

ISOLATION, PARTIAL CHARACTERIZATION, AND LOCALIZATION OF THE A AND B PROTEINS FROM THE TUBULAR ACCESSORY GLAND OF MALE *TENEBRIO MOLITOR*

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Abstract—In its fully differentiated state, the tubular accessory gland of the male mealworm beetle, *Tenebrio molitor*, synthesizes five groups of proteins (A, B, C, D₁ and D₂) which are easily distinguished from one another on two-dimensional pI-SDS polyacrylamide gels (Black *et al.*, 1982: *Devl Biol.* **94**, 106–115). In the present work, the A and B proteins have been isolated by preparative gel electrophoresis and Amicon ultrafiltration. The isolation procedure provided two fractions of interest: one contained a mixture of A and B proteins (A/B) and the other consisted of only B proteins. The major proteins in the A class have a molecular weight of 17,900 while those of the B class are 19,000 daltons in size.

Antibodies have been produced to the A/B mixture and to the B fraction. Ouchterlony immunodiffusion and straight line immunoelectrophoresis show that the A and B proteins share common immunological characteristics. The proteins from the tubular accessory gland were displayed on one dimensional SDS gels and electrophoretically blotted onto nitrocellulose paper. The antibodies to the A/B mixture recognize A and B bands on these gels. In addition, these antibodies show affinity for C proteins and another band of lower molecular weight.

Using the anti-A/B with techniques of immunodiffusion, straight line immunoelectrophoresis, and immunoblotting, we have identified the A and B protein in extracts of soluble proteins in the spermatophore. Furthermore, the A/B proteins have been localized by immunohistochemical techniques within the apical portions of the secretory cells of the tubular gland and also in the lumen of the spermatophore.

Key Word Index: A and B proteins, tubular accessory glands, *Tenebrio molitor*, protein isolation, immunological relationship, spermatophore, insect reproduction

INTRODUCTION

In many species of arthropods, the reproductive system possesses specialized accessory glands which play diverse roles. Many such glands secrete proteins and lipoproteins which form the walls of the spermatophore (Gerber *et al.*, 1971; Tuzet, 1977; Leopold, 1976; Chen, 1984; Happ, 1984). Secretions of the male tract of *Aedes aegypti* (Fuchs *et al.*, 1968; Fuchs *et al.*, 1969; Fuchs and Hiss, 1970; Hiss and Fuchs, 1972) and of *Drosophila funebris* (Baumann, 1974a, 1974b; Baumann *et al.*, 1975) are passed to the female during copulation and subsequently these substances act to reduce the mating receptivity of the female. In some species, male products enhance oviposition and egg maturation, as in *Melanoplus sanguinipes* (Friedel and Gillot, 1976), *Bombyx mori* (Yamaoka and Hirao, 1977), *Leucania separata* (Chao, 1981), and *Acanthocelides obtectus* (Huignard, 1974). Male products destined to supply nutrients to the eggs are passed from male to female *Melanoplus*

sanguinipes (Friedel and Gillot, 1977) and *Danaus plexippus* (Boggs and Gilbert, 1979). Male secretions may aid sperm maturation as in *Musca domestica* (Leopold and Degrugillier, 1973), saturniid moths (Shepherd, 1974a, 1974b, 1975) and ticks (Oliver and Shepherd, 1980) or they may initiate contractions in the female tract which aid sperm movement to the spermatheca as in *Rhodnius prolixus* (Davey, 1957, 1965).

There are only a few studies which attempt to define the precise chemical nature of these accessory gland secretions. The amino acid composition of the walls of the spermatophore have been reported in three species of arthropods: *Tenebrio molitor* (Frenk and Happ, 1976), *Chthonius ischonocheles* (Hunt and Legg, 1971), and *Spodoptera littoralis* (Navon *et al.*, 1983). Fuchs and his coworkers (Fuchs *et al.*, 1968, 1969; Fuchs and Hiss, 1970; Hiss and Fuchs, 1972) have isolated and partially characterized a protein from the tract of *Aedes aegypti* which accelerates oviposition and decreases receptivity of the female. The paragonial proteins of *Drosophila funebris*, which play similar roles, have been isolated and the amino acid content of two of them has been determined (Baumann, 1974a, 1974b; Baumann *et al.*, 1975). In the present paper, we describe some of the properties

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of accessory gland products from male *Tenebrio molitor*.

The male mealworm beetle, *Tenebrio molitor*, has two pairs of reproductive accessory glands: the bean-shaped accessory glands (BAGs) and the tubular accessory glands (TAGs). The developmental morphology and biochemistry of both gland pairs have been studied in some detail (Gadzama, 1971; Gadzama *et al.*, 1977; Happ *et al.*, 1977; Dailey *et al.*, 1980, Black *et al.*, 1982; Happ and Happ, 1982; Happ *et al.*, 1982; Dailey and Happ, 1983). The secretory products of the BAG appear to form the wall of the spermatophore (Gadzama and Happ, 1974; Frenk and Happ, 1976; Dailey *et al.*, 1980; Dailey, 1981; Happ *et al.*, 1982) while the TAG secretions form part of the soluble components of the spermatophore (Black *et al.*, 1982). This system of accessory glands and their products are one of the most thoroughly described in any insect.

Of particular interest here are the tubular accessory glands and their products. The TAGs undergo a process of maturation in the late pupa and the young adults, which culminates in the synthesis of four major groups of proteins distinguished by their apparent molecular weights after SDS-polyacrylamide gel electrophoresis (Happ *et al.*, 1977; Black *et al.*, 1982, 1984). Earlier work (Happ *et al.*, 1977; Black *et al.*, 1982) demonstrated changes in the patterns of accumulation and of leucine incorporation for two of these groups of proteins (A and B) during post-ecdysial development. The A and B classes of protein appear as distinct bands on one-dimensional SDS polyacrylamide gels. However, each class is heterogeneous with respect to isoelectric point as seen on two dimensional SDS-pI polyacrylamide gels (Black *et al.*, 1982). Antibodies to an homogenate of the TAG have been produced in rabbits. When these antibodies were applied in crossed immunoelectrophoresis and Ouchterlony immunodiffusion, the results indicated that the A and B proteins are antigenically similar and comprise part of the soluble fraction of the spermatophore (Black *et al.*, 1982). The work described below was aimed toward extending the initial observations made on these two groups of proteins. Their immunochemical and biochemical characterization has been expanded and the proteins have been traced to a specific compartment in the spermatophore. This has been accomplished by purification of the proteins using preparative electrophoresis and Amicon filtration and by production of specific antisera which allowed identification and localization of these proteins within the spermatophore.

MATERIALS AND METHODS

Animals

Mealworms, *Tenebrio molitor* L., were purchased from a commercial supplier and reared on chick feed (Purina Startena). At pupation, the animals were sexed, segregated into male and female groups, and maintained at 26°C. At adult ecdysis, animals were given sliced potato and maintained in small all-male groups at 26°C. Males at 8 to 12 days post-eclosion were used for all of the experiments described below.

Dissections of tubular accessory glands

Tubular accessory glands (TAGs) were dissected from

males submerged in phosphate buffered saline (Dulbecco and Vogt, 1954). The glands were cleaned of adhering fat body and tracheae and homogenized with a glass tissue grinder in glass-distilled water or in 0.03 M barbitol-HCl buffer. For immunological procedures and gel electrophoresis, the TAG homogenate was spun at 15,600 g in an Eppendorf model 5412 centrifuge for 3 min. The pellet was discarded and the supernatant was frozen at -20°C. Final protein concentrations ranged from 1 to 5 mg/ml.

Spermatophore collection

Spermatophores were collected from males which were individually maintained on a floor of coarse screen. Male *T. molitor* produce approximately one spermatophore every 24 hr (Dailey, 1981). Spermatophores (60-100) were collected daily from glass plates under the screen and homogenized either in water or barbitol buffer as described for the TAGs. After pelleting insoluble material by centrifugation at 15,600 g, the supernatant, with protein concentrations ranging from 0.5 to 3 mg/ml, was frozen at -20°C. Protein determinations were by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) from Sigma Chemical Company as a standard.

Isolation of the A and B proteins

Fifty to one hundred pairs of TAGs were homogenized in barbitol buffer as described above. The homogenate was spun at 10,000 g for 10 min in a Beckman J2-21 centrifuge (JA-17 rotor) to remove insoluble components. Bromophenol blue (final concentration, 0.001%) and sucrose (final concentration, 10%) were added to the supernatant.

The supernatant was applied to a preparative agarose gel containing 1% Biorad electrophoresis grade agarose in 0.03 M barbitol-HCl buffer at pH 8.6. The sample was overlaid with 5 ml molten agarose in the same buffer. The gel (28 × 22.5 cm) was run at 150 V (constant voltage from a Hoeffler PS 500 power supply) for 3.5-4 hr using the barbitol-HCl running buffer in a Hoeffler SE 600 series slab gel electrophoresis unit.

After electrophoresis, the gel was cut into 12 equal strips (each 2.3 cm) perpendicular to the plane of electrophoresis. Ten milliliters of glass-distilled water were added to each strip and the sample was homogenized for 3 min in a Brinkman polytron. The slurry was shaken overnight on a rotator and then poured into a 10 ml syringe packed with glass wool. The liquid was pumped off with an LKB 2120 Varioperpex II peristaltic pump, and this eluate was fast frozen with dry ice-acetone and lyophilized. The final samples were resuspended in 1 ml glass-distilled water and subjected to SDS-polyacrylamide gel electrophoresis (see Fig. 2). Slices 10 and 11, containing both A and B proteins, were combined to produce a fraction with both A and B (designated fraction A/B). Slice 12 contained only B proteins and the eluate was designated fraction B. Fractions A/B and B were dialyzed against one-tenth concentration PBS for 24 hr (3 changes).

Following dialysis, proteins in fractions A/B and B were applied to an Amicon X-50 ultrafilter to remove contaminants of over 50,000 daltons. The filtrates were fast-frozen and lyophilized. The dried samples were resuspended in glass-distilled water, divided into 50 µl aliquots, and stored at -20 or -70°C. Yields were monitored by the Lowry procedure and the composition of each fraction checked on 12% SDS polyacrylamide gels.

Preparation of antisera

Antibodies were produced against the A/B fraction and the B fraction after injection of each into female New Zealand rabbits using the method of Hurn and Chantler (1980). Two rabbits were used for each immunogen. Pre-immune sera was collected before any antigens were injected. The initial injection, containing 300 to 400 µg protein, was mixed with an equal volume of Freund's complete

vant (Gibco Laboratories). Each rabbit received four intramuscular injections: one in each thigh and one in each shoulder. Booster injections (400–800 µg protein in Freund's incomplete adjuvant) were given at 4 weeks and at 6 weeks after the initial injections.

The animals were bled from the lateral ear vein at 4–7 weeks after the initial injections. Three bleedings followed at monthly intervals. At the fourth month, all animals were bled by cervical dislocation and bled by cardiac puncture. For all bleedings, the whole blood was allowed to coagulate at room temperature and spun at 5000 g for 20 min. After standing overnight at 4°C, the sera were spun at 1000 g for 20 min to remove any remaining clots or blood cells. Some samples of antisera (anti-A/B and anti-B) were stored in 100 µl aliquots at –70°C. Other samples were treated with ammonium sulfate to produce a fraction enriched for immunoglobulins (Wier, 1964). For the latter technique, one volume of saturated ammonium sulfate (100%), pH 6.5, was added to the sera dropwise with gentle stirring. The sample was spun at 10,000 g for 10 min and the supernatant washed twice with 40% SAS. The pellet was then dissolved in PBS, reprecipitated with SAS, and washed with 40% SAS. The final pellet was dissolved in one-half the original volume in PBS. These immunoglobulin-enriched antisera were dialyzed against PBS (24 hr, three changes) and then desalted by passing the sample through a Pharmacia S column equilibrated with PBS. The final preparations of anti-A/B and anti-B were divided into 50 µl aliquots and stored at –70°C.

Antibodies to the soluble proteins of the TAGs were those previously described (Black *et al.*, 1982).

Electrophoretic techniques

One-dimensional sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was done on 12% gels (Laemmli, 1971). Samples (10–100 µg protein) were applied in a cocktail consisting of 2% specially pure SDS (BDH Chemicals), 10% glycerol, 0.001% bromophenol blue, 10% β-mercaptoethanol in 68.5 mM Tris [Tris(hydroxymethyl)aminomethane] containing 1 mM Na₂ EDTA, pH 8.3. Samples were heated at 100°C for 10 min and applied directly to the gel. The proteins were electrophoresed at 60 V (constant voltage) through a 5% polyacrylamide stacking gel at 100 V (constant voltage) through the running gel using 0.05 M Tris, 192 mM glycine, pH 8.3 as the running buffer. Molecular weight standards were obtained from the Sigma Chemical Company. The gels were stained overnight in 0.5% Coomassie brilliant blue R-250 in 50% methanol containing 10% acetic acid.

Following destaining, all polyacrylamide gels were soaked in 0.5% methanol containing 10% glycerol for 3 min and dried on filter paper using a commercial dryer (Hoeffer; Hoefer *et al.*, 1979).

Immunological procedures

Soluble proteins were analyzed by Ouchterlony immunodiffusion (Ouchterlony, 1964) and by straight line immunoelectrophoresis (Graber and Williams, 1953; Black *et al.*, 1982) in 1% Biorad agarose in 0.03 M barbitol-HCl buffer, pH 8.6. The agarose gels (2 × 80 × 60 mm) were poured on a glass slide (Marine Colloids Division). Twenty-four hours after the addition of the antisera, the straight line immunoelectrophoretic gels and the immunodiffusion gels were soaked in phosphate buffered saline with 0.02% Na₂SO₄ for 24 hr (three changes) and in glass-distilled water for 6 hr (three changes) to remove all the unreacted protein. The gels were covered with filter paper and dried under weights on a commercial slide dryer. After drying, they were stained for 10 min in 0.5% Coomassie blue in 45% methanol containing 10% acetic acid. The gels were destained by diffusion into glass-distilled water and then re-stained in 0.5% methanol containing 10% acetic acid.

Electrophoretic blotting

The blotting procedure was essentially that of Towbin *et al.* (1979). Proteins were subjected to electrophoresis on 12% SDS slab gels. The gel was removed from the electrophoresis apparatus and placed on a piece of Biorad nitrocellulose paper (0.45 mm pore size) and immersed in 25 mM Tris, 192 mM glycine, 20% methanol. The paper-supported gel was laid on a Dacron sponge and blotter paper. The gel was then covered with a second piece of blotter paper and the entire sandwich was placed into a cassette. The loaded cassette was put into a Hoefer Transfor unit (equipped with a Hoefer TE 50 power lid) with the nitrocellulose facing the anode. The chamber was filled with the same Tris-glycine-methanol (pH 8.3) which had been thoroughly degassed under vacuum. Electrophoresis was performed at 6–8 V/cm for 12–18 hr at 4°C with gentle stirring.

Following electrophoresis, the blots were initially soaked in 3% BSA in PBS for 2 hr at room temperature. The electroblots were washed three times (10 min each) in PBS followed by incubation in anti-A/B (diluted 1:1000) in 3% BSA, 10% fetal calf serum in PBS at room temperature for 2 hr. The control electroblots were treated with the same concentration of preimmune sera. The control and experimental electroblots were washed six times, 10 min each, in PBS. The washed electroblots were incubated for 2 hr at room temperature in the indicator antibody [goat anti-rabbit IgG (H and L) peroxidase conjugates, Miles Laboratories] which had been diluted 1:1000 in PBS. They were subsequently washed six times (5 min each) in PBS, once in 10 mM Tris-HCl, pH 7.4 (5 min) and soaked for 10–15 min in peroxidase substrate solution [25 µg 3,3'-dimethoxybenzidine (*o*-diansidine from Sigma Chemical) plus 0.01% H₂O₂ in 10 mM Tris-HCl, pH 7.4]. The reaction was terminated by washing the blots with glass-distilled water. After several additional washings in distilled water and PBS, the blots were photographed, dried under filter paper, and stored protected from light.

Immunohistochemistry

Glands were dissected from beetles and spermatophores were collected as described above and immediately thereafter, fixed in alcoholic Bouin's (Humason, 1967). The A and B proteins were localized using a modification of the method of Farr and Nakane (1982). Following fixation, glands and spermatophores were dehydrated through an ethanol series to xylene and embedded in Paraplast. Serial sections (5 µm thickness) were mounted on slides coated with Meyer's albumin. The sections were dewaxed with xylene and rehydrated through alcohol. After three washings (3 min each) in PBS, primary antibody (whole sera anti-A/B or preimmune sera) was placed gently onto the slides and then the slides were allowed to incubate for 3 hr at room temperature. At the end of the incubation, the slides were washed three times with PBS and subsequently incubated with a 1:10 dilution of affinity purified goat anti-rabbit IgG (H and L) peroxidase conjugates (Kirkegaard and Perry Laboratories, Inc.) in PBS. Two hundred microliters of substrate solution [0.02% diaminobenzidine (Sigma Chemical), 0.004% H₂O₂, in Tris-HCl, pH 7.6] was added to each slide. After a 10 min incubation, the slides were washed three times with PBS. The sections were dehydrated through an ethanol series to xylene and mounted in Permount. Sections were examined and photographed on a Zeiss Ultraphot II.

RESULTS

Isolation of the A and B proteins

The A and B proteins produced by the tubular accessory glands showed high electrophoretic mobility at pH 8.6 on 1% agar gels used for crossed line immunoelectrophoresis (Black *et al.*, 1982). This

Table 1. Purification of the A and B proteins from the tubular accessory glands of *Tenebrio molitor*

Fraction	Total protein (μ g)	n*	% Yield (\pm SD)
Initial homogenate	5600	7	100
Supernatant following centrifugation of initial homogenate†	4842	7	86.5 (6.4)
Combined agar fractions			
1-4	967	5	17.3 (7.2)
5-7	441	5	7.9 (3.3)
8-9	286	5	5.1 (2.0)
10-11	123	4	2.3 (1.2)
[12]	98	4	1.8 (0.9)
Fractions following Amicon X-50 ultrafiltration			
10-11	119	4	2.1 (0.6)
[12]	93	4	1.7 (0.4)

*Total number of samples used in standard deviation (SD) determination.

†Supernatant following centrifugation at 5000 g; considered soluble tubular accessory gland proteins.

characteristic mobility was the starting point for purification of these proteins. Electrophoresis of soluble proteins from homogenates of the TAGs on pH 8.6 preparative agarose gels yielded two broad bands with high mobility (Fig. 1). The agarose gel was cut into 12 equal segments. Each segment was homogenized, and the proteins eluted. Each eluate was displayed on 12% polyacrylamide gels (Fig. 2). Fractions 10 and 11 both of which contained mixtures of A and B proteins, were combined. Fraction 12 contained mostly B proteins. Fractions 10-11 (hereafter designated A/B) and fraction 12 (hereafter designated A) were passed through an Amicon X-50 filter to remove contaminants of high molecular weight. The protein profiles of each fraction during the purification sequence are shown in Fig. 3 and the yields from each step shown in Table 1.

The net weight of the combined A and A/B fractions was 4% of the total protein applied to the preparative agarose gel. On the basis of our previous determination (Black *et al.*, 1982) we found that the A and B proteins comprise 12% of the total in the TAGs, our yield from this two-step purification scheme is about 33%. As determined by mobility on SDS polyacrylamide gels, the molecular weights of the isolated proteins were 17,800 for A and 19,000 for B (Fig. 3) which is in good agreement with our earlier study. Gels without β -mercaptoethanol produced the same pattern and indicate the same molecular weights.

The A and B proteins are very similar in molecular weight and native charge, and our attempts to separate A class proteins from the A/B fraction were not successful. Cutting the preparative agarose gel into 24 slices instead of 12 provided the same results (i.e. fractions contained either pure B or an A/B mixture). Carboxymethyl cellulose ion exchange chromatography using 100 mM Tris-HCl (pH 7.8) and a continuous gradient of NaCl (5-500 mM) resulted in the selection of the A and B proteins in the presence of a number of other proteins at lower salt concentrations. TAG proteins were separated into several distinct fractions by precipitation with increasing concentrations of ammonium sulfate, but the A and B classes always co-precipitated.

Immunological comparisons of the A and B proteins from the tubular accessory gland homogenates, and spermatophore

As described in the Materials and Methods section the isolated A/B and B fractions were injected into rabbits for production of antisera. Assay of anti-A/B sera by Ouchterlony immunodiffusion demonstrated the immunological identities among A/B protein fraction, the B fraction, the TAG, the spermatophore (Fig. 4a). A parallel experiment using anti-TAG showed the same identities among A/B, B, spermatophore, and TAG (Fig. 4b).

Straight-line immunoelectrophoresis using the anti-TAG and the anti-A/B showed that the antibodies recognize no antigen of the BAG (low lanes in Fig. 5a and 5b). Ouchterlony immunodiffusion with both antibodies, directed against the homogenate of the BAG and against homogenate of the gut of the female (data not shown), likewise indicated that our antisera were specific to proteins found only in the TAG. Straight-line immunoelectrophoresis with anti-A/B and anti-TAG further demonstrates the identity between the antigens of TAG and the spermatophore (Fig. 5). In agreement with the Ouchterlony results, the common identity demonstrated by straight line immunoelectrophoresis indicate that proteins A and B are part of the spermatophore.

With both Ouchterlony immunodiffusion and straight-line immunoelectrophoresis, the reaction between anti-TAG and spermatophore showed a third precipitation arc that was neither A nor B protein (Figs 4 and 5). Since we have previously shown that our absorbed antibody to the homogenate of the TAG did not recognize the proteins of the BAG of the testes (Black *et al.*, 1982), this third precipitation arc may indicate the presence of the C or D proteins in the spermatophore.

Immunological detection of the A and B proteins in the TAG and the spermatophore after electrophoretic transfer to nitrocellulose

The protein profiles of soluble proteins from the TAG and from the spermatophore displayed on 12% polyacrylamide gels show significant similarities and differences (Fig. 6). There are proteins in the spermatophore which co-migrate with the A, B, C, and D classes of the TAG. After we had electrophoretically transferred the soluble proteins of spermatophore and the TAG to nitrocellulose sheets, we exposed them to anti-A/B. The distribution of the bound anti-A/B was determined by addition of goat anti-rabbit IgG peroxidase conjugates and peroxidase substrate. The resulting staining indicates that the A and B proteins comprise part of the soluble fraction of the spermatophore (Fig. 7).

This electroblotting procedure with anti-A/B also detected several additional bands in the homogenate of the TAG. The class C proteins in the gland homogenate were recognized by the antibody but a C band was not seen in the spermatophore lane (Fig. 7). A protein of lower molecular weight (A_1) was also recognized in the gland homogenates. Finally, there was a diffuse reaction with gland proteins of higher molecular weight. Thus the anti-A/B recognized a

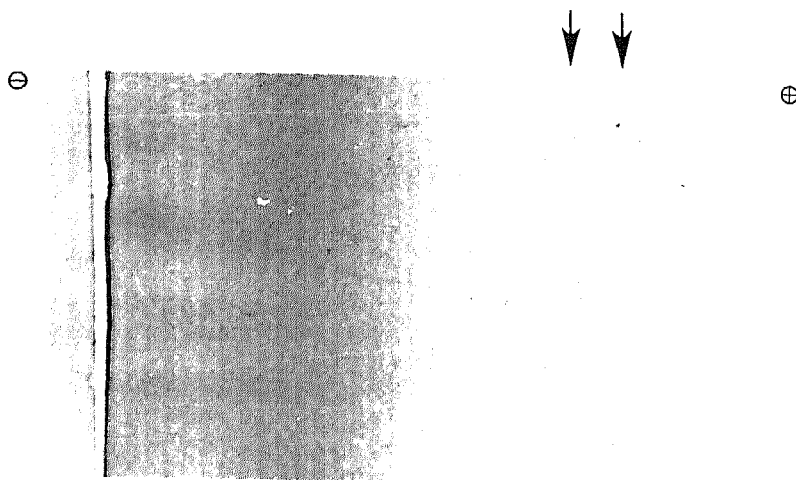


Fig. 1. Preparative gel electrophoresis of soluble proteins from mature tubular accessory glands at pH 8.6; arrows indicate the high mobility protein bands.

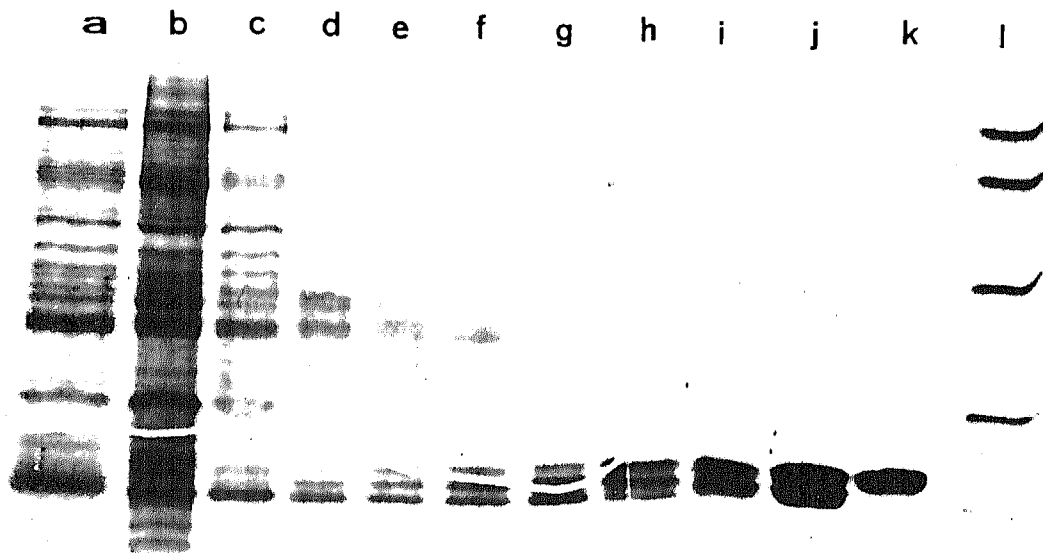


Fig. 2. SDS-polyacrylamide gel electrophoresis of tubular accessory gland soluble proteins eluted from preparative electrophoresis gel shown in Fig. 1; lanes a-k, preparative gel segments 2-12. Segment 1, which contained no stainable proteins, is not shown. Lane l, molecular weight standards.

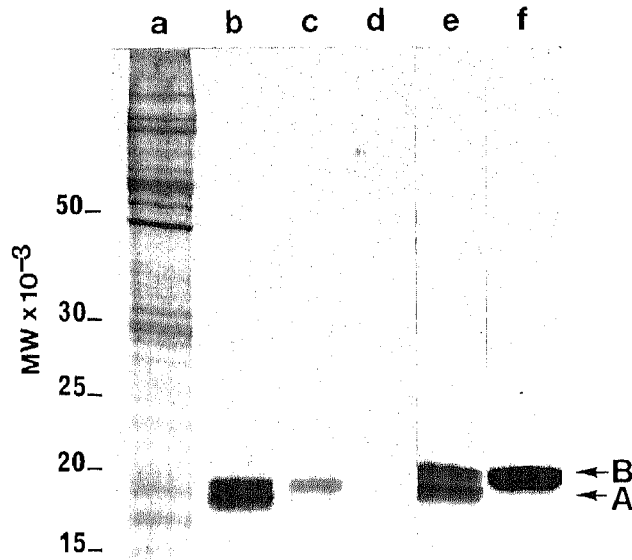


Fig. 3. SDS-polyacrylamide gel electrophoresis of the tubular accessory gland A and B proteins during different phases of purification; (a) soluble TAG homogenate, (b) fraction 10/11 before Amicon ultrafiltration, (c) fraction 12 before Amicon ultrafiltration, (d) high molecular weight proteins retained by Amicon X-50 filter, (e) fraction 10/11 after Amicon ultrafiltration, (f) fraction 12 after Amicon ultrafiltration; A and B refer to the classes of TAG proteins.

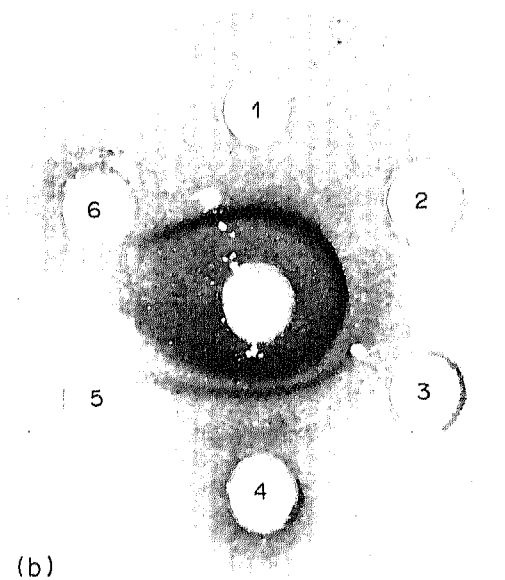
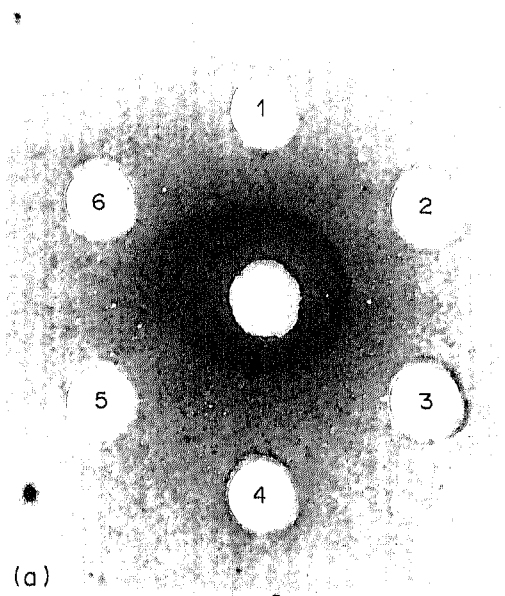


Fig. 4. Ouchterlony immunodiffusion of (a) anti-A/B (center well) versus (1) soluble TAG homogenate, (2) soluble spermatophore homogenate, (3) isolated B protein, (4) protein A/B mixture, (5) bean shaped accessory gland homogenate, (6) spermatophore insoluble homogenate and (b) anti-TAG (center well) versus (1) soluble TAG homogenate, (2) soluble spermatophore homogenate, (3) isolated B protein, (4) protein A/B mixture, (5) bean shaped accessory gland homogenate, (6) testes homogenate.

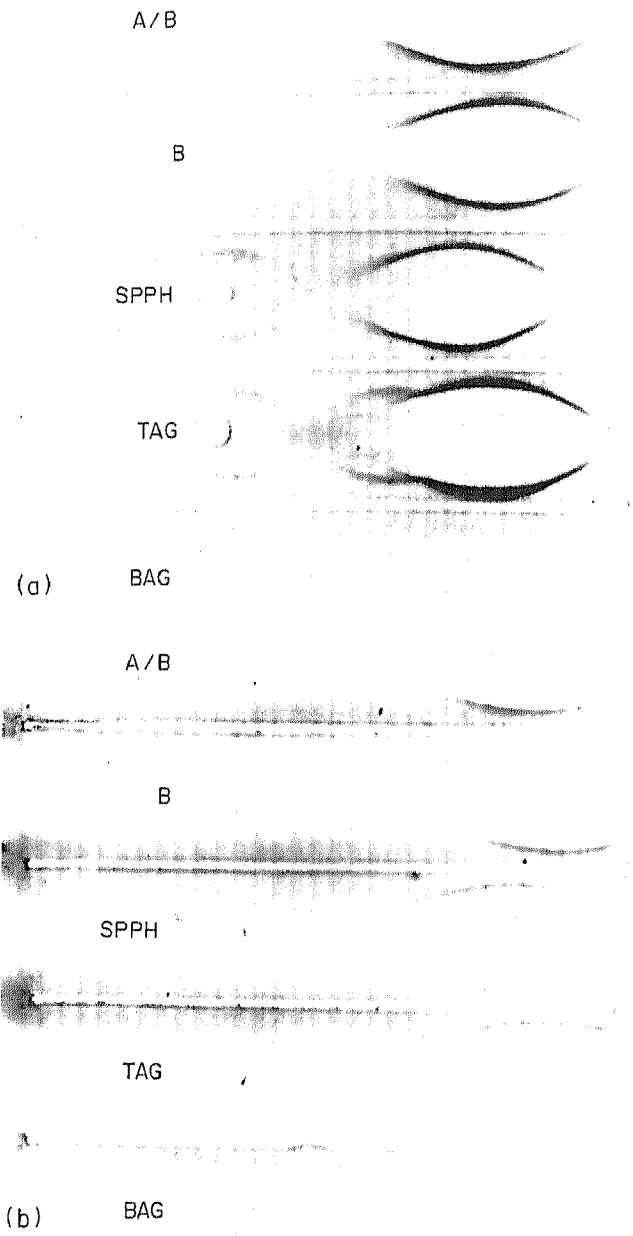


Fig. 5. Straight line immunoelectrophoresis of (a) mature tubular accessory glands (TAG), bean-shaped accessory glands (BAG), and spermatophore soluble proteins (SPPH) versus anti-TAG and (b) versus anti-A/B.

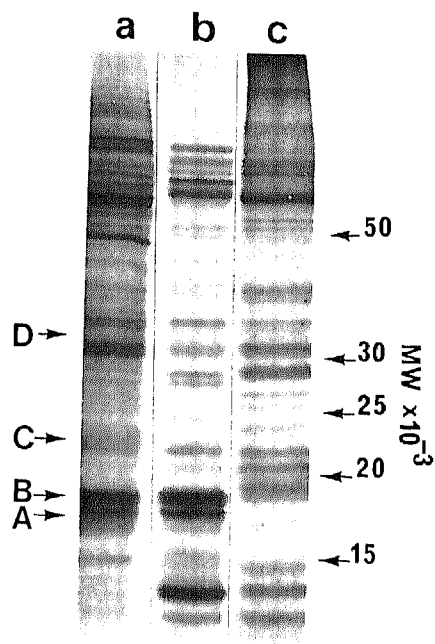


Fig. 6. SDS-polyacrylamide gel electrophoresis of soluble proteins from tubular accessory glands (a), soluble spermatophore proteins (b) and spermatophore insoluble proteins (c). A-A proteins, B-B proteins, C-C proteins, D-D proteins of the mature tubular accessory glands.

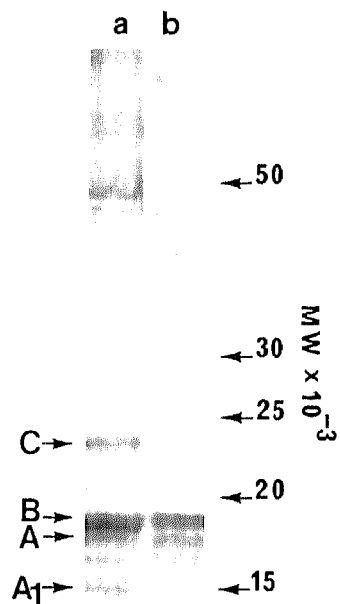


Fig. 7. Proteins from (a) soluble tubular gland homogenates and (b) soluble spermatophore homogenates have been electrophoretically transferred to $0.45 \mu m$ pore size nitrocellulose sheets from 12% SDS polyacrylamide gels, exposed to anti-A/B and then to goat anti-rabbit IgG-peroxidase, and visualized as described in Materials and Methods. A₁, A, B and C refer to the classes of soluble proteins.

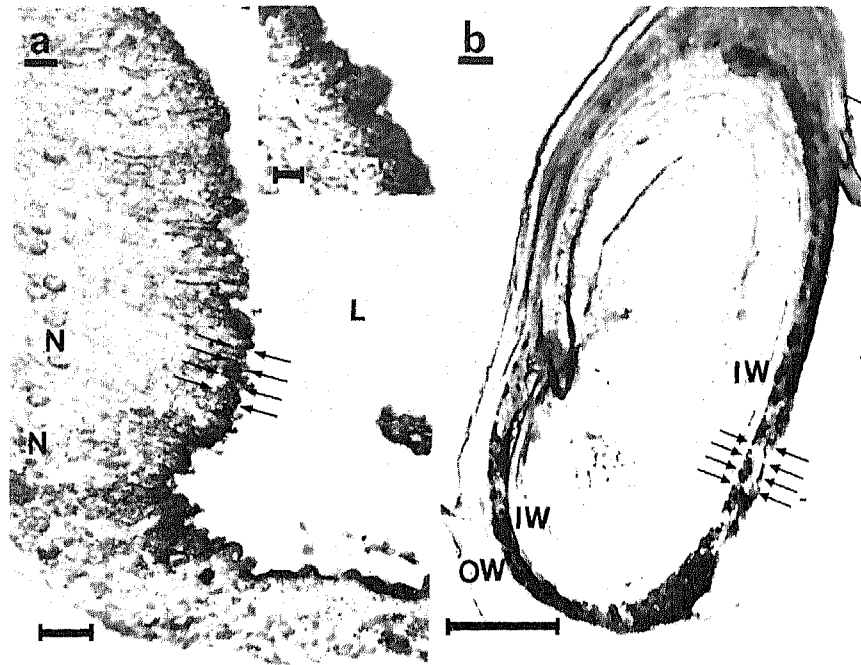


Fig. 8. (a) Immunohistochemistry of mature TAGs using anti-A/B and visualized using peroxidase conjugated secondary antibodies as described in Materials and Methods; L—gland lumen, N—nuclei, arrows indicate staining at apical portions of cells, bar is equivalent to $10\ \mu\text{M}$. Insert demonstrates the intense staining at the apical surface, bar is equivalent to $1\ \mu\text{M}$. (b) Immunohistochemistry of the spermatophore using anti-A/B; IW—invaginated wall, OW—outer wall, arrows indicate regions of intense staining, bar is equivalent to $10\ \mu\text{M}$.

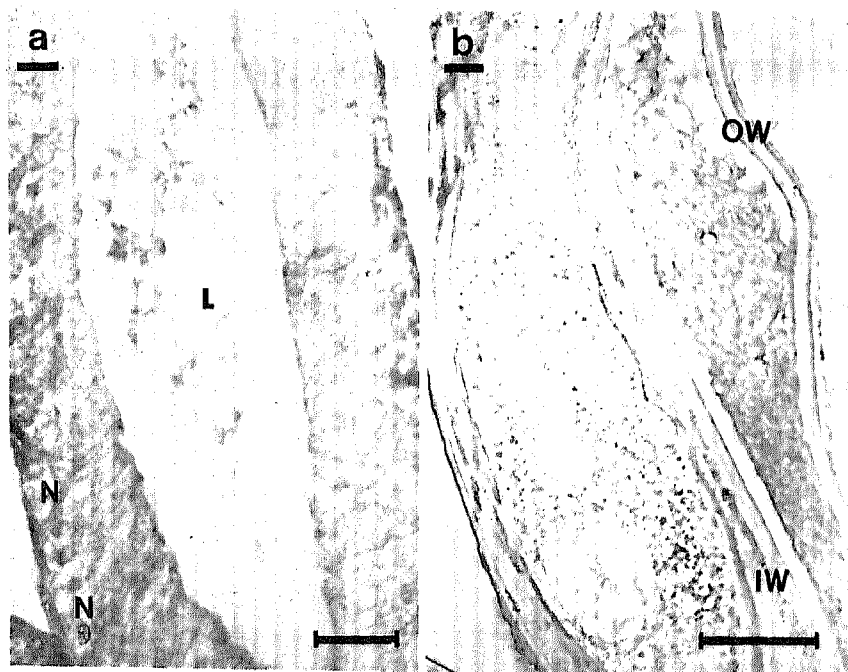


Fig. 9. Immunohistochemistry of (a) mature TAGs and (b) spermatophore using preimmune sera and peroxidase conjugated secondary antibody, bars are equivalent to $10\ \mu\text{M}$.

ler spectrum of antigens than the two major proteins used as immunogens.

Immunocytochemical localization of the A and B proteins in the TAG and the spermatophore

To determine the precise spatial distribution of the A and B proteins, anti-A/B was allowed to bind to affinity-purified sections of the TAG. The antibodies bound strongly to the secretory epithelium of the TAG (Fig. 8a). Staining was particularly intense in the apical portions of the cells which are known to be associated with secretory vesicles (Gadzama *et al.*, 1977). A similar experiment was carried out on sections of the spermatophore. The spermatophore is a long hollow tube with one end deeply invaginated. The sperm lie in the lumen between the outer wall and the invagination (Gadzama and Happ, 1974). The anti-A/B was localized in the lumen of the spermatophore (Fig. 8b). This result indicates that the A and B proteins comprise part of the seminal plasma which bathes the sperm within the spermatophore. When preimmune sera were substituted for anti-A/B in a control experiment, there was low background staining for peroxidase in the TAG and in the spermatophore (Fig. 9).

DISCUSSION

The A and B protein classes produced by the male accessory gland of male mealworm beetles are similar in size, isoelectric point, and electrophoretic mobility. But on the basis of the biochemical properties, the A and B protein classes seem quite distinct from one another. Their exact mobilities on SDS gels are not the same. The A proteins have a molecular weight of 17,900 daltons while the B proteins are larger, averaging 19,000 daltons. The immunochromatographic data demonstrate that there are significant similarities between the A and B proteins. In straight line immunoelectrophoresis using anti-A/B as the test antibody, both the A/B fraction and the B fraction produce but one precipitin arc. A slight difference in mobility in straight line immunoelectrophoresis in the spermatophore (Fig. 5) may reflect some modification of the proteins between the TAG and the spermatophore. In experiments using this antibody for Ouchterlony immunodiffusion, the A and B proteins are immunologically indistinguishable. If they shared only partial identity there should have been a characteristic identity spur on the plate. Since none was observed it can be concluded that A and B share common antigenic domains.

The antisera produced against the A and B proteins recognize other protein bands as well (Fig. 7). It is unlikely that there were major contaminants in the immunogen, since the samples used for the primary and secondary injections showed only A and B bands on SDS polyacrylamide gels. Rather we believe that the proteins giving rise to the 4 bands (A₁, A₂, B, and C) in lane a of Fig. 7 share immunological properties. This belief is in agreement with crossed immunoelectrophoretic data which demonstrated immunological relatedness between at least three groups of differentiation-specific proteins in the TAG (Black *et al.*, 1982). The specific nature of the inter-

relationships among these four groups of proteins awaits further study.

Immunocytochemical evidence showed that the A and B proteins are present in the secretory cells of the TAG and in the lumen of the spermatophore. It is not known whether the A or B proteins possess enzymatic activity. Since these soluble proteins are absent from the walls of the spermatophore and confined to the lumen of the structure, it seems unlikely that they serve in a structural role. However, the possibility remains that these proteins or other soluble components from the TAG may play a role in the maturation of spermatozoa in their expulsion from the spermatophore.

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