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HISTOLOGICAL STUDIES ON SEX PHEROMONE SOURCE IN FEMALE AMERICAN COCKROACH, *PERIPLANETA AMERICANA*

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ABSTRACT

Insects perceive the world through small molecules which carry information called as "Pheromones". Sex pheromone, in most insects is usually released by females to attract males. In American cockroach, *Periplaneta americana*, sex pheromone is produced in an adult female-specific cuticular abdominal gland located on the anterior of the last abdominal tergite. The basic structure of the cuticular abdominal gland was observed under the light microscope and the ultra-structural details were analysed with the help of Transmission and Scanning electron microscopic studies. The light microscopic analysis revealed that the anterior region of (*i.e.* tergal gland) American cockroach, observed to contain monolayer of epithelium, secretory vesicles and supportive cells. The secretory canal noted to contain a few secretory substances. The histochemical studies authentically proved that the secretory substance was protein in nature. Electron microscopic studies of cuticular abdominal gland revealed that the larger cells were noticed in the ventral part. The size of the nucleus was observed to be large round in shape. The nucleus is usually located in the basal region. The cells had a characteristic layer of microvilli just beneath the endo-cuticle. The endo-cuticle and the epi-cuticle had lamellae. The epi-cuticle was seemed to be denser than that of the endo-cuticle. During photo phase, the cells of cuticular abdominal gland became highly vesiculated with smaller vesicles that accumulated just below the microvilli. During scotophase, (*i.e.*, dark phase) pockets of granular material were appeared throughout the cytoplasm of each cell and also noted within the microvilli. Finally, the cuticular hairs were examined, which revealed that the extensions of the epicuticle had a hollow core and many pore canals.

Key Words: *Periplaneta americana*, Cuticular abdominal gland, Sex pheromone, Epi and endo cuticle.

INTRODUCTION

Insects perceive the world through small molecules which carry information (signature) for the recognition of potential mates, prey, and specific features of the environment, such as food sources, oviposition sites, etc. These information-carrying chemical compounds are referred as semiochemicals. Semiochemicals are subdivided into allelochemicals & pheromones depending on whether the interactions are interspecific or intraspecific. Intraspecific communications such as, sex attractants have been labelled as "Pheromones" (Nordlund and Lewis., 1976). Sex pheromone, in most insects is usually released by females to attract males. *i.e*., these chemical signals play a fundamental role in two important phases of their sexual behavioral sequences: attracting a sexual partner and mating behavior. Cockroaches are one of the most adaptable and successful insect (pest) groups to ever inhabit this planet. American cockroach, *Periplaneta americana*, has a wide geographic distribution throughout much of Asia and particularly in developing countries like India, China, and as is one of the few household insect pest species known (Barbara, 2000).

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A sex pheromone is produced in an adult female-specific cuticular abdominal gland located on the anterior of the last abdominal tergite in the female cockroach. In this area, the cuticle forms deep depressions in which a large number of cuticular orifices are located. The cuticular orifices are connected to secretory cells *via* cuticular ducts surrounded by duct cells.

Further, the cuticular abdominal gland exhibits a clear developmental maturation in relation to sexual maturation of the female (Sreng, 2006). The secretory cells of a newly formed gland in the imagine female are small and contain few secretory vesicles. The secretory cells in a mature cuticular abdominal gland are characterized by a large number of lucid secretory vesicles, abundant RER and SER, a large nucleus and a long, convoluted end apparatus which is lined with numerous microvilli.

MATERIAL AND METHODS

Insects

The American cockroaches (*Periplaneta americana*) were collected from the grocery shops during mid night (22-24Hrs.) using hand glove and kept in plastic containers with holes. Colonies of the cockroaches were maintained in 40cm x 30 x 10cm trays, covered with mosquito net under a 12L: 12D photo cycle at 27°C with access to dry mice feed and water. Adult male and female cockroaches with the size ranged from 3.5cm to 4.5cm were kept in the trays.

Histology

The histological techniques used were those described in Martoja and Martoja-Pierson, carried out by (Kannan, 1998). Following alcoholic Bouin's fixation, after dehydration and paraffin embedding, the preparations were sectioned at 7µm and stained with Masson's Trichrome.

Chemical Fixation with Formaldehyde

The selected tissues (cuticular abdominal glands) were cut into small pieces with the size of 3-5mm thick. They were fixed in 10% formalin and used for histochemical studies as per the standard protocol of (Kannan, 1998).

Staining

Hematoxylin and Eosin were used to stain the sections. Hematoxylin, a basic dye, stains nucleus into blue due to an affinity to nucleic acids in the cell nucleus; Eosin, an acidic dye, stains the cytoplasm pink. Toluidine blue a basic dye at pH 10 was used to stain some sections of female cockroach like clitellum and other regions as it stains peptide accumulates. The stained cockroach specimens were observed under trinocular research microscope (Olympus make) and micrographs were taken using Nikon Camera (L 22 series). The observed data were recorded and analyzed based on their histological features.

EM study for Ultra structure of Cuticular abdominal Glands

The methods used for ultra-structural studies of cuticular abdominal glands have been described previously (Sreng, 1984 & 1985). Gland fixation was carried out in a solution of 2% glutaraldehyde in 0.1M osmium tetroxide buffer (pH 7.4) for 16 h at 4°C u, followed by a post fixation of 1 h at 4°C using osmium tetroxide in the same buffer. Cuticular abdominal gland pieces were dehydrated in increasingly pure ethanol solutions, followed by propylene oxide, and embedded in Epon-Araldite resin. Sections were contrasted with a solution of uranyl acetate in 50% ethanol, then in lead citrate.

Scanning Electron Microscopic (SEM) Studies

The tissues of selected cuticular abdominal gland was primarily fixed in 2.5% glutaraldehyde (prepared in phosphate buffer, pH 7.2) for 5 hr. Subsequently, the selected glandular regions were cut into small pieces and fixed for overnight. Then, the specimens were washed three times in washing buffer (3% sucrose in 0.2M phosphate buffer) and fixed in the secondary fixative (1% osmium tetroxide prepared in phosphate buffer before two hours). Again the fixed specimens were thoroughly washed in washing buffer (01. M phosphate buffer saline). Dehydration was made by passing through cold acetone, 30,50,70,80.90 and 95% solution in which the tissues were kept for 15 minutes in each grade. The specimens were then dried with liquid CO_2 using POLARONE-300 apparatus. Metal coating of the SEM specimens was performed under Argons gas. Using gold as a target, a coating of about 35nm thickness was obtained under the following conditions: Current: 2.5 mA; Pressure:0.5 mbar; Gas (Atmosphere): Argon; Distance between cathode and specimen: 30mm and Time: 60 seconds. Finally, the gold coated specimens were observed with a Jeol JSM – 820 electron microscope at 10kv.

Transmission electron microscopy

Small sections (1±2 mm^2) were cut from the cuticular abdominal gland and localized the various components of secretory cells/vesicles. Dissected tissue was placed in buffered 2Mglutaraldehyde (0.05 M phosphate buffer, pH 6.8) at 22.8ºC for 2 hr. Following primary chemical fixation, the samples were washed for 1hr in six changes of the buffer, post-fixed in buffered 2M osmium tetroxide for 2 h, rinsed in buffer, dehydrated in an acetone series, and infiltrated with Spurr's low viscosity embedding medium. In an attempt to optimize the chemical fixation and resin infiltration, two variations of this procedure were also used. First, microwave irradiation was performed in a domestic Amana Microwave Oven (Amana Refrigeration, Inc., Amana, IO) at 500 W. A beaker containing 400 ml of distilled water was placed in the rear of the oven to act as the heat sink.

Vials containing dissected tissue in the buffered glutaraldehyde were irradiated continuously at a 'high' setting for 30 s and then transferred to fresh fixative at room temperature. All subsequent washing, post-fixation, dehydration and infiltration were done as described above. Secondly, tissues were fixed in a mixture of 3% glutaraldehyde, 1.5% formaldehyde and 1.5% acrolein in 0.1 M sodium Cacodylate buffer (EM Sciences, Ft. Washington, PA). Following Fixation, the samples were washed the Cacodylate buffer, post-fixed for 1 hr in 1% Sorenson's phosphate buffered osmium tetroxide, washed in buffer, dehydrated in an alcohol series and infiltrated in Spurr's medium as described above. Silver-grey sections of selected areas of the tissue were cut on a Reichert/AO Ultra cut (Leica, Deerfield, IL) microtome with a Diatome (Diatome US, Fort Washington, PA) diamond knife and mounted on 400 mesh grids.

The ultra-thin sections were stained with 2.5% uranyl acetate in a 1:1 ratio of ethanol/water solution for 1hr, and then with 3% lead citrate for 5 min. After observing the cells in the Toluidine blue staining under compound microscope, thin sections were viewed in either a Hitachi H-500 or a Hitachi H-7000 transmission electron microscope operating at 75 kV with a 30 mm objective aperture. Images were recorded on Kodak film using a 2 s exposure.

RESULTS

The selected tissues of American cockroaches, collected from the grocery shops during mid-night (22-24hr.) were used for histological studies. The present work describes the histo-morphology of female cuticular abdominal gland whose secretions contained sex attractant pheromones which played a significant role in precopulatory sexual behaviour in American Cockroach. In the present investigation, we noticed a distinctive cuticular histological modification in the abdominal glands of cockroaches, such as tufts of setae, ridges and fossae. The anterior region of the cuticular abdominal gland was undulating and slacking and normally covered by 9^{th}tergite of the female cuticular abdominal gland. The female has cuticular abdominal glands located on the anterior zone of 10^{th}tergite covering a 1.8 mm^2 surface area. The cuticular surface of the anterior zone contained numerous glandular orifices which were observed to be very undulating and slack. Thus, the preliminary observation under light and dissection microscopy clearly authenticated the occurrence of glandular structure underneath the cuticular region of abdomen of female cockroach.

The light microscopic analysis revealed that the anterior region of cockroach, observed to contain monolayer of epithelium, secretory vesicles and supportive cells. The secretory canal noted to contain a few secretory substances.

The histochemical studies authentically proved that the secretory substance was protein in nature. Furthermore, the present investigation explored the glandular cells, which were identified on the epithelium. The present finding positively showed the occurrence of secretory glands aside the monolayer of epithelium (Fig.2). The supporting cells were observed as elongated cells. Cross section of anterior region (segment 5-10) as described in Fig 1A, visualised the deposition of secretory substances in between the supportive and epithelial layers. Under high magnification (400X), the anterior region found to contain a cluster of glandular cells (Fig.1B).

The secretory vesicles of anterior region contained a limited amount of sex pheromone in the interior which is observed to be dark bluish in colour while staining with Toluidine blue. The storage vesicles of anterior glands are lined with collagen fibres with endodermal cells. The inner part of the secretory vesicle was noticed just like a mitochondrial structure (Fig. 1B).

Similar kind of light microscopic studies had been under taken in the posterior region of American cockroach. These histochemical studies with a specific stain known as Toluidine blue clearly demonstrating that the secretory canal was completely filled with mucus, partially digested debris and sex pheromones. Interestingly, micrographic observation significantly proved that sex pheromones are also released through the anal region and it was found to have distinguished canal system to release the excretory substances (Fig.3). The notification of sex pheromone in posterior region *i.e.*, the anal region may supposed to receive the secretory substance either through alimentary canal or through the mucus secretion of American cockroach. The region adjacent to the secretory cells was highly dominated by the collagen fibres. Even at high magnification (400X), the glandular cells did not possess considerable amount of protein component while staining with Toluidine blue. The occurrence of glandular cells was a unique feature of cuticle (Fig. 3A and B). On the other hand, monolayer cuticle was surrounded with non-glandular epithelium and here also the secretory cells were noticed.

The portion of cuticle was completely occupied by different kinds of glandular tissues, with sex pheromone secreting cells. The light micrograph showed in Fig.1 experimentally authenticated that the cuticle region was found to secrete mucus component through a perfect secretory pathway that released the sex pheromone. Thus, the histochemical studies documented that sex pheromones were glandular origin and accumulated at higher level in posterior as well as in the cuticle region. From these observations, it was concluded that the pheromonal source of American cockroach was restricted to cuticle, thorax and posterior region.

Electron Microscopic Study

In order to describe the actual source of sex pheromones in American cockroach, histological and histochemical observations were made in cuticular abdominal gland. The present investigation under Scanning

electron microscopy explained the occurrence of cuticular abdominal gland, secretory vesicles, secretory cells and secretions (Fig.4). The secretory cells had been deeply stained with Haematoxylin-Eosin and Toluidine blue. Significant amount of protein was noticed in the secretory cells, when compared to vesicles and glandular region.

Electron microscopic studies of cuticular abdominal gland revealed that the larger cells were noticed in the ventral part. The size of the nucleus was observed to be large round in shape. The nucleus is usually located in the basal region (Fig.2 and 5). The cells had a characteristic layer of microvilli just beneath the endo-cuticle. The endo-cuticle and the epi-cuticle had lamellae. The epi-cuticle was seemed to be denser than that of the endo-cuticle. During photo phase, the cells of cuticular abdominal gland became highly vesiculated with smaller vesicles that accumulated just below the microvilli (Fig.3). Thin layers of electron opaque material were noticed at the tips of the microvilli next to the endo-cuticle. No obvious Golgi bodies were observed in those cells. During scotophase,(*i.e.,* dark phase) pockets of granular material were appeared throughout the cytoplasm of each cell and also noted within the microvilli **(**Fig.5**).** The cytoplasm was very rich in rough endoplasmic reticulum and mitochondria. In addition, intercellular canals with distinct desmosomes were of common occurrence. No electron opaque band between the microvilli and the endo-cuticle was present in scotophase cells.

Finally the cuticular hairs were examined, which revealed that the extensions of the epicuticle had a hollow core and many pore canals (Fig.4). Short-filamentous material also appeared in the central cavity of the cuticular hairs and in the pore canals (Fig.4). In narrow folds of the cuticle, the cytoplasm, particularly the apical microvilli, was found in the cavity formed by the endo-cuticle (Fig.4).

The narrow cells of the gland also had prominent nuclei. Basal portions of these cells showed invaginations that appeared to arise from the basement membrane and continued as inter an intracellular canals towards the apex of the cell. Some of these canals contained electron opaque granules that appeared to accumulate towards the apex of the cell (Fig. 5). The cytoplasm of these cells had numerous mitochondria and free ribosomes and a few lipid droplets besides a number of autophagic bodies or lysosomes. Apically the cells exhibited the layer of microvilli, similar to that in the larger cells. In scotophase, numerous desmosomes associated with the intracellular canals became prominent (Fig.5).

Fig. 1. The Haematoxylin and Eosin straining of the cuticular gland are identified in the abdominal region of female American Cockroach. The panel A shows the microscopic view of the entire gland and the secretory vesicles of cuticular abdominal gland are noticed on the peripheral region with a cluster of glandular tissue which effectively uptakes the dark pink colour strains. By contrast, the ducts of the glands are opaque in nature. The secreted materials are accumulating on the middle lumen in the form of dense granules with the lipid droplets like vacuoles. The second panel (B) makes it crystal clear that the secretory vesicles have batches of granulated cells with proper path for the release of compound from the gland to the environment through the cuticle. Magnification power is 400X

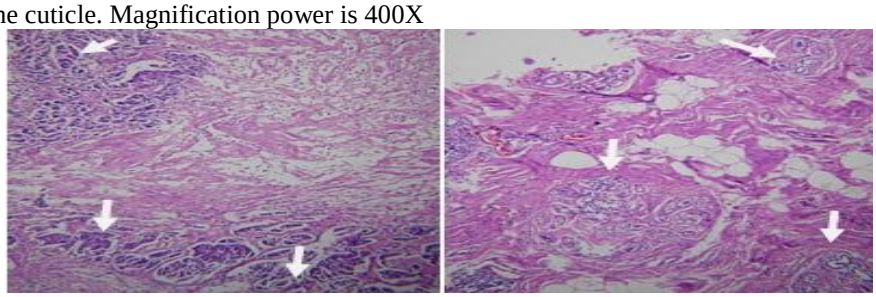


Fig. 2. Ultra-thin sectioning and triple staining with Haematoxylin-Eosin and Toluidine blue to identify the granular matters of secretory vesicular cells present in the cuticular abdominal gland of American cockroach. This light micrograph showed two different types of cells in a pocket like structures where the pheromones are believed to be stored in the cuticular abdomina1 gland of female cockroach.Magnification power is 400X

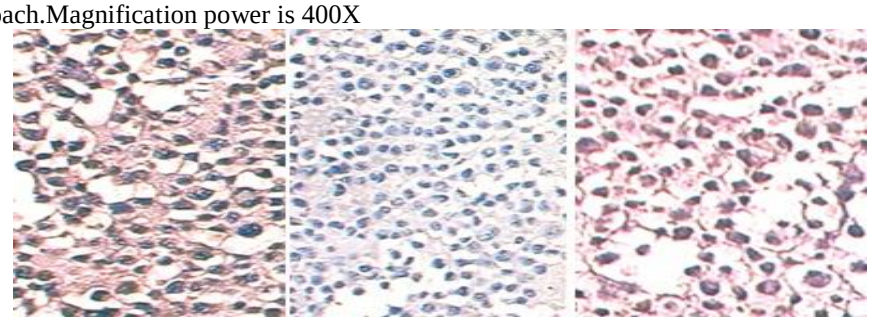


Fig. 3. Histological analysis of the two distinguished regions of the cuticle reveals that the epi cuticle did not contain the granulated substance and the endo cuticle regions found to have rich amount of the granulated substances. Further, the black colour arrow pin-point the secretory path in the apical region as well as in the middle part of the secretory endo cuticle. The white colour arrow demote the secreted substances which were found to be rich in endo cuticular region (Fig.B) than that of the epicuticular region (Fig.A)Magnification power is 400X.

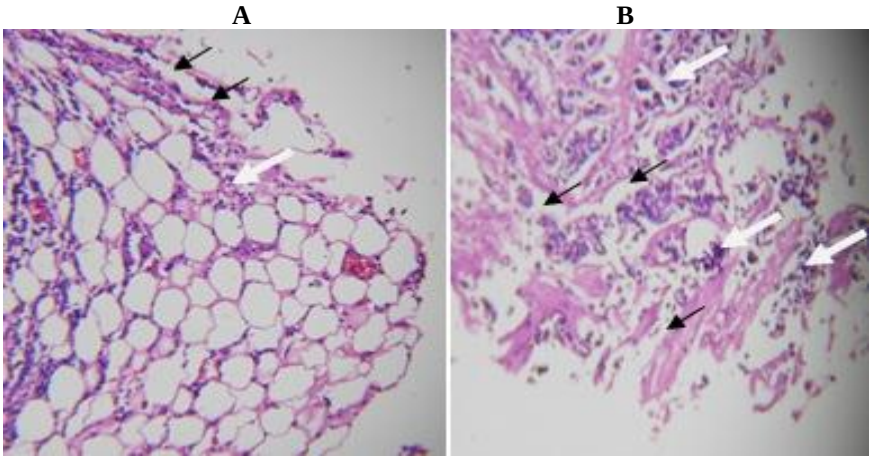


Fig. 4. The Scanning Electron Micrograph of the cuticular abdominal gland of female American Cockroach. The cuticle surface is fully occupied by the lipid droplets which are suspected as pheromone sources in lower form of organisms like insects. Magnification power is 70,000X.

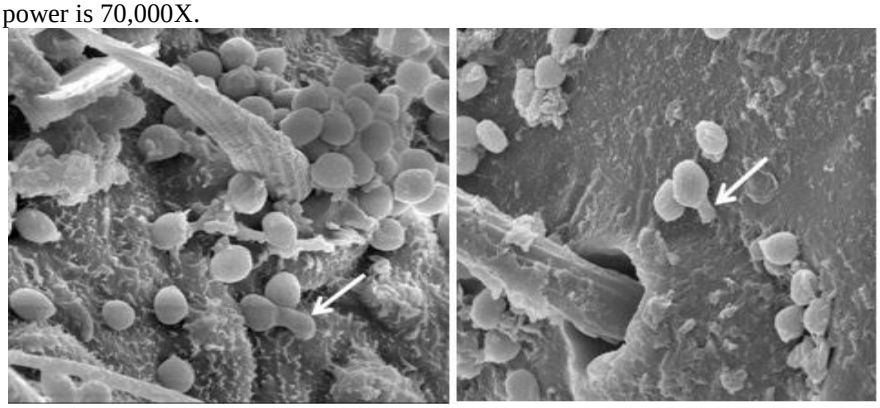
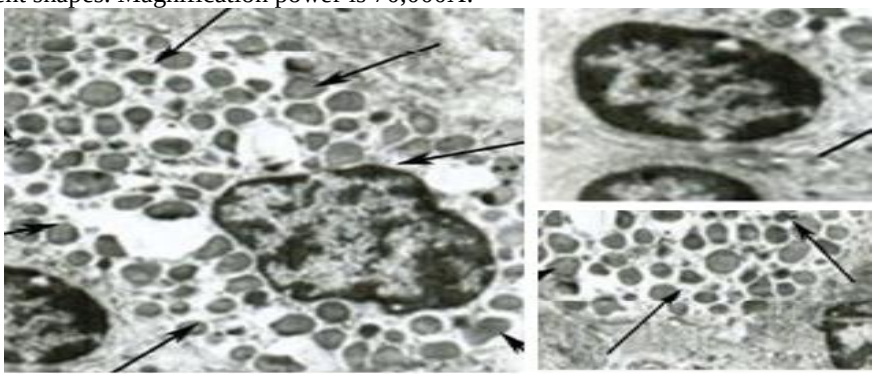


Fig. 5. Transmission Electron Micrograph of the secretory cells in cuticular abdominal glands of female American cockroach reveals the occurrence of prominent nucleus with secretory substances. The First showed the occurrence of amorphous granules with prominent nucleus. In the nuclear region, several condensed chromatin regions were localized in the form of dense regions in the nucleoplasm. Interestingly, the granules are observed to be in a polymorphic condition. This may be due to the existence of the pheromonal compounds in different intermediates stages of their biochemical pathway. Hence, they look like in different shapes. Magnification power is 70,000X.



DISCUSSION AND CONCLUSION

Most studies on the ultra-structure of pheromone glands in Lepidoptera described only one type of glandular cell. Hallberg and Subchev (1997) reported two cell types in the moth *Theresimima ampelophaga*, namely gland cells and wrapping cells. In American cockroach, we also observed two distinct types of glandular cells that shared common features; the apical microvilli and pockets of granular material. Microvilli containing a core of smooth endoplasmic reticulum have been reported in *C. fumiferana* (Percy., 1974). Extensive basal invaginations were also reported in pheromone gland cells of *T. ampelophaga* (Hallberg and Subchev, 1997). Lipid spheres of varying sizes have been reported in the cytoplasm of pheromone gland cells in *C. fumiferana* (Percy, 1974), and *Argyrotaenia velutinana* (Feng and Roelofs, 1977). Our findings are in agreement with the previous reports to justify the occurrence of lipid droplets and granulated substances in the cytoplasm of pheromone secreting cells.

The histological analysis of cuticular abdominal gland of female cockroach shows the secretory vesicles, secretory cells and secretion. The epidermal cells show glandular cells which secrete granulated substances and there are some special gland cells among the endodermal layer of cells. The endodermal glandular cells are supposed to have the function of secretion of pheromonal component which is responsible for sexual organization in female cockroaches. Histological and ultra-structural studies have revealed the presence of a variable and complex structure of epidermis in insects. In these organisms, the epidermis consists of a mono layered epithelium containing glandular, supporting ciliated and sensory cells. The glandular cells secrete mucus.

In many invertebrates, especially the Lepidopteron female moths secrete species-specific sex pheromones which are biosynthesized in pheromone glands namely modified inter-segmental membrane that resides between the 8th and 9th abdominal segments in many Lepidopteron species (Percy-Cunningham and Donald., 1987; Bendib and Minet, 1998). However, knowledge of the universality and diversity in structures of the pheromone glands is limited (Raina *et al*., 2000). Besides the glands, or more precisely pheromone-producing regions, are generally difficult to isolate, pheromone analyses are routinely performed using isolated whole terminal abdominal segments (Fukuzawa *et al*., 2006).

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