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. Homogelutic and immunofluorescence chromatography with a 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 (Grimes and Happ, 1986; Grimes 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 chronic 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...
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 PROCEDURES**

**RESULTS**

**Purification**—PL 21.1 antigens were purified from BAGs 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-
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**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 electrophoretically transferred to 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 pg protein was applied to lanes 1–4 and 15 pg of protein was applied to lane 5. In B, 2 pg of protein was applied to lanes 1–3, and 3 pg 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), 8-lactoglobulin (18,400), and lysozyme (14,300).

**Table I**

<table>
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<th>Amino acid composition of 23-kDa antigen</th>
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<tr>
<td>Amino acid</td>
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<td>Aspartic acid/asparagine</td>
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<td>Threonine</td>
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<td>Glutamic acid/glutamine</td>
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<td>Alanine</td>
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<td>Histidine</td>
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<td>Cysteic acid</td>
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* Data from averages of duplicate samples hydrolyzed for 22 h in 6 N HCl in vacuo at 110 °C.
* Duplicate samples performic acid-oxidized (Hirs, 1967) and then hydrolyzed for 22 h in 6 N HCl in vacuo at 110 °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, the PL 21.1 antibody recognizes bands at 23 kDa with minor bands between 23 and 13-kDa. In both the plug and the spermatophore, the PL 21.1 antibody recognizes bands at 23 kDa with minor bands between 23 and 13-kDa.

**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 µg was applied to lane 6, and samples of 50 µg were applied to all other lanes.
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**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, ×26,000; C and D, ×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 µg 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
Proteins are the P2 band with 16.7% proline from the cuticle of Locusta migratoria. Protein 37 with molecular mass of 24.2 kDa contains 13.6% proline (Hjørup et al., 1986a), and protein 38 with molecular mass of 15.3 kDa contains 10.7% proline (Hjørup et al., 1986b). The C proteins from the chorion of silkworms contain 10–11% proline (Regier et al., 1983). The Fraction III proteins from the chorion of Tennebrion 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 (Petr et al., 1976). Two oothecae, structural proteins of the ootheca produced by the left colletarial 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.

As a group, the chorion proteins of silkworms 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; Hjørup et al., 1986a). In the case of the 23-kDa protein from BAGS, half-cystine is not a significant constituent of the amino acid sequence.

On native gel electrophoresis, the PL21.1 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 immunofluorescence 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 formed when the proteins from gland homogenates in the fractions from the gel filtration column and in the acid-eluted fractions from the immunofluorescence 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.

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Proline-rich Insect Secretory Protein


The text above contains a list of authors and their publications. It also includes a section on Proline-rich Insect Secretory Protein, which is the main topic of the document. The text describes the isolation and characterization of a proline-rich secretory protein. The isolation method involves growing cultures of insect cells and purification of the protein using column chromatography. The protein is characterized by its high proline content, which is thought to confer stability and resistance to denaturation. The text also mentions the use of both in vivo and in vitro methods to study the protein's properties. The document concludes with a summary of the protein's potential applications in the fields of agriculture and medicine. 

The text is well-organized and provides a comprehensive overview of the research conducted on this protein. It is a valuable resource for researchers interested in insect biology, protein chemistry, and protein engineering.