

## A MONOCLONAL ANTIBODY AGAINST A STRUCTURAL PROTEIN IN THE SPERMATOPHORE OF *TENEBRIO MOLITOR* (COLEOPTERA)

KARIN A. GRIMNES and GEORGE M. HAPP

Department of Zoology, University of Vermont, Burlington, VT 05405, U.S.A.

(Received 30 July 1985; revised and accepted 12 October 1985)

**Abstract**—Monoclonal antibodies were produced against insoluble proteins of spermatophores of *Tenebrio molitor*. One hybridoma clone produced antibody which recognized two antigens (29.4 and 27.6 kd mol. wt) in the bean-shaped accessory reproductive glands (BAGs) and in the secreted precursor of the insoluble fraction of the spermatophore. At least two molecular weight variants which differed by 1.5–1.7 kd mol. wt daltons are present. Processing accompanies secretion into the BAG lumen, with a reduction of about 4000 daltons in apparent molecular weights.

The amount of target antigen during reproductive maturation, its localization and its transport were studied using Western blotting and immunohistochemistry. The monoclonal antibody recognized a protein present in one of the eight secretory cell types (type 3) of the BAG, in the secretory production of this gland, and in discrete layers of the spermatophore ejected from the male. This specific probe, and others currently being produced, will facilitate detailed studies on the process of spermatophore formation.

**Key Word Index:** *Tenebrio molitor*, accessory glands, differentiation, spermatophore, monoclonal antibodies

### INTRODUCTION

Many species of insects transfer semen within spermatophores (Tuzet, 1977). The biochemistry and assembly of these structures have been relatively little studied (Happ, 1984; Chen, 1984). We aim to explore the assembly processes for the spermatophore of *Tenebrio molitor*. This spermatophore is cylindrical; the sperm lie in a lumen which is bounded by an outer wall and an inner core (Gadzama and Happ, 1974). Most, if not all of the structural proteins are derived from the reproductive accessory glands of the male and are molded into the multilayered wall and core within the ejaculatory duct (Dailey *et al.*, 1980; Happ *et al.*, 1982).

There are two pairs of reproductive accessory glands in male mealworm beetles—the bean-shaped accessory glands (BAGs) and the tubular accessory glands (TAGs; Gadzama, 1972; Poels, 1972; Gerber, 1976). The TAGs secrete soluble proteins which are mixed with the sperm in the lumen of the spermatophore (Black *et al.*, 1982). The organized wall and the core are largely derived from the less soluble products of the BAGs (Gadzama, 1972; Frenk and Happ, 1976; Dailey *et al.*, 1980; Happ *et al.*, 1982). Within this gland are eight types of secretory cells, each of which has a morphologically distinct secretory product. The secretions of each cell type are coherent; they accumulate in patches within the lumen of the glands. The patches become drawn out into layers which flow posteriorly from the BAG and are transformed into a pre-spermatophore mass within the ejaculatory duct (Gadzama, 1972; Dailey *et al.*, 1980; Dailey and Happ, 1983). The morphology of the secretions alters further during flow into and along the ejaculatory duct and during the molding of the walls and core. On the basis of

morphology alone, it has proved difficult to track cellular secretions to their final site in the spermatophore.

It is our goal to employ monoclonal antibodies as probes for the structural proteins of the spermatophore of mealworm beetles. With such antibodies, it should be possible to analyze the processes of biosynthesis and export, the mechanisms of assembly of the spermatophore, and the development of the glands which produce the spermatophore. In the work described below, our specific aims were: (1) to isolate a hybridoma clone which secretes antibody against proteins which are common to the BAG and the spermatophore; (2) to identify the target antigen, the cell type which synthesizes the antigen in the BAG, and the time of appearance of antigen during the differentiation of the gland; and (3) to trace the movement of antigen from the secretory cells of the gland into the pre-spermatophore mass in the ejaculatory duct.

### MATERIALS AND METHODS

#### *Animals*

Mealworm beetle larvae were obtained from a commercial supplier or from laboratory cultures and maintained on Purina Chick Chow. At pupation, the animals were segregated, sexed and maintained at 26°C until eclosion (usually 8 days later). Newly emerged adults were maintained at 26°C and fed potato.

#### *Preparation of protein samples*

Proteins were obtained from the spermatophore, from the BAG and other tissues, and from the semisolid secretory products in the lumen of the BAG. If males are not allowed access to females, they eject a spermatophore each day. These discarded dried spermatophores were collected. Because of the large amount of material required for these

studies, only dried spermatophores were used. Groups of 200 were homogenized in distilled water and spun at 15,000 g (3 min) in an Eppendorf microfuge. The supernatant was designated as the soluble spermatophore fraction. The pellet was washed twice and became the insoluble spermatophore fraction. The glands and other tissues were dissected from the beetles and homogenized in distilled water.

Protein concentrations were determined using the procedure of Lowry *et al.* (1951) with bovine serum albumin as the standard.

#### *Gel electrophoresis and Western blotting*

Slab gel SDS-electrophoresis (12% gels) followed the methods of Laemmli (1970). Two dimensional electrophoresis involved an isoelectric focusing (IEF) tube gel followed by an SDS slab gel (12% polyacrylamide) for the second dimension (O'Farrell, 1975). IEF and slab gel samples were prepared as described by Black *et al.* (1982). Prestained molecular weight markers (Bio-Rad) were run on each gel and were used to calculate the molecular weight of the antigen by regression analysis. Molecular weights were corrected by comparison of prestained standards to unstained molecular weight markers (Sigma).

Proteins were transferred from 1D or 2D slab gels onto nitrocellulose sheets (0.45  $\mu$  pore size, Millipore) using a Bio-Rad Trans-Blot Cell and following the methods of Towbin *et al.* (1979) and Burnette (1981). After transfer (12 hr at 30 V, 4°C), the nitrocellulose filter was placed in 5% fetal calf serum in phosphate-buffered saline (PBS) for 30 min. The blot was rinsed with PBS and placed in 5% fetal calf serum containing the monoclonal antibody (diluted 1:100, or 1:10,000 depending on strength of antibody) for 2 hr. After rinsing three times in PBS the blot was placed in a solution of 5% fetal calf serum containing a 1:500 dilution of peroxidase-labelled rabbit anti-mouse IgG (H and L Chains, Miles Labs). The blots were rinsed five times in PBS, and developed in 10 mM Tris, pH 7.4, containing 25  $\mu$ g/ml *O*-dianisidine (Sigma) and 0.01% H<sub>2</sub>O<sub>2</sub> for up to 15 min. Blots were then rinsed in distilled water, air-dried and photographed. Positive bands were stained orange-brown.

#### *Enzyme-linked immunosorbent assay (ELISA)*

An enzyme-linked immunosorbent assay was used to screen sera and clones. The protocol was based on the ELISA methods of Voller *et al.* (1979). Two hundred microliters of antigen solution (5  $\mu$ g/ml or serial dilutions thereof) were bound to Immulon II microtiter plates (Dynatech, Alexandria, Virginia) for 12–24 hr at 4°C. The plates were rinsed 5 times with phosphate buffered saline (PBS-T) containing 0.05% polysorbitan monolaurate (Tween-20) and blocked with 1% normal rabbit serum (200  $\mu$ l) for 1 hr. After rinsing, the wells were filled with 200  $\mu$ l of PBST, the serum or hybridoma supernatant was added (50–10  $\mu$ l) and allowed to incubate for 6 hr. The plates were rinsed 5 times and the peroxidase conjugated antibody was added (200  $\mu$ l/well, 1:5000 dilution) and allowed to bind for 12 hr at room temperature. After washing 10 times the plates were developed in 0.1 M phosphate-citrate buffer pH 5.0 containing 0.034% *O*-phenylene diamine (Sigma) and 0.012% H<sub>2</sub>O<sub>2</sub>. After 20 min the reaction was stopped by adding 50  $\mu$ l of 2 N H<sub>2</sub>SO<sub>4</sub> to each well, and absorbance was read at 490 nm using a BIO-TEK ELISA Reader Model EL-307.

#### *Immunization*

Six-week-old Balb/c female mice were injected intraperitoneally with 0.1 ml of an emulsion of equal volumes of Freund's complete adjuvant and 1.0  $\mu$ g/ $\mu$ l BAG plug protein (50  $\mu$ g/animal). After 4 weeks, the mice were bled by tail bleeding and injected with 50  $\mu$ g BAG plug protein in Freund's incomplete adjuvant.

Blood was screened for antibodies against BAG proteins and insoluble spermatophore proteins using the ELISA. The

animals with the highest titer (positive at >1:10,000 dilution) was boosted with 100  $\mu$ g of BAG protein in dH<sub>2</sub>O.

#### *Fusion*

Three days after boosting, the mouse was killed by cervical dislocation and the spleen was removed, and torn apart in 5.0 ml of serum free Opti MEM (Gibco). The cells were pelleted (300 g, 10°C, 5 min), and incubated in 0.17 M NH<sub>4</sub>Cl, pH 7.0 (10 min, 0°C) to lyse red blood cells. Spleen cells were pelleted and resuspended in media, counted and mixed with an equal number of logarithmically growing SP2/0.Ag14 myeloma cells. Cells were co-pelleted and the media was decanted from the pellet. After tapping to dissociate the pellet, 0.5 ml 50% polyethylene glycol (Sigma, mol. wt 1300–1600 diluted with media) was added slowly over 1 min with occasional shaking. Five milliliters of media were added over 3 min and the mixture was centrifuged as above. The pelleted cells were resuspended in 50 ml HAT Opti-MEM (4% Myoclon serum, Gibco; 1  $\times$  10<sup>-4</sup> M hypoxanthine; 3  $\times$  10<sup>-5</sup> M thymidine; and 5  $\times$  10<sup>-7</sup> M aminopterin, all from Sigma), dispensed into five 96-well Falcon tissue culture plates (0.1 ml/well) and cultured in a humidified incubator (NAPCO, model 5300) containing 7% CO<sub>2</sub> and 93% air at 37.5°C.

#### *Screening and cloning*

After 7 days, the plates were observed and 50  $\mu$ l of media were removed from each well containing growing cells and tested by an ELISA against insoluble spermatophore proteins. Positive cultures were transferred to 24-well plates in HT Opti-MEM, allowed to grow to 50% confluency and screened again with the ELISA. Supernatant from positive colonies was tested for epitope specificity against electroblots of TAG, BAG and spermatophore proteins. Colonies of interest were cloned by limiting dilution (0.5 or 1.0 cells/well) onto plates containing a feeder layer of intraperitoneal macrophages (100  $\mu$ g/well of macrophages from one Balb/c mouse in 50 ml Opti-MEM). Growing colonies were screened again by ELISA and positives were moved to 60 mm tissue culture dishes and then to 100 mm tissue culture plates.

#### *Cell storage and antibody production*

An aliquot of cells was frozen in Opti-MEM containing 4% calf serum and 7% DMSO (spectro grade, Kodak) and moved to a liquid nitrogen tank. Hybridoma cells were injected intraperitoneally into several Balb/c mice pretreated with 0.1 ml pristane (Sigma) for ascites fluid production. Cells were also allowed to overgrow in petri plates, after which the media was removed and frozen as an antibody source.

#### *Immunohistochemistry*

Immunohistochemical methods are primarily those of Farr and Nakane (1981). The BAGs, TAGs, seminal vesicles and ejaculatory duct of *T. molitor* form one anatomical complex. This complex was dissected and fixed in 0.5% glutaraldehyde (EM Grade, Ladd Research) in 0.1 M NaPO<sub>4</sub> buffer pH 7.4 for 2 hr then stored overnight in buffer without glutaraldehyde. The tissue was dehydrated in alcohols, transferred to xylene, and imbedded in Paraplast. Serial transverse sections (5  $\mu$ m thick) were cut and mounted on a slide coated with Mayer's albumin. Slides were thoroughly dried on a warming tray, deparaffinated, hydrated and placed in PBS containing 2.5% normal rabbit serum (PBS-R) for 1 hr. Next, the slides were incubated with 10  $\mu$ l ascites fluid in 3 ml of PBS-R for 2 hr, rinsed (3 times, 10 min each) and incubated with a 1:60 dilution of peroxidase-labelled rabbit anti-mouse IgG in PBS-R for 2 hr. After washing (3 times, 10 min each) the slides were developed with 50 mM Tris pH 7.6 containing 0.02% 3,3'-diaminobenzidine and 0.004% H<sub>2</sub>O<sub>2</sub> for 10–20 min. Slides were rinsed, dehydrated, through alcohols, passed

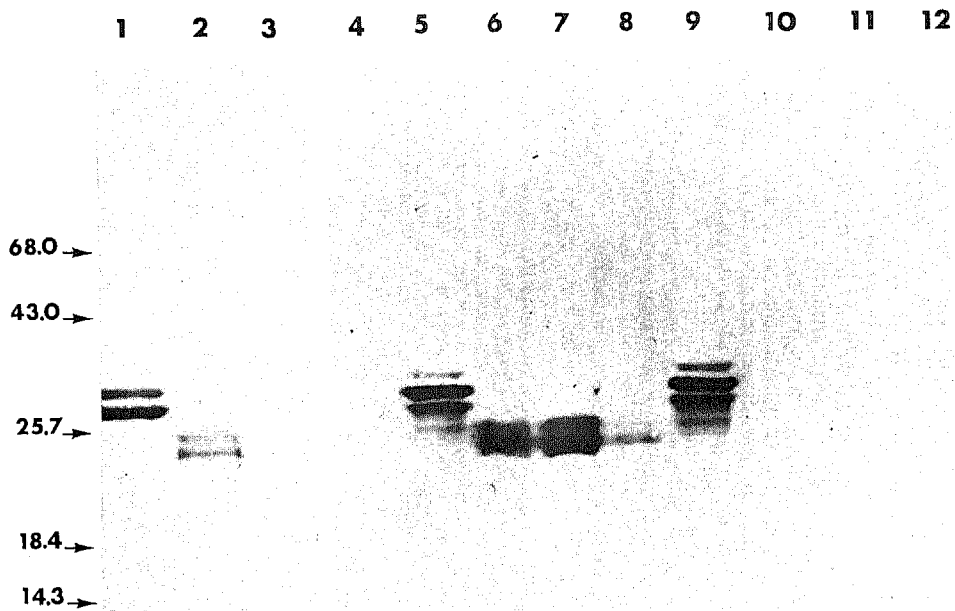


Fig. 1. Tissue-specific expression of PL3.4 antigen. Western blot of a SDS gel of protein samples immunodeveloped with PL3.4 antibody: (1) BAG, (2) water-insoluble spermatophore, (3) TAG, (4) seminal vesicle, (5) BAG, (6) plug, (7) water-insoluble spermatophore, (8) water-soluble spermatophore, (9) BAG, (10) testis, (11) hemolymph, (12) fat body. Each well contained 100  $\mu$ g total protein. Molecular weights to the right are in kilodaltons.

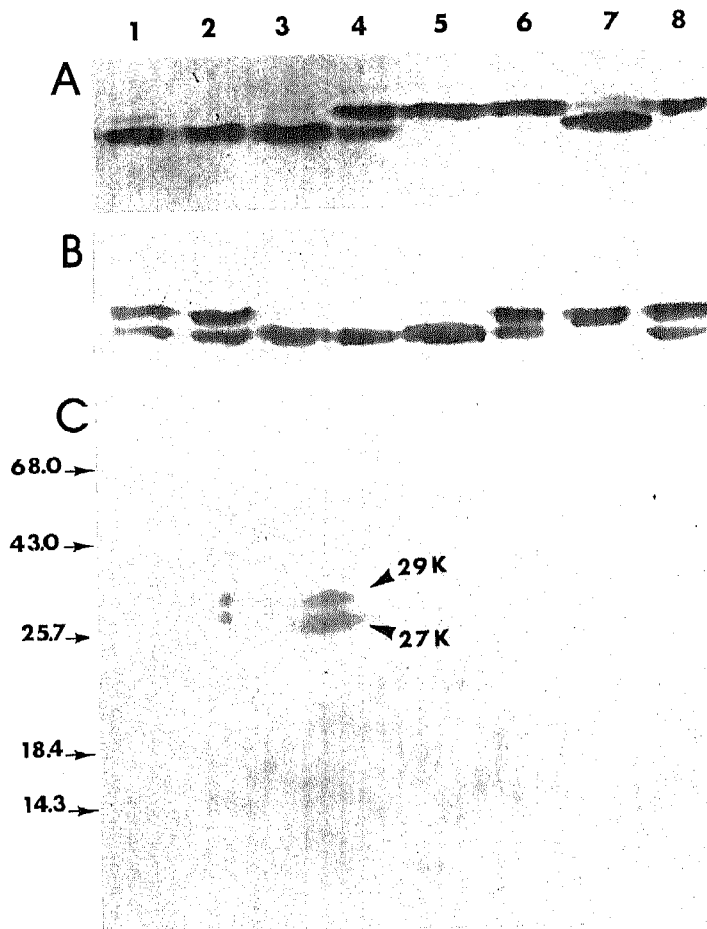


Fig. 2. Variation and 2D gel electrophoresis of PL3.4 antigen. Western blot of (A, B) 1 dimensional SDS gel and (C) 2 dimensional pI-SDS gel of BAG proteins, immunodeveloped with PL3.4 antibody: A (wells 1-8) BAGs from single animals; B (wells 1-8) BAGs from single animals; (C) pI-SDS gel of BAG protein; pH gradient from 4.4 (right) to 8.0 (left). Arrows indicate location and molecular weight of two common antigens. Each sample contained 100  $\mu$ g total protein. Blots were immunodeveloped with PL3.4 antibody. Molecular weights to the right are in kilodaltons.

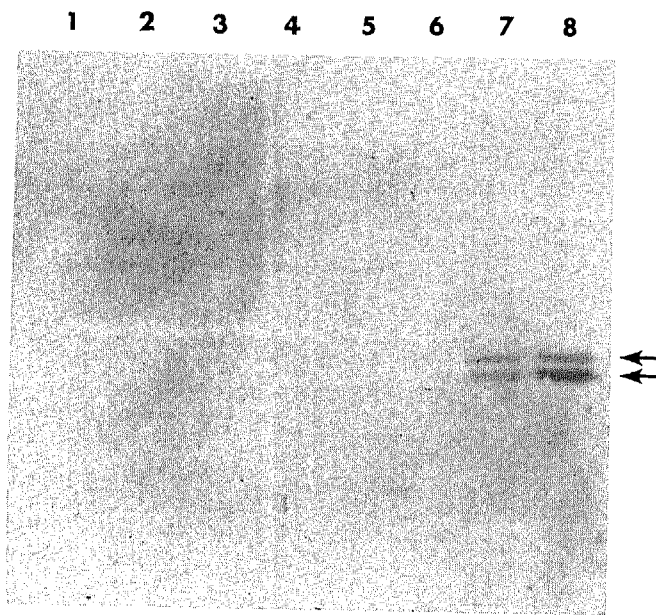


Fig. 3. Age-related expression of PL3.4 antigen. Western blot of a SDS gel of BAG proteins from animals of different ages, immunodeveloped with PL3.4 antibody: (1) 0-day pupae; (2) 4-day pupae; (3) 6-day pupae; (4) 7-8-day pupae; (5) 0-day adult; (6) 2-day adult; (7) 4-day adult; (8) 8-day adult. Each sample contained 100  $\mu$ g total protein. Arrows indicate location of two antigen bands.

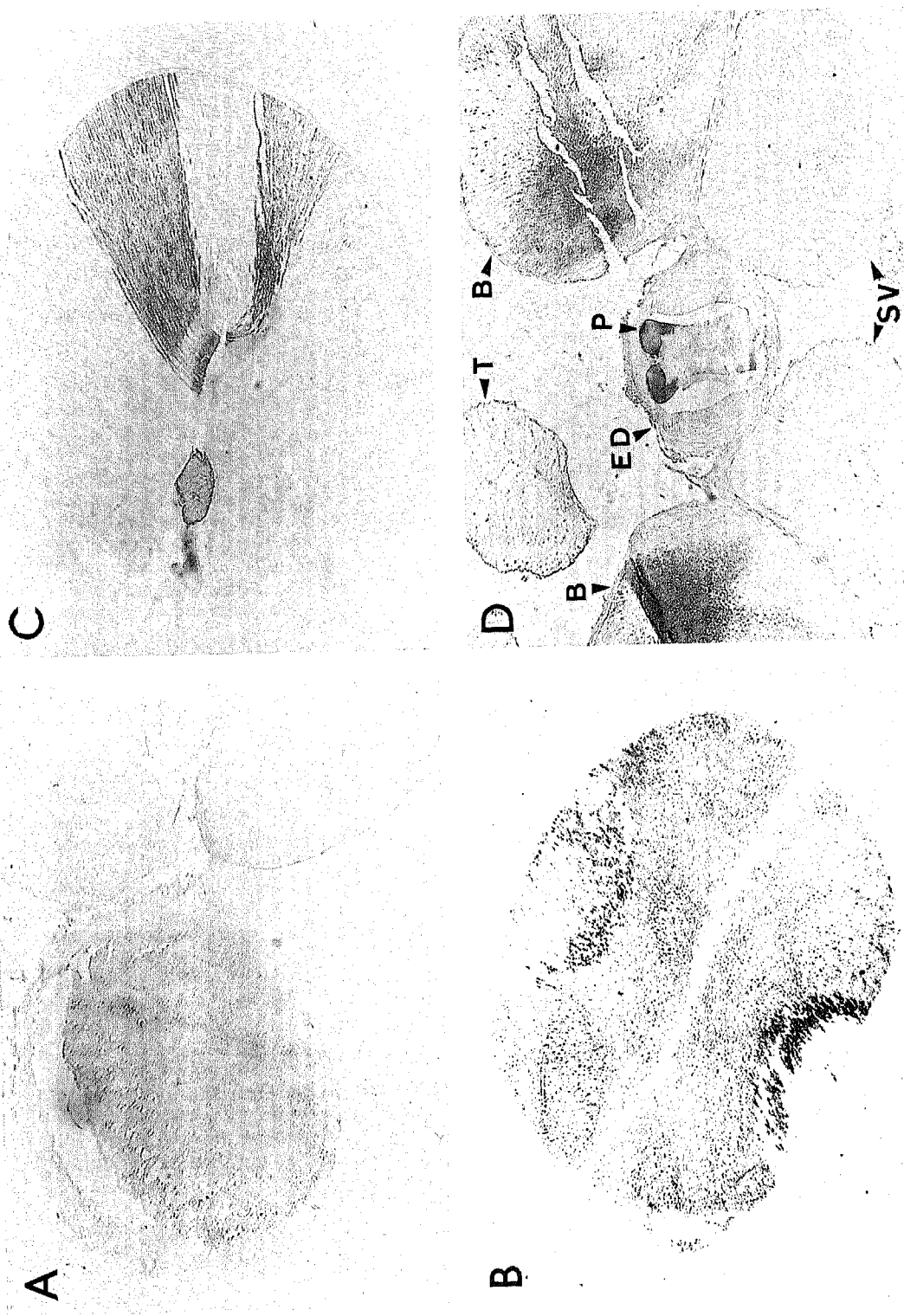


Fig. 4. Location of PL3.4 antigen in BAG. Immunohistochemical development of 8-day old adult BAG cross-sections and point of convergence of all glands, using PL3.4 antibody, peroxidase conjugate and DAB: (A) anterior section of BAG, non-immune serum; (B) anterior section of BAG, PL3.4 antibody; (C) posterior section of BAG PL3.4 antibody; (D) section through point of convergence (junction) of the reproductive accessory glands developed with PL3.4 antibody; B, BAG; ED, ejaculatory duct; P, penial plug; SV, secretory vesicle; T, TAG.  $\times 320$ .

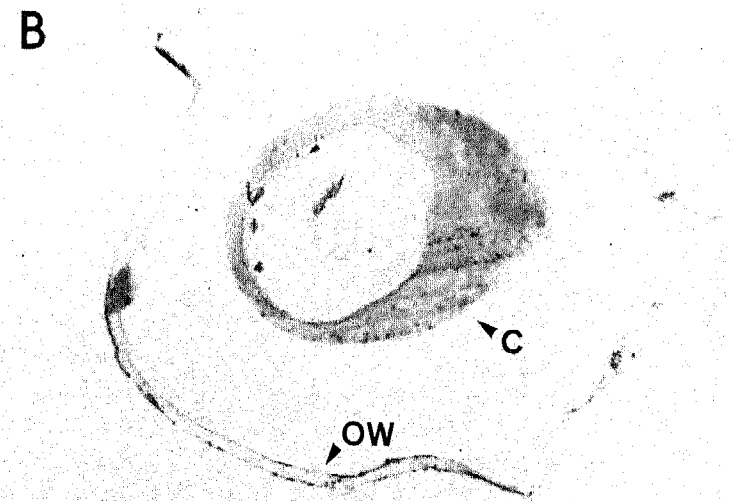
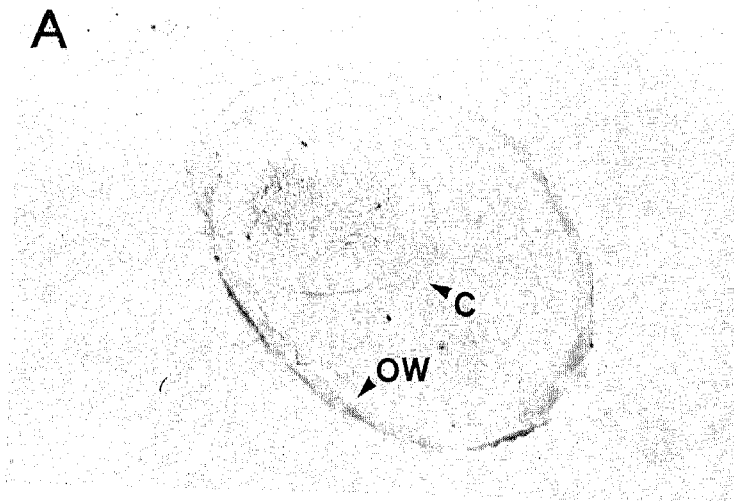


Fig. 5. Location of PL3.4 antigen in spermatophore. Immunohistochemical development of spermatophore cross-sections with PL3.4 antibody, peroxidase conjugate and DAB: (A) non-immune serum; (B) PL3.4 antibody; C, core or central cylinder; OW, outer wall of spermatophore.  $\times 1280$ .

through xylene, mounted in DPX (BDH Chemicals, England) and observed on a Zeiss Ultraphot. Controls for primary antibody (normal mouse serum), background binding (lack of primary or secondary antibody) and endogenous peroxidase (no secondary antibody) were all performed simultaneously on representative sections.

## RESULTS

### Isolation of hybridoma clone PL3.4

Mice were immunized with homogenates of the BAG secretory products and with homogenates of the spermatophore. After fusion of the lymphocytes of these mice with myeloma cells, the resulting hybridomas were screened by an ELISA against homogenates of BAGs, TAGs and insoluble spermatophore proteins. Culture medium from positive wells was used as the primary antibody source for Western blots of gels run with the same three samples. Cells from wells that yielded both a positive ELISA and a distinct pattern of bands on the blots were cloned by limiting dilution. At the present writing, 18 stable clones which secrete antibodies against antigens from the spermatophore and/or the accessory glands have been isolated. One of these, PL3.4, was produced after immunizing the mouse with a suspension of the plug of secretory product from the lumen of the BAG and is the subject of this report. The antibody secreted by clone PL3.4 is referred to as PL3.4 antibody and the target antigen recognized by this antibody is referred to as PL3.4 antigen.

### Clone PL3.4 recognizes two antigens in the BAG and in the spermatophore

Antibodies secreted by PL3.4 were used in Western blots of SDS-gels run with homogenates of the BAGs, the TAGs and spermatophores (Fig. 1, wells 1-3). The PL3.4 antibody detected two bands in the homogenates of the BAGs and of the spermatophore. The pair of antigens detected in the BAG appear to have lower mobility on our SDS gels than the bands in the spermatophore. No antigens in the TAG homogenate are recognized by this antibody.

In order to investigate the distribution of the PL3.4 antigens, a wider range of tissues was immunoblotted (Fig. 1, wells 4-12). In each case, tissues from many animals were pooled to produce the sample applied to the SDS gel. The PL3.4 antigens were detected strongly in the water-insoluble fraction of the spermatophore. In addition, they were seen in the BAGs, and in secretory plugs dissected from the lumens of the BAGs. They were not detected in other tissues, including the testes and the seminal vesicles, both of

which contribute protein to the spermatophore, nor were they identified in fat body or hemolymph.

The two antigen bands in the pooled samples could represent a pair of proteins in every gland or two genetic variants that might occur together or singly in individual beetles. To investigate the distribution of the antigens in single BAGs, individual glands were dissected and homogenized, and 60 µg of protein from each BAG were run on SDS gels. Analysis of the resulting blot revealed that an individual BAG may contain either one or both of the target antigens (Fig. 2A, B). Pooled samples of 10 or more BAGs, plugs, or spermatophores have always yielded both bands. The data suggest that, as already described for certain TAG secretory proteins (Grimnes and Happ, 1985), the two antigens recognized by PL3.4 antibodies may represent two genetic variants in our population of *T. molitor*.

Molecular weights of the antigens were calculated by comparing mobility of the bands recognized by the antibody with the regression equation calculated for mobility against size for pre-strained protein standards transferred to the same blot (Table 1). In the homogenates of the BAG, the larger band has a mol. wt of 29.4 kilodaltons (kd), and the smaller protein has a mol. wt of 27.6 kd. There is an apparent difference of about 1.7 kd between the two bands found in pooled samples of the BAGs, and there is a similar difference between the members of the pair in the plugs and in the spermatophores (mol. wt of 25.1 and 23.4 kd).

The pair of bands in the BAGs appear to be about 4 kd larger than those in the plug or in the spermatophore. Plug and spermatophore bands are indistinguishable from one another. After the plug has been removed from the lumen of the BAG, only trace amounts of the faster migrating antigens can be detected in BAG homogenates.

To characterize more fully the antigens in BAG and spermatophore, homogenates were applied to two dimensional pI-SDS polyacrylamide gels. The PL3.4 antigens were detected by immunodevelopment of blots. From homogenates of BAGs, two spots with pIs of 7.6-7.7 and molecular weights of 27 and 29 kd were prominent (Fig. 2C). But when homogenates of spermatophores were similarly processed, no spots were visible on blots in three separate experiments. Previous results show most antigen in the spermatophore is present in the water-insoluble fraction (Fig. 1, well 7), so we suspected that this anomalous result might follow from failure of some antigens to migrate into non-denaturing gels.

### The antigens detected by PL3.4 are differentiation-specific

Homogenates of BAGs from animals of increasing age (from 0 day pupae to 10 day adults) were run on an SDS slab gel, and the resulting gel was electroblotted and developed (Fig. 3). The antigen was detected strongly in adult animals of 4 days or older, although traces of antigen appeared in glands from 2-day-old adults. This is consistent with data from 2D fluorographs which show onset of synthesis for differentiation-specific proteins occurs between 0 and 2 days of adult life (Happ *et al.*, 1982).

Table 1. Molecular weights of PL3.4 antigens in the BAG and spermatophore of *T. molitor* (mol. wt  $\times 10^{-3}$  daltons)

	n	$\bar{x}$	$\pm$ SE
<i>Bag</i>			
High band	6	29.42	0.39
Low band	6	27.60	0.40
<i>Spermatophore</i>			
High band	5	25.12	0.48
Low band	5	23.52	0.48

### Immunocytochemical localization of the antigens

Spermatophores and the reproductive tracts of mature males were fixed in glutaraldehyde, embedded in wax and serial sectioned. The antibody produced by PL3.4 recognized antigens in the BAG, the plug and the spermatophore (Figs 4 and 5), thus confirming the earlier results with Western blots (Fig. 1). No significant staining was seen in the TAGs, the seminal vesicles, or the vasa deferentia. The staining within the BAG was not uniform, and the antigen-containing cells ran in a well-defined band on the mid-dorsal, lateral and mid-ventral surfaces of each gland. From the serial sections a pattern of staining could be reconstructed, which was remarkably similar to the distribution of cell type 3, one of the 8 cell types previously reported (Dailey *et al.*, 1980; Dailey and Happ, 1983).

Within the cells of the BAG, the secretory plug and the ejaculatory duct, the chromagen was localized within discrete zones (Fig. 4C, D). These results suggest that there are coherent blocks of secretory product associated with each cell type of the BAG. In the spermatophore, the antigen was most concentrated in the superficial zones of the outer wall and in an intermediate layer of the inner core (Fig. 5B). The staining patterns observed with the antibody support our earlier hypothesis concerning coherent and organized material flow which was based only on toluidine blue staining of the BAG and plug (Dailey *et al.*, 1980). The secretions from cell type 3 are not blended with those of other cells into a homogenous mass—rather they flow as a part of an ordered array of secretions which moves through the lumen of the gland, into the ejaculatory duct, and finally into particular layers of the spermatophore.

### DISCUSSION

Several workers have presented convincing evidence that some proteins of male accessory glands are synthesized in the insect fat body [*Melanoplus sanguinipes* (Gillot and Friedel, 1976); *Leptinotarsa decemlineata* (Peferoen and De Loof, 1984)]. Such proteins are subsequently transported through the hemolymph to be taken up by accessory glands and then passed into the spermatophore or seminal fluids. In *Rhodnius prolixus*, accessory glands appear to be the source of most secretory proteins (Barker and Davey, 1982). In the present study with *T. molitor*, PL3.4 antigen was detected in the BAG but not in either fat body or hemolymph samples. The results are consistent with the synthesis of PL3.4 antigen within the type 3 secretory cells of the BAG.

Our data indicate that two types of post-translational modification take place after synthesis of the polypeptide for PL3.4 antigen. A change in electrophoretic mobility suggests a decline in molecular weight (estimated at 4 kd) at the time of exocytosis. A shift in solubility begins in the BAG and appears to be complete before exocytosis. Post-translational modifications which could account for these changes include proteolytic cleavage, formation of disulfide links, and the addition of lipids, sugars, phosphates, or sulfates. Although these modifications are known to occur within insects, we do not yet know which processes account for the observed

changes in PL3.4 antigen. We suspect proteolytic cleavage is the most likely cause of the change in molecular weight.

In cuticle, chorion, and spermatophore, the assembly of molecular building blocks takes place in bounded extracellular spaces. For both cuticle and chorion, assembly occurs within the relatively inaccessible areas above the epidermis (Hepburn, 1985) or surrounding the oocyte (Petri *et al.*, 1976), respectively. In contrast to these systems, spermatophore assembly in *T. molitor* involves relatively large amounts of secretion which are stored for some hours in the lumen of the BAG and then flow posteriorly to be molded into the spermatophore within the ejaculatory duct (Dailey *et al.*, 1980). Since the secretory product can be dissected from the BAG, components can be biochemically analyzed before and after assembly reactions take place. We believe the spermatophore of *Tenebrio molitor* will be a useful model system, not only for studies of spermatophore formation in insects but also for analyses of general mechanisms of assembly in extracellular spaces.

PL3.4 and other monoclonal antibodies currently being developed are specific markers with which we will follow the flow of antigens from the cytoplasm of the secretory cells to the assembled spermatophore. Furthermore, these antibodies will assist in the isolation of specific proteins from the glands and from the spermatophore which can then be subjected to biochemical analysis. Of particular interest will be the modification of the proteins as the semisolid mass becomes transformed into the spermatophore.

The monoclonal antibodies are powerful tools for the analysis of accessory gland development and its control. With these reagents, we plan to measure the content of differentiation-specific antigens during normal development and after manipulation with developmental hormones *in vivo* and *in vitro*. We hope to extend our investigation of accessory gland development to the molecular level. Such studies will involve cloning of genes of *T. molitor* and messenger-derived cDNAs in expression vectors within bacterial hosts. After inducing the expression of the cloned DNA, we hope to use the monoclonal probes to screen these libraries for the expression of the corresponding antigens and thus to identify the clones which code for spermatophore proteins.

*Acknowledgements*—We thank David Boraker and Laurie Eldar for their helpful technical advice, Connie Bricker for the immunohistochemical photography, and Ruth Goodridge for typing the manuscript. This work was supported by a grant to G. M. Happ from the National Institute of Health (AI-15662).

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