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PURIFICATION AND PROPERTIES OF MICROVITELLOGENIN OF MANDUCA SEXTA ROLE OF JUVENILE HORMONE IN APPEARANCE AND UPTAKE.

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Microvitellogenin, a female specific protein found in hemolymph and eggs of adult female Manduca sexta (tobacco hornworm moth) has been purified to homogeneity. It is a 31,000 dalton protein lacking covalently bound carbohydrate. It appears in the hemolymph near the time of adult eclosion. The appearance of microvitellogenin in adult hemolymph is not dependent upon the presence of juvenile hormone. Uptake of both vitellogenin and microvitellogenin are greatly reduced in the absence of juvenile hormone.

Most adult female insects produce in the fat body a large glycolipoprotein, vitellogenin, which is taken up by an endocytotic process into the ovary and deposited in the oocyte as the major yolk protein, vitellin (1,2). Vitellins and vitellogenins from different insects are generally quite similar, in that they consist of large apoproteins (~180,000 daltons), moderately sized apoproteins (~40,000 daltons) and 10 percent lipids, among which diacylglycerols and phospholipids predominate (3).

In many insects the synthesis of vitellogenin and its uptake by the ovaries, are under the control of juvenile hormone (2,3). In the tobacco hornworm moth, Manduca sexta, vitellogenin can be synthesized in the fat body and sequestered by ovaries in the absence of juvenile hormone. However, the eggs never reach full size and are resorbed unless juvenile hormone is supplied (4,5).

Oocytes contain several other proteins as well, some of which may be passively taken up from the blood by endocytosis, and others are synthesized by the ovaries themselves (1,6). In Hyalophora cecropia, a relatively small protein, also female specific, is found in both hemolymph and eggs (6).

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Originally called "reluctin" (1), the protein has now been renamed microvitellogenin (6). We have recently discovered microvitellogenin in the hemolymph and eggs of M. sexta. In this paper we report the purification and characterization of the protein. Furthermore, we have performed experiments to determine whether production and sequestration of microvitellogenin are under juvenile hormone control.

MATERIALS AND METHODS

Animals. Adult M. sexta came from the eggs kindly supplied by Dr. J. P. Reinecke and Dr. J. Buckner, U. S. Department of Agriculture, Fargo, ND. The animals were raised according to Kramer et al. (7).

Extraction of Egg Proteins. Eggs were dissected from the abdomens of females and homogenized in 10 mM Na-phosphate buffer pH 6.8 containing 50 mM glutathione and 20 mM DFP[++].

Collection of Hemolymph. An average of 35 adult female animals, 1-3 days after eclosion, were used in each hemolymph preparation. Bleeding procedures and hemolymph collection were performed according to Shapiro and Law (8). The hemolymph was centrifuged at 12,000 g for 5 min to remove hemocytes.

Ultracentrifugation. Separation of lipophorin from other hemolymph proteins was achieved by ultracentrifugation in a NaCl/KBr density gradient (9). 18.3 ml of the subphase (characterized by its blue color) was collected from the bottom of the centrifuge tube by means of a hypodermic syringe.

Gel Permeation Chromatography. The volume of the hemolymph subphase was reduced to approximately 2.0 ml by ultrafiltration through a YM10 Amicon membrane. Gel permeation chromatography was performed on a 2.5 cm by 1 m Sephadex G-75 column equilibrated with phosphate buffered saline (PBS, 0.15 M Na-phosphate, 0.10 M NaCl, 0.1 M EDTA and 0.02 percent (w/v) NaN_3 pH 7.0). Fractions (3.2 ml each) were collected and the absorbance of proteins in each fraction was read at 230 and 280 nm respectively by means of a Perkin-Elmer Lambda 3 Spectrophotometer. An aliquot of every third fraction from the G-75 column was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Cation Exchange. Gel electrophoresis showed that approximately 20 ml of G-75 eluate contained microvitellogenin. The fractions were pooled and the volume reduced to 1.0 ml by ultrafiltration. The ultrafiltrate was dialyzed against 10 mM Na-succinate pH 5.4 and applied to a SP-Sephadex C-25 cation exchange column (8 ml bed volume) equilibrated in the same buffer. After washing to remove nonadsorbed protein, microvitellogenin was eluted from the column with 0.1 M NaCl pH 6.0.

Affinity Chromatography. Microvitellogenin from the cation exchange was dialyzed against buffer (0.2 M Tris HCl, 0.5 M NaCl, 10 mM CaCl, 10 mM $MgCl_2$ and 0.02 percent (w/v) NaN_3, pH 7.4), and loaded on to a conconavalin A Sepharose (Pharmacia) column (8 ml bed volume) equilibrated in the same

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[++] Abbreviations used: DFP, diisopropylphoshorofluoridate; Con A, conconavalin A; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Vg_L, large vitellogenin apoprotein; Vg_S, small vitellogenin apoprotein; V_L, large vitellin apoproteins; V_S, small vitellin apoprotein; mVg, microvitellogenin, Apo III, apolipophorin III.

buffer. Microvitellogenin was eluted with the buffer, while contaminating glycoproteins remained bound to the column.

Adsorption Chromatography. Fractions from affinity chromatography were pooled, dialyzed against 30 mM Na-phosphate pH 6.8 and loaded on hydroxylapatite (Bio-Gel HT, Bio-Rad) column (13 x 1.5 cm) equilibrated in the same buffer. Microvitellogenin was eluted from the column at the rate of 16 ml/hr by means of a 30-500 mM Na phosphate gradient (total vol. 500 ml). Conductivity of each fraction was measured by a Radiometer (Copenhagen) conductivity meter, and salt concentration in millimoles deduced from a standard curve. Microvitellogenin was eluted at a salt concentration of 140 mM.

Molecular Weight Estimation. The molecular weight of microvitellogenin was estimated by gel filtration using a Sephadex G-75 column calibrated with protein standards, and by its electrophoretic mobility relative to that of Bio-Rad protein standards on SDS-PAGE.

Amino Acid Analysis. Duplicate samples were hydrolyzed in 6N HCl at 110°C in vacuo for 24, 48, and 72 hr. Cysteine and cystine were determined as cysteic acid after performic acid oxidation (10). Tryptophan was determined by amino acid analysis preceded by mild hydrolysis in 3N mercapto-ethanesulfonic acid (22 h, 110°C) (11). Analyses were performed on a Dionex D-300 amino acid analyzer using the standard column and three-buffer system suggested by the manufacturer, with an additional sodium citrate buffer (0.2 N Na^+/pH 7.4) added for tryptophan elution after the B buffer. Peaks were directly integrated on a Hewlett-Packard 3388A integrator.

PAGE. Electrophoresis-SDS-PAGE was performed on 4-15 percent gradient gel slabs (1.5 x 18 x 20 cm) made with a BRL gradient maker. Electrophoresis was performed according to Laemmli (12). The gels were either stained with Coomassie Blue R-250 for protein or with periodate-Schiff reagent for glycoproteins (13).

Decapitation. Twenty-seven pupae at day 9 before eclosion were used. Before and during surgery, the animals were anesthesized by chilling on ice. Cherbas buffer (14) containing 50 mM glutathione and 0.1 percent (w/v) streptomycin was used as the dissecting medium. Seventeen pupae were decapitated, the wound filled with dissecting medium and sealed according to Harvey and William (15). In the sham controls (10 animals), a window was cut through the dorsal side of the pro- and meso-thorax. The cuticle and muscle were removed and the wound sealed as previously described (15).

RESULTS AND DISCUSSION

Previous workers (4,5,16,17) failed to detect microvitellogenin in crude hemolymph samples because of the presence of interfering proteins of similar molecular weight. After gel permeation chromatography, however, detection is much easier. Fig. 1 shows SDS-PAGE gels of fractions from the hemolymph of female and male adult M. sexta. The female hemolymph contains the two vitellogenin apoproteins that are associated with the ~500,000 dalton vitellogenin, as well as the 31,000 dalton microvitellogenin. All three polypeptides are absent in the male hemolymph. Microvitellogenin is also present in egg homogenates (Fig. 2).

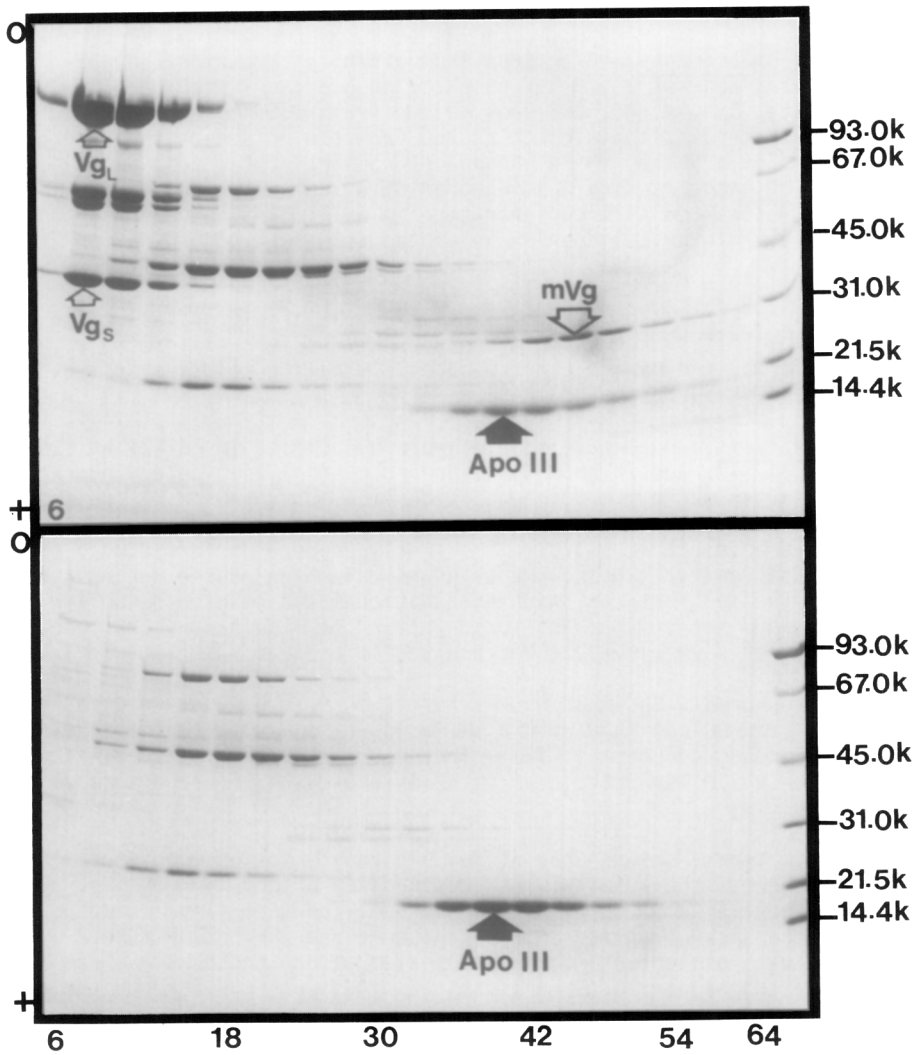

Figure 1. SDS-PAGE of fractions from Sephadex G-75 gel permeation chromatography. Crude hemolymph was subjected to KBr gradient ultracentrifugation to remove the lipoprotein, lipophorin (8). The subphase was applied to the G-75 column. Above, adult female; below, adult male. Vg_L represents the large vitellogenin apoproteins (185,000 and 175,000 daltons); Vg_S is the small apoprotein (45,000 daltons) (16); Apo III is apolipophorin III (8); mVg is microvitellogenin. The positions of the molecular weight markers are indicated at the right.

Purification of microvitellogenin from adult female hemolymph by the procedures described here yielded homogeneous material. Fig. 3 shows the SDS-PAGE gels of samples taken at various stages in the purification. The amino acid analysis of material from the final stage of purification is shown

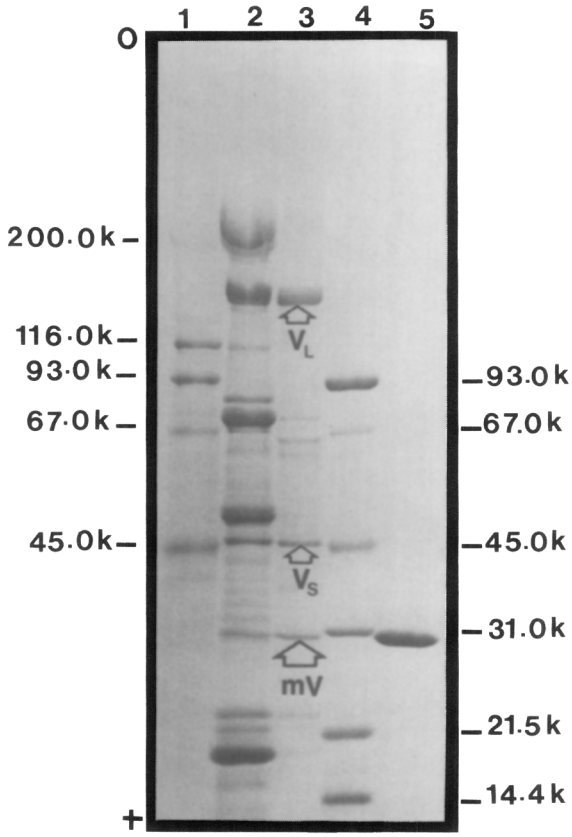

Figure 2. SDS-PAGE of egg homogenate and crude hemolymph. 1, molecular weight markers; 2, crude hemolymph; 3, egg homogenate; 4, molecular weight markers; 5, microvitellogenin purified from egg homogenate; V_L, large vitellin apoproteins; V_S, small vitellin apoproteins; mV, microvitellin.

in Table 1. This composition is significantly different from that of M. sexta vitellogenin (16).

Unlike vitellogenin, microvitellogenin is not a glycoprotein, since it was not retained on Con A Sepharose and fails to react with the periodate-Schiff reagent. Fig. 1 shows that the protein has the same mobility as the carbonic anhydrase standard (M_r = 31,000) on SDS-PAGE. On a calibrated Sephadex G-75 column the molecular weight was also estimated at 31,000.

Animals were decapitated prior to adult eclosion as a means of removing the corpora allata and thus the source of juvenile hormone (4,5,17).

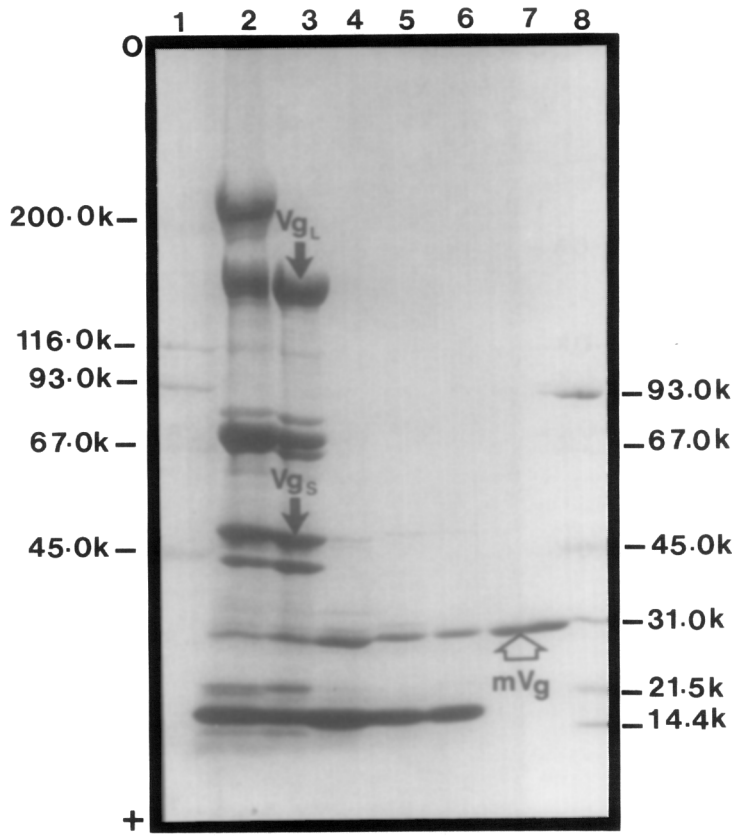

Figure 3. Purification of microvitellogenin. SDS-PAGE of microvitellogenin preparations at various stages of purity. 1, molecular weight markers; 2, crude female hemolymph; 3, subphase from density gradient centrifugation; 4, after gel permeation chromatography; 5, after SP-Sephadex chromatography; 6, after passage over con A Sepharose; 7, after adsorption chromatography on hydroxylapatite; 8, molecular weight markers.

Hemolymph taken during the operation did not have microvitellogenin, as shown by SDS-PAGE. Following eclosion, microvitellogenin was present in about the same amounts in the hemolymph of both sham operated control animals and decapitated animals. Thus, juvenile hormone is not required for the appearance of microvitellogenin in adult hemolymph.

Ovaries were dissected from both control and decapitated animals. The ovaries of decapitated animals had only small oocytes, some of which were in the process of resorption, as described by Nijhout and Riddiford (5,6). The ovaries were homogenized and the soluble proteins were subjected to SDS-PAGE. Both vitellin and microvitellogenin were present in both egg extracts, but

Table 1

Amino Acid Composition of Microvitellogenin from M. sexta Hemolymph

Amino Acid	Number of Residues per 32,000	Mol Percent
Aspartic Acid	43	14.8
Threonine	11	3.7
Serine	20	6.9
Glutamic Acid	26	9.0
Proline	8	2.8
Glycine	23	7.9
Alanine	23	7.9
Valine	20	6.9
Methionine	7	2.4
Isoleucine	17	5.9
Leucine	21	7.2
Tyrosine	12	4.2
Phenylalanine	11	3.8
Histidine	7	2.4
Lysine	15	5.2
Arginine	20	6.9
Tryptophan	5	1.7
Cysteine	1	0.3
TOTAL	290	99.8

their amount was greatly diminished in the egg extract of the decapitated animals. A notable difference was the absence of the lipoprotein, lipophorin, in the eggs of the decapitated animal. In addition, at least two novel polypeptides were present in egg extracts of decapitated animals. As some of the eggs were in the process of resorption, it is possible that both the absence of lipophorin and the appearance of the new polypeptides is the result of proteolysis. Further study will be necessary to decide if this is the case.

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REFERENCES

1. Telfer, W. H., Rubenstein, E. and Pan, M.-L. (1981) in "Regulation of Insect Development and Behavior" (Sehnal, F., Zabza, A., Menn, J. J. and

Cymborowski, B., eds.) Wroclaw Technical University Press, Wroclaw, Poland, pp. 637-654.
2. Engelmann, F. (1979) Adv. Insect Physiol. 14, 49-108.
3. Hagedorn, H. H. and Kunkel, J. G. (1979) Annu. Rev. Entomol. 24, 475-505.
4. Nijhout, M. M. and Riddiford, L. M. (1974) Biol. Bull. 146, 377-392.
5. Nijhout, M. M. and Riddiford, L. M. (1979) Intern. J. Invertebrate Reprod. 1, 209-219.
6. Telfer, W. H. and Kulakosky, P. C. in "Invertebrate Reproduction", (Engels, W., Went, D. and Clark, W., eds.) Vol. 3, Elsevier, Amsterdam (1974).
7. Kramer, K. J., Sanburg, L. L., Kezdy, F. J. and Law, J. H. (1974) Proc. Natl. Acad. Sci. USA 71, 493-497.
8. Shapiro, J. P. and Law, J. H. (1983) Biochem. Biophys. Research Communs. 115, 924-931.
9. Shapiro, J. P., Keim, P. S. and Law, J. H. J. Biol. Chem. submitted.
10. Hirs, C. H. W. (1976) Methods Enzymol. 11, 59-62.
11. Penke, B., Ferenczi, R. and Kovacs, K. (1974) Anal. Biochem. 60, 45-50.
12. Laemmli, U. K. (1970) Nature 227, 680-685.
13. Kapitany, R. A. and Zebrowski, E. Z. (1973) Anal. Biochem. 56, 361-369.
14. Cherbas, P. (1973) Ph.D. Thesis, Harvard University, Cambridge, Massachusetts.
15. Harvey, W. R. and Williams, C. M. (1961) J. Insect Physiol. 7, 81-99.
16. Mundall, E. C. and Law, J. H. (1979) Comp. Biochem. Physiol. 63B, 459-468.
17. Imboden, H. and Law, J. H. (1983) Insect Biochem. 13, 151-162.