pheromone which accelerates ovarian development (Takayanag *et al*. 1986). Thus, the presence of PBAN like peptide in male might possibly secrete pheromones that might help in ovary development and or other functions also. However, the function of PBAN like neuropeptide in male insects is still obscure, further exploration is required for understanding the male physiology.

Another protein named arylphorin subunit β from hexamerin group of proteins are storage proteins, belong to hemocyanin superfamily were identified in our study. Arylphorin, a storage protein with aromatic amino acids is found in all the stages of an insect's life cycle, especially synthesized in the fat body (Scheller *et al*. 1990, Chandrasekar *et al*. 2009). The synthesis of arylphorin is mediated by hormonal regulation of 20-hydroxyecdysone reported in *Corcyra cephalonica* (Manohar *et al*. 2010, VenkatRao *et al*. 2016). Arylphorins are involved in diverse functions during metamorphosis and reproduction (Telfer *et al*. 1983, Chandrasekar *et al*. 2009), among the other functions the hexamerins and arylphorins present in insect hemolymph, appear to play a role in immunity (Burmester 1999, Campbell *et al*. 2008). The hexmerins (HEX70a) is suggested to serve as an aminoacid source for the production of yolk proteins in honey bee (Martins *et al*. 2011). The expression of arylphorin in MAG secretions is reported in *Glossina morsitans* (Scolari *et al*. 2015), though the function of this protein expressed in MAG is not clear. The presence of arylphorin in MAG secretions and the transfer of this protein to females during mating might involve in any of the functions discussed earlier, however, precise information for the function needs further exploration.

Among the various protein classes identified from MAG secretions protease and protease inhibitors are also detected widely in insects (Gillott 2003, Avila *et al*. 2011, LaFlamme and Wolfner 2013). In our study antitrypsin was detected in MAG of *H. armigera*. Antitrypsin is a protease inhibitor that belongs to serpin superfamily (serine protease inhibitors). α1-antitrypsin is also present in *Drosophila melanogaster* MAG secretions (Wolfner *et al*. 1997). Hence, the balanced distribution of antitrypsin in the seminal plasma plays a major role during the spermatozoa formation by preventing the gradual deterioration of spermatozoa, essential, for determining male sterility. In vertebrates it plays a major role in immunity. In humans α1-antitrypsin contributes in inactivation of elastase responsible for the polymorphonuclear leucocyte, a marker for inflammation in the male genital tract as a result of which the quality of spermatozoa deteriorates (Fung *et al*. 2004, Lebig *et al*. 2010). In boar's seminal plasma the presence of α1-antitrypsin also contributes in improving the spermatozoa quality (Strzezek *et al*. 1995). The protein C inhibitor from the serpin family is found abundantly in the seminal plasma (Gonzales 2001). The disruption of the protein C inhibitor gene was reported to cause impairment during spermatogenesis and infertility in mice (Uhrin *et al*. 2000).

The protein Juvenile hormone acid O-methyltransferase (JHAMT) detected from MAG of *H. armigera* belongs to a protein class sesquiterpenoids (Gilbert *et al*. 2000). JHAMT is an enzyme that plays a crucial role in catalyzing the final

step of Juvenile hormone (JH) biosynthesis by converting the JH acids or inactive precursors of JH to the active form in corpora allata of Lepidoptera (Shinoda and Itoyama 2003). Further it was reported that when JHAMT was knocked down there was no JH production in insects and in turn affects insect development (Minakuchi *et al*. 2008, Dominguez and Maestro 2018). JH is suggested to play a vital role in controlling many biological processes in insects, such as, development, growth and reproduction (Gilbert *et al*. 2000). The transfer of JH from male to females during mating was reported in *Heliothis virescens*, suggesting its role in the stimulation of egg maturation in females (Park *et al*. 1998).

Another protein named sorbitol dehydrogenase (SDH) identified in this study is a zinc containing enzyme belongs to a group of proteins within the dehydrogenase family. In energy metabolism (especially, polyol pathway) SDH along with aldose reductase (AR) plays a vital role in the conversion of glucose to fructose (King and Mann 1958). In the first step of the pathway glucose is reduced to sorbitol by AR in the presence of an electron donor, NADPH (nicotinamide adenine dinucleotide phosphate). In the second step the sorbitol obtained is oxidized to fructose by SDH in the presence of an electron acceptor NAD^+ (nicotinamide adenine dinucleotide) (Kinoshita and Nishimura 1988). The resulting ATPs (adenosine triphosphates) are utilized by the tissues for specific functions. In drosophila the enzyme SDH is found to be in higher concentration in males during the late pupal and early adult stages (Bischoff 1978). In the accessory glands of male *Glossina morsitans* SDH enzyme was detected to be potentially involved in sperm motility (Scolari *et al*. 2015). Furthermore, in *Bombyx mori* females the SDH was reported to be involved in terminating of diapause in eggs (Yaginuma and Yamashita 1979). However, the identification of SDH in MAG of *H. armigera* in our study suggests playing a vital role in spermatogenesis as well as sperm motility in the female reproductive tract.

The initiation of translation in eukaryotes requires various factors collectively called eukaryotic translation initiation factor (eIFs). Among the translation initiation factors, eIF3 is a large heteromeric protein complex that plays a central role in the initiation process by binding to the initiator Met-tRNA (methyonyl tRNA) to the freely available 40S subunits in order to generate the stable 43S preinitiation complex (Kapp and Lorsch 2004, Hinnebusch 2006). In zebra fish the subunit eIF3h, encoded by two genes (eIF3ha and eIF3hb) serves as a regulator during embryonic development (Choudhuri *et al*. 2010). The detection of eIFs was confirmed in the seminal fluid of *Callosobruchus maculates* indicating its role in metabolism (Bayram *et al*. 2017). In this study we have detected eIF3h from the MAG, however, the protein is nonsecretory in nature, the presence of the protein spot in virgin male but the absence of this in mated suggests its role in protein metabolism.

The protein, V-type proton ATPase subunit F (V-ATPaseF) identified in *Helicoverpa armigera* is an electrogenic proton pump involved in the acidification of lumen of both intra and extra cellular compartment (Sun-Wada *et al*. 2003). The acidic pH generated by V-ATPase is important for the regulation of a wide range of biological processes such as secretion, membrane fusion and signaling pathway (Sun-Wada *et al*. 2015). V-ATPase was detected in the seminal fluid content of mosquito speculating to be transferred to females and might be involved in regulating the release of vacuolar content within the female reproductive tract (Sirot *et al*. 2011). However, the functions based on tissue specific expression of V-ATPaseF are not well understood. Probably the V-ATPaseF of *Helicoverpa armigera* is involved in similar functions as in the case of mosquitoes.

Table 2 Classification based on functional prediction of proteins

Predicted protein function	Protein name	Protein family	References
Protease inhibitor	Antitrypsin	Serpin	Fung *et al*. (2004) Lebig *et al*. (2010)
Ovary development / metabolism	Bombyxin B-12	Insulin	Orikasa *et al*. (1993) Chen *et al*. (1996) Iwami *et al*. (1996)
	Bombyxin-related peptide A		
	Male origin PBAN-type neuropeptides	Pyrokinin	Takayanag *et al*. (1986) Jacquin *et al*. (1994)
	Male origin PBAN-type neuropeptides		
Spermatogenesis	Sorbitol dehydrogenase	Dehydrogenase	Kobayashi *et al*. (2002) Scolari *et al*. (2015)
	Arylphorin subunit beta	Hemocyanin	Chandrasekar *et al*. (2009)
Olfaction	General odorant-binding protein 1	PBP/GOBP	Sun *et al*. (2012)
Metabolism	Eukaryotic translation initiation factor 3 subunit H	eIF-3 subunit H family	Hinnebusch (2006) Bayram *et al*. (2017)
	Juvenile hormone acid O-methyltransferase	Methyltransferase superfamily	Shinoda and Itoyama (2003)
	V-type proton ATPase subunit F	V-ATPase F subunit family	Sun-Wada *et al*. (2015)

Seminal fluid proteins are naturally occurring behavior modifying components that are involved in the regulation of a wide range of biological processes. These proteins are known to influence female reproductive behavior and could be exploited for the development of protein based biopesticides. The present study on MAG proteins from *H. armigera*, will provide an insight on the role played by individual proteins influencing reproduction and metabolism. Further, tracking these proteins within the female reproductive tract is required for the precise understanding of the mechanism involved within the female reproductive tract.

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