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Synthesis, Distribution, and Levels of an Egg Lipoprotein from the Apple Snail *Pomacea canaliculata* (Mollusca: Gastropoda)

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ABSTRACT The site of synthesis of mollusc lipoproteins is hitherto unknown and was investigated for perivitellin 2 (PV2), an egg lipoprotein found in the freshwater snail *Pomacea canaliculata*. Tissues (albumen gland, gonad–hepatopancreas complex, and muscle) from vitellogenic females were incubated in vitro with ^{14}C-leucine at 25°C for 12 hr. At the end of incubation, soluble proteins from tissue homogenates and medium were analyzed for de novo protein synthesis by electrophoresis and HPLC, and radiolabeled proteins were quantified by liquid scintillation. Two albumen gland radiolabeled proteins (67 and 31 kDa) co-migrated with the subunits of PV2, and they represented 6.0% of the total labeled protein in that tissue. Western blot analysis confirmed the presence of PV2 only in the albumen gland. In vivo experiments where adult females were injected with ^{3}H Leucine revealed that PV2 was not present in hemolymph. ELISA analysis in all tissues of the snail confirmed the PV2 presence only in the albumen gland and developing eggs with levels of 26 and 98 mg/g protein, respectively. Therefore, the albumen gland is the only site for PV2 synthesis, and no extra-gland synthesis, circulation, or accumulation could be found. PV2 subunits were further characterized analyzing N-terminal sequences which showed no homology with other proteins. *J. Exp. Zool. 290:00–00, 2002.* © 2002 Wiley-Liss, Inc.

During yolk synthesis primary oocytes increased their size by accumulating vitellus. This vitellus is usually composed of proteins, lipids, and carbohydrates, and it represents the energy source for the developing embryo. The major egg-yolk proteins are either called vitellins if they are solely proteins or lipovitellins if they possess associated lipids (Wallace, '67), the latter being classified as high or very high density lipoproteins (HDL or VHDL) according to their hydrated density. Mollusc lipovitellins have been isolated from the eggs of the bivalve *Pecten maximus*, the cephalopod *Sepia oficialis* (Lee, '91), and more recently from the gastropod *Pomacea canaliculata* (Garin et al., '96).

Vitellogenesis in vertebrates generally occurs outside the ovary (termed heterosynthesis), vitellins being synthesized as vitellogenins in liver, secreted into blood, and taken up by oocytes. Vitellogenesis in invertebrates has been well studied in insects and more recently in crustaceans, being essentially similar to that in vertebrates, although different tissues are the source of circulating vitellogenins in hemolymph. Nevertheless, some insects and crustaceans have both heterosynthetic as well as autosynthetic yolk production (Kanost et al., '90; Fainzilber et al., '92). In comparison, little is known about this process in Molluscs. In cephalopods (De Jong-Brink et al., '83) and some gastropods (Barre et al., '91; Bride et al., '92) heterosynthesis seems to be the main yolk-forming process. Gastropod vitellins have been described in the oocytes of *Helix* (Barre et al., '91), *Helisoma* (Miksys and Saleuddin, '86), *Lymnaea,* and *Planorbis* (Bottke, '86). Unlike this general mechanism, most gastropods have a perivitelline fluid, mainly synthesized by accessory glands of the female reproductive tract called the albumen glands, which represents the major source of nutrients for the embryo. Therefore, proteinaceous yolk granules found in oocytes or in the embryos developing from egg cells provided with perivitelline fluid do not serve the purpose of nutrient storage, but they function as primary lysosomes in charge of perivitelline fluid digestion instead (Raven, '72; De Jong-Brink et al., '83; Wourms,

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'87; Bretting et al., '92). *P. canaliculata* belongs to this latter group where yolk is a minor source of nutrients to the embryo. This freshwater snail is a plague of rice crops in Asia, and it is the vector of the human meningoencephalitis. In an effort to understand its biochemistry during embryogenesis we have been studying the perivitelline fluid. Two lipoproteins (perivitellin-1 (PV1) or ovorubin, and perivitellin-2 (PV2)) and one lipoprotein fraction (PV3) were isolated (Garin et al., '96), representing 57.0%, 7.5%, and 35.5% of the egg total proteins, respectively. Considering their hydration densities, PV1 and PV2 perivitellins fall into the VHDL range. PV2 is a high molecular mass particle of 400 kDa. The major lipid classes of PV2 are free sterols and phospholipids, and they also have significant quantities of energy-providing triacylglycerides and free fatty acids. This particle is composed of two subunits of 67 and 31 kDa (Garin et al, '96). The lipid class composition of this particle suggests it may play an important role both as structural and energy source, which was confirmed following the dynamics of this lipoprotein through embryogenesis (Heras et al. '98). In the present work we determined the site of synthesis, mobilization and storage of PV2, and we further characterized its protein moiety in an effort to understand the energy metabolism during embryogenesis.

MATERIALS AND METHODS

Snails

Adults of *P. canaliculata* were collected in streams or ponds near the city of La Plata, Buenos Aires Province, Argentina. Eggs were collected from females raised in our laboratory or in the wild between November and April of consecutive seasons. All egg masses employed had embryos developed to no more than the morula stage. Embryo development was checked in each egg mass microscopically.

Tissue extracts

Albumen gland, gut, stomach, lung, muscle, and digestive gland–gonad complex were dissected from 3–4 females, repeatedly rinsed with ice-cold 20 mM Tris-HCl, pH 6.8, containing 0.8 μM aprotinin (Trasylol, Mobay Chemical Co., New York), and homogenized in a Potter-type homogenizer (Thomas Sci., Swedesboro, NJ). The ratio of buffer/sample was kept 5:1 v/w. Fertilized eggs were homogenized in the same fashion. The crude homogenates were then sonicated for 15 sec and centrifuged sequentially at 10,000*g* for 30 min and at 100,000*g* for 60 min. The pellet was discarded and the supernatant stored at –70°C until analysis. Hemolymph was collected by cardiac puncture with a 1-mL syringe with a G22 needle. By this procedure it is possible to obtain 2–6 mL from each adult. Protein content was determined by the method of Bradford ('76) using bovine serum albumin as standard.

PV2 isolation and purification

The egg soluble protein obtained by the above procedure was dialyzed for 24 hr against BrNa; d = 1.017 g/mL. The dialyzed sample was layered over BrNa, 1.26 g/mL, and ultracentrifuged at 207,000*g* at 10°C for 22 hr, on a Beckman L8M instrument (Beckman, Palo Alto, CA). A tube layered with BrNa, d =1.07 g/mL, in lieu of the sample was used as a blank for density calculations. After the run, 19 aliquots of 200 μL were collected from the top of the tubes. Absorbance at 280 nm was performed on each aliquot to obtain the protein profile. Refractive index of the blank tube aliquots was determined with a refractometer (Bausch & Lomb, New York), and converted to g/mL using tabulated data (Lindgren, '75).

A serial purification was done using a Merck-Hitachi high-performance liquid chromatograph (HPLC) (Hitachi Ltd., Tokyo, Japan) with an L-6200 Intelligent Pump and an L-4200 UV detector set at 280 nm. The sample was analyzed in a Mono Q HR 10/10 (Amersham-Pharmacia, Uppsala, Sweden) using a gradient of 0–1 M NaCl in a 20 mM Tris-HCl, pH 8.5, buffer. The PV2 peak was then further purified by size-exclusion chromatography (Superdex 200 HR 10/20, Amersham-Pharmacia, Uppsala, Sweden) using an isocratic gradient of 50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.6. Purity of the single peak obtained was checked by native PAGGE.

Anti-PV2 rabbit serum preparation

Antibodies directed against purified PV2 were prepared in rabbits. Animals were given multiple subcutaneous injections of about 1.5 mg of PV2 emulsified in Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO). A booster injection with about 1.5 mg antigen mixed with Freund's incomplete adjuvant was administered after 4 weeks. Two weeks later, the rabbits were bled through cardiac puncture. The collected blood was allowed to clot for 30 min at room temperature and then overnight (4°C). After centrifugation the serum obtained was stored at –70°C and used for

the immunochemical techniques. The specificity of the antiserum was verified by immunoblotting against all the fractions obtained from tissues. The antiserum reacted only with the PV2 fraction.

Gel electrophoresis

Total proteins of each fraction were measured by the method of Bradford et al. ('76). Ultracentrifugation fractions purified by HPLC were dialyzed against 20 mM Tris-HCl, pH 7.0, and concentrated by CentriPrep membrane concentrators with a MW 10,000 cut-off (Amicon, Beverly, MA). Non-dissociating electrophoresis was performed by a 4–20% polyacrylamide gradient (PAGGE) (Davis, '64; Margolis and Wrigley, '75). Protein subunits and apoproteins were analyzed by sodium dodecyl sulfate (SDS)–PAGGE using a gradient of 4–23% acrylamide (Laemmli, '70). The gels were stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co.). Molecular weights were calculated as previously described (Garin et al., '96).

Immunoblotting

For Western blots, proteins were separated by SDS–PAGGE or native electrophoresis and electroblotted for1.5 hr at 12 V (Trans-Blot SD Semi Dry Transfer Cell, Bio-Rad Laboratories, Hercules, CA) from the unstained gel to nitrocellulose membranes using 39 mM Tris, 48 mM Gly, pH 9.2, 20% MeOH buffer (Towbin et al., '77). After being blocked overnight at 4°C with 3% (w/v) non fat dry milk in 10 mM Tris-HCl , pH 7.4, 0.15 M NaCl, the membranes were incubated with the anti-PV2 serum (1:1,000) for 2 hr. Specific antigens were detected by goat anti-rabbit IgG horseradish peroxidase conjugate (1:3,000) using 4-chloro-1-naphthol, 0.5 mg/ml (Bio-Rad) in a Tris-saline buffer (TBS) in the presence of H_2O_2.

Enzyme-linked immunosorbent assay (ELISA)

In order to minimize PV2 titer variations related with either egg developmental stage or albumen gland cycle, samples were obtained from eggs at morula stage and from the gland of vitellogenic females.

The procedure was based on the assay of Engwall and Perlmann ('72).

The standard curve was prepared using purified PV2. Nunc-Immunoplate Maxisorp microtiter plates were loaded with 50 µL/well of the PV2 standard (0–10 ng) dissolved in a buffer containing 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6 (coating buffer). Samples of hemolymph, gut, lung, stomach, albumen gland, digestive gland–gonad, muscle, and eggs were diluted with the coating buffer. Aliquots of 50 µL were pipetted into the wells (100 ng protein/well) and incubated at 37°C for 90 min. The antigen solutions were then shaken out, and each well was filled with 300 µL of PBS, pH 7.4, containing 1% (w/v) non-fat dry milk. The plates were incubated at room temperature for 2 hr and subsequently washed three times with 0.05% (v/v) Tween in PBS. The anti-PV2 rabbit serum diluted in PBS-Tween (1:10,000) containing 1% non-fat dry milk was poured into each well, and plates were incubated overnight at 4°C and washed three times as above. Goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) diluted (1:250) in PBS–0.05% Tween–1% non-fat dry milk was added to each well (50 µL) and incubated at room temperature for 2 hr. After four washes as before, 50-µL aliquots of substrate solution, ABTS, and H_2O_2 (Bio-Rad Laboratories) were added to each well, and the plates were incubated at room temperature for 15 min. After color development, the reaction was stopped by addition of 2% oxalic acid (50 µL) and the absorbance was read at 415 nm on an EL-307C microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). One percent nonfat dry milk in PBS was used in all assays as a negative control and blank. All samples were analyzed in triplicate, and the values were averaged.

In vitro tissue incubations

To investigate the tissue site of PV2 synthesis, gonad–digestive gland complex, muscle, and albumen gland were dissected from vitellogenic females, rinsed with sterile incubation medium, weighed, and cut into 3 pieces. Each piece (approximately 50 mg, 0.2–0.4 cm^3) was weighed and incubated separately in 1 mL of modified *Helisoma* medium (Kater and Mattson, '88) composed of 5 mM HEPES, pH 7.3, 40 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2{\cdot}2H_2O$, 1.5 mM Cl_2Mg, 50 U/mL penicillin, and 50 U/mL streptomycin containing ^{14}C-Leu (315 µCi/nmol, New England Nuclear; final specific activity 1 µCi/ml). Incubation vials were placed in a water bath at 24°C and gently agitated for 12 hr. Control incubations were carried out with media containing labeled leucine but no tissue. After the incubation period, tissues were washed repeatedly with medium without the tracer and homogenized as described above. Aliquots were analyzed for total protein (Bradford, '76). Radioactivity of homogenates was quantified

in a Pharmacia LKB Wallac 1219 Rackbeta liquid scintillation spectrometer (Uppsala, Sweden). PV2 lipoprotein was isolated form homogenates by HPLC or electrophoresis as described above. Radioactivity incorporated into protein fractions separated by HPLC was either measured by Radio-HPLC (Radiomatic Flo-one beta, Packard, Meriden, CT) or by collecting the peaks and counting the radioactivity by liquid scintillation spectrometry. Radioactivity incorporated into protein fractions separated by electrophoresis was quantified by liquid scintillation spectrometry after digestion of the gel slices with H_2O_2.

In vivo incubations

Vitellogenic females were injected in the foot with 100 μL of *Helisoma* salts containing 10μCi of ^{3}H-Leu (New England Nuclear) and left in aquaria at 26μC for 18 hr. After that period, hemolymph was collected from each specimen and stored at –70°C for analysis.

N-Terminal sequence

Purified PV2 was sequenced at Laboratorio Nacional de Investigación y Sevicios en péptidos y proteínas (LANAIS-PRO, Universidad de Buenos Aires—CONICET). The system used was an Applied Biosystems 477[a] Protein/Peptide Sequencer interfaced with an HPLC 120 for one-line phenylthiohydantoin amino acid analysis.

RESULTS

Distribution of PV2 in different tissues

The presence of PV2 was evaluated in homogenates of most tissues of the apple snail: albumen gland, gut, stomach, lung, digestive gland–gonad complex, hemolymph, muscle, and egg cytosol using native PAGGE and SDS–PAGGE. Figure 1A shows a minor band with molecular mass 400 kDa in the albumen gland that corresponded to native PV2, although the major protein is the 300-kDa ovorubin. This band was immunoreactive to anti PV2 antiserum as shown by Western blots (see below). Under dissociating conditions (Fig. 2) PV2 was also identified by its two subunits of 31 and 67 kDa that were present in albumen gland samples. The 31-kDa subunit overlaps with ovorubin subunits (28, 32, and 35 kDa), giving a strong band in the gels.

Incorporation of ^{14}C Leu into PV2

A pilot experiment was performed to determine the time course of incorporation, adjust pH, tracer concentration, and select incubation time and medium. Microscopic inspection of tissues after 12 hr of incubation was performed to check for necrosis (data not shown). The results indicate that tissues were viable under these conditions. A number of different media composition were tried and the best composition was found to be the modi-

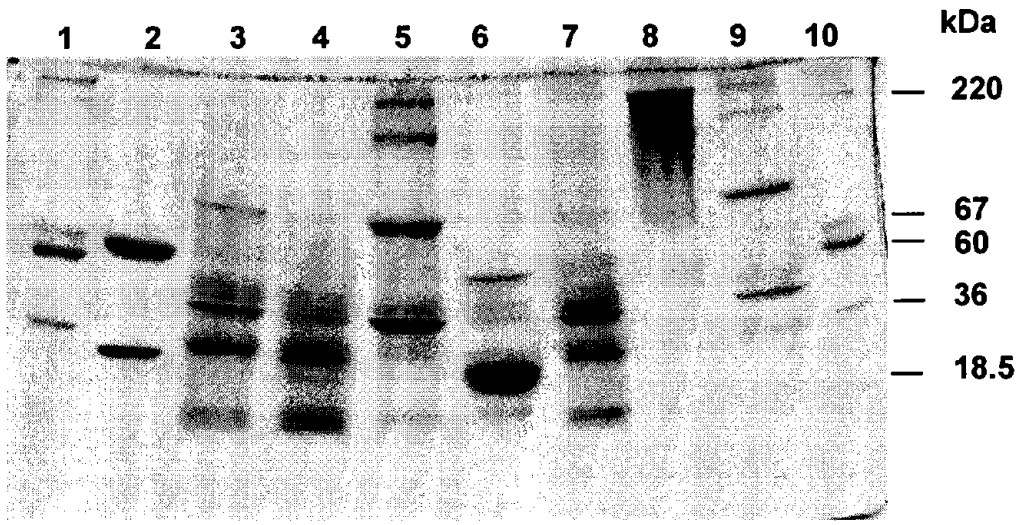

Fig. 1. Native gel electrophoresis (A) and Western blot (B) of tissue and perivitelline 2 samples from *P. canaliculata*. Gel A was prepared using an acrylamide gradient of 4–20% w/v; gel B was prepared using an acrylamide gradient of 4–23% w/v. Lanes 1 and 10, STD; lane 2, purified PV2; lane 3, gut; lane 4, gonad–digestive gland complex; lane 5, muscle; lane 6, albumen gland; lane 7, stomach; lane 8, hemolymph; lane 9, lung. Proteins were revealed by Coomassie blue staining. STD: high molecular weight standards (Pharmacia). Native proteins: thyroglobulin (MW 669,000), ferritin (MW 440,000), catalase (MW 232,000), lactate dehydrogenase (MW 140,000), and albumin (MW 67,000).

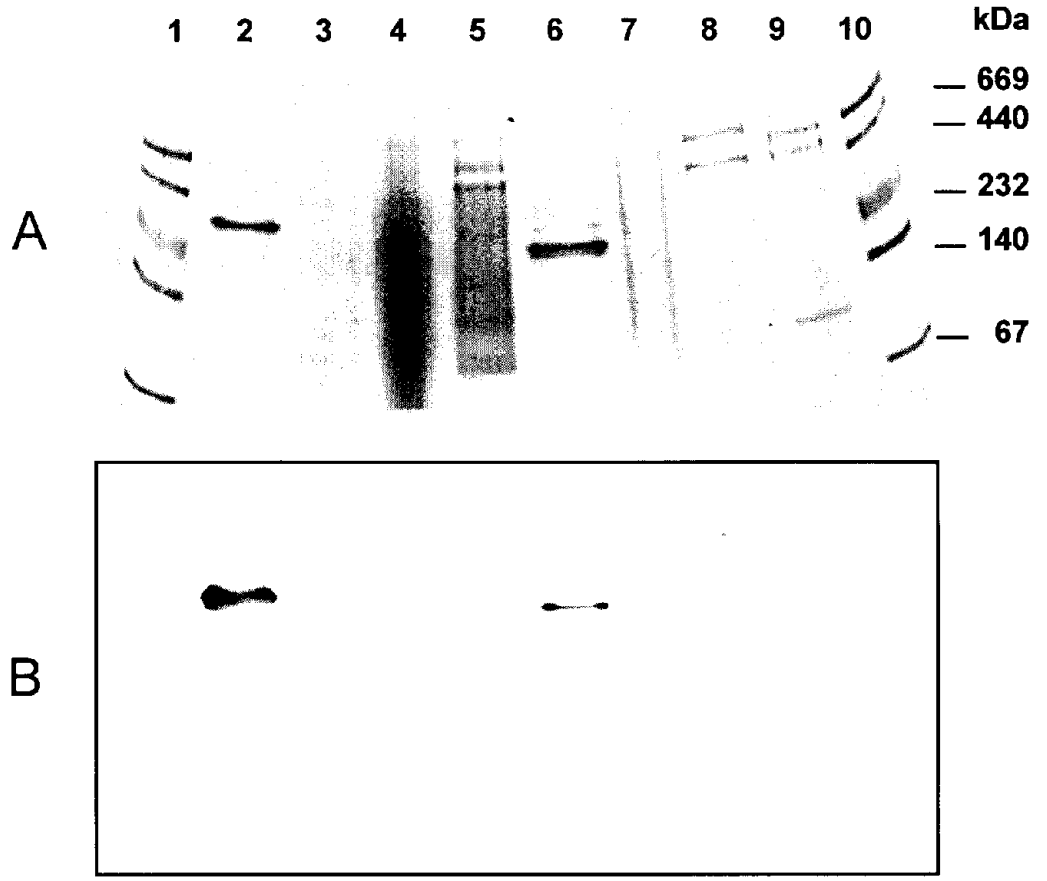

Fig. 2. Dissociating gel electrophoresis of tissues and perivitelline 2 samples from *P. canaliculata*. Gel was prepared using an acrylamide gradient of 4–23% w/v. Lanes 1–10: see Fig. 1. Proteins were revealed by Coomassie blue staining. STD: ferritin half-unit (MW 220,000), albumin (MW 67,000), catalase (MW 60,000), lactate dehydrogenase (MW 36,000), and ferritin (MW 18,500).

fied *Helisoma* salts, buffered with HEPES, as mentioned in Materials and Methods. Based on the time course of incorporation, a 12-hr incubation period was selected for further analysis of PV2 synthesis.

Tissue proteins from incubations were separated by PAGGE and HPLC. All tissue incorporated the tracer into proteins (Table 1). As mentioned above, protein bands that co-migrated with native PV2 were present in albumen gland (Fig. 2). Proteins of similar size were present in the gonad–hepatopancreas complex. As specific activity was not enough to perform autoradiography analysis, each gel lane was sliced, digested, and quantified by liquid scintillation counting. Results showed that the only tissue incorporating ^{14}C-Leu into a protein of PV2 MW was albumen gland. Radio-HPLC analysis of proteins showed a radiolabeled protein peak that eluted at PV2 elution volume in albumen gland and in gonad–digestive gland complex, but only albumen gland protein was immunoreactive to anti-PV2 antiserum. Western blot analysis using anti PV2 antiserum as probe revealed that the albumen gland contained immunoreactive proteins (Fig. 1B) and that immunoreactivity was not observed in homogenates of either muscle, gut, stomach, lung, or gonad–digestive gland complex.

TABLE 1. Percentage of radioactivity incorporated into PV2 protein fraction after 12-hr incubation with ^{14}C-Leu[1]

Tissue	PV2[2]	Other proteins
Albumen gland	5.75 ± 1.1	94.25
Digestive-gonad complex	ND	100
Muscle	ND	100

[1]Values are the mean of HPLC and electrophoresis data ± SD (n = 2). PV2 identity was confirmed by western blot analysis; ND, not detected.
[2]Analyzed together with PV3.

Incorporation of [^{3}H]Leu into PV2

When vitellogenic females were injected with labeled leucine, all tissue extracts contained la-

TABLE 2. PV2 lipoprotein levels[1]

Tissue	Soluble protein (mg/mL)	PV2 (% P/P)
Egg	8.6	9.8
Albumen gland	12.0	2.6
Hemolymph	11.0	ND
Gonad-digestive gland	5.5	ND
Muscle	1.9	ND
Gut	2.3	ND
Stomach	4.5	ND

[1]Values are the mean of duplicate analyses; ND, not detected.

beled proteins. As the aim of this experiment was to assess the presence of circulating perivitellogenins, hemolymph was particularly analyzed. HPLC and electrophoretic analysis of radiolabeled hemolymph indicate that no protein with PV2 characteristics was present. Western blot analysis of unlabeled hemolymph showed that hemolymph did not contain any PV2-immunoreactive protein, though some faint cross-reactivity with other hemolymph protein was present.

PV2 titer in different tissues by ELISA

This method had a linear relationship from 0 to 12 ng of purified PV2, 15 min after the addition of the substrate solution. When the samples were analyzed, well-to-well variation was similar to the variation in the standard curve.

Perivitellin was not detected in hemolymph, muscle, gut, lung, stomach, or gonad–digestive gland complex. It was only present in albumen gland and egg cytosol (Table 2).

N-Terminal sequence

N-terminal amino acid sequence was determined in both subunits (Table 3). The sequence homology was checked in Swiss Prot database, but no homology with any known sequence was found.

DISCUSSION

The most largely studied group of aquatic invertebrates, with respect to hemolymph, ovarian, and egg lipoproteins, is the crustaceans. Lipoproteins have been characterized in many species of different groups such as lobsters, crabs, horseshoe crabs, shrimps, prawns, and crayfish, which have mostly HDL and in some cases VHDL lipoproteins (Lee, '91; Yepiz-Plascencia et al., 2000, and references therein). Unlike crustaceans, little is known in Molluscs about egg reserve lipoproteins, and the few available reports focus mainly on the lipid and apoprotein composition of these lipoproteins (Fuji, '60; Garin et al., '96; Heras et al., '98). All lipoproteins of the perivitelline fluid of the freshwater prosobranch *P. canaliculata* fall into the VHDL and HDL categories (Garin et al., '96). This characteristic, together with the presence of associated pigments, is basically the only structural feature in common with other invertebrate and vertebrate lipovitellins (Lee, '91) because it is not pertinent to consider homologies between this perivitelline lipoproteins and yolk lipovitellins based on their functional analogy. For PV2, this assumption was confirmed by the N-terminal amino acid sequence of PV2 that was compared with crustacean apolipoproteins and with other related and non-related proteins. There was no sequence homology between PV2 apolipoproteins or any other published sequence.

TABLE 3. N-terminal amino acid sequences of PV2 subunits

	1	5	10	15
67-kDa subunit	A R V X P K I V P G L D K L R V G V			
31-kDa subunit	F T S V K M P R D E H W P Y N			

Many organisms including insects, crustaceans, and vertebrates possess vitellogenins (Vg), female-specific lipoproteins that appear in the hemolymph during vitellogenesis. Therefore we studied the possibility that *P. canaliculata* females would have a PV2 Vg. There was no evidence of circulating PV2 in vitellogenic females as determined by either labeling hemolymph proteins in vivo with ^{3}H-Leu or by Western blot and ELISA analysis of hemolymph proteins. In a previous study using electrophoresis, Garin et al. ('95) did not find any vitellogenin in the hemolymph of *P. canaliculata*. Barre et al. ('91), using antibodies, showed the presence of molecules immunologically similar to vitellins in the hemolymph and glandular cells of the hepatopancreas of *Helix aspersa*, suggesting that in this snail some egg Vg would be synthesized in the hepatopancreas and mobilized through the hemolymph to the vitellogenic egg. There is no other report in gastropods, and although a vitellin-like lipoprotein was mentioned for the bivalve *Pecten maximus,* no evidence of the corresponding vitellogenin was found (Zagalsky, '72).

In the present research, the albumen gland incorporated radiolabeled leucine into PV2 subunits. The other potentially synthesizing tissues examined, including the gonad–digestive gland complex, did not show any labeled or immunoreactive PV2-like lipoprotein. Albumen gland synthesis of PV2, quantified together with a minor perivitellin (PV3), was 5 times higher than ovorubin which, in contrast, is the most abundant egg lipoprotein

in *P. canaliculata* (results not shown). However, the three lipoproteins taken together do not account for more than 7% of the total protein synthesized after 12-hr incubation of the gland. This result would suggest that in the apple snail all perivitellus proteins are synthesized in the albumen gland, something that could be termed a heterosynthetic mechanism sensu lato. In a study about a pulmonate albumen gland secretion in vitro, Morishita et al. ('88) found that the albumen gland of the pulmonate *Helisoma duryi* secretes three perivitellins of 288, 278, and 238 kDa, which do not coincide with *P. canaliculata* PV2 MW. Other gastropods, such as the garden snail *H. aspersa* show a heterosynthetic mechanism, the vitellogenins being synthesized in the digestive gland that surrounds the ovotestis and being transported by hemolymph to vitellogenic oocytes (Barre, '91; Bride et al., '92). In the freshwater snails *Lymnaea* and *Planorbis* other vitellin–vitellogenin proteins have been identified, such as the iron reserve ferritin considered to be their major yolk component. It was found that the yolk ferritin is also produced by the digestive gland of these snails (Bottke '86; Bottke et al., '88). Vitellogenesis in crustaceans and insects is generally heterosynthetic, although some organisms also have different strategies for the synthesis of their egg lipoproteins that seem to be species-specific (Yano and Chizei, '87; Browdy et al., '90; Kanost et al., '90; Fainzilber et al., '92; Shafir et al., '92). In the apple snail, perivitellins would be secreted by the albumen gland together with the other perivitellus components and be directly layered over the fertilized oocyte as it passes through the oviduct. Additional comparative work on different families and species of gastropods is required to further confirm the strategies for egg reserve protein synthesis in this complex group.

In crustaceans the source of Vg in hemolymph is still not yet clear. Some data point out an extra-ovarian origin, being later on taken up by the ovary where it constitutes at least part of the vitellins (Vt). This is accepted for most crustacean and insect species. However, several reports show that crustacean Vt is also synthesized within the ovary. In this case, Vg may originate from either controlled secretion by the ovary (Yano and Chizei, '87) or passive leakage from oocytes in the process of resorption (Shafir et al., '92). There is also evidence that the subepidermal connective and adipose tissues may produce lipovitellin in some crustaceans (Kerr, '69; Aiken and Waddy, '80; Tom et al., '87).

ELISA analysis confirmed PV2 presence only in the albumen gland and developing eggs with levels of 26 and 98 mg/g protein, representing 2.6% and 9.8% of the total protein, respectively. PV2 is the least concentrated of the three lipoprotein fractions of perivitellus and holds 3.75% lipids, which corresponds to 10.1% of the total lipids for the egg. The composition and amount of lipids suggested *P. canaliculata* egg lipoproteins would play a role in providing structural components and metabolic precursors for the developing embryo, and they should not be considered only as energy sources for the embryo as lipovitellins are. In fact, following the dynamics of this lipoprotein through embryogenesis, Heras et al. ('98) showed that PV2 was consumed by embryos as a source of both structural and energetic molecules.

Therefore, we can conclude that the albumen gland is the only site for the PV2 synthesis in the apple snail, as no extra-gland synthesis, circulation or accumulation could be demonstrated in the present work. A detailed study on the structure, amino acid sequence, and lipid-binding properties of perivitellin lipoproteins would establish patterns and homologies with other proteins of great evolutionary interest, and this is the subject of ongoing research in our laboratory.

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