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Qualitative Analysis of Accessory Glands Secretory Proteins in Different Species of *Drosophila*

Suhasini L. Kudupali[1], Shivanna N.[2,*]

[1]Department of Zoology, Karnatak University, Dharwad, India
[2]Department of Applied Genetics, Karnatak University, Dharwad, India
*Corresponding author: drnshivanna@rediffmail.com

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Abstract Qualitative analysis of male accessory gland secretory proteins in 11 species of *Drosophila* was analyzed using SDS-PAGE technique and their molecular weight was calculated with the help of marker proteins, the molecular weight ranges from 12-134 kD. The accessory gland secretions of *D. virilis* and *D. hydei* reveal more number of protein fractions. *D. rajasekari* showed less number of ACP fractions. Out of 14 protein fractions of *D. virilis* 5 were glycosylated whereas in *D. hydei* none of the fractions were glycosylated. In *D. rajasekari* 2 fractions were glycosylated. In *D. bipectinata, D. malerkotlina* and *D. ananassae* even though the accessory glands produced 7, 8 and 11 protein fractions only one protein fraction is glycosylated. These results showed that the number of ACP fractions and their glycosylation is species specific.

Keywords: *drosophila, accessory gland, qualitative analysis, CBB stain, glycosylated fractions, PAS stain*

1. Introduction

The accessory glands arise from a special set of cells in the male primordium of the genital disc whose developmental fate is determined by the male sex determination pathway during the third instar larvae. It plays an essential role in insect reproduction. Insect have yielded a variety of basic information about ultrastuctural, physiological and biochemical analysis. In many insects the main function of accessory gland is the production of spermatophore for sperm transfer from male to female genital tract during mating [1,2].

Accessory glands secretory protein (ACP) plays an important role in fertile reproduction. ACP's are major components of *Drosophila* seminal fluid. The seminal fluid of *Drosophila melanogaster* contains over 80 proteins and peptides, which are transferred along with sperm from male to female during mating [3]. Seminal proteins induce morphological and behavioural changes in mated females.

Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) is a relatively simple rapid low cost and high sensitive tool used for the separation of proteins and to determine their molecular weight. The separation of proteins by electrophoresis is based on the fact that charged molecules will migrate through a matrix upon application of an electrical field. It is a well known fact that proteins are dissociated into their constituent polypeptide chains by detergents like SDS. SDS is a denaturing and solubilizing agent; induce conformational changes in the proteins [4].

The SDS-PAGE analysis and quantity of proteins ejected during mating has been analyzed in *D. melanogaster, D. n. nasuta, D. s. neonasuta, D. rubida, D. pararubida, D. varians* and *D. ananassae* [5,6]) and also qualitative and quantitative correlations of male accessory gland secretions has been analysed in *D. nasuta* sub group [7].

Periodic Acid Schiff (PAS) staining is widely used in the detection of glycoprotein. Glycoprotein plays an important role in numerous developmental and physiological processes, especially in the nervous system. The glycosylated protein fraction varies in different species of *Drosophila* [5,8].

In spite of more than 110 species in *melanogaster* species group, which consists of sibling, sympatric and closely related species, the information about ACP is available only of *D. melanogaster*. Therefore to know the nature and characteristics of ACP in different species, species belonging to various groups/ subgroups of *Drosophila* were taken for the present study.

2. Materials and Methods

2.1 Fly stocks

D. melanogaster, D. simulans, D. mauritiana, D. ananassae, D. varians, D. bipectinata, D. malerkotliana and D. rajasekari, belonging to melanogaster species group, D. virilis and D. texana, belonging to virilis species group and D. hydei belonging to repleta species group were obtained from Drosophila stock centre, Department of Zoology, University of Mysore, Mysore, India for the present work. The stocks were maintained using wheat cream agar medium and prepared as per the procedure described by Shivanna et al., [9]. The cultures of Drosophila were maintained at a constant temperature of 22 ± 1℃ and humidity of 70-80%.

2.2 Protein Sample Preparation

The virgin male and female flies were separated within 1 hour of their eclosion from the cultures and they were kept in a separate vial and aged for 7 days. After 7 days the flies were etherized and accessory glands from adult male were dissected in physiological saline (0.7% of NaCl). Glands were isolated and fixed in 95% ethanol. The ethanol fixed glands were washed in a mixture of methanol and chloroform (1:1) and dried at 37℃ for 15 minutes. The sample buffer about 40 µl (0.623 M Tris-HCl, pH 6.8, 1% sodium dodecyl sulphate (SDS), 1% β-mercaptoethanol, and 10% glycerol) was added to each sample to dissolve the glands. The eppendorf tube containing the sample was boiled for about 10 minutes in boiling water bath and centrifuged at 1000rpm for 10 minutes.

2.3 Electrophoresis of Protein Sample

SDS polyacrylamide gel of (T=13.7% C=3.5%) 1.5mm thickness was prepared. 40µl of protein sample was loaded separately into each slot of the stocking gel (3.8 ml of distilled water, 3.8ml of 0.5M Tris HCl, pH 6.8, 118µl of 20% SDS, 3.8ml 0f 22% acryl amide, 100µl of 10% APS and 8µl of TEMED). Electrophoresis was performed at room temperature with 60V for 1 hour. Later it was increased to 80V until the dye migrated to 7cm in the running gel (16.8ml of distilled water, 6.9ml of 1.5M Tris-HCl pH 8.8, 208µl of 20% SDS, 16.8ml of 22% acryl amide, 184µl of 10% APS, 18.4µl of TEMED). The gel was then removed and stained with Coomassie Brilliant Blue R-250 for three hours and destained in a mixture of 25% methanol and 7.5% acetic acid.

2.4 Staining of Glycoproteins

To identify the glycosylated protein fractions of the ACPs, the gel was stained employing the procedure described by Segrest and Jackson [10]. The gel was fixed overnight in PAS fixative solution (40% ethanol, 5% glacial acetic acid and 55% distilled water). The gels were then treated with periodic acid (0.7%) solution covering the gels for 2-3 hours. This was followed by treatment with sodium metabisulfate (0.2%) with one solution change after 30 minutes, and then the gels were transferred to Schiff's reagent. Colour develops in 12-18 hours at room temperature and thereafter the gels were stored at 4℃ and photographed.

Molecular weight of each protein fractions was calculated using the marker proteins (phosphorylase-97,400 Da, Bouines serum albumin-66,000 Da, ovalbumin-43,000 Da, Carbonic anhydrase-29,000 Da, Soybean Trypsin Inhibitor-20,100 Da, Lysozyme-14, 300 Da) with the help of log graph.

3. Results

Figure 1 shows the SDS-PAGE patterns of accessory gland protein in different species of *Drosophila*. Table 1 embodies the number of major and minor accessory gland protein fractions, less than and more than 50 kD fractions. The molecular weight of each accessory gland protein fractions in different species of *Drosophila* were recorded along with glycosylated fractions (Table 2).

Table 1. Major and minor accessory gland protein fractions in different species of *Drosophila*

Species	Number of ACP fractions		> 50 KD fractions	< 50 KD fractions
	Major	Minor		
D. melanogaster	7	4	6	5
D. simulans	3	8	4	7
D. mauritiana	3	7	3	7
D. ananassae	4	7	5	6
D. bipectinata	2	5	2	5
D.malerkotliana	3	5	2	6
D. virilis	6	8	4	10
D. texana	4	5	3	6
D. hydei	8	6	6	8
D. varians	7	6	4	9
D. rajasekari	2	3	2	3

Table 2. Molecular weight of accessory gland protein fractions in different species of *Drosophila*

D. melanogaster	134	69	**68**	**62**	55	52	**39**	28	26	**20**	**18**	-	-	-
D. simulans	89.1	75	**68**	59	46	38	34	28	26	24	**19**	-	-	-
D. mauritiana	97	**71**	**58**	38	32	30	28	26	24	**20**	-	-	-	-
D. ananassae	97	83	73	65	52	39	27	23	**21**	19	18	-	-	-
D. bipectinata	72	62	49	29	26	**21**	19	-	-	-	-	-	-	-
D.malerkotliana	61	59	45	33	28	27	**22**	20	-	-	-	-	-	-
D. virilis	81	**66**	**60**	54	46	43	38	33	**29**	**28**	**22**	20	18	17
D. texana	113	66	61	**39**	33	**27**	**25**	22	12	-	-	-	-	-
D. hydei	126	89	84	72	64	58	40	38	30	26	24	22	18	13
D. varians	97	74	68	59	46	40	32	**28**	**24**	**22**	21	18	17	-
D. rajasekari	100	**63**	29	**22**	10	-	-	-	-	-	-	-	-	-

Bold number indicates PAS positive protein fractions

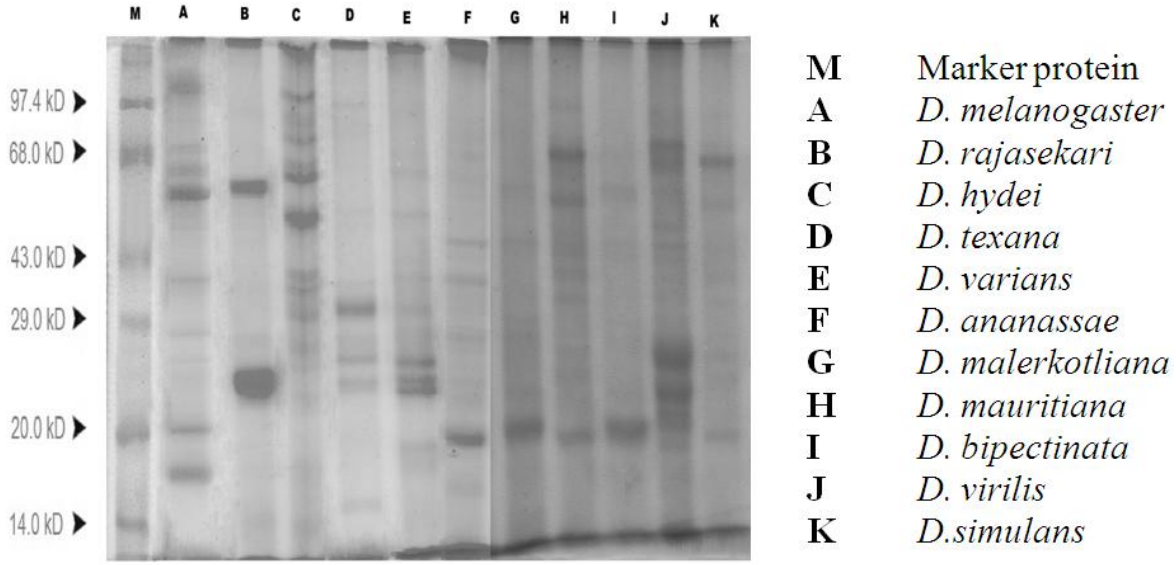

Figure 1. Coomassie Brilliant Blue stained SDS-PAGE patterns of accessory glands secretory proteins in different species of *Drosophila*

Among the sibling species, the ACP in *D. melanogaster* has composed of highest number of major protein fractions (7) with molecular weight of 134, 69, 68, 62, 39, 20 and 18 kD. In *D. simulans*, the secretions are composed of 3 major fractions having molecular weight 68, 59 and 19 Kd, and *D. mauritiana* is composed of three major protein fractions with molecular weight of 200, 71 and 58 kD.

Among sympatric species (*D. ananassae, D. bipectinata, D. malerkotliana* and *D. rajasekari*), it was found that the proteins of *D. bipectinata* composed of 2 major protein fractions with molecular weight of 62 and 21 kD. *D. ananassae*, consists of 4 major ACP fractions with molecular weights of 52, 39, 27 and 21 kD and *D. malerkotliana* is composed of 3 major protein fractions with molecular weight of 61, 59 and 22 kD and *D. rajasekari* has 2 major fractions with molecular weight of 63 and 22 kD. *ananassae* subgroup species, *D. varians* contains 7 major ACP fractions with molecular weights of 68, 59, 40, 32, 28, 24 and 22kD.

Among the closely related species *D. virilis* is composed of 6 major protein fractions with molecular weight of 66, 60, 29, 28, 22 and 20 kD. *D. texana* has 4 major protein fractions with molecular weight of 39, 27, 25 and 12 kD. *D. hydei* is composed of 8 major protein fractions with molecular weight of 126, 89, 84, 72, 64, 58, 40 and 38 kD.

Glycoprotein stains pink with fuchsin on a clear background. Out of 11 fractions 5, 2 and 1 protein fractions are glycosylated in *D. melanogaster, D. simulans* and *D. ananassae* respectively. In *D. mauritiana* out of 10 only 3 fractions are glycosylated. In *D. bipectinata* and *D. malerkotlina* only one fraction is glycosylated out of 7 and 8 fractions. Out of 14, 5 are glycosylated in *D. virilis*. In *D. texana* and *D. varians* 3 fractions, in *D. rajasekari* 2 protein fractions are glycosylated whereas in *D. hydei* none of the major protein fractions are glycosylated (Table 2 and Figure 2).

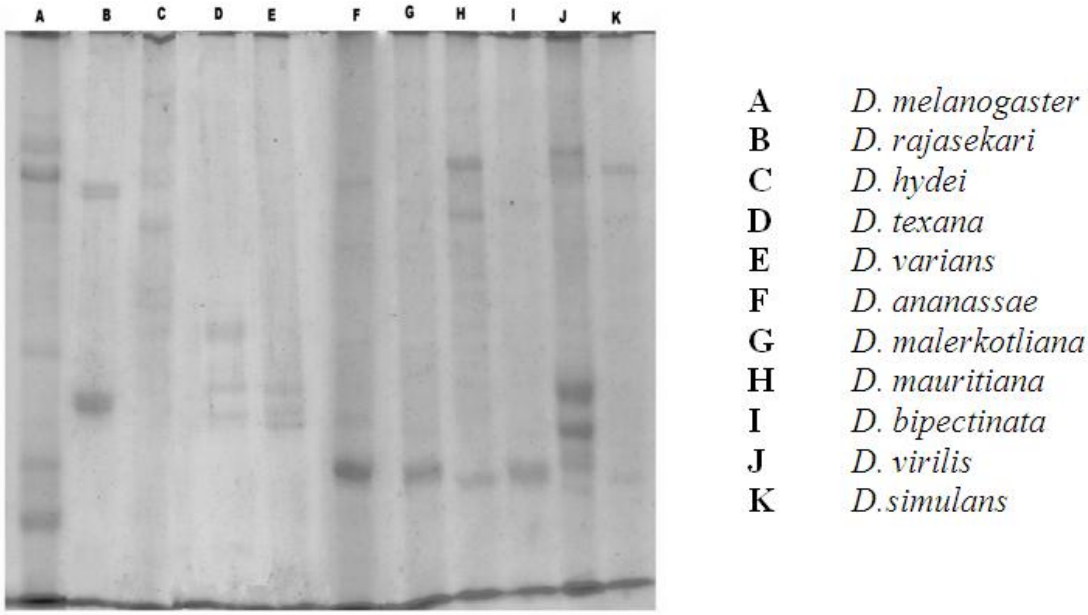

Figure 2. Periodic acid-Schiff stained SDS-PAGE patterns of accessory glands secretory proteins in different species of *Drosophila*

4. Discussion

Drosophila has been used as a model organism for over a century. It is the most valuable organism in biological research, particularly in genetics, developmental biology and reproductive biology. Reproductive system of male *Drosophila* accessory gland is a bilobed structure composed of a single layer of cell surrounding a lumen [11]. Newly emerged male requires 12 hours to synthesize their glandular secretions. In *D. melanogaster* the accessory gland secretory proteins have been found in glandular lumen even in the pupae [12]. The reproductive tract tissue of male produces seminal fluid and it is transferred to female during mating, it induces numerous physiological and behavioral changes like, decreased receptivity to remating, affecting sperm storage parameters etc., [13]. For maximum synthesis and accumulation of secretion the accessory gland requires 7-15 days depending on the type of species, therefore for the present investigations 7 days aged flies were used. Whereas for *D. virilis, D. hydei* and *D. texana* 15 days old flies were used [14]. Accessory gland secretion is generally composed of carbohydrates, lipids, amino acids and amines. The major components of accessory gland secretion are proteins [15].

The secretory proteins of *D. melanogaster* on 7.5 % and 10% SDS polyacrylamide gel yielded a minimum of 12 and more than 40 fractions with molecular weight ranging from 12 to 175 kD respectively [1]. In *immigrans* species group, *D. n. nasuta* consists of 10 fractions with molecular weight ranges from 14 to 120 kD, in *D. s. neonasuta* one more fraction with molecular weight of 92 kD, *D. rubida* and *D. pararubida* accessory gland secretion comprises of about 8 and 9 fractions with molecular weight ranges from 12 to 90 kD [5]. *D. nasuta* sub group species consists of 4 to 18 major protein fractions. Based on their mobility, the major protein fractions are categorized into 3 groups and their molecular weights ranges from 14 to 92 kD [16]. *D. ananassae* accessory gland secretory protein consists of 12 fractions with molecular weight ranges from 15 to 200 kD. In *D. n. nasuta, D. s. neonasuta, D. rubida* and *D. pararubida* the low molecular weight fractions are not glycosylated [5].

Among the *melanogaster* species group *D. rajasekari* has lowest (5) whereas *D. varians* has highest (13) number of protein fractions. *D. virilis* has the highest (14) and *D. texana* has lowest (9) protein fractions which belonging to *virilis* species group. *D. hydei* has highest (14) number of fractions. Species like *D. melanogaster, D. simulans* and *D. ananassae* has similar (11) number of protein fractions which belongs to same group, whereas *D. virilis* and *D. hydei* belongs to different group has similar (14) number of protein fraction, this indicates that the number of fractions is not similar in species belonging to same group or different group. Though the number of fractions is similar in species belonging to same or different group their molecular weights are different. Thus there is no uniformity in number of fractions and their molecular weights in different species. The glycosylated fractions among all the species is also not similar it ranges from 0 to 5 in different species. Earlier reports and the present study on 11 species of *Drosophila* revealed that the variations in molecular weight, number of fractions and their glycosylation are unique to the species and this shows that, they are species specific.

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