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Constitutive Exocytosis of Seminal Fluid Proteins in Male Accessory Glands and Ejaculatory Duct of *Drosophila melanogaster*

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Male accessory glands and ejaculatory duct of *Drosophila melanogaster* are reproductive organs which synthesize secretory seminal proteins. Several products of these organs involved in egg laying, receptivity, and sperm stability or storage were isolated from their lumens. Despite their secretory process play an important role, exocytosis pathway in these organs is not well known. In the present study, we characterized secretory protein profiles and determined their secretory mechanisms. Eight accessory gland secretory proteins and two ejaculatory duct secretory proteins were detected in their lumens. All these proteins were constitutively synthesized in these organs and secreted to their lumens. Secretion of newly synthesized proteins initiated at about 1 h after synthesis, and reached the peak at 4 h after synthesis. It seems that secretion of the proteins may occur via constitutive exocytosis pathway.

Male reproductive organs of insect consist of accessory glands, ejaculatory duct, seminal vesicles, and testis (Fig. 1). In *Drosophila melanogaster,* the seminal fluid produced from the accessory glands and the ejaculatory duct is transferred to female by mating (Chen et al., 1988; Monsma and Wolfner, 1988), thereby mediating physiological and behavioral changes in the mated female (Bertram et al., 1992). These responses include increase of egg laying (David, 1963), decrease of receptivity to further mating (Manning, 1967; Hihara, 1981), and sperm stability or storage (Chen, 1984). It has also been suggested that the products may be involved in species isolation mechanisms (Chen, 1976; Fuyama, 1983).

Because of the various roles of seminal fluid, the studies on the accessory glands and the ejaculatory duct have been focused on their functions and gene regulation. Genomic and cDNA clones for some male-specific transcripts made in the accessory glands have been isolated (Chen et al., 1988; Monsma and Wolfner, 1988; Herndon and Wolfner, 1995). The sex-peptide is the representative one synthesized in the accessory glands. It is transferred to the female, and affects courtship response or egg-laying (Chen et al., 1988). The putative product of the *mst355a* (Monsma and Wolfner, 1988), another accessory gland-specific transcript, shows an interesting sequence homology to egg-laying hormone from *Aplysia californica* (Herndon and Wolfner, 1995), and also affects egg-laying. The *mst315, mst316,* and *mst57Da-c* were isolated from the accessory glands (Dibenedetto et al., 1990; Simmerl et al., 1995).

The products of the ejaculatory duct are relatively unknown. Three proteins have been found in the ejaculatory duct lumen: glucose dehydrogenase (Cavener and MacIntyre, 1983), esterase-6 (Richmond and Tepper, 1983) and andropin (Samakovlis et al., 1991).

Although most of these tissue-specific products show secretory property, the processes of their secretion have not been elucidated well. Electron microscopic and protein analyses showed that the accessory glands and the ejaculatory duct have active vacuoles filled with secretory materials (Federer and Chen, 1982; Stumm-Zollinger and Chen, 1985). However, it is not known how the lumen proteins are secreted from Golgi apparatus and how much time is taken to secrete them.

Secretory process from Golgi apparatus to the exterior of the cell can be divided into two pathways (Burgess and Kelly, 1987). One is constitutive pathway, in which most of proteins are destined for immediate delivery to the cell surface. Transported vesicles normally leave from the *trans*-Golgi network in a steady stream. The membrane proteins and the lipids in these vesicles provide new components for the plasma membrane, while the soluble proteins inside the vesicles are secreted to the extracellular space. The other is regulatory exocytosis pathway, which is adopted

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by special cells that produce neurotransmitters or digestive enzymes rapidly on demand.

The studies on exocytosis have revealed that Ca^{2+} and protein kinase C (PKC) are important factors which largely affect regulatory exocytosis pathway (Miyata et al., 1989). Cyclic AMP and protein kinase A (PKA) are also known to affect some kinds of exocytosis (Macrae et al., 1990; Sammak et al., 1992; Hansen and Casanova, 1994). Recently, some studies have proposed that constitutive exocytosis pathway could also be regulated by Ca^{2+}, PKC, and cAMP (Miller and Moore, 1991; Brignoni et al., 1995; Chavez et al., 1996).

Male accessory glands and ejaculatory duct of *Drosophila* are good model systems for exocytosis study. The glands and the duct are secretory organs containing the lumen filled with secretory proteins.

In view of importance of the accessory glands and the ejaculatory duct on reproduction, we determined their secretory properties of seminal fluid proteins. Secretory protein profiles were examined. The secretory process, secretory time, and the effects of temperature on exocytosis in these organs were also characterized.

Materials and Methods

Drosophila culture

Flies were reared in uncrowded half-pint glass bottle on standard medium (cornmeal, sugar, agar, and yeast) containing propionic acid as mold inhibitor. They were maintained at 70-80% humidity and 24±1℃ under photoperiodic regime (12L : 12D).

Exocytosis assay

Continuous labeling: Three male accessory glands and ejaculatory ducts were isolated from 10-day-old virgin flies. The dissected organs were incubated in 10 μl MOPS buffer (10 mM MOPS, pH 7.0, 80 mM NaCl, 10 mM KCl, 0.2 mM $MgCl_2$, and 0.1 mM $CaCl_2$) containing 2 μCi of [^{35}S] methionine for 0.5-4 h on a piece of parafilm which was mounted on a petridish. Just after labeling, the organs were washed and then their lumen fluids were collected.

Short term labeling: To estimate the time interval between synthesis and secretion, three or five glands were labeled with [^{35}S] methionine for 15 min. Then the glands were washed with fresh MOPS buffer for 3 times, and incubated for an appropriate time in 10 μl MOPS buffer. To determine the effect of temperature on exocytosis, the glands were labeled with [^{35}S] methionine for 15 min at 24℃ or 37℃ and washed with fresh MOPS buffer for 3 times. Then, they were incubated for additional 2 h at appropriate temperature.

Preparation of secretory proteins and SDS-PAGE: The prepared organs were punctured with a fine needle. The glands immediately emptied themselves by secretion through active contraction of the glandular epithelium. The empty glands were washed with MOPS buffer. The lumen fluid and the empty glands were separately stored at -70℃ until analyzed. The samples were boiled for 5 min in SDS sample buffer and separated in 10% or 15% polyacrylamide gel according to Laemmli (1979) with slight modifications. The bands were visualized by Coomassie blue staining.

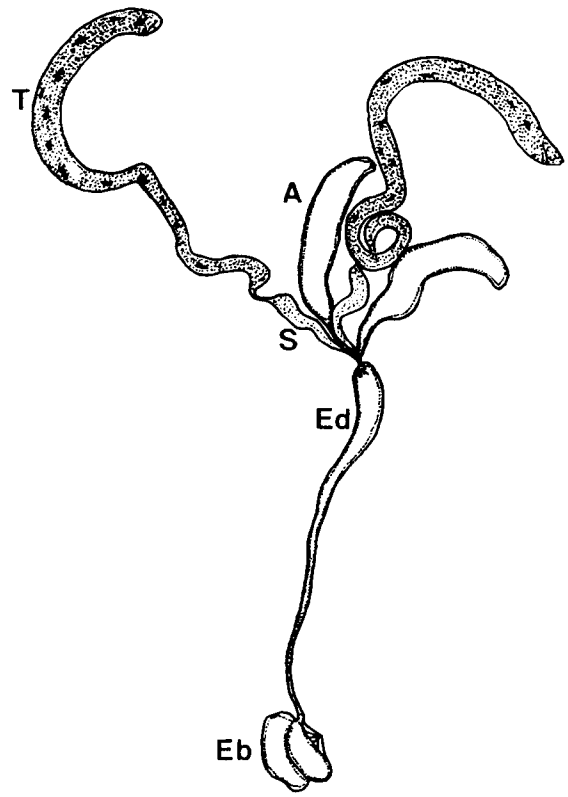

Fig. 1. Male reproductive organs of *Drosophila melanogaster*. T, testis; S, seminal vesicle; A, accessory gland; Ed, ejaculatory duct; Eb, ejaculatory bulb.

Scanning and Data processing

To measure the degree of protein secretion, the gel was dried and exposed to X-ray film for 1 d. The autoradiogram was scanned and analyzed with a computer program (Adobe photoshop version 3.0).

Results

Profiles of secretory proteins

The fully grown virgin male flies (10-day-old) were used to examine the profiles of secretory proteins. The results are shown in Fig. 2A. About 8 proteins were detected from the accessory glands lumen fluid. Two of them (number 4 and 6 in Fig. 2A) were observed from the ejaculatory duct lumen fluid.

Implication of constitutive protein secretion

The secretory proteins were also detected in the

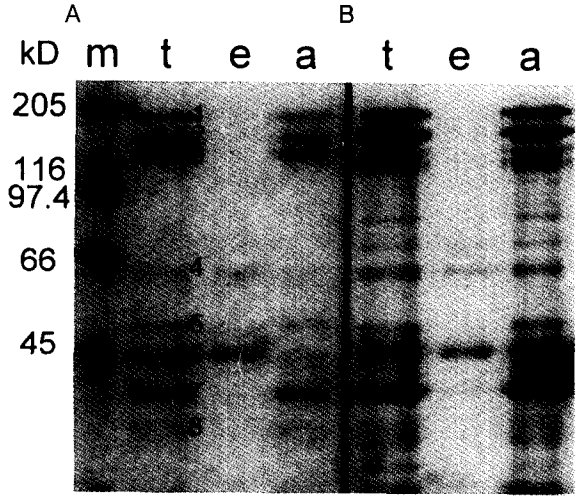

Fig. 2. Secretory proteins of accessory glands and ejaculatory duct. Five pairs of the glands were labeled with [^{35}S] methionine for 15 min and washed. After more 2 h incubation at room temperature, their lumen fluid was collected and analysed. A, 10% SDS-PAGE and Coomassie blue stain. t, total secretions from accessory glands and ejaculatory duct; e, ejaculatory duct secretion; a, accessory gland secretion; m, molecular weight protein standards. B, Autoradiogram of A.

lumen of the organs by autoradiograph after [^{35}S] methionine labeling (Fig. 2B). The accessory glands and the ejaculatory duct had additional bands of more than 12 and 5 proteins from autoradiography, respectively, although the bands were not thick. Continuous labeling assay was also performed to identify the exocytosis pathway of these organs. Protein secretion was initiated at 1 h after labeling. The concentrations of secreted proteins were increased in proportion to incubation time up to 4 h (Fig. 3A). The secretion levels were measured using two proteins, M_r 194 kDa and M_r 45 kDa (corresponding to numbers 1 and 6 in Fig. 2A, respectively), as representative proteins of the accessory glands and the ejaculatory duct, respectively (Fig. 3B and C). The secretion rates of these proteins had some similarity. The slopes of linear regression imply that the secretion levels of two proteins increased according to incubation time.

Temperature dependence of protein secretion

Despite that constitutive protein secretion was examined in the accessory glands and the ejaculatory duct, we could not exclude the possibility that the protein secretion resulted from damaged Golgi apparatus. So we examined protein secretion at a reduced temperature (4℃) to confirm whether protein secretion occurrs through bioactive mechanism, such as exocytosis. Although proteins were normally synthesized and accumulated in their epithelial cells (Fig. 4A, lane 8), their secretions were almost blocked after 2 h incubation at 4℃ (Fig. 4A, lane 2). However, the organs secreted all the accessory gland and the ejaculatory duct secretory proteins at room temperature (Fig. 4A, lane 1).

Protein secretion occurred normally at 19℃ (data

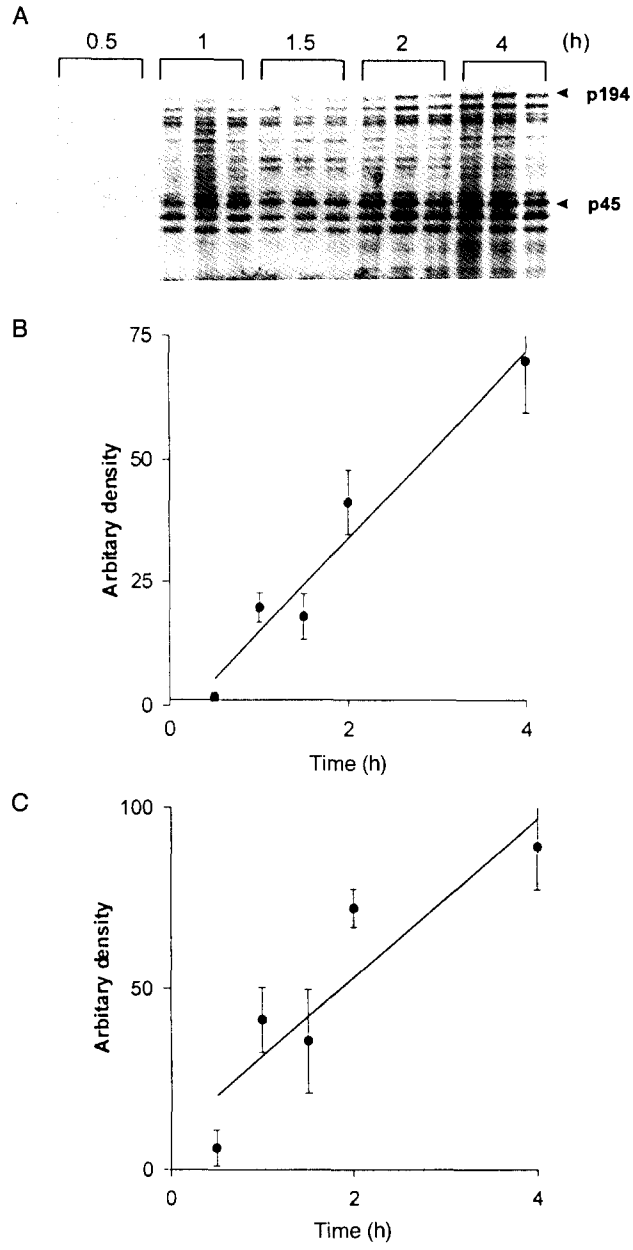

Fig. 3. Continuous labeling and protein secretion. Male accessory glands and ejaculatory duct were incubated in 10 μl MOPS buffer containing 2 μCi of [^{35}S] methionine for 0.5, 1, 1.5, 2, 4, 6 h. A, Autoradiogram, B, Secretion rate of p194, C, Secretion rate of p45. The results are mean±S.D. (n=3).

not shown). However, protein secretion slightly decreased at 37℃ and two major heat shock proteins (Hsps) appeared (Fig. 4A, lane 3). From comparison with imaginal wing disc of third instar larva, the two Hsps were identified as Hsp83 and Hsp70 (Fig. 4A, lanes 4 and 5). On the other hand, small Hsps including Hsp22, 23, 26, 27 were not secreted from the organs. It is possible that high temperature (37℃) damages the gland cells. So we assayed protein secretion at normal temperature (24℃) after labeling of the organs for

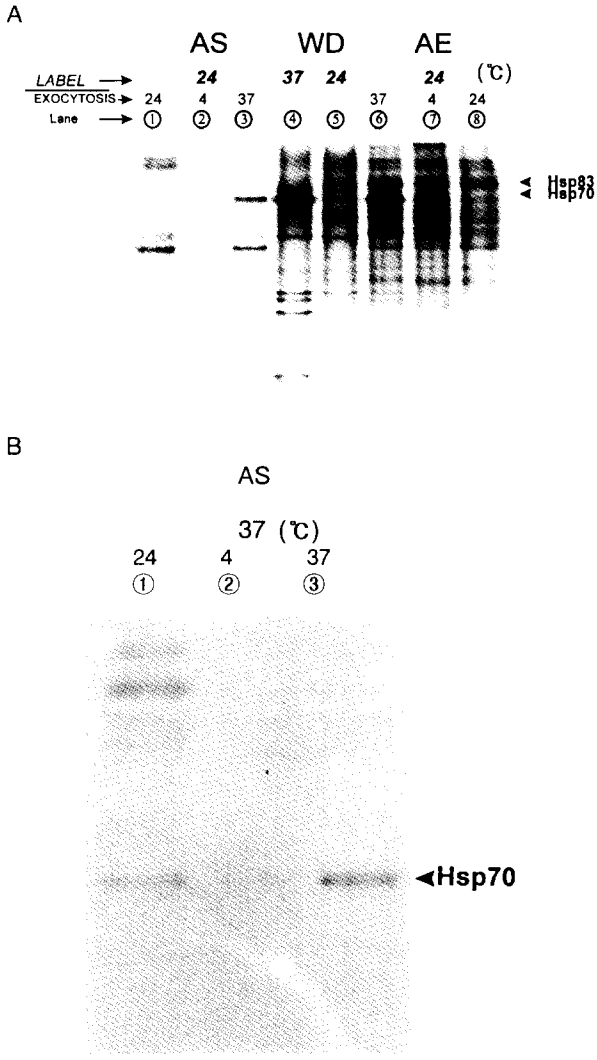

Fig. 4. Effect of temperature on protein secretion. Male accessory glands and ejaculatory duct were labeled with [^{35}S] methionine for 15 min at 24℃ or 37℃ and washed. After more 2 h incubation at 24℃, 4℃, or 37℃, their lumen fluid was collected and analysed. A, 15% SDS-PAGE. Lanes 1-3, secretory proteins of the glands collected after 24℃ labeling and different temperature protein secretion; lane 4, imaginal wing disc of third instar larva incubated at 37℃ for 30 min; lane 5, imaginal wing disc of third instar larva incubated at 24℃ for 30 min; lanes 6-8, epidermis of the glands collected after 24℃ labeling and different temperature protein secretion. B, 10% SDS-PAGE. Secretory proteins of accessory glands and ejaculatory duct collected after 37℃ labeling and different secretion temperature. Arrow heads indicate heat shock protein 70. AE, accessory glands and ejaculatory duct epithelial extract; AS, accessory glands and ejaculatory duct secretion; WD, wing disc of third instar larva.

15 min at 37℃. Hsp70 was also detected from the gland fluid (Fig. 4B, lane 1). On the other hand, the secretion was blocked at 4℃ in the same manner (Fig. 4B, lane 2).

Elapsed time of secretion after synthesis

In order to estimate the elapsed time of secretion after synthesis, the organs were incubated for a consecutive time course after labeling for 15 min at room temperature. Little secretory proteins were detected from the organs which were punctured immediately after labeling (Fig. 5A, lanes 1-3). At 1 h after labeling, only a little proteins were detected (Fig. 5A, lanes 4-6). Interestingly, the levels of secretory proteins were dramatically elevated in the lumen fluid at 2 h after labeling

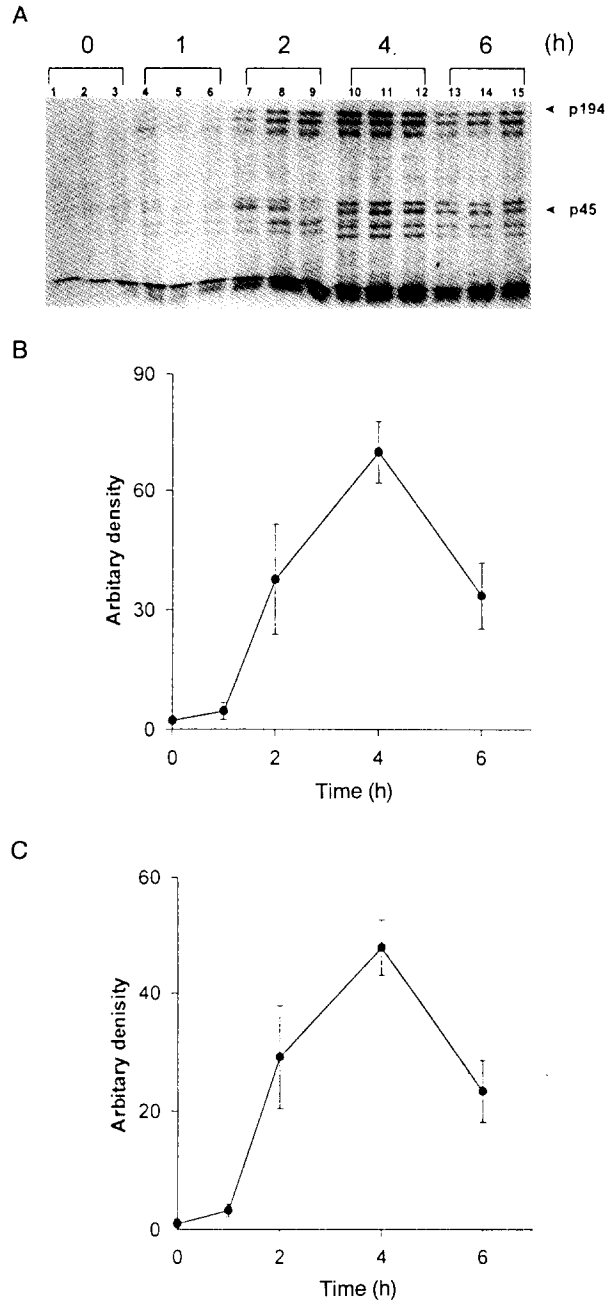

Fig. 5. Determination of elapsed time of secretion after synthesis. Male accessory glands and ejaculatory duct were labeled with [^{35}S] methionine for 15 min and washed. After incubation for consecutive time course, 0, 1, 2, 4, and 6 h, their lumen fluid was collected and analysed. A, Autoradiogram of secreted proteins. B, Protein secretion level of 194 kDa accessory gland protein. C, Protein secretion level of 45 kDa ejaculatory duct protein. The results are mean±S.D. (n=3).

(Fig. 5A, lanes 7-9). The levels of secretion reached the peak at 4 h after labeling (Fig. 5A, lanes 10-12). Secretions of p194 and p45 also showed similar patterns (Fig. 5B and C).

Discussion

In the present study, the secretions of lumen fluid from male accessory glands and ejaculatory duct were assayed with an *in vitro* organ culture system. From [^{35}S] methionine incorporation, we detected more than 20 proteins secreted from accessory gland and more than 5 proteins secreted from ejaculatory duct. The continuous labeling assay showed that the concentrations of secretory proteins increase in proportion to the labeling time, which means that these organs secrete the lumen proteins by constitutive exocytosis pathway.

Lowering the temperature to 4℃ caused a complete block of exocytosis. On the other hand, protein secretion occurred normally at 18℃ (Data not shown). In case of mammalian cells, a very rapid decrease of secretory rate (60-70%) was observed between 37℃ and 25℃ (Beaudoin and Mercier, 1980) and no exocytosis was shown at 4℃ (Takayama et al., 1994).

In order to estimate the interval between synthesis and exocytosis of the lumen proteins, the short term labeling assay was carried out. The assay revealed that protein secretion was initiated from about 1 h after synthesis, and increased gradually up to 4 h, and then decrease of labeled proteins were initiated. The time courses of protein secretion by these organs were similar to exocytosis by dissociated lactating mammary acini cell (Turner et al., 1992).

At 37℃ incubation, Hsp83 and Hsp70 were secreted. A recent study has shown that some strains of yeasts are able to produce secretory Hsps (Tsiomenko et al., 1997), however, no Hsp has been reported as secretory in *Drosophila*. Their roles in seminal fluid are not clear. However, it appears that they might protect seminal fluid proteins from any abnormal conditions containing heat shock.

We selected two proteins to probe secretory properties of the accessory glands and the ejaculatory duct. They showed a similar exocytosis pattern in elapsed time and temperature-dependency. Male accessory glands are composed of two morphologically distinct secretory cell types, 'main' and 'secondary' cells (Bertram et al., 1992). The secretory cell type of ejaculatory duct is not clear yet. The results that these organs might have the same exocytosis mechanism, in spite of their different cell types.

Male reproductive behavior of *Drosophila* might account for the constitutive exocytosis of these organs. Male accessory glands and ejaculatory duct are small at eclosion and filled with seminal fluid in proportion to age during 7 d after eclosion (for the review, see Ashburner, 1989). After this time, they are full of seminal fluid and this condition lasts at least 15 d after eclosion in virgin flies. This seminal fluid is transferred to the female fly during mating by squeezing of the proteins. Their synthesis is increased rapidly after mating. They recover their shape almost completely about 8 h after mating. One *Drosophila melanogaster* male may sire 10,000 to 14,000 progenies in his life. So the accessory glands and the ejaculatory duct should be filled with seminal fluid constantly. This might be a reason why proteins are synthesized and secreted via a constitutive exocytosis in these organs.

Acknowledgements

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