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Short Communication

Vitellin and Vitellogenin in the Soldier Bug, *Podisus maculiventris*: Identification With Monoclonal Antibodies and Reproductive Response to Diet

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A 171,000 M_r polypeptide of *Podisus maculiventris* (Say) (Heteroptera: Pentatomidae) that constituted 16% of the protein in eggs also constituted up to 25% of the protein in hemolymph of fed females. It was identified as the major or sole apoprotein of vitellogenin. Eggs contained major polypeptides of 171, 106, and 51 kDa. The hemolymph polypeptide was identified with a polypeptide (vitellin) in egg extracts by comparing molecular weights, specificity of occurrence in fed females, and immunological reactivities. Females, starved for 5 days after eclosion to assure complete previtellogenic development, produced vitellogenin within a day after feeding on larval *Galleria mellonella*, and within 4 days after feeding on an artificial diet. Appearance of vitellogenin preceded ovarian growth by 2–3 days. Two monoclonal antibodies raised against egg proteins of *P. maculiventris* were selected for their strong reaction against egg extract and female hemolymph and null reaction against male hemolymph. Only one 170-kDa band in egg and hemolymph reacted with the antibodies on denaturing Western blots. These monoclonal antibodies are being used to develop an enzyme-linked immunosorbent assay (ELISA) to quantitate reproductive response of females to diets of differing quality. Arch. Insect Biochem. Physiol. 44:130–135, 2000. **Published 2000 Wiley-Liss, Inc.**[†]

Key words: vitellin; vitellogenin; monoclonal antibody; artificial diet; ELISA; oogenesis; fecundity

Abbreviations used: 4CN = 4-chloro-1-naphthol; ELISA = enzyme-linked immunosorbent assay; HRP = horseradish peroxidase; mAb = monoclonal antibody; PBS = phosphate-buffered saline (0.15 M NaCl, 50 mM sodium phosphate, pH 7.2); SDS = sodium dodecyl sulfate; TBST = Tris-buffered saline (20 mM Tris, 0.5 M NaCl, pH 7.5) with 0.05% Tween-20; Vg = vitellogenin; Vn = vitellin.

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INTRODUCTION

Vitellogenesis is a major component of oogenesis in insects. Vitellogenesis comprises the synthesis in fat body of the yolk protein precursor, vitellogenin (Vg), its secretion into the hemolymph, and transport to the follicles, which selectively bind and internalize it into oocytes as vitellin (Vn) (Hagedorn and Kunkel, 1979; Raikhel and Dhadialla, 1992). In Heteroptera, there is clear evidence for this mechanism of egg development in such species as the bean bug *Riptortus clavatus* (Shinoda et al., 1996), *Rhodnius prolixus* (Wang and Davey, 1993), *Oncopeltus fasciatus* (Rankin and Jackle, 1980), and the lygaeid *Spilostethus pandurus* (Ibanez et al., 1992). Oviposition, the final outcome of oogenesis, is measured on a daily or cumulative basis as fecundity. Fecundity is the most common measure of reproductive fitness in insect colonies or of reproductive response to feeding on artificial diets. To shorten the time required to develop artificial diets or to assess fitness of reared populations, we propose to determine the reproductive state of female predators using an enzyme-linked immunosorbent assay (ELISA) as a predictor of fecundity. Although ELISAs have been developed to determine prey content of predaceous Heteroptera (Hagler et al., 1991, 1992a,b), the use of an ELISA to determine reproductive potential is apparently unexplored. The present work describes the biochemical basis for such an ELISA using a hemipteran predator, *Podisus maculiventris*, that has been successfully employed for augmentative biological control in both field and greenhouse and for which several artificial diets have been developed (De Clercq and Degheele, 1993; De Clercq et al., 1998; Greany and Carpenter, 1998).

MATERIALS AND METHODS

Insects and Diets

P. maculiventris were reared at 27±2°C, 65±5% RH, under a 14:10 (L:D) photoperiod. Except for diet comparison experiments, all stages were fed on fifth instar larval *Galleria mellonella* that had been heat-treated for 15 sec at 56°C to prevent web-spinning, reared as described by Jindra and Sehnal (1989). For diet comparison, all stages were fed fresh daily on either a meat-based artificial diet (Greany and Carpenter, 1998) supplemented with ground beef (Cohen, 1985), or on *G. mellonella* larvae. Artificial diet was prepared by blending ground beef (28.5 g, approxiamtely 30% fat content) and fresh chicken egg yolk (26.0 g); combining powdered tissue culture medium (3.6 g; SF-900, Gibco BRL Life Technologies, Inc.), L-glutamine (0.6 g), sodium bicarbonate (0.03 g), and gentamicin (0.1 ml of 50 mg/ml solution, Sigma) in 80 ml deionized water; adding 28.5 g ground beef liver and the 85-ml SF-900 mixture to the beef/egg yolk mixture and homogenizing; and readjusting the pH to 6.8 with 10% KOH. Diet was encapsulated in Parafilm for presentation (Greany and Carpenter, 1998). Individuals in these experiments were kept separate, except for mating on day eight. To measure ovarioles, females were dissected ventrally in saline and ovarioles (7 of 14/female) were measured by ocular micrometer under a dissecting microscope.

Electrophoresis and Blotting

SDS-PAGE was run at room temperature on polyacrylamide minigels (BioRad, Richmond, CA). Hemolymph was collected from each insect only once. For the diet comparison study, 6 µl of hemolymph were collected into 40 µl of PBS, mixed 1:2 with sample buffer containing SDS and β-mercaptoethanol, boiled 5 min, and 15-µl samples were run on 10% SDS gels. For egg samples, twenty eggs (440 µg each) were homogenized in 333 µl PBS, centrifuged at 15,000 rpm for 10 min in a microcentrifuge, the pellet was re-extracted twice by the same procedure, and supernatants were pooled. Soluble protein was determined by BCA assay (Pierce Co., Rockford, IL) to be approximately 4 mg/ml, averaging approximately 45% of total egg mass. Gels were stained with Coomassie Brilliant Blue R in methanol/acetic acid/water (40:10:50). For other gels, hemolymph was diluted directly into sample buffer. Gradient gels (4–15%) were used for comparison of eggs and hemolymph samples and for M_r determinations of polypeptides, and were stained with colloidal Coomassie stain (BioSafe, BioRad Co.). For Western blots, 7.5% SDS-PAGE gels were used. Molecular weights were interpolated on a spreadsheet program (Excel, Microsoft, Bellevue, WA) from linear regressions of protein standards, using R_f vs. log-

transformed molecular weights. Digital scans and integration of egg polypeptides were performed with Scion Image software (Scion Corp., Frederick, MD; vers. Beta-3B) from digital photographs taken with a Kodak (Rochester, NY) DC260 camera and fluorescent light box. Scans were run on black and white TIFF image files following conversion from a color JPEG file. For blotting, samples were run on 7.5% SDS-PAGE gels with 30 μg of egg protein or 0.185 μl of hemolymph per lane. Polypeptides were transferred from the gels onto nitrocellulose membrane (BioRad) overnight at 5°C and 200 V constant voltage. Membranes were cut into strips, blocked 1 h in 1% nonfat dry milk (Carnation, Glendale, CA) in TBST, and incubated 1 h in 16 ml TBST containing 1 ml of purified 2D5 or 1C11 mAb. Bound mouse IgG was detected by incubating in HRP-conjugated goat anti-mouse serum in TBST (1:3,000) (Sigma, St. Louis, MO) 1 h and in 4CN substrate 10–15 min.

MAb Production and Purification

Hybridomas were produced, screened by ELISA for IgM- and IgG-specific activity against egg extract, and cloned by the University of Florida Hybridoma Core Lab, Gainesville, FL. A crude homogenate in PBS (20 eggs/ml, 1.0 mg/ml of protein by BCA assay) of freshly laid (<24 h) eggs, stored at 85°C until used, served as the immunogen. Fifteen micrograms were injected into a BALB/c mouse, which was boosted six times over a 4-month period with 30-μg aliquots from the same homogenate. Following the final boost, IgG isotype IgG2B (kappa light chain) predominated. Clones active against egg and female hemolymph proteins and inactive against male hemolymph proteins were selected in a secondary screen of cell supernatants using dot blots. Supernatants from 1-L cultures of cloned cells were collected and 30–200 ml were passed through a 5-ml HiTrap protein G column (Amersham-Pharmacia, Piscataway, NJ), rinsed with 25 ml of 20 mM/pH 7.0 sodium phosphate, and IgG was eluted with 15 ml of 0.1 M glycine HCl, pH 2.7. The column was reequilibrated and the process was repeated until each liter had been purified. Gel electrophoresis showed two bands at approximately 50,000 and 20,000 M_r, indicating IgG purity.

RESULTS

SDS-PAGE comparison of polypeptides in hemolymph from fed (gravid) and unfed females, eggs, and male hemolymph identified one putative apoprotein of vitellogenin, at 171 kDa. The band appeared only in hemolymph of gravid females (Fig. 1A, lane 2) and in egg extracts (Fig. 1A, lane 4), but not in hemolymph from unfed females (Fig. 1A, lane 3) or males (Fig. 1A, lane 5). In fed females, it constituted 23% of total lane density. Egg extracts contained major polypeptides at 171 kDa (16% total lane density), 106 kDa (18%), and 51 kDa (20%). In eggs, the 171 and 106 kDa bands appeared with preceding lighter bands of about 9 and 5% of total density. At least five other minor bands were evident (Fig. 1B).

In a diet comparison study, females and males were fed as both nymphs and adults on either artificial diet or *G. mellonella*. The female-specific 171 kDa polypeptide appeared in *G. mellonella*-fed females on day 6, within a day after feeding, at 16% of total protein (Fig. 2). It reached maximum density (25% of total protein) on days 8 and 9. When fed on the artificial diet, the 171 kDa polypeptide appeared in female hemolymph on day 9 as 18% of total protein, 4 days after feeding commenced. It reached a maximum at 22% of total protein on day 10. As indicated by the timing of increase in ovariole length, ovarian development followed the appearance of the Vg band by 2–3 days, and ovarioles from diet-fed females showed much slower increase in size with vitellogenic growth (Fig. 2).

Hybridoma cell lines that showed strong immunological activities against egg extracts in a preliminary ELISA were screened in a secondary test against egg extract, hemolymph from gravid females, and hemolymph from males on dot blots. The cell supernatants showed no significant activity against male hemolymph, strong activity against egg homogenate, and variable activity against hemolymph from gravid females. The two cloned cell lines showing the strongest activity against eggs and female hemolymph, 1C11 and 2D5, were selected for production. Supernatants from these clones were reacted against Western blots of SDS-PAGE gels of egg homogenate and female hemolymph. The two mAbs reacted iden-

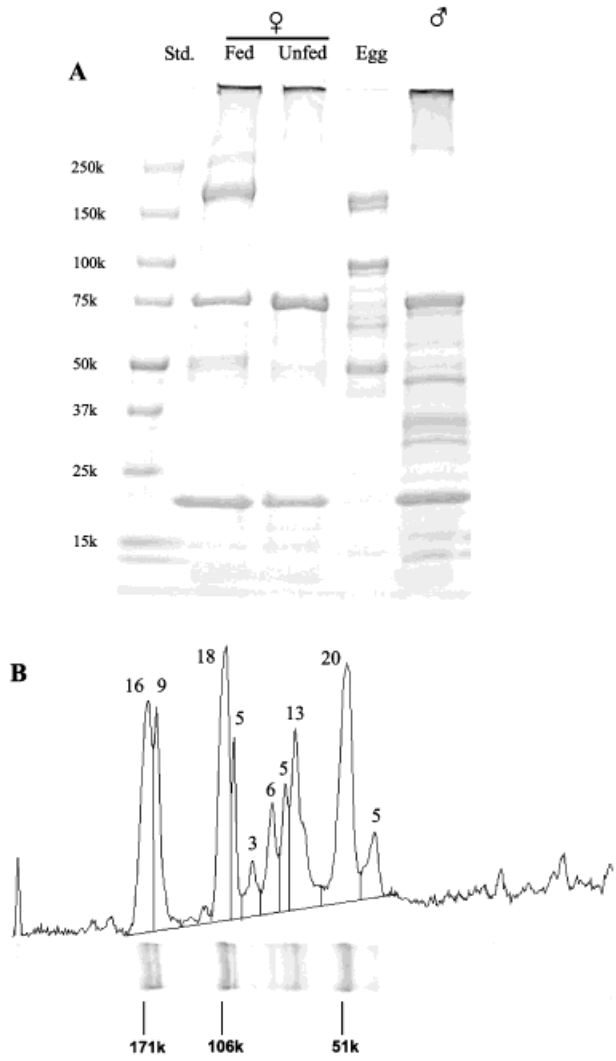

Fig. 1. SDS-PAGE (4–15% gradient gel) of female and male hemolymph and egg extract, and density analysis of egg polypeptides. **A:** Std = Molecular weight standards (BioRad High Precision); Fed = hemolymph from fed female (gravid, 10 d post-eclosion); Unfed = hemolymph from unfed female (<1 d posteclosion); Egg = egg extract; ♂ = hemolymph from male (10 d post-eclosion). **B:** Photographic density analysis of egg polypeptides. Numbers above peaks indicate the percent of total lane density of marked peaks.

tically with respect to each other, whether on blots of SDS gels or native gels. A single immunoreactive polypeptide at 170 kDa, comparable to the Vg observed on coomassie-stained gels, was identified in egg and hemolymph on blots from SDS gels (Fig. 3).

DISCUSSION

We have identified a Vg in the hemolymph of *P. maculiventris* by electrophoretic and immunological comparison with egg yolk polypeptides. Following 5 days of starvation, the major female-specific polypeptide appeared in hemolymph within a day of feeding on *G. mellonella* larvae. It ran on SDS-PAGE coincident with a 171 kDa egg polypeptide. Two mAbs, developed against egg extract and selected for their specificity to female hemolymph, were reactive against a hemolymph

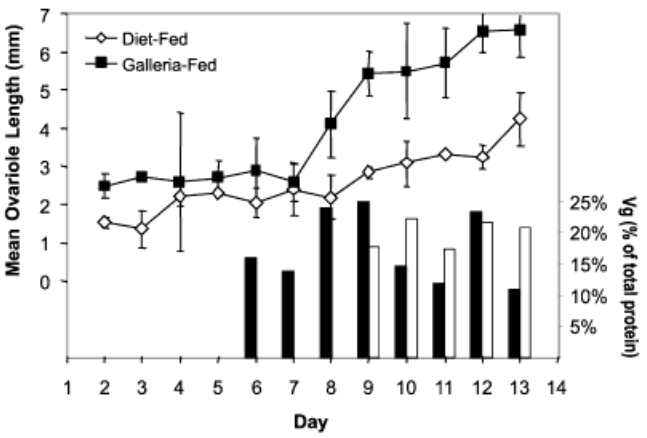

Fig. 2. Growth of ovarioles (means ± SD; n=3) and relative content of Vg in hemolymph from females fed *G. mellonella* larvae or an artificial diet. Vg as percent of total hemolymph protein was densitometrically estimated from SDS-PAGE. Open bars = diet-fed; filled bars = *Galleria* fed.

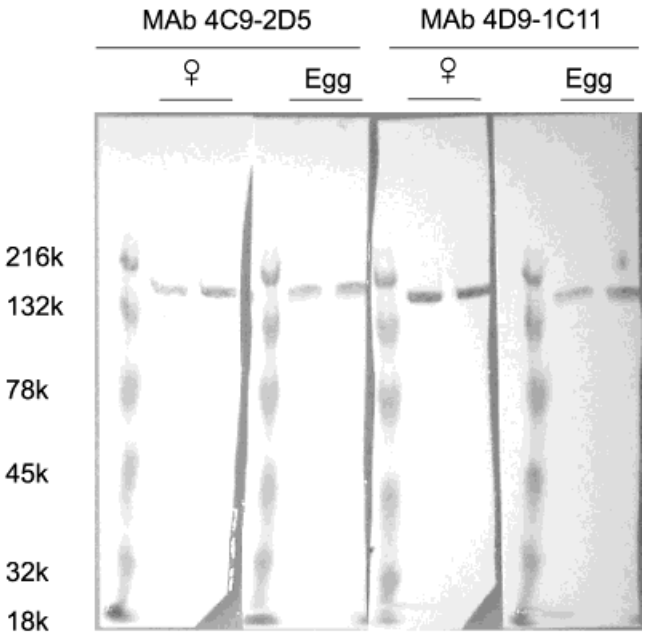

Fig. 3. Western blots of two purified mAbs (2D5 and 1C11) reacted against: ♀ = female hemolymph; Egg = egg homogenate. Molecular weight standards, **lanes 1, 4, 7**, and **10**, are BioRad Rainbow markers.

polypeptide of the same molecular weight (170 kDa) as the reactive band in eggs. This indicates that the reactive polypeptide in eggs is the same or very similar to that in hemolymph. We therefore identify it as an apoprotein of Vg.

Vgs from other heteropterans have been identified. Vgs and Vns from two Pyrrhocoridae, *Pyrrhocoris apterus* (Socha et al., 1991) and *Dysdercus koenigii* (Venugopal and Kumar, 1999) have been isolated. In *P. apterus*, a single Vg contained two apoproteins of 186 and 150 kDa, while two distinct *D. koenigii* Vns contained 3–4 apoproteins of 116, 92, 62, and 40 kDa. In Lygaeidae, an mAb against *Lygus hesperus* egg proteins reacted with polypeptides of 150, 140, 123, and 64 kDa (Hagler et al., 1991). *Spilostethus pandurus* expressed three Vg polypeptides that crossreacted with ovarian proteins at 176, 166, and 156 kDa (Ibanez et al., 1992). *Oncopeltus fasciatus* contained two Vg polypeptides of 200 and 68 kDa. Only the 68 kDa apoprotein was found in eggs (Rankin and Jackle, 1980). From Alydidae, *Riptortus clavatus* yielded two distinct yet immunologically related groups of Vg/Vns (Shinoda et al., 1996). The major polypeptides common to both Vg and Vn were 170, 160, 120, 54, and 50 kDa. In *Rhodnius prolixus*, Vn polypeptides were 180, 158, 50, and 46 kDa (Oliveira et al., 1995). In all of these cases including *P. maculiventris*, except *D. koenigii*, a large polypeptide between 170 and 200 kDa and averaging 180 kDa is a common feature.

Increased titers of vitellogenin preceded ovarian development, and the response was diet-dependent. In other studies, cumulative fecundity of *P. maculiventris* reared on the same artificial diet was about 44% of those reared on *G. mellonella* over a 50-day period (Greany, unpublished results). Diet development is now limited by knowledge of nutritional requirements of any one species. A more systematic approach will expedite success in rearing (Cohen, 1992), e.g., one in which diet components are initially deduced from nutritional profiles of prey, and quality control of resulting insects predicts their vigor in the field. Using mAbs with demonstrated activity against Vg and Vt, ELISAs are being developed to predict fecundity and shorten a test that now takes months down to days following feeding on test diets. This will save time and allow completion of many more studies. In cases where some diet components are limited, e.g., by the necessity to preparatively isolate them from an organism, the need to supply diet for a month or more may be prohibitive when a small-scale test over several days would be practicable. In any case, increased rates of diet development will result from improved means of assessing the effects of nutritional treatments. Quality control in insect rearing will also benefit from more efficient means of assessing fecundity.

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