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Male Accessory Gland Derived Factors Can Stimulate Oogenesis and Enhance Oviposition in *Helicoverpa armigera* (Lepidoptera: Noctuidae)

Zhao-Yang Jin and He Gong*

State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Academia Sinica, Beijing, China

In *Helicoverpa armigera*, female moths began to lay eggs on the third day after emergence. Mating stimulated earlier egg maturation/oogenesis ($P = 0.002$) and oviposition ($P << 0.01$). We established a suitable bioassay model for the influence of male accessory glands (MAG) on the physiology of virgin females: Crude extracts of MAG (2- to 3-day-old) were injected into 2-day-old virgin females, and the injected females were dissected 20 h after mating. It was shown that crude extracts of MAG stimulated earlier egg maturation ($P < 0.001$) and oviposition (the oviposition ratio was more than 2 times the ratio of the control). Proteinaceous components in crude extracts purified by fractionation and sub-fractionation in reverse phase high performance liquid chromatography also stimulated earlier egg maturation ($P < 0.01$) and ovipositon (more than 2 times the ratio of the control), and we called them the oogenesis and ovipostion factors (OOSF). With SDS-PAGE, the molecular mass of the bands from OOSF was estimated to be between 55–66 KD. Arch. Insect Biochem. Physiol. 46:175–185, 2001.

Key words: MAG; purification; oogenesis and oviposition stimulating factors

INTRODUCTION

During copulation, male insects transfer sperm and seminal fluid to females and cause behavioral and physiological changes in mated female insects (reviewed by Leopold, 1976; Gillott, 1988; Chen, 1984, 1996; Wheeler, 1996; Wolfner, 1997). The males of many insect species not only provide nourishment for females in the form of glandular secretions (reviewed by Leopold, 1976), but also supply specific factors at mating that may be released into the hemolymph of the females or stay in the genital tract of females to suppress the subsequent mating of females or trigger oviposition, either transiently or permanently (Bali et al., 1996, Park et al., 1998a; reviewed by Leopold, 1976; Wolfner, 1997).

Abbreviations used: BSA = bovine serum albumin; CA = corpora allata; DU = ductus ejaculatoris duplex; JH = juvenile hormone; MAG = male accessory glands; OOSF = oogenesis and oviposition stimulating factors; OSP = oviposition stimulating protein; OSS = ovulation stimulating substance; PBS = phosphate buffered saline; PSP = pheromonostastic peptide; RP-HPLC = reverse phase high performance liquid chromatography; SP = sex peptide; SPX = ductus ejaculatoris simplex; T&SV = testes and seminal vesicles.

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*Correspondence to: He Gong, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Academia Sinica, Beijing 100080, China. E-mail: gongh@panda.ioz.ac.cn

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Much of the seminal fluid comes from the secretions of male accessory glands (MAG), which contain inorganic ions, amino acids, proteins/peptides and related derivatives, and hormonal substances such as juvenile hormone (JH) or ecdysteroids (reviewed by Leopold, 1976; Chen, 1984; Gillott, 1988; Wolfner, 1997).

Numerous studies have demonstrated that MAG plays an essential role in insect reproduction. So far, it has been found that substances in MAG are transferred to females as energy sources (Osanai et al., 1986, 1987), nutrients (Craig, 1967; Boggs and Gilbert, 1979; Boggs, 1981; Greenfield, 1982), hormones (Shirk et al., 1983; Dahm et al., 1976; Park et al., 1998a), and/or active proteinaceous factors (Kingan et al., 1993, 1995; Yi and Gillott, 1999; reviewed by Kubli, 1992; Chen, 1996; Wolfner, 1997) to influence the physiology and behavior of mated females, which include: alleviating attraction to males; depressing subsequent mating; enhancing oviposition; stimulating oogenesis; transferring, storing, and utilizing sperm; and shortening life span.

Research identifying the active factors in MAG, so far, has been reported mainly for dipteran insects. Baumann et al. (1975) reported the isolation of a 27–amino acid residue peptide from MAG of *Drosophila funebris* that was responsible for the termination of receptivity in the females. In *Drosophila melanogaster,* more than 16 Acps (accessory gland proteins) have been identified (reviewed by Wolfner, 1997), including SP (sex-peptide) (Chen et al., 1988; Schmidt et al., 1993; Moshitzky et al., 1996; Soller et al., 1997; Fan et al., 1999; reviewed by Kubli, 1992) and Acp26Aa (Herndon and Wolfner, 1995; Lung and Wolfner, 1999; Heifetz et al., 2000; reviewed by Wolfner, 1997), which both increase eggs laid. Moreover, SP can depress the receptivity of mating. In *Drosophila suzuki,* two SP-like factors (OSS and SP_{DS}) were found (Ohashi et al., 1991; Schmidt et al., 1993). Active factors have been purified and analyzed in two other speices: pheromonostastic peptide (PSP) in *Helicoverpa zea* (Kingan et al., 1993, 1995) and oviposition stimulating protein (OSP) in *Melanoplus sanguinipes* (Yi and Gillott, 1999).

The cotton bollworm, *Helicoverpa armigera,* is a well-known pest of a variety of field crops. Therefore, it is important that we study its reproductive biology and find a feasible way to control this pest from a reproduction perspective. However, in *H. armigera*, little is known of the role of its MAG in the reproductive biology and of the components contained in MAG. Therefore, this study was conducted to examine the role and functions of MAG in *H. armigera* and to gain an understanding of the proteins from the MAG.

MATERIALS AND METHODS

Insect Rearing

H. armigera were reared on an artificial diet (Wu and Gong, 1997) in the laboratory under a reversed 14:10 h (light:dark) photoperiod and at 27 ± 1°C. Insects were sexed during the pupal stage, and then males and females were placed in different screen cages (25 × 25 × 25 cm) for emergence. Adult males that emerged during the last scotophase were recorded and then placed in another cage (one batch per day). Individual females for injection bioassay that emerged during the last scotophase were also recorded, and collected separately in a plastic cup (diameter: bottom, 4.5 cm; top, 7 cm; height: 9cm). Moths were fed 10% (V:V) honey solution on cotton balls.

Female Single Mating Experiment

This experiment was conducted to compare the ovipositions between virgin females and single mated females. Males and females that emerged in the last scotophase were collected in different cages. The following evening, females were divided into two groups (A: females for pairing with males; B: virgin females as a control) according to the ratio of 3/2 (A/B). Thrity virgin females of group B were transferred separately to a plastic cup. During the last 3 h of this scotophase, 40 virgin females of group A were paired with 60 virgin males (2–3 days old); then, later, 30 mating pairs were gently removed and covered with a glass disk on a table separately. Upon completing mating, males were discarded; females were transferred to a plastic cup (one moth per cup) with a small piece of white gauze serving as an oviposition substrate. Moths were also fed 10% (V:V) honey solution on cotton balls. Every day, the mated and virgin females were moved to new cups at the end of scotophase, and the eggs were counted. Moreover, the day of onset of egg laying was recorded for every moth. Counting eggs did not end until

moths died. The results from females living less than 5 days were not considered. The data of fecundity (the total number of eggs laid by a female in a life) and the eggs laid on the day of onset of oviposition were analyzed by an independent-samples *t*-test with SPSS 10.0 software.

Protein Samples Preparation for Electrophoresis

The reproductive systems of 48-h-old virgin males were removed carefully in a drop of ice-cold phosphate buffered saline (PBS), and then the fat body and the tracheoles were discarded. MAG, ductus ejaculatoris duplex (DU), testes (T) and seminal vesicles (SV), and ductus ejaculatoris simplex (SPX) of five male adults were cut, cleaned with PBS, and separately pooled into different microcentrifuge tubes. The above samples were homogenized manually on ice. The homogenates were centrifuged at 12,000*g* for 25 min at 4°C, and the pellet was similarly extracted with 50 μl PBS two times. All three supernatants were pooled. The tissue extracts were either used immediately or stored at –70°C until use. The same steps were carried out for the MAG from 0- or 24-h-old virgin male adults.

Preparation of Lyophilized Crude Extracts for Bioassay

Two- to three-day-old males were dissected to take MAG in ice-cold distilled water. Immediately after the MAG was taken out, it was rinsed in clean distilled water quickly, and then collected. Two hundred pairs of MAG were pooled and homogenized manually on ice. The homogenate and the subsequent rinses (three rinses for homogenizer with 300 μl distilled water) were pooled and centrifuged at 12,000*g* for 25 min at 4°C. The fat layer at the top was carefully removed. The pellet was similarly extracted two more times with 200 μl distilled water. All three supernatants were pooled and frozen. Subsequently, the supernatants were lyophilized (Flexi-Dry™ μP) and stored at –70°C until use.

Partial Purification

Lyophilized crude extracts were prepared as above from 400 pairs of MAG per batch. The extracts were redissolved in 300 μl ice-cold protein preparation buffer composed of: 1 mol/l hydrochloric acid, 5% formic acid, 1% trifluoroacetic acid (TFA), and 1% sodium chloride (Bennett, 1986). The solution was then centrifuged at 12,000*g* for 25 min at 4°C and the pellet was similarly extracted two more times with 150 μl ice-cold protein preparation buffer each time. The three supernatants were pooled and immediately stored at –20°C until use. The supernatant was reduced in volume by approximately one third by the lyophilizing (Flexi-Dry™ μP) prior to fractionation through reverse phase high performance liquid chromatography (RP-HPLC). RP-HPLC fractionation of the supernatant was performed on Vydac C4 column (cat. no. 214TP54) using a TFA-acetonitrile gradient system (Schmidt et al., 1993) and two solvents: A, 0.05% TFA; B, 95% acetonitrile containing 0.05% TFA; gradient: 0–15% B in 10 min, 15–31% B in 80 min, 31–50% B in 10 min, and 50–100% B in 5 min; flow rate: 0.7 ml/min. The column was loaded with the supernatant containing a maximum of 50 equivalent pairs of MAG. The eluted fractions were pooled every 5 min beginning with minute 8 and dried separately by SpeedVac Concentrator. The active fractions among them were further fractionated and dried as described above for further bioassay. The active fractions and the active subfractions were analyzed by electrophoresis with silver staining.

Electrophoresis

Tissue extracts (see Female Single Mating Experiment) were examined by SDS-PAGE on 12% polyacrylamide gels (Laemmli, 1970). Between 1/10 and 1/6 insect equivalents of different tissue extracts were applied to sample lanes, respectively. To compare the change of protein concentration of MAG extracts during adult *H. armigera* development stage, the same equivalents of AG from 0-, 24-, and 48-h virgin male adults were added to sample lanes, respectively. After electrophoresis, gels were stained with Coomassie Blue R-250. The molecular weights of the bands were determined by comparison with the electrophoretic mobilities of a series of mid-range protein standards (Promega) and high-range standards (Sigma).

The active fractions and the active subfractions were analyzed by SDS-PAGE electrophoresis (Laemmli, 1970) with 12% polyacrylamide gels. One and three equivalent pair MAG of the fractions and the subfractions were applied to

sample lanes, respectively. Upon completing electrophoresis, the gel was stained with a protein silver staining kit (Pharmacia Biotech), according to the instructions of the manufacturer.

Determination of the Total Protein Concentration in the MAG

Proteins from the MAG were obtained as detailed in Female Single Mating Experiment. The solute was 1.15% KCl solution. The total protein concentration was determined using Bradford's method (1976) with bovine serum albumin (BSA) as a standard.

Bioassay

To demonstrate the influence of MAG extracts or its proteinaceous factors on egg maturation and oviposition, we used an injection method. Lyophilized crude extracts, eluted fractions, or subfractions (the eluted fractions of the second RP-HPLC) were redissolved, respectively, in 400 μl sterile saline chilled on ice. Virgin females (48-h-old) were placed for 5 min at 4°C and then immediately anesthetized with CO_2. The anesthetized insects were transferred on ice, and 3-μl sample solution (about 1.5 equivalent pairs of MAG) was injected into the female abdominal cavity through the intersegmental membrane. Females were allowed to recover for 15 min at 4°C after injection and then were placed back into the incubator. Moths were dissected 20 h after injection. The number of chorionated eggs retained in the ovaries was counted, and the females that laid eggs or not were also recorded. As a control, the same amount of sterile saline was injected.

Data Analysis

Data for chorionated eggs in the ovaries was analyzed by an independent-samples *t*-test with SPSS 10.0 software. The oviposition ratio was calculated as:

$$r = \frac{\text{number of injected females laying eggs}}{\text{total number of injected females}}\%$$

RESULTS

Female Single Mating Experiment

Both fecundity and oviposition on the first day of laying eggs increased. The fecundity per mated female was (mean ± SE) 1,207.8 ± 110.7, in contrast to 714.5 ± 100.7 in virgin female (P = 0.002); the number of eggs on the first day of oviposition per moth was 191.7 ± 22.7 for mating, 32.5 ± 6.4 for virgin ($P << 0.01$) (see Table 2). All female moths began to lay eggs on the third day post-emergence (see Table 2).

Electrophoresis

Different Reproductive Tissues in Male Moth

As illustrated in Figure 1a, the separation of the proteins from the MAG, T&SV, DU, or simplex on polyacrylamide gels indicated a minimum of 30 proteins/polypeptides. Three major protein bands were found in the MAG sample (bands 1–3, Fig.1a). The molecular mass of band 1 was in the region of 21.5–31 KD, while both bands 2 and 3 were below 14.4 KD. In the "DU" lane, bands 4–6 were the three major proteins; the molecular masses of bands 4 and 5 belonged to the region of 66.2–97.4 KD. Among the bands in the "T&SV" lane, bands 7–10 were the most prominent. Band11 in the "SPX" lane of molecular mass less than 14.4 Kd was the most concentrated and was distinct from bands in other male reproductive tissues. The bands of the OOSF lane showed between 55–66 KD, which differed from the major protein band (with the molecular mass less than 31 KD) of the active fractions (Fig. 1c and d).

Protein Accumulation of MAG

SDS-PAGE of MAG extracts from 0-, 24-, or 48-h-old virgin males showed that bands 2 and 3 were very faint on the gels 0 h after eclosion (Fig. 1b) and increased significantly from 0–24 h and less significantly from 24–48 h. For virgin males, the total protein concentration of MAG increased significantly from 0–24 h, and increased at a lower rate during 24–48 h (Table 1).

Partial Purification of Factors That Stimulate Earlier Oogenesis and Oviposition

As illustrated in Figure 2, virgin females injected with crude extracts produced 434.64 ± 22.36 (mean ± SE) chorionated oocytes in ovaries, which was significantly higher ($P < 0.001$) than the control, which produced 325.12 ± 17.06 mature eggs; Similar phenomena were also observed in females injected with fractions [eluted between 18–23 min

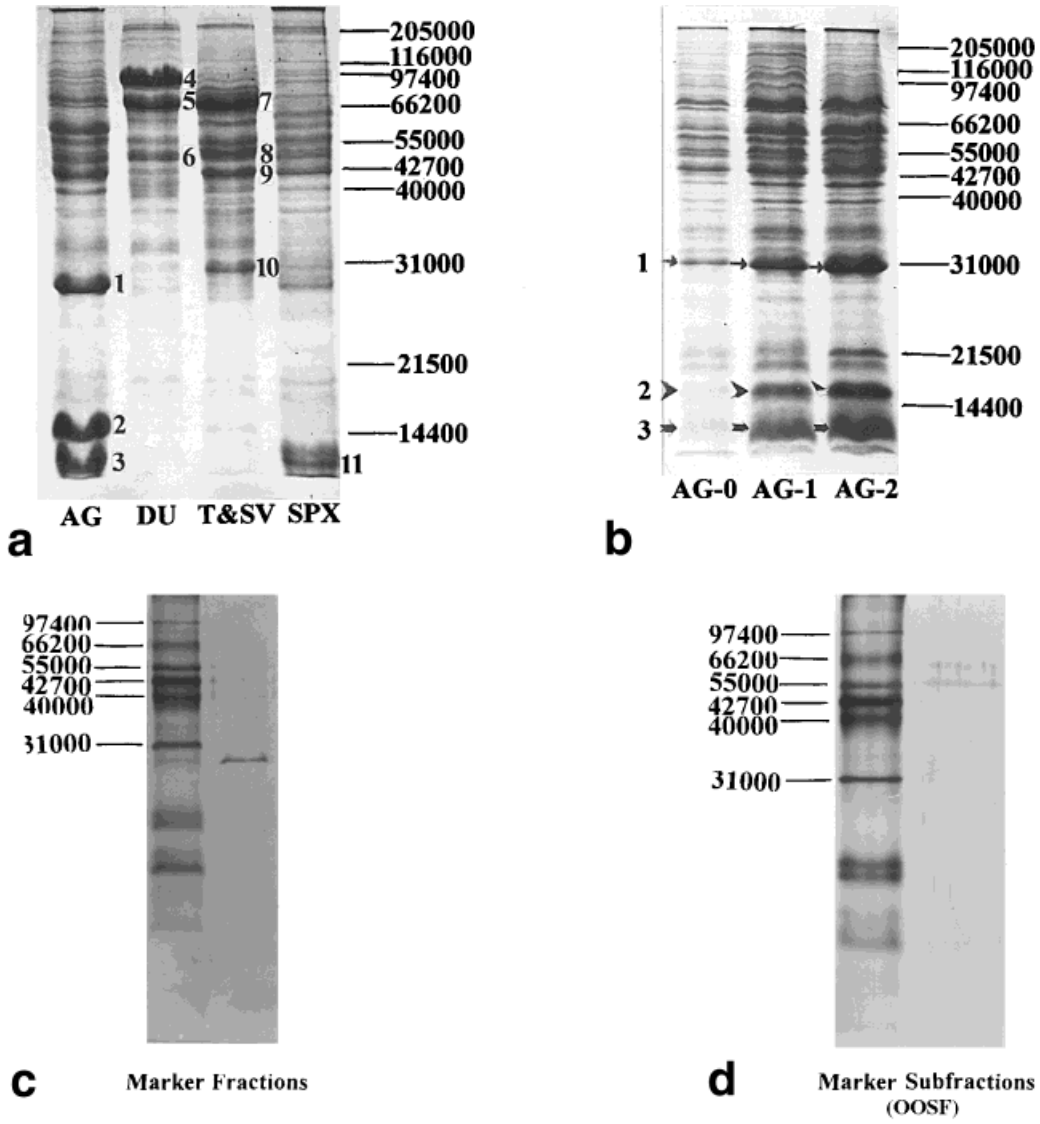

Fig. 1. **a**. Separation of proteins from different reproductive tissues extracts of virgin adult male *H. armigera* (48-h-old) by SDS-PAGE. MAG: male accessory glands; DU: ductus ejaculatoris duplex; SPX: ductus ejaculatoria simplex; T&SV: testes and seminal vesicles. **b**. Proteins extracted from MAG of virgin adult male *H. armigera* from different developmental stage. AG-0: 0-h-old accessory gland extract; AG-1: 1-day-old accessory gland extract; AG-2: 2-day-old accessory gland extract. **c**. Separation of proteins from 18–23-min fractions by SDS-PAGE with silver staining. Marker: Protein molecular weight standard; Fractions: 18–23-min fractions. **d**. Separation of proteins from 20.5–22-min subfractions by SDS-PAGE with silver staining. Marker: the protein molecular weight standard; Subfractions: 20.5–22-min subfractions.

(see Fig. 4) of the first RP-HPLC separation] or subfractions [eluted between 20.5–22 min (see Fig. 5) of the second separation], which caused 477.05 ± 24.18 and 450.67 ± 23.14 chorionated eggs, respectively, whereas the females injected with saline contained 380.4 ± 26.56 (P = 0.011) and 347.6 ± 26.38 mature eggs ($P < 0.01$) (Fig. 2). The crude extracts, fractions, or subfractions also promoted oviposition earlier (Fig. 3). The oviposition ratios of females injected with crude extracts, fractions, or subfractions were 64, 47.6, or 54.2%, whereas in their controls the ratios were 24, 23.8, or 21.7%, respectively. We call the subfractions "OOSF" to indicate that they contain oogenesis and oviposition stimulating factors.

DISCUSSION

Just as in other insects (Ramaswamy et al., 1997; Park et al, 1998a; reviewed by Leopold, 1976;

TABLE 1. Mass of Proteins in the AG of Virgin Males*

Age(h)[a]	Total proteins (μg/pair, mean ± SE)
0	61.81 ± 3.607 (n = 5)
24	114.03 ± 3.607 (n = 5)
48	126.96 ± 3.607 (n = 5)

*Data was analyzed by one way ANOVA with SPSS 10.0 software ($P << 0.01$).
[a]Age of virgin males is hours after emergence.

Chen, 1984), mating not only increases the fecundity of female *H. armigera* (Hou, 1998; Table 2, $P = 0.002$), but also significantly increases the number of eggs laid on the first day of oviposition ($P << 0.01$) (Table 2). These results suggest that some male factors that change the physiology of females were transferred to the mated females during mating. The lyophilized crude extracts of MAG were shown to stimulate earlier egg maturation and oviposition of virgin females (Figs. 2 and 3). The substance from MAG is attributable to the above-mentioned male factors, and one of the functions of MAG on the female is to stimulate earlier egg maturation and oviposition. Furthermore, the fractions and subfractions (OOSF) also have the same function (Figs. 2 and 3). This suggests that the proteinaceous factor (OOSF) is the male factor. Since the volume of OOSF used for injection was so little that it had little effect on egg maturation as a nutrient, stimulating oogenesis and ovipositon earlier should be controlled at least by a non-nutritive mechanism.

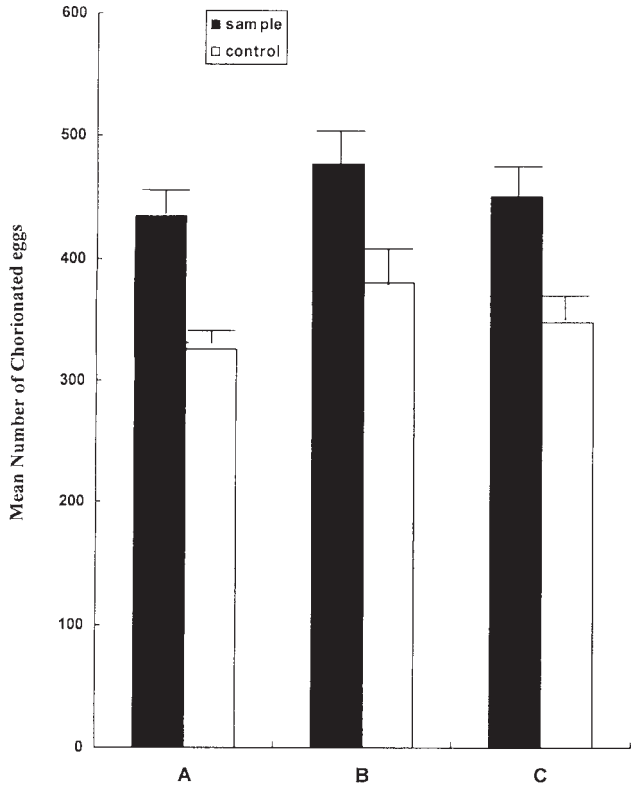

Fig. 2. Number of chorionated eggs in ovaries of female *H. armigera* 20 h after injection. Data for chorionated eggs was analyzed by independent-samples T test with SPSS 10.0 software ($P < 0.01$). **A**: Number of eggs in ovaries of female injected crude extract (n = 25)/control (n = 25); **B**: number of eggs in ovaries of female-injected 18–23-min fractions (n = 19)/control (n = 20); **C**: Number of eggs in ovaries of female-injected 20.5–22-min subfractions (n = 221)/control (n = 20).

In the present study, we developed a bioassay for studying the physiological effects of MAG on female *H. armigera* moths. We introduced two observable indexes: chorionated eggs in ovaries (the index of oogenesis) and eggs laid (the index of ovipostion) as introduced in other insects (Chen et al., 1988; Bali et al., 1996; Park et al., 1998; Heifetz et al., 2000).

Generally, male *H. armigera* become sexually mature by 24 h after emergence and fully mature by 48 h post-emergence (Hou, 1998; this study). During this period, the accumulation of proteins in MAG was significantly increased after eclosion (Fig. 1b, Table 1). Therefore, we assayed MAG materials from 2- to 3-day-old males. Female *H. armigera* begin to lay eggs on the 3rd day post-emergence and most of them mate after 48 h of age (Hou, 1998; this study). Previously, our laboratory showed that vitellogenesis occurs 7–12 h after eclosion, and the concentration of Vg in hemolymph reached a peak on the fourth day after eclosion (Ding, 1997). Moreover, by dissection, we found that most of the substance in the spermatophore was depleted 24 h after mating. Since, in most insects, the effect of the secretions from the MAG or its proteinaceous factor, such as SP in *D. melanogaster*, on physiology and behavior is temporary (reviewed by Leopold, 1976; Chen, 1984, 1996; Kubli, 1992; Wolfner, 1997), we selected the injection time of 48 h post-emergence of females, and the dissection time of 20 h post-injection. For control experiments, because egg maturation was almost unaffected by proteins as nutrients through the injection (O'Meara and Evans, 1977; Bali et al., 1996; Eberhard, 1996), we only used saline as our injection solution for control. The protein concentration of OOSF was much lower than that of crude extracts, but the activity of OOSF proved to be almost the same as that of the crude extracts (Figs. 2–5).

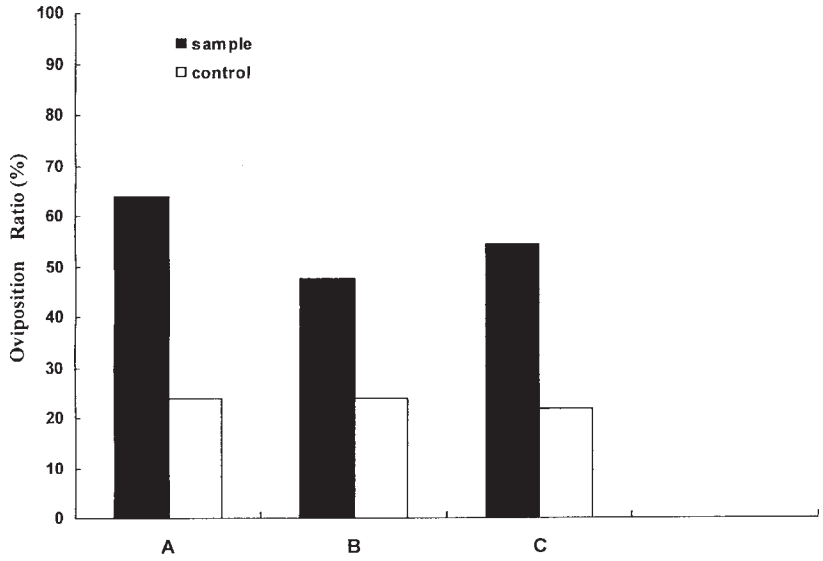

Fig. 3. The oviposition ration of female 20 h after injection. **A**: Ratio of females injected crude extract (n = 25)/control (n = 25); **B**: Ratio of female injected 18–23-min fractions (n = 20)/control (n = 20); **C**: Ratio of female injected 20.5–22-min subfractions (n = 24)/control (n = 24).

Like other insects (Kaulenas, 1992), the proteins of MAG and other male reproductive tissues were more complex in *H. armigera* (Figs. 1a and 4) and the major protein bands in them differed from each other (Fig. 1a). Most of their proteins contributed to form spermatophore (Kaulenas, 1992), although the relationship among them in reproduction is still uncertain. Among the variety of proteins from MAG of *H. armigera* (Fig. 1a,b), there also exist one or more proteinaceous factors that stimulate oogenesis and promote oviposition earlier (Figs. 2 and 3). In the course of an arduous effort to purify and characterize the active factors, we found that the partially purified OOSF exhibited activities similar to those of MAG extracts (Figs. 2, 3).

It is widely accepted that both mating and the MAG (including its proteinaceous factors) stimulate egg maturation and oviposition. In various species of insects, juvenile hormone (JH) regulates reproduction in adult females, and also in many Lepidoptera (including *H. armigera*) (Ding, 1997). JH is crucial for egg maturation and/or production (Shu et al., 1998; Ramaswamy et al., 2000). In *Heliothis virescens*, Ramaswamy's group (Ramaswamy et al., 1997, 2000; Zeng et al. 1997; Park et al. 1998a,b; Shu et al., 1998) studied the relationships of the six factors: mating, JH, JH-esterase, MAG, corpora allata (CA), and egg maturation and production. They demonstrated that in *H. virescens*, mating resulted in the transfer of JH contained in MAG from male to female and stimulated the mated females' CA to biosynthesize JH, while it concurrently inhibited the JH degradative system. They proposed that the co-occurrence of these three mechanisms accounts for the post-mating increase in hemolymph JH titers of mated females, resulting in stimulation of egg maturation and production. In *D. melanogaster*, JH has been shown to be involved in yolk protein synthesis and uptake by the ovary (Postlethwait and Handler, 1979; Bownes, 1994). Moshitzky et al. (1996) found that SP stimulated JH synthesis in the mature CA in vitro, and, later, Soller et al. (1997) clarified that SP also stimulated the accumulation of yolk in oocytes. In *H. armigera*, Fan et al. (1999, 2000) demonstrated that SP of *D. melanogaster* and its N-terminal fragments—$SP_{1\text{-}11}$

TABLE 2. Effect of Single Mating on Fecundity, the Oviposition on the First Day of Laying Eggs of Female Cotton Bollworms (Mean ± SE)

Females	n	Fecundity	Oviposition on the first day of laying eggs	Onset of egg laying
Single mating	23	1,207.8 ± 110.7	191.7 ± 22.7	3 days old
Virgin	25	714.48 ± 100.7	32.5 ± 6.4	3 days old

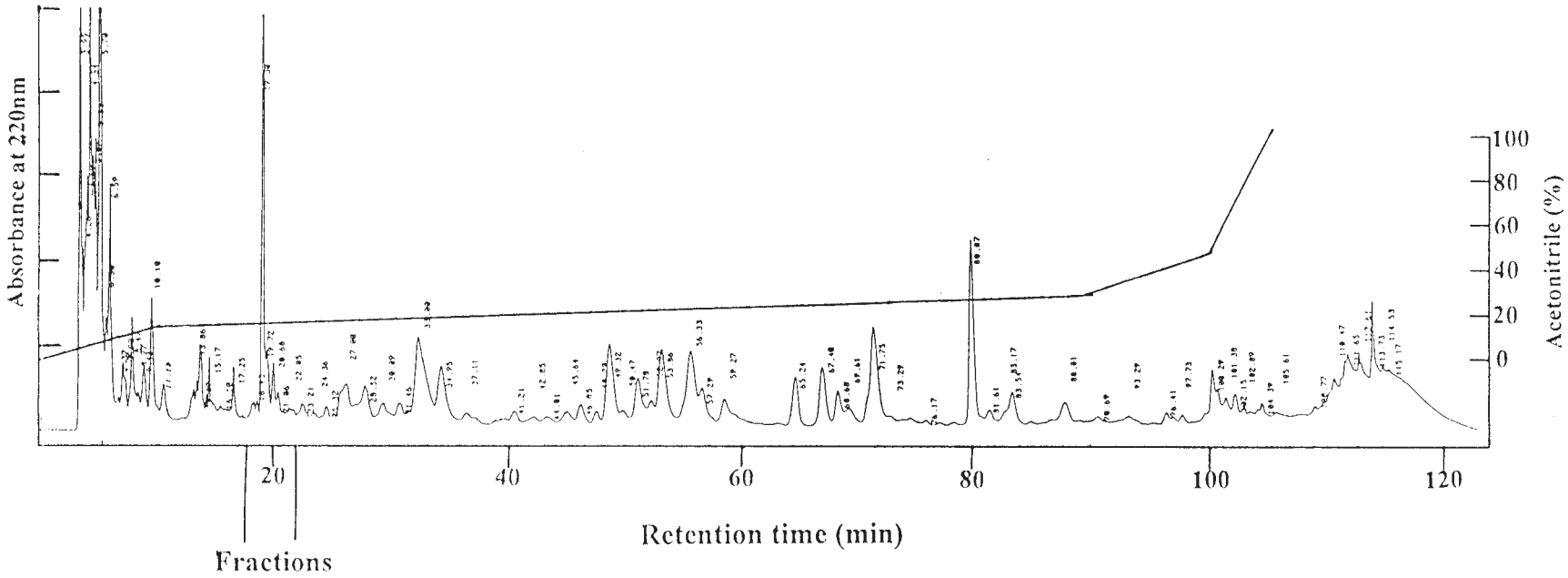

Fig. 4. RP-HPLC separation of extracts from MAG of *H. armigera*. "Fractions" has oviposition stimulating and oogenesis enhancing activity.

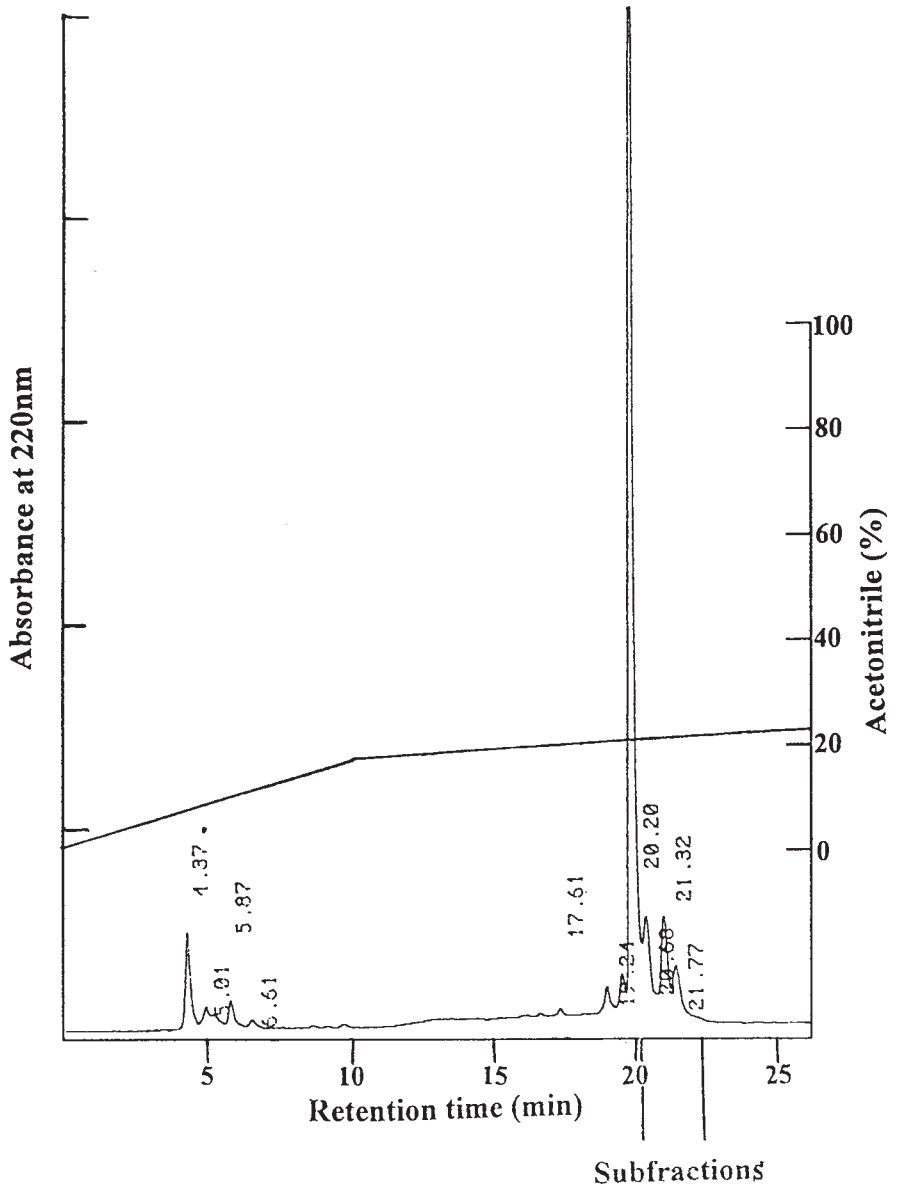

Fig. 5. Second RP-HPLC separation of "Fractions" with activity. "Subfractions" also has oviposition stimulating and oogenesis enhancing activity.

and $SP_{1\text{-}22}$ significantly activated *H. armigera* CA to biosynthesized JH in vitro. In this study, mating and OOSF were shown to stimulate oviposition and egg maturation earlier ($P < 0.01$) (Figs. 2 and 3; Table 2). Additionally, in *H. armigera*, by Western blotting, we found that only a protein band from MAG with SDS-PAGE had a special reaction against polyantibodies of SP from *D. melanogaster* (kindly provided by professor E. Kubli of the University of Zürich, Switzerland), and also that only a protein band with the same molecular weight from a soluble substance in fresh spermatophore had the same result. Moreover, the band disappeared in the soluble substance of spermatophore 1 day postmating (our unreported data). Therefore, this suggests that in *H. armigera* there may exist at least one mechanism of regulation: mating transfers proteinaceous factors (for example, OOSF) of male adult to the female. Then, the factor(s) may regulate the titer of JH (we call it the "transfer station") in female hemolymph and, finally, through the increase of the titer of JH, realize their behavior/physiological function(s), to stimulate egg maturation and egg production.

So far, the molecular characteristics of five proteins/peptides related to oviposition from MAG in several insects (Chen et al., 1988; Ohashi et al., 1991; Schimidt et al., 1993; Herndon and Wolfner,

1995; Yi and Gillott, 1999) have been determined. Except for SP_{DS} in *D. suzukii* (which shares great similarity in gene sequence with SP in *D. melanogaster*), other proteins/peptides show little similarity with each other and the molecular masses vary from 3.99 KD (OSS) to 60 KD (OSP). In this study, the molecular mass of the bands of OOSF was between 66 and 55 KD. This indicates that there may exist two possible reasons for different molecules with similar function: one is the fast evolution in the reproduction field and the other is that their targets are different. For example, Acp26Aa (Heifetz et. al., 2000) and OSP (Yi and Gillott, 1999) may act on ovaries, while SP is suggested to act directly on CA (Moshitzky et al., 1996).

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