

Purification and Characterization of a Proline-rich Secretory Protein That Is a Precursor to a Structural Protein of an Insect Spermatophore*

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The spermatophore or sperm sac of *Tenebrio molitor* (yellow mealworm beetle) is an acellular structure composed mostly of structural proteins, termed spermatophorins. The proteins are derived from the bean-shaped accessory reproductive glands of the male and are assembled into the multilayered structure within the ejaculatory duct. Homogenates of the secretory plug from this gland were used as immunogens for the production of monoclonal antibodies, including one identified as PL 21.1 which recognizes an antigen in the gland and the spermatophore. With the aid of gel filtration and immunoaffinity chromatography with a PL 21.1, we isolated a glandular secretory protein that is a precursor to a spermatophorin with similar electrophoretic mobility. On native polyacrylamide gels, the antigen from gland homogenates has an apparent molecular mass of 370 kDa. On sodium dodecyl sulfate gels, the antigen from the gland and that from the spermatophore have apparent molecular masses of 23 kDa. According to immunoblots of sodium dodecyl sulfate gels, the 23-kDa glandular antigen is organ-specific and adult-specific. By immunocytochemistry with PL 21.1, we found the antigens to be restricted to secretory vesicles of only one cell type in the gland and to a discrete layer in the outer wall of the spermatophore. The 23-kDa secretory antigen is distinguished by being high in glutamic acid/glutamine (15.4%) and in proline (25.2%).

spermatophores consist of an outer wall, divisible into several layers, and various internal zones that may contain sperm and seminal fluids or that may play roles in ejecting semen from the package. With transmission electron microscopy, at least some layers in the wall of a beetle spermatophore can be seen to be formed of parallel filaments (Gadzama and Happ, 1974; Bricker and Happ, 1985). By extraction of the spermatophores of mealworm beetles (*Tenebrio molitor*) under denaturing conditions, we solubilized proteins which vary in their molecular mass from 14 kDa to over 100 kDa (Happ *et al.*, 1982).

Spermatophorins, the structural proteins of spermatophores (Happ, 1987), are derived from secretory products of male accessory reproductive glands. In order to identify individual spermatophorins during the formation of the spermatophore, we have produced monoclonal antibodies to some of these secretory proteins of *Tenebrio*. In this species, as in many others, the male accessory glands are composed of several types of secretory cells. With immunocytochemistry at the light and electron microscopic levels, we have followed two antigens from the cells of origin to the wall of the spermatophore (Grimnes and Happ, 1986; Grimnes *et al.*, 1986). For the three cases we have studied, the cell-specific antigens do not blend together, but each remains confined to a discrete coherent patch in the lumen of the gland. In the ejaculatory duct, each patch is molded into a thin layer. It appears that the secretions from a single cell type are targeted toward particular sites of the final spermatophore. Such a morphological chronicle of antigen distribution is useful for an understanding of the overall strategy of spermatophore assembly. However, we believe that the most interesting aspects of spermatophore formation are the interactions among proteins which govern linking of monomers into aggregates and filaments.

For spermatophores, as indeed for cuticle, very little is known of the mechanisms by which molecular precursors assemble to form filaments. Since spermatophores contain mostly protein, the mechanisms may be easier to study than for cuticle which consists of both proteins and chitin. In order to understand aggregation of the proteins into filaments and layers, it is necessary first to characterize the spermatophorins. The amino acid compositions of mixtures of spermatophorins have been reported from homogenates of the spermatophore of two species of insects, the mealworm beetle (Frenk and Happ, 1976) and a moth (Navon *et al.*, 1983). In both cases, the crude hydrolysate was distinguished by the presence of high levels (13–30%) of proline. With the aid of a monoclonal antibody, we report here the first purification and characterization of a secretory protein which is incorporated

In insects, acellular structures like the exoskeleton are assembled in extracellular space from secreted precursors. Among the most intriguing such structures are spermatophores, elaborate packages for transfer of sperm from male to female (Mann, 1984). Their composition, assembly, and physiological function are receiving increasing attention (Tuzet, 1977; Chen, 1984; Happ, 1984).

The cuticle of the body wall, which is also the insect skin, has been the most intensively studied of the large extracellular structures (Hepburn, 1985). Spermatophores are not made of cuticle; but like cuticle, they are highly organized. Typical

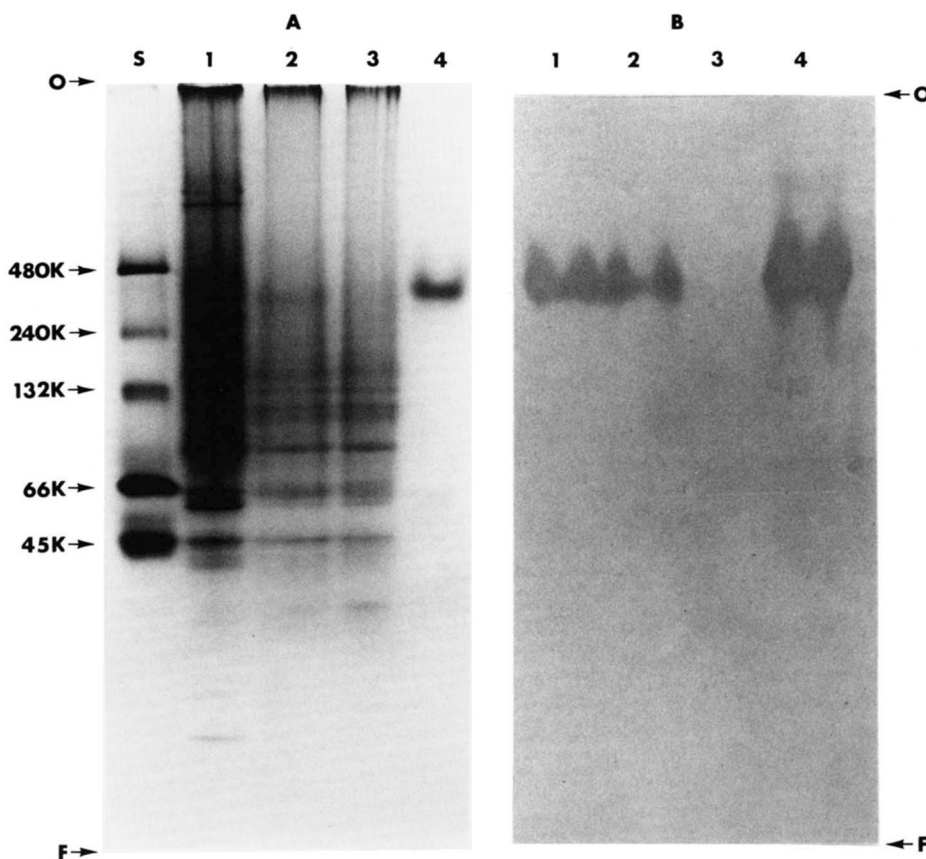
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FIG. 3. Native polyacrylamide slab gel electrophoresis and corresponding immunoblots of PL 21.1 antigens at various stages of purification. *A* shows a gel stained with Coomassie Blue. Electrophoresis on a 4–20% gradient was at a constant 70 V (5 °C) until the tracking dye (bromphenol blue) reached the end of the gel. For *B*, proteins from a similar gel were electroblotted onto nitrocellulose and reacted with PL 21.1 monoclonal antibody. Bound mouse immunoglobulins were visualized by exposure to rabbit anti-mouse IgG coupled to peroxidase followed by application of diaminobenzidine and hydrogen peroxide. *Lane 1* was loaded with crude BAG homogenate, and *lane 2* was loaded with pooled immunoreactive fractions from Sephadex G-150. Fractions from the immunoaffinity column were applied to *lane 3* (nonabsorbed) and *lane 4* (acid-eluted). For *A*, 20 μ g of protein was applied to each lane. For *B*, 3 μ g of protein was applied to *lanes 1–3* and 3 μ g of protein was applied to *lane 4*. Molecular weight standards (*lane S*) are: urease tetramer (480,000), urease dimer (240,000), bovine serum albumin dimer (132,000), bovine serum albumin monomer (66,000), and ovalbumin (45,000). *O*, origin; *F*, front.



into the wall of an insect spermatophore. In this case, the secretory protein is unusual in that over 25% of its amino acid residues are proline.

EXPERIMENTAL PROCEDURES¹

RESULTS

Purification—PL 21.1 antigens were purified from BAGs² of male adults by gel filtration, monoclonal antibody affinity chromatography, and extraction from an SDS-polyacrylamide gel. The elution profile from Sephadex G-150 revealed that the antigen was found after the major protein peak which eluted with the void volume (Fig. 1). Fractions containing antigen were pooled and applied to an Affi-Gel immunoaffinity column. Most of the proteins passed directly through the column; and, after a high salt wash, the antigen was eluted with 0.2 N acetic acid (Fig. 2).

Native gels of the acid-eluted fraction (Fig. 3A, *lane 4*) show a single major band and a minor one of lower molecular weight. Western blotting of such a native gel (Fig. 3B) with PL 21.1 antibody showed that the major protein band in *lane 4* is recognized by the antibody, whereas the minor band is not recognized. Furthermore, the Western blot demonstrates the presence of PL 21.1 antigen in the homogenate (Fig. 3B,

lane 1), the antigen-positive fractions from gel filtration (Fig. 3B, *lane 2*), and the absence of an immunoreactive band from fractions that did not bind to the affinity column (Fig. 3B, *lane 3*). According to regression analysis based on mobility relative to the standards, the apparent molecular weight of the immunoreactive band is 370,000.

On Coomassie-stained SDS gels, the acid-eluant from the immunoaffinity column (Fig. 4A, *lane 4*) shows a single major band and also some faint bands of high mobility. According to regression analysis, the molecular mass of the major band is 23 kDa and that of the minor one is 13 kDa. A strong 23-kDa band is also seen in the homogenate of BAGs (Fig. 4A, *lane 1*) and in the immunoreactive fractions after gel filtration (Fig. 4A, *lane 2*), but it is absent from fractions that did not bind to the immunoaffinity column (Fig. 4A, *lane 3*). With the exception of the 23-kDa band, stained proteins in *lane 2* are present in *lane 3*. Western blotting shows that the PL 21.1 antibody binds to the 23-kDa band (Fig. 4B) in all fractions where it is visible after Coomassie staining (Fig. 4A). In addition, the minor band seen at 13 kDa after acid elution (Fig. 4A, *lane 4*) and faint bands between 23 and 13 kDa are immunoreactive. In order to purify the 23-kDa protein, the band corresponding to that peptide was extracted from a preparative SDS gel. The extracted material contained only the 23-kDa band after Coomassie staining or after Western blotting (*lanes 5* of Fig. 4, *A* and *B*). The 23-kDa band stained with periodic acid-Schiff's reagent, indicating the presence of covalently bound carbohydrate. A similar extraction procedure was attempted to purify the 13-kDa band, but no distinct 13-kDa band was seen on an SDS gel after the extraction.

Amino Acid Analysis—The amino acid composition of the 23-kDa antigen is shown in Table I. The most noteworthy feature is the high proline content (25.4%). In addition, the protein contains significant amounts of glutamic acid/gluta-

¹ Portions of this paper (including "Experimental Procedures" and Figs. 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-3253, cite the authors, and include a check or money order for \$3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

² The abbreviations used are: BAGs, bean-shaped accessory glands; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay.

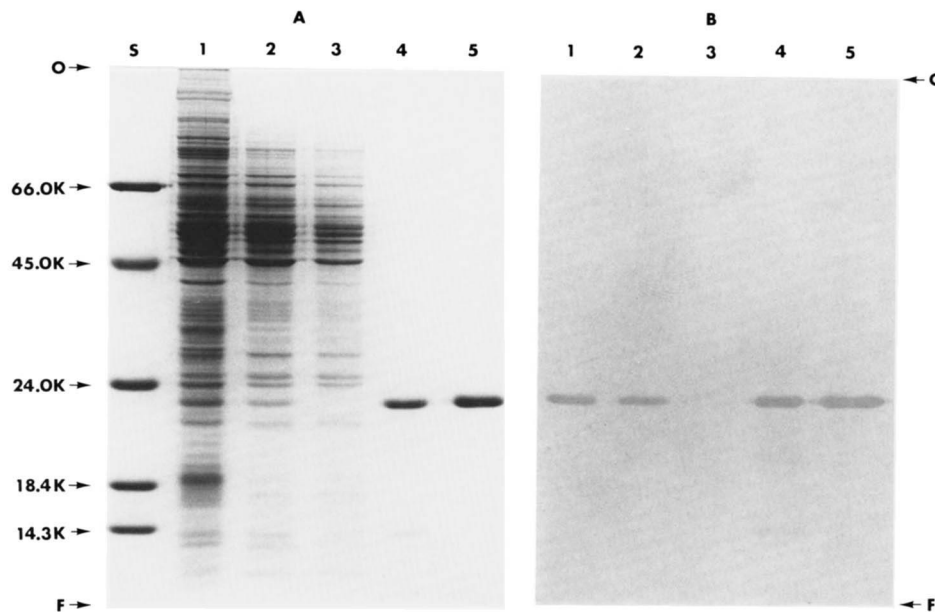


FIG. 4. SDS-polyacrylamide slab gel electrophoresis and corresponding immunoblots of PL 21.1 antigens at various stages of purification. *A*, lanes 1–5 show a gel stained with Coomassie Blue. Electrophoresis on a 12% slab gel was at a constant 100 V (5 °C) for 5 h. For *B*, lanes 1–5, proteins from a similar gel were electroblotted onto nitrocellulose and reacted with PL 21.1 monoclonal antibody. Bound immunoglobulins were detected as described for Fig. 3. Lanes 1 were loaded with crude BAG homogenate. Lanes 2 were loaded with pooled immunoreactive fractions from Sephadex G-150 column. Fractions from the immunoaffinity column were applied to lane 3 (nonabsorbed) and lane 4 (acid-eluted). Lanes 5 show material of 23 kDa which had been eluted from an earlier 12% SDS gel. In *A*, 10 μ g protein was applied to lanes 1–4 and 15 μ g of protein was applied to lane 5. In *B*, 2 μ g of protein was applied to lanes 1–3, and 3 μ g of protein was applied to lanes 4 and 5. Molecular weight standards (lane *S*) are: bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000), β -lactoglobulin (18,400), and lysozyme (14,300).

TABLE I
Amino acid composition of 23-kDa antigen

Amino acid	mol % ^a
Aspartic acid/asparagine	6.9
Threonine	3.9
Serine	3.5
Glutamic acid/glutamine	15.4
Proline	25.2
Glycine	5.7
Alanine	5.9
Valine	4.5
Methionine	0
Isoleucine	5.4
Leucine	4.0
Tyrosine	4.0
Phenylalanine	3.2
Histidine	1.7
Lysine	3.2
Arginine	3.5
Cysteic acid ^b	4.1
Tryptophan	ND ^c

^a Data from averages of duplicate samples hydrolyzed for 22 h in 6 N HCl *in vacuo* at 110 °C.

^b Duplicate samples performic acid-oxidized (Hirs, 1967) and then hydrolyzed for 22 h in 6 N HCl *in vacuo* at 110 °C.

^c ND, not determined.

mine (15.4 mol %) and cysteine (4.1 mol %), but it completely lacks methionine.

Localization of PL 21.1 Antigen—Tissues from male adults 5 days after ecdysis were homogenized and examined by Western blotting (Fig. 5). The PL 21.1 antibody recognizes bands from BAGs, secretory plug (mass of secretion dissected from the lumen of BAG), and the spermatophore. In BAGs (Fig. 5, lane 1), the major band is at 23 kDa with minor bands between 23 and 13-kDa. In both the plug and the spermatophore

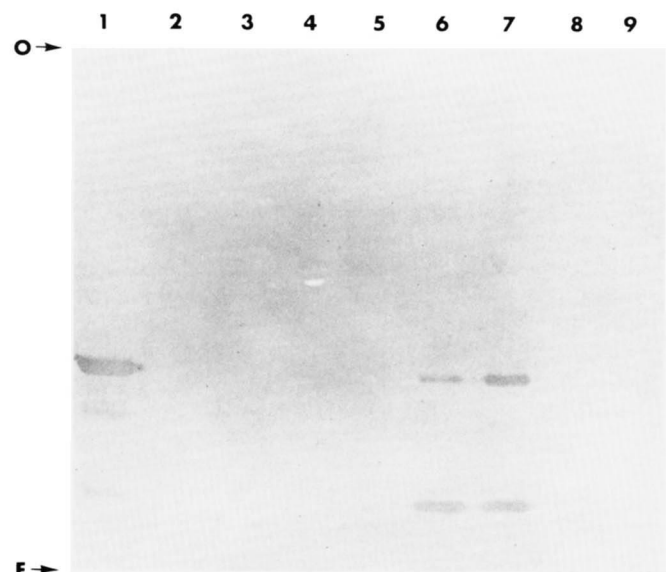


FIG. 5. Immunoblot of SDS-polyacrylamide gel of organ homogenates and secretions. Samples were collected from males 5 days after adult ecdysis. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 12% gel (100 V for 5 h) and transferred electrophoretically to nitrocellulose. The nitrocellulose sheet was incubated with PL 21.1 antibody followed by rabbit anti-mouse IgG coupled with peroxidase, and the sites of IgG binding were visualized with diaminobenzidine plus hydrogen peroxide. Lane 1, BAG; lane 2, tubular accessory gland; lane 3, ejaculatory duct; lane 4, vas deferens and seminal vesicle; lane 5, testis; lane 6, secretory plug from BAG; lane 7, spermatophore; lane 8, fat body; lane 9, hemolymph. A protein sample of 30 μ g was applied to lane 6, and samples of 50 μ g were applied to all other lanes.

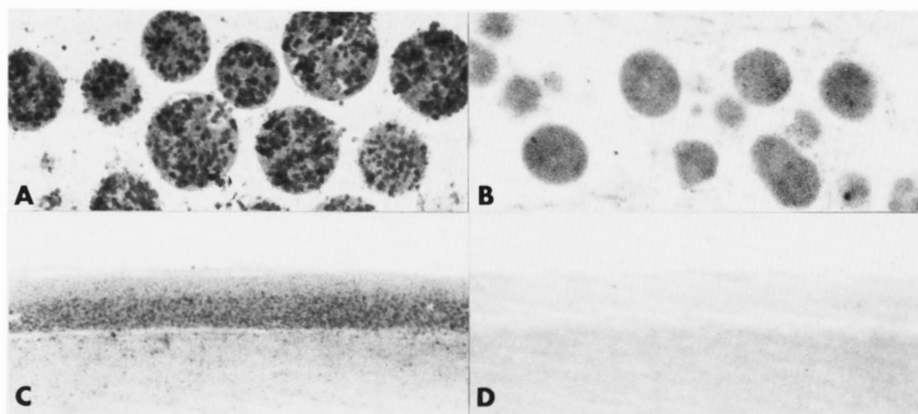


FIG. 6. Immunocytochemical localization of PL 21.1 antigens. Tissues were fixed in formaldehyde/glutaraldehyde and embedded in resin, and thin sections were soaked in primary antibody. After successive exposure to biotinylated anti-mouse IgG and avidin/biotin/peroxidase conjugate, the sites of antibody binding were detected by staining with diaminobenzidine (A and C). Sections through cell type 4 (A and B) demonstrate that the antigen is concentrated in the secretory vesicles (A) of the experimental cells but is absent from the control cells (B) which were not exposed to primary antibody. Antigen is also present in a discrete layer of the wall of the spermatophore (C) which is not visible in the unstained control (D). Magnifications: A and B, $\times 26,000$; C and D, $\times 12,000$.

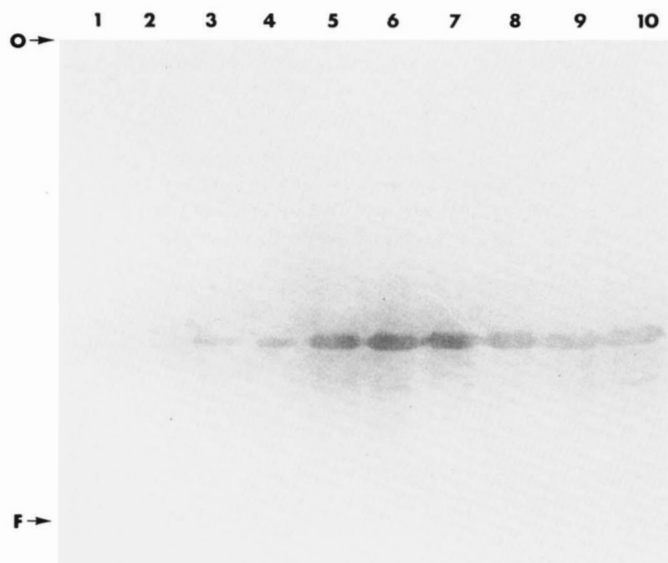


FIG. 7. Immunoblot of SDS-polyacrylamide gel of BAG homogenates at various ages. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 12% gel (100 V for 5 h) and transferred electrophoretically to nitrocellulose. The nitrocellulose sheet was incubated with PL 21.1 antibody followed by rabbit anti-mouse IgG coupled with peroxidase, and the sites of IgG binding were visualized with diaminobenzidine plus hydrogen peroxide. Lane 1, 0-day pupa; lane 2, 5-day pupa; lane 3, 8-day pupa; lane 4, 0-day adult; lane 5, 1-day adult; lane 6, 2-day adult; lane 7, 3-day adult; lane 8, 5-day adult; lane 9, 7-day adult; lane 10, 9-day adult. Protein samples of 50 μ g were applied to all lanes.

phore (lanes 6 and 7), two strongly reactive bands are seen at 23 and 13 kDa. No antigens were recognized in the homogenates of tubular accessory glands, seminal vesicle, or testes, all of which contribute to the spermatophore. No reactive antigens were detected in fat body or spermatophore.

Immunohistochemistry was used to localize the antigens within BAG and the spermatophore. In BAG, the PL 21.1 antigens were confined to the secretory granules within secretory cell type 4 (Fig. 6A). In the spermatophore, the antigens were distributed in a discrete zone of the outer wall (Fig. 6C).

Developmental Profile of Antigen Accumulation—Homogenates of BAGs from animals of increasing ages, from 0-day

pupae to 9-day adults, were subjected to electrophoresis on an SDS slab gel and to Western blotting. In this age series, the first traces of the 23-kDa antigen were detected in 8-day pupae (Fig. 7). Strong accumulation of antigen at 23 kDa and weaker reactions at lower masses were evident in homogenates of glands from 1-day adults and from older animals (Fig. 7).

DISCUSSION

PL 21.1 antibody recognizes an antigen in homogenates of the spermatophore which was restricted to a narrow zone in its thick outer wall. We designate this spermatophorin of molecular mass 23 kDa as "Sp23." Since PL 21.1 antigens were found only in homogenates of BAGs but not in other organs that contribute proteins to the spermatophore (Fig. 5), we conclude that Sp23 originates from the 23-kDa antigen in BAG. On the basis of immunoreactivity with PL 21.1 antibody and of relative mobility on SDS gels, the 23-kDa proline-rich protein from BAGs is indistinguishable from the corresponding spermatophorin, Sp23, to which it gives rise. It is possible that subtle secondary modifications of the 23-kDa secretory antigen might occur during its secretion and incorporation in the layers of the spermatophore.

A minor immunoreactive band with apparent molecular mass of 13 kDa is noticed on blots of SDS gels. Several other very weakly immunoreactive bands are also detected between 13- and 23-kDa protein bands from the fresh homogenate of BAG and from the acid eluant of the immunoaffinity column. An attempt was made to extract the 13-kDa band from the polyacrylamide gel, but it was unsuccessful. Since the 13-kDa band and the faint bands between 13- and 23-kDa are more prominent in physiologically aged samples (secreted plug and spermatophore) than in BAG homogenates (Fig. 5), they may represent degradation products of the 23-kDa proline-rich protein.

The 23-kDa antigen from BAG contains 25.2% proline (Table I). The best known proline-rich protein is collagen, which contains not only 10–25% proline but also 30–35% glycine (Ashhurst, 1985). On the basis of its low glycine content (5.7%), it is unlikely that the 23-kDa antigen from BAG is related to collagen.

The presence of relatively high amounts of proline has been reported in insect structural proteins isolated from cuticle, egg shells, vitelline membranes, and egg cases (oothecae). It

has been shown that cuticular proteins contain about 10% proline residues (Andersen, 1979). Notable among cuticular proteins are the P2 band with 16.7% proline from the cuticle of pupal cecropia (Willis *et al.*, 1981) and two proteins, designated 37 and 38, which were purified from the cuticle of *Locusts migratoria*. Protein 37 with molecular mass of 24.2 kDa contains 13.6% proline (Højrup *et al.*, 1986a), and protein 38 with molecular mass of 15.3 kDa contains 10.7% proline (Højrup *et al.*, 1986b). The C proteins from the chorion of silkmoths contain 10–11% proline (Regier *et al.*, 1983). The Fraction III proteins from the chorion of *Tenebrio* contain 16.9% proline, and the insoluble residue from the chorion of *Gryllus mitratus* contains 19.5% proline (Kawasaki *et al.*, 1975). The vitelline membrane of *Drosophila* is also rich in proline (Petri *et al.*, 1976). Two oothecins, structural proteins of the ootheca produced by the left colleterial glands of female American cockroaches, contain 18 and 25% proline (Pau *et al.*, 1971). A partial NH₂-terminal amino acid sequence of the 23-kDa protein (23 of the first 30 residues) supports the composition obtained after acid hydrolysis: 6 of 23 identified amino acids are proline.³

As a group, the chorion proteins of silkmoths contain all 20 naturally occurring amino acids (Regier *et al.*, 1983), and the chorion is stabilized by disulfide bridges. In contrast, many cuticular proteins are low in cysteine and lack methionine altogether (*e.g.* Fristrom *et al.*, 1978; Højrup *et al.*, 1986a). In the case of the 23-kDa protein from BAGs, half-cystine is present but methionine is absent (Table I).

On native gel electrophoresis, the PL 21.1 antibody recognizes only one antigen with an apparent molecular mass of 370 kDa from gland homogenates in the fractions from the gel filtration column and in the acid-eluted fractions from the immunoaffinity column. In the latter case, there is but one significant band stained with Coomassie Blue (Fig. 3). When the same sample is run on SDS gels, the predominant component is the 23-kDa proline-rich protein (Fig. 4). These data suggest that the 370-kDa antigen is composed of a 16-mer. We cannot prove conclusively that such a quaternary structure is actually found in BAG, much less in the wall of the spermatophore, or whether it is an aggregation artifact. But the fact that it is the only species seen in all fractions indicates that it is likely to be the physiological form in BAG. In the cuticle of at least one beetle, Hackman (1972) has argued for noncovalent aggregation among cuticular proteins. For the spermatophore and for cuticle, these reported examples of aggregation may be early steps in the formation of larger structures.

It is difficult to dissect apart the steps involved in secretion and incorporation of proteins into insoluble extracellular structures. The processes of forming filaments, networks, or sheets must involve many small steps. It is possible that the protein constituents of the aggregates undergo various secondary modifications, as for example the transformations of tropocollagen molecules into collagen (Walton, 1981). In the case of another secretory antigen of BAG, we discovered that a change in electrophoretic mobility accompanies its export from the cells into the lumen of the gland (Grimnes and Happ, 1986). For the 23-kDa proline-rich secretory protein considered in the present paper, no change in apparent molecular mass was noted during its secretion and incorporation into the spermatophore.

With monoclonal antibodies, we have traced three different secretory antigens from BAG to the wall of the spermatophore (Grimnes and Happ, 1986; Grimnes *et al.*, 1986; this paper). In all three cases, the secretions from different cell types do

not co-mingle but instead remain segregated from one another as each patch of secretion from BAG is molded into a particular layer of the spermatophore. These three examples suggest that some layers of the spermatophore are cell-specific. If such is the case, the spermatophore could be formed by the parallel assembly of eight distinct molecular networks derived from eight different cell types. It will be of great interest to compare the aggregation of the precursors, the processes of alignment, and the mechanisms for stabilization as each of the macromolecular networks are constructed in extracellular space to yield the final spermatophore.

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Supplementary material to "Purification and Characterization of a Proline-rich Secretory Protein that is a Precursor to a Structural Protein of an Insect Spermatophore"

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This supplement provides Experimental Procedures and Figs. 1 and 2.

EXPERIMENTAL PROCEDURES

Animals—Larvae of *Tenebrio molitor* were obtained from a commercial supplier (Rainbow Mealworms, Compton, California) and reared as previously described (Grimes and Hopp, 1985).

Protein Samples—Dissections were performed in ice-cold phosphate-buffered saline (PBS; 8.2 mM Na₂HPO₄, 1.5 mM K₂HPO₄, 140 mM NaCl, 26 mM KCl, pH 7.4), blotted on filter paper, and collected in 1.5 ml microcentrifuge tubes. Organs were disrupted in a Kontes homogenizer (10-20 µl of ice cold PBS per mg tissue). For hemolymph collection, the cuticle of the abdomen was gently torn and the expressed blood was collected in 25 µl Drummond microcaps. When males do not have access to females, they spontaneously eject spermatophores. These discarded, dried spermatophores were collected from the rearing boxes and used as a source of protein. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin (Sigma Fraction V) as the standard.

Production of monoclonal antibody—Homogenates of the secretory plug, dissected from bean-shaped accessory glands (BAGs), were injected into female Balb/c mice. Immunization, fusion, enzyme-linked immunosorbent assays (ELISAs), screening, cloning, ascites production, and cell storage were performed as previously described (Grimes and Hopp, 1986). The hybrid cells were screened by ELISA against homogenates of the BAGs, tubular accessory glands (TAGs), and spermatophore. Culture medium from the positive wells was used as the primary antibody source for Western blots of SDS gels run with the same three samples. During screening of the hybridomas, one clone, designated PL 21.1, was recovered which produced an antibody against a peptide band of Mr 23k in the BAGs and the spermatophore but not the TAGs. The antibody produced by this clone and the target antigens recognized by it are referred to as PL 21.1 antibody and PL 21.1 antigens, respectively. The immunoglobulin fraction from PL 21.1 ascites was purified on Affigel Protein A according to the MAPS protocol of BioRad. The eluate containing the immunoglobulin was dialyzed against one change of 100 volumes of bicarbonate buffer (200 mM NaHCO₃, 300 mM NaCl, pH 8.0).

Immunohistochemistry—Ultrastructural localization of PL 21.1 antigens was performed on BAGs and spermatophores. BAGs were dissected in cold PBS and quickly transferred to fixative at 4°C. Fixation was in 100 mM sodium phosphate, pH 7.4, containing either 1% (v/v) formaldehyde-0.5% (v/v) glutaraldehyde or 4% formaldehyde and 0.1% glutaraldehyde. Fresh spermatophores were obtained by interrupting copulation 30 seconds after its commencement. Spermatophores and BAGs were fixed for 2 h, dehydrated through graded alcohols, and embedded in Epon 812. Thin sections (silver) were cut with a diamond knife on a Reichert OMU-2 ultramicrotome and collected on nickel grids. Sections were etched for 5 min in 5% (v/v) H₂O₂. After soaking in blocking serum (horse), sections were incubated in primary antibody (IgG purified from ascites and diluted 1/50 or 1/100 in PBS containing 0.1% (v/v) horse serum for 30 min, in biotinylated anti-mouse IgG for 30 min, in avidin-biotin-peroxidase conjugate for 60 min (Vectastain of Vector Laboratories), and finally stained with 0.05% (w/v) diaminobenzidine - 0.01% H₂O₂ for 10 min (Hsu et al., 1981; Chlids, 1983).

Gel electrophoresis and Western blotting—Native polyacrylamide gel electrophoresis used 4-20% gradient slab gels. The resolving gel (375 mM Tris-HCl, pH 8.8) was overlaid with a 2% stacking gel (125 mM Tris-HCl, pH 6.8). Electrophoresis was run at 5°C at a constant 70 V. The separating buffer was 50 mM Tris-HCl, 384 mM glycine, pH 8.3. SDS-PAGE was according to Laemmli (1970) with a 12% running gel and a 3% stacking gel. Gels were stained with Coomassie blue for proteins (Black et al., 1982) and with periodate-Schiff's for carbohydrates (Kapitany and Zebrowski, 1973). Molecular weights on native and denaturing gels were estimated by reference to a regression line of log molecular weight vs. relative mobility. Reference proteins of known molecular weight were from Sigma. Western blotting followed the protocol previously described (Grimes and Hopp, 1986).

Preparation of immunoaffinity column—PL21.1 immunoglobulin, purified through the MAPS protocol, was coupled to Affi-Gel 10 (BioRad) by the method of Staehlin et al. (1981). To block unreacted sites, the gel slurry was mixed with an equal volume of 100 mM glycine (adjusted to pH 8.0 with NaOH). After reaction at room temperature for 1 hr, the gel slurry was washed with PBS until free of glycine. The column was stored in Buffer B (see below).

Purification of PL 21.1 antigens—All procedures were carried out at 5°C or in an ice bath. All buffers for gel filtration and immunoaffinity chromatography contained 0.05% (v/v) Tween 20 and 0.02% (w/v) NaN₃. One gram of BAGs (approximately 300 BAG pairs) from male adults 6-10 days after ecdysis was homogenized with 10 ml of Buffer A (25 mM Tris-HCl, 150 mM NaCl, pH 7.5). The homogenate was centrifuged at 14,000g for 15 min and the supernatant saved. The precipitate was washed with 5 ml of buffer A and re-centrifuged. First and second supernatants were combined and applied to a column of Sephadex G-150 (2.5 x 110 cm) equilibrated with Buffer A. Proteins were eluted with Buffer A.

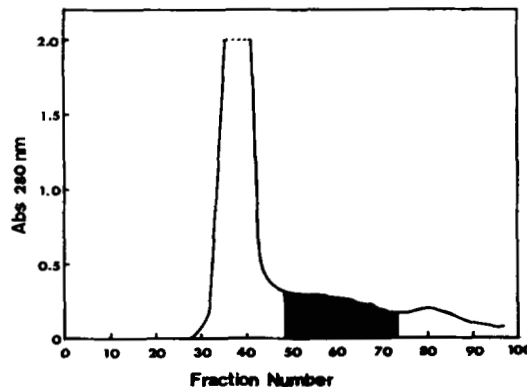


Fig. 1. Gel filtration chromatography of PL 21.1 antigens. Shaded area indicates fractions from Sephadex G-150 column which contained PL 21.1 antigens according to immunoblot procedures.

Fractions from the G-150 column which contained PL 21.1 antigens (detected by immuno-dot-blot) (Fig. 1) were pooled and applied to the Affi-Gel 10 column (0.9 x 2 cm) which had been linked to antibody PL 21.1 and equilibrated with Buffer B (15 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 140 mM NaCl, 3 mM KCl, pH 7.3). After non-absorbed proteins were eluted by washing with 30 ml of Buffer A and ionically-bound proteins were eluted with 20 ml of Buffer C (25 mM Tris-HCl, 500 mM NaCl, pH 7.5), the column was briefly washed with 10 ml of Buffer D (150 mM NaCl). Finally, the specifically-absorbed antigens were eluted with 20 ml of Buffer E (0.2 M acetic acid, 150 mM NaCl) (Fig. 2). After the A₂₈₀ declined to baseline levels, the column was washed with Buffer B and stored in that buffer. The early fractions eluted with Buffer E contained PL 21.1 antigen. Positive fractions were pooled, dialyzed against two changes of 1 l of distilled water, lyophilized, and the residue dissolved in 0.5 ml of 10 mM sodium phosphate at pH 7.0.

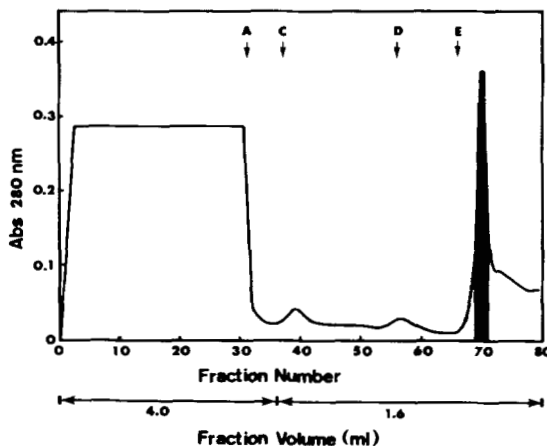


Fig. 2. Chromatography on Affigel-10 column containing affinity-purified PL 21.1 antibody. Bound material eluted with 0.2 M acetic acid (shaded fractions) contained PL 21.1 antigens.

The sample was applied across the width of a 12% polyacrylamide slab gel buffered to pH 7.0 with 100 mM sodium phosphate and the gel was run at 50 mA for 15 hr using sodium phosphate running buffer. After completion of the electrophoresis, the protein band of 23k was located by staining narrow lateral strips of the gel and the protein band excised from the remainder of the gel. The gel slice was placed in 20 ml of 10 mM sodium phosphate buffer (pH 7.0) and homogenized in a Polytron for 2 min. The slurry was shaken for 2 hr on a rotator at room temperature and then filtered through a cotton pad. The pad was washed with 20 ml of distilled water. Filtrate and wash were combined and dialyzed for 3 hr against 5 changes of 2 l of distilled water. After lyophilization, the residue was dissolved in a small volume of distilled water and stored at -70°C.

Amino acid composition—Duplicate samples were hydrolyzed in 6N HCl at 110°C in *vacuo* for 22 hr. Cysteine was determined as cysteic acid after performic acid oxidation (Hira, 1967). Analyses were performed on a Varian Vista 1400 HPLC amino acid analyzer in the Department of Medical Biochemistry at the University of Vermont. Tryptophan was not detectable by these methods.