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Research article

STUDIES ON THE MALE ACCESSORY SEX GLANDS AND EFFECT OF JUVENILE AND ECDYSONE HORMONE ON THE AQUATIC BEETLE, *CYBISTER TRIPUNCTATUS (OL.)*

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ABSTRACT: In *Cybister tripunctatus* male accessory sex glands (MASG) are paired, long, coiled and tubular structures extending into the abdominal cavity and open into the lateral ejaculatory duct of its own side. The wall of the MASG is composed of a layer of tall columnar epithelial cells and the lumen is filled with granular secretory material. The histochemical studies revealed the presence of abundant quantity of DNA in the nuclei, RNA in the nuclei and cytoplasm suggesting protein synthesis in the epithelial cells of MASG in adult male. The histochemical tests suggested that the secretory material accumulated in the cells and lumen of the MASG is a mixture of protein, carbohydrate and lipid. The biochemical analysis showed that the secretory material of MASG composed of large quantity of protein and little amount of carbohydrate and lipid. The SDS – PAGE separated in all 17 protein bands ranging from 18.5 to 205.000 in molecular weight. Tropical application of JH-III on the mature male caused significant increase while that of β - ecdysone showed reduction in the total protein concentration of MASG.
Key words – *Cybister tripunctatus*, juvenile hormone, ecdysone, male accessory sex glands

INTRODUCTION

The structure mechanism and functions of the male accessory sex glands (MASG) have been reviewed [1,2,3,4]. The male accessory glands secrete the secretion for the production of spermatophore, seminal fluid, activation of spermatozoa stimulation of oviposition and antifedent material [2,5].The presence of protein and carbohydrate as major component of accessory gland secretion has been reported in accessory gland of many Coleopteran insects [6,7,8]. The chemical nature of MASG secretion in insects has been analyzed as the mixture of proteins, mucopolysaccharides and lipids [2, 4]. The protein pattern of the MASG secretion has reported [9, 10]. Protein pattern with large number of protein band have been observed in extracts of accessory glands of Coleoptera [11]. The present investigation deals with structure, analysis of chemical nature and the effect of JH and ecdysone on MASG in the dytistid beetle, *Cybister tripunctatus.*

MATERIAL AND METHODS

Large numbers of beetles were collected from the local ponds and were acclimatized in the small cemented tank in the premises of the PGTD of Zoology, Nagpur (India). The adult males were separated and used for the present study.

Histology

The MASG were dissected in saline from the adults, fixed in aqueous Bouin's fluid for 18-24 hrs at room temperature, were dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin. Sections were cut at 4 μm and stained with Heidenhain's iron haematoxylin-eosin.

Histochemical methods

The glands were fixed in Carnoy's fixative or 10% formalin for 6-8 h, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax (60-62^{0}C). 4 µm thick sections were treated with Feulgen reaction for DNA, Brachet's Toluidine blue test for RNA, Mazia et al., mercury bromophenol blue (Hg-BPB) reaction for protein and Hotchkiss's periodic acid Schiff's reagent (PAS) techniques for carbohydrates. For lipid, Chieffelle and Putt's Sudan black-B (SBB) techniques was applied on the 10% calcium formol fixed frozen sections [12].

Biochemical estimation

The MASG were removed from the adults. The MASG were separated, tracheae and fat body were removed and homogenized at 0^0C for 5 min in different volumes of ice-cold distilled water, Ringer's solution and 0.25 M sucrose solution separately. For lipid, the MASG were homogenized in 20 ml of chloroform, methanol (2:1 v/v) containing 0.01% butylated hydroxyl-toluene (BHT) as an antioxidant and centrifuged at 3000 rpm for 15 min. Total concentration of DNA and RNA was estimated by Burton's Diphenylamine [13] and dische-Orcinol [14] methods respectively. The procedure of Lowry et al.[15] was followed for the estimation of total protein. Total lipid was estimated using the method of Frings and Dunn [16]. The method of Dubois et al. was used for the estimation of total carbohydrates [17].

Electrophoresis

The 1 mm 3% stacking gel (pH 6.8) was followed by a 10 cm 10% separating gel (pH 8.8) with 1% SDS. MASG from adult male beetles were dissected out and cut into pieces, homogenized and centrifuged as mentioned above and the supernatant was used as the sample. 50 µl of clear supernatant was mixed with 50 µl of treatment buffer (Tris-2.5 ml, pH 6.8, SDS-4 ml, Glycerol-2 ml, 2-Mercaptoethanol-1 ml, distilled water-0.5 ml and a pinch of Bromophenol blue).The samples were heated for 5 minutes in a water bath. The mixture was cooled and its 20 µl , 30 µl , 35 µl and 40 µl was loaded in each well of the stacking gel with a micropipette. Standard wide range molecular weight marker protein was also run together. The gel was stained with Coomassiv brilliant blue for 2 hrs and destaining was done with a mixture of methanol-acetic acid-distilled water until the bands on gel became clear. The molecular weight of the protein bands with regard to the marker proteins was estimated with the help of the Densitometer [18].

Application of JH III and β-ecdysone

In order to study the hormonal control of accessory gland secretory activity, the 10 mg JH III and 1mg β-ecdysone (Sigma, U.S.A.) dissolved separately in 1 ml of cold acetone were topically applied to three groups of newly emerged male beetles *Cybister tripunctatus* with the help of Hamilton's CR- 700 constant rate syringe (USA) . The groups of treated adults were sacrificed after interval of 1hr, 2hr, 4hr, and 6hr respectively. Beetles of first group were treated with equal quantity of cold acetone along and were served as the control insects while the second group with JH III and third with β-ecdysone. The Male accessory glands were homogenized separately and the supernatant was used for the estimation of total protein concentration of MASG.

RESULTS

Histology

The MASG are paired, 21.4± 0.8mm long, coiled and tubular structures. They open into the lateral ejaculatory ducts. Each accessory gland is divisible into two parts, anterior bean shaped reservoir and posterior long coiled tubular part (Fig.1). The wall of the MASG is composed of an outer circular muscle layer and an inner layer of epithelium. The circular muscle layer is thinner in tubular part of MASG. The epithelium consists of the tall columnar cells with prominent nuclei and dense cytoplasm. The nuclei are elongated and measure about 12μm in diameter. The secretion of the epithelial cells is discharged into the lumen in the form of fine secretory droplets. Internal surface of the epithelium is devoid of cuticular intima suggesting the MASG as the mesadenia (Fig 2. A, B)

Histochemistry

Intense reaction in the nuclei of the epithelial cells was observed after the Feulgen and Toluidine blue tests suggesting presence of abundant quantity of DNA and RNA (Fig 2. C, D). The cytoplasmic inclusion in the epithelial cells as well as the secretory material in the lumen was reacted intensely with the Hg-BPB, PAS and SBB tests, suggesting protein, carbohydrate and lipids in the secretory material of MASG (Fig 2.E, F).

Biochemical observations

The total concentration of protein, carbohydrate and lipid in MASG extract of adult male were analysed (Table 1). The biochemical analysis suggests protein and carbohydrate to be a major component of the accessory gland secretion in *Cybister tripunctatus*.

Electrophoretic separation of proteins

SDS-PAGE analysis of the MASG secretions showed separation of 24 protein bands ranging from 18.5 to 205.000 in molecular weight (Fig.3). Among these, six proteins of 18.5, 37.1, 41.4, 48.3, 91.5 and 205 kDa molecular mass were stained intensely representing major class of MASG proteins distinctly.

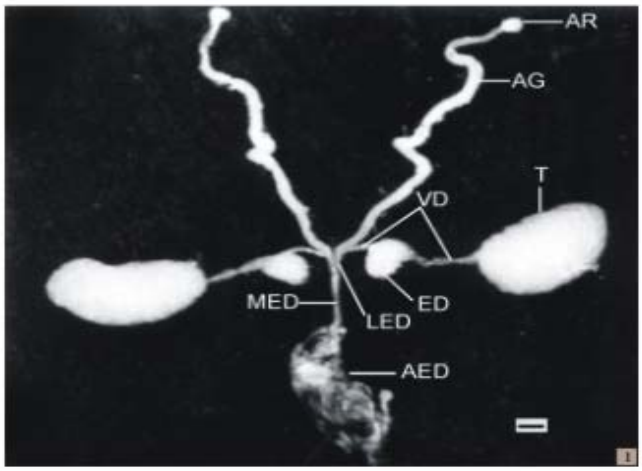

Figure 1:- Male reproductive system (Bar - 1 mm)

Abbr.
AED - aedeagus, Ag - accessory gland, Ar - accessory reservoir, ED - epididymus, LED - leteral ejaculatory duct, MED- median ejaculatory duct. T - testis, VD - vasa deferentia

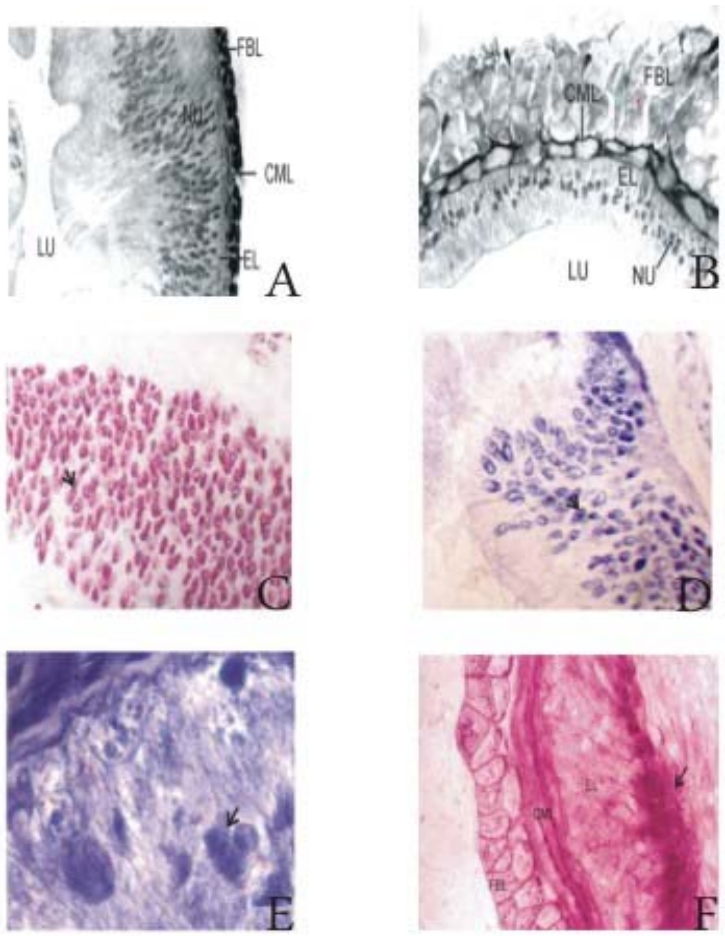

Figure 2 :- Histological and Histochemical staining of the transverse section of male accessory sex gland.
A, B - Histological staining of accessory gland and reservoir showing histological structures, FBL- Fat body layer, NU- Nucleus, CML-Circular Muscle Layer, EL - Epithelial Layer, LU- Lumen, X 200 FeH-E;
C - Feulgen reaction showing DNA (arrow) X300;
D - Toluidine blue showing RNA (arrow) X300;
E - Mercury bromophenol blue showing protein (arrow) X640;
F - Periodic acid schiffs showing carbohydrate (arrow) X300

Application of JH-III and β-ecdysone on protein concentration

The topical application of JH-III caused significant rise in the concentration of protein in MASG than that in the control insects after a period of 1hr, 2hr, 4 hr and 6hr (Table 2). No change in total protein concentration was observed in control insects, the elevated concentration of total proteins in the JH III treated insects after 4hr and 6hrs remained constant. On the other hand treatment of β- ecdysone resulted significant reduction in protein concentration after 1 hr, 2hr, 4hr and 6 hr from that in the control insect.

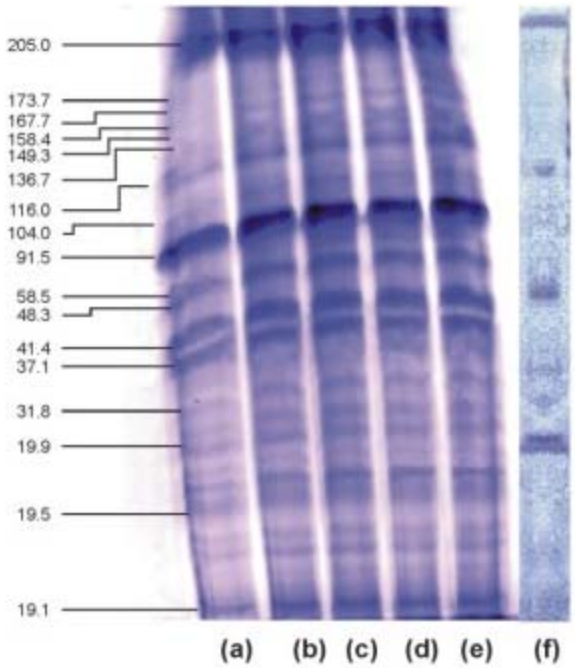

Figure 3:- SDS-PAGE analysis of various protein bands of the male accessory sex gland: (MASG) during (a) February, (b) April, (c) May (d) June, (e) July and (f) standard molecular weight marker proteins (205-6.500kD).

Table 1. Biochemical analysis of MASG (adult)

Substances	Concentration ((µg/mg)
Protein (µg/mg)	361.8 ± 18.8
Carbohydrate (µg/mg)	3.76 ± 7.5
Lipids (mg/100ml)	102.33 ± 4.92

± - SE of mean value

Table 2. Protein concentration (µg/mg)

Treatment	JH III		Ecdysone	
	Experimental	Control	Experimental	Control
1hr	420 ± 0.039	367 ± 8.2	355±0.31	367 ± 8.2
2hr	*425 ± 0.188	371 ± 1.0	*350± 0.078	371 ± 1.0
4hr	*436 ± 0.108	371 ± 1.0	*341 ± 0.31	371 ± 1.0
6hr	*436 ± 0.108	370 ±18.8	*325 ± 0.07	370 ±18.8

*P = 0.05, ± - SE of mean value

DISCUSSION

In the adult male beetle *Cybister tripunctatus* the MASG are evident as pair of long coiled tubular structures composed of columnar epithelium covered externally with circular muscle layer and enclosing internally a large lumen but without internal cuticular intima suggesting their mesodermal origin. Ectadenia and mesadenia are known to occur in polyphagan Coleoptera like *Lytta nuttali* [7] and *Tenebrio molitor* [19, 20, 21], while only mesadenia are reported in *Dytiscus marginalis* [22] and are also found in *Cybister tripunctatus.* The mesadenia in *Cybister tripunctatus* are the tubular glands resembling with the mesadenia in other Coleopteran insects [4, 7, 19, 20, 21]. The male accessory sex gland of the mesodermal origin is also observed in many insects [2].

Histochemical reactions demonstrated Hg-BPB positive proteins, Pas positive carbohydrates (muco-polysaccharides) in epithelial cells of MASG of *Cybister tripunctatus* indicating homogeneous mucopolysaccharide –protein mixture, where protein and carbohydrate appear to be a major component. Mucopolysaccharide – proteinaceous secretion of male accessory sex gland is also reported in *Schistocerca gregaria, Locusta migratoria* and *Glossina*, [23,24,25] and *Apis cerana indica* [26]. Their presence has also been reported in the secretion of accessory gland of many coleopteran insects [6, 7, 8, 27].

The biochemical analysis showed that proteins are the major constituent of the secretions, while carbohydrates and lipids make smaller contributions to the MASG secretory material of *Cybister tripunctatus* and thus supporting the observations of [28] for *A. mellifera.*

The MASG secretion containing large number of proteins has been reported in Lepidoptera i.e., 49 to 50 in *Spodoptera litura* [10], 32 in *Opisina arenosella* [29] and 19 bands in *Antheraea mylitta* [30]. Electrophoretic separation of MASG in *Cybister tripunctatus* showed in-all 17 distinct protein bands varying from each other in their molecular weight suggesting multifunctional role of accessory gland secretion such as spermatophore formation, sperm nourishment and provision of nutrients to the female to modify female fecundity and receptivity [4]. The MASG secreting a large number of proteins and performing various functions has been investigated by several workers [2,4,31,32,33].

Topical application of juvenile hormone caused stimulation of secretory activity in epithelial cells and showed significant rise in protein in MASG of *Cybister tripunctatus* supporting the earlier workers suggesting stimulatory effect of JH III on protein synthesis [27,34,35,36,3738]. In *Oryctes rhinoceros, in vitro* development and secretory activity of male accessory gland occurred after the corpus allatum added to a culture [39].

In *Cybister tripunctatus,* β-ecdysone exerted an inhibitory action on MASG. In *Tenebrio molitor* and *Bombyx mori,* ecdysteroids were found stimulating the development of MASG at the pupal stage but acting adversely during the adult stage [40, 41]. Male accessory gland rudiment in *Oryctes rhinoceros* showed only proliferation of cells *in vitro* in presence of 20-hydroxyecdysone but no tubular development of the glands and thus indicating an inhibitory action [42].

The present observation, therefore, strongly suggest that the JH is stimulating while the β-ecdysone is inhibiting the secretory activity of MASG in the dytistid beetle, *Cybister tripunctatus.*

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