Male accessory gland secretory proteins in *nasuta* subgroup of *Drosophila*: Nature and SDS-PAGE patterns

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Received 16 February 1999; revised 21 April 1999

Male accessory gland secretory proteins in seven members of *Drosophila nasuta* subgroup were analyzed by SDS-PAGE in combination with different staining techniques such as CBB-R250, Silver, PAS, PAS-silver and zinc-imidazole reverse staining. Based on coomassie blue patterns the protein fractions could be classified in to 3 major groups namely group I, group II as well as group III: with high molecular weight fractions falling into group I and low molecular weight fractions into group III. All the three groups of fractions are post-translationally modified by way of glycosylation and group III fractions are found to be highly glycosylated. Fractions of groups I and II when localized with silver stain and group III fractions when localized with PAS-silver stain appear yellow; suggesting that they are sialo-glycoproteins. A 40 kD fraction of group II shows differential staining property with zinc-imidazole stain in closely related species namely *D. n. nasuta* and *D. n. albomicans*. Analysis of this protein fraction in F1 males of an interspecific cross revealed that it is synthesized by X-chromosomal gene.

SDS-PAGE has become one of the most widely used techniques for resolving and determining the apparent molecular mass of protein subunits. The SDS solubilizes insoluble proteins, making possible the analysis of otherwise insoluble mixtures. This technique can be combined with a number of other techniques inorder to obtain a variety of important information on the chemical properties of specific protein.

The *nasuta* subgroup of *Drosophila immigrans* group consists of an assemblage of closely related, morphologically almost identical species having different degrees of reproductive isolation\(^5\)^\(^6\). Various members of the subgroup have been analyzed to understand their cytogenetic interrelationships\(^4\)^\(^6\) as well as the extent of chromosomal, isozyme and tissue specific secretory protein differentiation\(^7\)^\(^11\).

The accessory gland of *Drosophila* plays an important role in reproduction. This secretory tissue of the male genital tract provides multiple components to the ejaculate, including short peptides and longer prehormone like molecules with the potential to be cleaved into bioactive peptides\(^12\)^\(^15\) and is a mixture of proteins, aminocids and carbohydrates\(^16\). SDS-PAGE analysis of male accessory gland secretory proteins in *D. melanogaster* has revealed the existence of a complex pattern that could be resolved into 40 fractions in one-dimensional and 85 fractions on two-dimensional gels\(^12\).

A preliminary SDS-PAGE analysis of the accessory gland secretory proteins in only two species of *nasuta* subgroup has revealed that the patterns are much simpler than those of *D. melanogaster*\(^18\). Present investigations, which includes the study of the tissue specific protein namely, male accessory gland proteins were undertaken to analyze their patterns, nature and to localize some of the genes responsible for their synthesis among seven members of *D. nasuta* subgroup.

Materials and Methods

Stocks — For the present study, we have employed seven members of *nasuta* subgroup of *Drosophila* namely *D. nasuta nasuta* (Coorg, India; Stock No. 201.001), *D. n. albomicans* (Okinawa, Japan; Stock No. 202.002), *D. n. kepulauana* (Sarawak, Stock No. 203.001), *D. kohkou* (Thailand, Stock No. 204.001), *D. sulfurigaster sulfurigaster* (Queensland, Stock No. 205.001), *D. s. albosirimata* (Cambodia; Stock No. 207.001) and *D. s. neonasuta* (Mysore, India; 206.001). All these stocks were obtained from Drosophila stock centre, University of Mysore, Mysore. Care was taken to maintain uniformity with regard to space, the density of the populations, amount of food and moisture in the experimental...

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cultures of all the members used in the present analysis. All the experimental cultures were maintained on standard wheat cream agar medium seeded with yeast at 20°C±1°C.

Sample preparation—Unmated males were isolated from the above-mentioned cultures (obtained through synchronized eggs) within 2-3 hr of their eclosion from the puparium. They were transferred to vials containing fresh media and were aged for 7 days. The accessory glands isolated from single individuals were fixed in separate eppendorf tubes containing 95% ethanol. The secretions which were condensed in the form of a plug in the ethanol fixed glands were then isolated by dissecting and discarding the gland tissue with fine entomological needles. These secretions were later washed in a mixture of methanol, chloroform (1:1) and dried at 37°C. 20 μl of sample buffer (consisting of 0.625M Tris HCl pH 6.8, 2% SDS, 5% β-Mercaptoethanol and 10% Glycerol) was then added to each of the tubes containing a pair of secretions and were left overnight for solubilization of sample. Before applying these samples in to the gel slots, they were heated in a boiling water bath for 10 min., cooled and centrifuged for 10 min. at 1000 rpm.

Electrophoresis —13.4% SDS Polyacrylamide gels were prepared (C=3.5%) and the samples were loaded into each slot with Bromophenol blue as the tracking dye. The gel, after electrophoresis was processed to localize the proteins either by Coomassie Brilliant Blue R-250, or silver stain or PAS stain or PAS-silver stain or zinc-imidazole reverse staining. Further, photographs of zinc-imidazole reverse stained gels were taken by following the procedure of Hardy et al. We have used 12.5, 8, 8 and 15 μl of sample for CBB staining, silver staining, PAS-silver staining and zinc-imidazole reverse staining respectively; while secretions from 4 pairs of glands in 20 μl formed the sample for PAS staining.

Chromosomal localization—For the chromosomal localization of major accessory gland secretory protein fractions, virgin females and unmated males were isolated from the cultures of D. n. nasuta and D. n. albomicans within 3 hr of their eclosion from the pupal case. They were aged for 5 days before the reciprocal crosses were conducted to get F1 generation. Unmated F1 males were isolated and accessory gland samples were prepared as mentioned above and electrophoresed along with the samples prepared from the males of parental stocks, for comparison.

Results

The objective of the present study was to analyze patterns and the nature of accessory gland secretory proteins in D. nasuta subgroup by subjecting them to different staining techniques.

CBB Staining — After staining the electrophoresed gels with coomassie brilliant blue, we could measure the OD values of various protein fractions with the help of Bio-Rad Gel Doc 1000, based on which, we could arbitrarily classify them into “major” [peak area (Optical Density/mm) ≥ 0.02] and “minor” (peak area < 0.02) fractions. Further, the major fractions could be distinguished into 3 groups namely group I, II and III with high molecular weight fractions falling into group I and the low molecular weight fractions into group III (Figs 1&2). Table I embodies information with regard to the number of major protein fractions in each species and their approximate molecular weights.

Silver staining — Only group I and group II fractions could be localized by silver staining; while group III fractions could not be localized under recommended conditions (Figs 1&2). Further, it was observed that fractions of group I and II stained yellow with silver.

PAS staining — In order to localize glycoproteins, the gels were processed and treated with Schiff’s reagent. We have found that all the three major groups of protein fractions were PAS positive. Group III fractions stained heavily owing to the amount of protein and the extent of glycosylation; whereas a
reduction in the staining intensity was observed in case of proteins of group I and II (Fig. 3).

PAS-silver staining — By following the double staining method (PAS-silver), we could stain even the group III fractions, which could not be localized by only the silver stain (Fig. 4). In this procedure also, we observed that all three groups appeared as yellow bands.

Zinc-imidazole reverse staining — Very important observations could be made when the gels were subjected to reverse staining. Staining with zinc-imidazole resulted in a complex pattern of white (positive) and transparent (negative) bands. We found that all the three groups of major fractions that stained with CBB were positive with zinc-imidazole except 40 kD fraction in case of D. n. albonicans and 39 kD fraction in D. s. sulfurigaster, D. s. albostrigata & D. s. neonatasu. These fractions stain negatively. Among the four species of frontal sheen complex of D. nasuta subgroup namely D. n. nasuta, D. n. albonicans, D. n. kepulauana and D. kohkoa, the 40 kD fraction stains negatively only in D. n. albonicans while it appears as a white (positive) band in the other three. Further, we could identify large number of negatively stained or transparent bands between Group I and II and in the region above group III fractions on the gel. Four transparent bands (appearing black in Fig. 5) were observed above group II fractions in all the species without any variation in the mobility. In case of D. n. nasuta and D. n. kepulauana a transparent band localized below group II fractions, shows slight variation in mobility. Further, in D. n. kepulauana, D. kohkoa and all the three species of orbital sheen complex namely D. s.

![Fig. 2](image1.png)

![Fig. 3](image2.png)

**Table I**—Accessory gland secretory protein fractions in *Drosophila nasuta* subgroup (Based on Coomassie Brilliant Blue staining)

<table>
<thead>
<tr>
<th>Species</th>
<th>Total no. of major fractions</th>
<th>kD values of secretory proteins of groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I (&lt;100-60kD)</td>
</tr>
<tr>
<td>D. n. nasuta</td>
<td>8</td>
<td>94,92</td>
</tr>
<tr>
<td>D. n. albonicans</td>
<td>7</td>
<td>94</td>
</tr>
<tr>
<td>D. n. kepulauana</td>
<td>6</td>
<td>94</td>
</tr>
<tr>
<td>D. kohkoa</td>
<td>4</td>
<td>90</td>
</tr>
<tr>
<td>D. s. sulfurigaster</td>
<td>7</td>
<td>94</td>
</tr>
<tr>
<td>D. s. albostrigata</td>
<td>7</td>
<td>94</td>
</tr>
<tr>
<td>D. s. neonatasu</td>
<td>7</td>
<td>94</td>
</tr>
</tbody>
</table>

*Polymorphic
sulfurigaster, D. s. albostrigata as well as D. s. neonasuta a transparent band above their respective group III fractions could be localized (Fig. 5).

**Chromosomal localization** — For the chromosomal localization of 40 kD fraction of group II in D. n. albomicans, we took the advantage of cross fertility between D. n. nasuta and D. n. albomicans and also the differential staining property of this 40 kD fraction in them. The analysis of samples prepared from F1 males in comparison with the ones obtained from their male parents revealed that the 40 kD fraction of group II in D. n. nasuta and D. n. albomicans is X-linked (Fig. 6). The basis of our observation leading to the identification of the fraction produced by X-chromosomal gene is schematically represented in Fig. 7.

**Discussion**

The successful application of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as a technique for resolving complex mixtures of proteins of wide molecular weight range has greatly facilitated the biochemical analysis of these macromolecules. The detection and analysis of these gel-resolved proteins is possible by different staining techniques. In the present investigations, an attempt has been made to study the biochemical properties (nature) of the accessory gland secretory proteins in *nasuta* subgroup by subjecting them to five different staining techniques. The results of CBB R-250 staining revealed mobility variation in the profiles of mostly group III fractions in different species employed in the present study. The variation in mobility of proteins on SDS-PAGE may be due to post-translational modifications or due to change in the sequence of even a single aminoacid.

Each version of silver stain differs in its ability to stain proteins. While some proteins stain perfectly with silver, others such as acidic proteins, phosphoproteins and highly glycosylated proteins may stain weakly or does not stain at all. The results of silver staining have shown that the group I and II fractions stain yellow, while group III fractions could not be localized. To detect these group III fractions we therefore employed a recent PAS-silver double staining technique which has been developed for staining highly glycosylated proteins. The localization of group III with this technique suggests that they are highly glycosylated. Even the results of PAS staining are in accordance with this observation;
wherein we could localize the group III fractions in just one hour after placing the gel in Schiff’s reagent while 16-18 hr were needed to localize the fractions of group I and II. Therefore, the results of these three staining techniques namely silver, PAS-silver and PAS indicate that all the three groups of accessory gland secretory proteins in the species of the nasuta subgroup are glycosylated and group III fractions are highly glycosylated. “The glycosylation of proteins increases thermal stability and resistance to proteolytic degradation enabling thermophilic adaptation”\textsuperscript{29}. As these proteins are transferred to female during mating\textsuperscript{14,15} this glycosylation may probably help in storage of these secretions in the female reproductive tract. Perusal of the literature reveals that in D. melanogaster also, the accessory gland secretions are glycosylated\textsuperscript{19,31}. However, the extent of glycosylation has not been documented. In the present study, the results of PAS staining suggest that there is 100% glycosylation of all the secretory protein fractions in nasuta subgroup species.

Dzandu et al.\textsuperscript{32}, have demonstrated that the sialoglycoproteins stain yellow with silver. Deh et al.\textsuperscript{33}, have shown that sialoglycoproteins with high amount of O-glycosidically linked carbohydrate moieties stain yellow with silver in SDS-polyacrylamide gels. Further, we have observed that all the secretory fractions appear yellow, when localized with silver and PAS-silver methods, suggesting that they probably are sialoglycoproteins. However, desialization of the samples by using neuraminidase is in progress to confirm whether yellow staining is due to presence of sialic acid.

Proteins electrophoresed in the absence of SDS may be detected by imidazole-zinc salts as complex patterns of both negatively (transparent) and positively (white) stained bands. It is proposed that the "positive or negative staining of protein fractions is a consequence of their particular interactions with the staining reagent"\textsuperscript{24}. "SDS when removed, is also expected to generate complex protein staining patterns\textsuperscript{23}. In the present investigations, localization

\textbf{Cross - I}

\begin{align*}
D. n. nasuta \ & \ \otimes \ & \ X \\
(Xn40kD White / & Xn40kD White) \\
\downarrow \\
Hybrid \ & \ \delta \ & \ \delta
\end{align*}

\textsuperscript{(Xn40kD White /Ya)}

Shows the white 40 kD fraction of D. n. nasuta

\textbf{Cross - II}

\begin{align*}
D. n. nasuta \ & \ \delta \ & \ \delta \\
(Xn40kD White/ & Yn) \\
\downarrow \\
Hybrid \ & \ \delta \ & \ \delta
\end{align*}

\textsuperscript{(Xa40kD Transparent /Ya)}

Shows the transparent 40 kD fraction of D. n. albomicans

\textbf{Fig. 7—Scheme for the identification of accessory gland secretory protein gene on X-chromosome.}


\textbf{a = Accessory gland secretions (Coomassie Blue stained)}

\textbf{b = Accessory gland secretions (Silver stained)}

\textbf{* = Fractions that are not stained with silver.}

\textbf{> = X-chromosomal fraction}

\textbf{? = Polymorphic fraction}
of accessory gland secretory proteins with zinc-imidazole revealed complex band patterns consisting of three groups of positively stained bands (which are also CBB positive) alternating with negatively stained bands. We have observed that a 40 kD fraction of group II stains negatively in D. n. albomicans while in case of D. n. nasuta the protein fraction with same molecular weight stains positively. D. n. nasuta and D. n. albomicans belong to frontal sheen complex of the nasuta subgroup and are cross fertile2,35. We took the advantage of cross fertility of these two members of the subgroup and the differential zinc-imidazole staining property of 40 kD protein, to analyze the nature of this fraction in the F1 hybrid male of reciprocal crosses conducted between D. n. nasuta and D. n. albomicans. Such an analysis revealed that if the F1 male inherits the X-chromosome of D. n. albomicans (cross: D. n. nasuta X D. n. albomicans ? ? ?) the 40 kD fraction will be negatively stained. If the F1 male inherits the X-chromosome of D. n. nasuta (cross: D. n. nasuta X F1 male) the 40 kD fraction in the accessory gland secretions of F1 male will be positively stained. The results suggest that the 40 kD fraction localized by zinc-imidazole staining technique is synthesized by gene located in the X-chromosome. In D. melanogaster all Acp (Accessory gland protein) genes are localized on autosomes and it has been proposed that the absence of Acp genes from the X-chromosome might be related to their male limited expression. Further, it has been suggested that autosomal placement of Acp genes has been advantageous because the genes could be expressed at high levels with out needing to acquire dosage compensation regulation36. On the contrary, present study has provided the first evidence of existence of X-chromosomal genes involved in the synthesis of accessory gland secretory protein fractions though their expression is male limited.

Post-translational modifications involving the addition of carbohydrate groups not only confer important physical properties such as conformational stability, protease resistance, charge and water binding capacity but also play an important role in biological recognition where sequence diversity provides signals for protein targeting and cell-cell interactions37. In Drosophila, the secretions of the male accessory gland enter the female genital tract along with the sperms during mating. They cause an elevation in the rate of egg laying, decrease the receptivity to mating, decrease the life span and play an important role in storage as well as efficient utilization of sperm in the female14,15,16,31,38,39. Extensive post-translational modifications of these proteins as seen in D. nasuta subgroup probably ensure their functioning in the female genital tract, which is an alien environment for these secretions.

Acknowledgement
This study was financially supported by The Department of Science & Technology, New Delhi. We thank the Chairman of our department for the facilities. The encouragement and valuable suggestions of Prof. H. A. Ranganath of our department and Prof. W.-E. Kalisch, Institut für Genetik, Ruhr Universität Bochum, Germany, are gratefully acknowledged. We also thank Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ) and Deutscher Akademischer Austauschdienst (DAAD), Germany for donating equipment and chemicals (to SRR).

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