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The major egg reserve protein from the invasive apple snail *Pomacea maculata* is a complex carotenoprotein related to those of *Pomacea canaliculata* and *Pomacea scalaris*

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ABSTRACT

Snails from the genus *Pomacea* lay conspicuous masses of brightly colored eggs above the water. Coloration is given by carotenoproteins that also which play important roles in protection against sun radiation, stabilizing and transporting antioxidant molecules and helping to protect embryos from desiccation and predators. They seem a key acquisition, but have been little studied. Here we report the characteristics of the major carotenoprotein from *Pomacea maculata* and the first comparison among these egg proteins. This particle, hereafter PmPV1, represents ~52% of perivitellin fluid protein. It is a glyco-lipo-carotenoprotein responsible for the bright reddish egg coloration. With VHDL characteristics, PmPV1 apparent molecular mass is 294 kDa, composed of five non-covalently bound subunits of pI 4.7–9.8 and masses between 26 and 36 kDa whose N-terminal sequences were obtained. It is a glyco-lipo-carotenoprotein scarcely lipidated (<1%) but highly glycosilated (13% by wt). Lipids include phospholipids, free fatty acids and carotenoids; mannose and galactose predominate over other monosaccharides. Main carotenoids are esterified and non-esterified astaxanthin (71 and 25%, respectively). Carotenoid removal does not seem to affect the structural characteristics of the oligomer, while deglycosilation reduces subunit number from five to a single one. The carotenoid–protein association protected the former against oxidation. PmPV1 cross reacts with polyclonal antibodies against the PcOvo, the major carotenoprotein from *Pomacea canaliculata*. The characterization of PmPV1 allows the first comparisons among snail carotenoproteins and further highlights the importance of these perivitellins in the reproductive strategy of *Pomacea*.

1. Introduction

The genus *Pomacea* is considered the most derived clade of ampullarids (Caenogastropoda: Architaenioglossa) (Hayes et al., 2009b) and is the most speciose. It contains several species of invasive freshwater snails that have become major pests in humid tropics and subtropics of the world (Hayes et al., 2008). The two most widespread species, *Pomacea canaliculata* (Lamarck 1822) and *Pomacea maculata*, Perry, 1810, formerly *Pomacea insularum*, (d'Orbigny 1835) (Hayes et al., 2012) are closely related and difficult to distinguish morphologically though genetically distinguishable species (Rawlings et al., 2007; Hayes et al., 2008; Matsukura et al., 2008). Both species occur sympatrically in invaded areas as well as in their original range in Paraná and Uruguay River basins of Argentina, although *P. canaliculata* extends further south (Martín et al., 2001). Until recently, studies of these snails were wrongly referred both to a single widespread and highly variable species, *P. canaliculata*, considered one of the world's 100 worst invasive species (Lowe et al., 2000).

Native from South America, the apple snail *P. maculata* possess a high reproductive rate which, coupled to an opportunistic consumption pattern, gives this species invasive capacity (Burks et al., 2010). Moreover, its large size (up to 165 mm in adults shell length (Hayes et al., 2012) and its taste for aquatic plants suggest a high potential for ecological and economical damage for wetlands and crop fields (Carlsson et al., 2004; Youens and Burks, 2008). It has already invaded North America and E and SE Asia and more recently southern Europe, damaging wetlands and rice fields (Barnes et al., 2008; López et al., 2010). *P. maculata* is a vector for the nematode parasite *Angiostrongylus cantonensis* that causes the human eosinophilic meningitis disease (Qvarnstrom et al., 2013; Teem et al., 2013) and therefore the dispersion of *P. maculata* could pose a threat to public health. All these

Abbreviations: ASX, astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′dione); Glc, glucose; Gal, galactose; Xyl, xylose; GlcNAc, *N*-acetyl-glucosamine; Fuc, fucose; Man, mannose; PVF, perivitellin fluid.

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facts stress the need to study its reproduction biochemistry, currently completely unknown. These snails lay conspicuous calcareous egg masses in trunks, plants or other surfaces above the water, a synapomorphy usually accompanied by bright colors (Hayes et al., 2009a). This is an unusual reproductive strategy for an aquatic organism because eggs are thus exposed for several days to sunlight, high temperatures, and predators (Heras et al., 2007).

Embryos of ampullariids rely for energy and nutrients on the perivitellin fluid (Heras et al., 1998). It has been determined that their perivitellin fluid proteins, called perivitellins, are not only a source of nutrients for the embryo, but also play several other roles as part of the defense system against environmental factors and predators (Dreon et al., 2007; Heras et al., 2008; Dreon et al., 2010; Frassa et al., 2010; Ituarte et al., 2012; Dreon et al., 2013). Moreover, recently the first apple snail egg proteome was characterized in *P. canaliculata* revealing several other new functions for perivitellins that are awaiting functional studies (Sun et al., 2012).

Among perivitellins, carotenoproteins seem to always be present in those ampullariids that resent aereal oviposition, particularly the genus *Pomacea*. Carotenoid-binding proteins are commonly found in invertebrates. Their carotenoids form non-covalent complexes with proteins giving tissues a variety of colors. Carotenoproteins are primarily found in reproductive structures (gonads and eggs) (Cheesman et al., 1967). The ovaries and egg yolk carotenoproteins usually form a water-soluble complex, and are frequently associated with sugars and lipids, forming glyco-lipo-carotenoprotein particles (Zagalsky, 1985). Remarkably hardly any studies have been made on Mollusks carotenoproteins, namely the reports in one Polyplacophora, five bivalves, and five gastropods (Allen, 1977; Cheesman et al., 1967; Czeczuga, 1983; Euler et al., 1934; Goodwin and Taha, 1950; Heras et al., 2007; Ituarte et al., 2008; Nakadal, 1960; Paparo and Murphy, 1978; Yang et al., 1994; Zagalsky, 1972). However, the nature of carotenoproteins complexes and structural aspects in mollusks as a whole has been well studied only for the eggs of two apple snails, *P. canaliculata* and *Pomacea scalaris.* In both species they provide nutrients and are involved in embryo photoprotection against sun radiation, stabilizing and transporting antioxidant molecules and helping to protect embryos from desiccation and predators (Dreon et al., 2004a,b, 2007; Ituarte et al., 2008). Besides, it is thought that the reddish or pinkish color they provide to the eggs would be a warning (aposematic) coloration, advertising predators of the egg biochemical defenses (Heras et al., 2007). However, they have also evolved other functions in apple snails which are species specific. For instance, the carotenoprotein of *P. scalaris* eggs (PsSC) is a lectin (Ituarte et al., 2012), while that of *P. canaliculata* (PcOvo) is an antinutritive/antidigestive molecule (Dreon et al., 2010).

It is accepted that the acquisition of carotenoproteins was a key adaptation that allowed ampullarids to lay clutches out of the water. All ampulariids that lay their eggs below the water line studied so far do not possess colored eggs (Heras et al., 2007).

Here we report the isolation and characterization of the major egg carotenoprotein from *P. maculata* and compare its composition, N-terminal sequences and immunological features and capacity to protect carotenoids with those of other *Pomacea* snails to ultimately provide a broader vision on mollusk carotenoproteins and their relation with the successful aerial egg laying strategy of the genus. We also report that the spectral differences among carotenoproteins provide a mean to differentiate eggs from *P. canaliculata* from those of *P. maculata*, two spatially overlapping species in both their native and their invaded areas.

2. Materials and methods

2.1. Sample collection

Adults of *P. maculata* were collected in the Paraná River in San Pedro 33°39′35.97″ S; 59°41′52.86″ W, Buenos Aires province, Argentina and kept in the laboratory. Voucher specimens were deposited in the Museo de La Plata Collection (MLP 13749). Eggs were collected within 24 h of laying, and kept at −20 °C until processed.

2.2. Isolation and purification of the major carotenoprotein

Egg masses were weight and homogenized with a Potter type homogenizer (Thomas Sci., Swedesvoro, NJ) on ice-cold 20 mM Tris–HCl, pH 7.4 buffer (keeping a 4:1 v/w buffer:sample ratio) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The crude homogenate was centrifuged at 10,000 *g* for 20 min in an Avanti JE centrifuge (Beckman, Palo Alto, CA). The pellet was discarded and supernatant was centrifuged at 100,000 *g* for 50 min on a Beckman L8M centrifuge with a fixed angle rotor 70.1 Ti. The pellet was discarded and the supernatant (from now on:perivitellin fluid (PVF)) was layered on NaBr $\delta = 1.28$ g/mL and ultracentrifuged at 207,000 *g* for 22 h on a Beckman L8M with a swinging bucket rotor SW 60 Ti (Beckman, Palo Alto, CA, USA). A blank with buffer was ultracentrifuged in parallel using buffer instead egg soluble fraction. Gradient was aliquoted in 200 μL fractions from the top of the tube. Absorbance of each aliquot was determined at 280 nm in an Agilent 8453 UV/Vis diode array spectrophotometer (Agilent Technologies, Waldbronn, Germany) to obtain the protein profile. Refractive index of the blank tube aliquots was determined with a refractometer (Bausch & Lomb, Inc., Rochester, NY, USA), and converted to density using tabulated values (Orr et al., 1991). NaBr was washed from colored fractions with homogenation buffer using Amicon Ultra membrane concentrators with a 50,000 MW cut off (Amicon, Beverly, MA, USA) and then separated by anionic exchange liquid chromatography in a HPLC system (Agilent technologies, 1260 infinity) using a Mono-Q column (Amersham-Pharmacia, Uppsala, Sweden). All purification steps were done at 4 °C. Purity steps were checked by polyacrylamide gel electrophoresis as described below. The protein purified following this method is hereafter named PmPV1. To avoid confusion with the major PVF proteins of other species of *Pomacea*, perivitellin names were given using following Sun *et al* (2012) criteria, that is beginning with a species specific prefix ("Pm" for *P.maculata*).

2.3. Spectrophotometric analysis

Absorption spectra of *Pm*PV1 was recorded between 350 nm to 650 nm in an Agilent 8453 UV/Vis diode array spectrophotometer (Agilent Technologies).

2.4. Protein quantification and gel electrophoresis

Total protein was quantified following the method described by Lowry et al. (1951) using BSA as standard. Native polyacrylamide gel electrophoresis (PAGE) was performed in 4–20% gradient polyacrylamide gels in a miniVE Electrophoresis System (GE Healthcare, Life Science), high molecular weight standards (Pharmacia) were run in the same gels. Subunits were separated by SDS-PAGE in 4–20% gradient polyacrylamide gels containing 0.1% SDS; samples were denatured at 95 °C, with dithiotreitol and β-mercaptoethanol treatment (Laemmli, 1970). Low molecular weight standards (Pharmacia) were used and gels were stained with Coomassie Brilliant Blue R-250 (Sigma Chemicals).

2.5. Lipid content and lipid classes

Lipids were extracted using a chloroform–methanol mixture following the Bligh and Dyer (1959) method. Quantity of total lipids was obtained gravimetrically until constant weight was achieved. Qualitative analysis was performed by thin layer chromatography (TLC). The separation was conducted with two different solvent systems in the same TLC plate (Merck, Darmstadt, Germany). First, chloroform:acetic acid:

water (50:45:5 v/v/v) for polar lipids and then, hexane:diethyl ether: acetic acid (80:20:1.5 v/v/v) for neutral lipids. Standard lipids were run in parallel to identify lipid classes. Lipids were revealed with anisaldehyde (Fuchs et al., 2011).

2.6. Carbohydrate content

Total hexose content was determined following the sulfuric-phenol colorimetric method described by Dubois et al. (1956), using D-glucose (Sigma-Aldrich, St. Louis, MO, USA) as standard. A standard curve was measured between 35 and 200 μg/mL. Samples (final volume 150 μL) were treated with an aqueous 5% (w/v) phenol solution (150 μL) before concentrate H_2SO_4 (750 μL) was added. After 30 min at 37 °C absorbance was read at 485 nm in an Agilent 8453 UV/Vis diode array spectrophotometer.

2.7. Monosaccharide composition

Lyophilized glycoprotein (50 μg of total sugar) was hydrolyzed by heating in the presence of methanolic HCl. Volatile derivatives of methyl glycosides were prepared from the dried methyl glycosides using Tri–Sil (2:1:10 (v/v/v) hexamethyldisilazane/trimethylchlorosilane/pyridine) (Manzi, 1995). Silylated samples were analyzed by gas chromatography coupled with a flame ionization detector (GC) using an HP6890 capillary GC (Hewlett Packard, Palo Alto, CA, USA) fitted with a HP-5 column (5% phenyl methyl siloxane): 30 m (length), 0.25 mm (I.D), 0.25 μm (film) (Hewlett Packard). Conditions and temperatures were: FID 250 °C; injector 180 °C. The column temperature was programmed as follows: initial temperature 50 °C held for 3 min; increased at a rate of 20 °C min^{-1} to a temperature of 170 °C, a second ramp of 6 °C min^{-1} to a final temperature of 250 °C. The flow was set at 2 mL/min. The carrier gas was helium at a pressure of 20 psig and hydrogen and air pressures were 13 and 23 psig, respectively. A mix of standards containing glucose (Glu), galactose (Gal), xylose (Xyl), *N*-acetyl-glucosamine (GlcNAc), fucose (Fuc), and Mannose (Man) (Sigma-Aldrich) was prepared and run in parallel as well as independent from each other.

2.8. Two dimensional gel electrophoresis (2-DE)

Two-dimensional electrophoresis was carried out with immobilized pH gradient (IPG)-isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension (Görg et al., 1988). The IEF was performed using an Ettan IPGphor III (GE Healthcare) and 7 cm linear pH 3–10 Immobiline dry strips (GE Healthcare). Rehydration of the strip and loading of the samples (22 μg protein) were carried out overnight at room temperature in a dilution buffer (0.002% w/v bromophenol blue, 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% v/v, IPG Buffer 3–10 linear (GE Healthcare). After IEF, the Immobiline dry strips were equilibrated at room temperature for 20 min in a buffer containing: 75 mM Tris–HCl, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue and 1% w/v DTT, and then 20 min in the same buffer with 4.5% w/v iodacetamide in place of DTT. For SDS-PAGE second dimension, the IPG strips were sealed on the top of 1.5 mm thick 12% polyacrylamide gels, with MW standards (GE Healthcare) run in parallel. Vertical electrophoresis was carried out at 30 mA. Gels were stained with a colloidal suspension of Coomassie Brilliant Blue R-250 (Sigma-Aldrich).

2.9. Chemical deglycosylation

The protein was deglycosylated using trifluoromethanesulfonic acid (TFMS) (Sigma-Aldrich) as previously described by Ituarte et al. (2010). Aliquots containing 1 mg of lyophilized protein were incubated for 3 h on ice with 150 μL of pre-cooled 10% anhydrous anisole in TFMS; after incubation, the acid was neutralized by adding 60% pyridine drop wise, keeping the samples at about −15 °C using chilled 96% ethanol. Addition of water to the neutralized mix caused protein precipitation which was transferred and washed few times with ddH_2O and finally redissolved in 7 M urea, 2 M thiourea, 2% CHAPS. Results were analyzed by SDS-PAGE.

2.10. Carotenoid analysis

Carotenoids were extracted from PmPV1 and PcOvo with ice cold acetone keeping a ratio sample:acetone 1:4 v/v. After a brief centrifugation, dissolved carotenes were transferred to another tube and the precipitated protein dissolved in 20 mM Tris–HCl pH 7.4. The extraction was repeated twice. The carotenoid-containing phase was extracted with 3 volumes of hexane. After a brief centrifugation, hexane upper phase containing the carotenoids was collected and dried under a stream of N_2. Analytical high performance TLC (HPTLC) was carried out on Silicagel G 60 plates (Merck, Darmstadt, Germany) using hexane/acetone, 80:20 (v/v) as mobile phase. Carotenes were identified comparing R_f with *P. canaliculata* known carotenes (Dreon et al., 2004b), and astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′ dione) (ASX) standard (Sigma). The nature of the carotenoids was also checked by their spectral characteristics. The relative amount of each was calculated densitometrically on the HPTLC plates using ImageJ 1.46 software (Schneider et al., 2012). Besides, the apo-carotenoprotein was analyzed by PAGE.

2.11. N-terminal sequence

Subunits of purified PmPV1 were sequenced by Edman degradation at the Laboratorio Nacional de Investigación y Servicios en Péptidos y Proteínas (LANAIS-PRO, Universidad de Buenos Aires—CONICET). The system used was an Applied Biosystems 477a Protein/Peptide Sequencer interfaced with an HPLC 120 for one-line phenylthiohydantoin amino acid analysis. N-terminal sequences were compared with other *Pomacea* N-terminal sequences using the multiple sequence alignment program CLUSTAL 2.1 (Larkin et al., 2007).

2.12. Immunoblotting

Proteins were transferred from SDS PAGGE 4–20% gels onto nitrocellulose membranes (Amersham) in a Mini Transblot Cell (Bio RadLaboratories, Inc.), using 25 mM Tris–HCl, 192 mM glycine, 20% (v/v) methanol, pH 8.3 buffer. After blocking for 2 h at 37 °C with 5% (w/v) nonfat dry milk in PBS-Tween, the membranes were incubated overnight at 4 °C with the anti-sera dilutions against PcOvo in 3% (w/v) nonfat dry milk in PBS-Tween. Specific antigens were detected by goat anti-rabbit IgG horseradish peroxidase conjugate (BioRad Laboratories, Inc.). Immunoreactivity was visualized by electro-chemiluminiscence.

2.13. Protective effect of PmPV1 on its carotenoids

A 2 mL aliquot of purified apo-PmPV1 (1.2 mg/mL) was incubated with 100 μL of standard solution of astaxanthin (430 μg/mL) in DMSO. After incubation for 1 h in the dark at 26 °C with shaking, reconstituted PmPV1 was cleaned from unbound astaxanthin on a Sepharose G-25 column (Amersham-Pharmacia, Uppsala, Sweden) (1 × 10 cm), pre-equilibrated with 0.02 M Tris–HCl 0.3 M NaCl buffer, pH 8.5. The eluted fractions were monitored for their absorption at 280 nm. The PmPV1 containing fractions were pooled and stored at 4 °C overnight until analysis. The integrity of the reconstituted PmPV1 was checked by PAGE.

1 mL solutions containing PmPV1, reconstituted PmPV1 or free astaxanthin were exposed to fluorescent light, air and 35 °C for 470 min. Sample absorbance was monitored at 492 nm (λ max of astaxanthin in DMSO) every 30 min. DMSO (100 μL) was added to control tubes.

Differences between samples were analyzed by a two-way ANOVA with Bonferroni post-test using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1. Isolation of P. maculata perivitellins

Egg masses weighted around 13 g and had approximately 10 cm length (Fig. 1a). After NaBr gradient ultracentrifugation of the egg soluble fraction three protein peaks were observed (Fig. 1b): a yellow fraction with a relative density of 1.13–1.16 g/mL (HDL) which was called PmPV3; a reddish fraction at the bottom of the tube with a relative density of 1.23–1.28 g/mL (VHDL), which was called PmPV1 and, a third colorless protein fraction with a density of 1.17–1.22 g/mL which was called PmPV2. As mentioned in Methods, perivitellin names were given following Sun *et al* (2012) criteria. Fig. 1c shows the hydration density and protein profile of the gradient of the tube indicating the location of the three fractions.

To investigate the protein composition of these three protein fractions a native gel electrophoresis was performed (Fig. 1c and inset). PmPV1 is a single protein band that corresponds to the major soluble protein of the perivitelline fluid; PmPV2 is also comprised by a single particle, while PmPV3 corresponds to an heterogeneous fraction.

3.2. Carotenoproteins spectral behavior

Fig. 2 shows the absorption spectrum for PmPV1 absorbs in a wide range of the visible spectra (350-650 nm). The spectrum shows two absorption maxima at 430 nm and 504 nm (the latest previously reported by Clark, R. J. H. et al. (1980). PmPV1 maximum absorption differs from the absorption maximum of the other two carotenoproteins as was shown in Table 1.

3.3. Biochemical composition of PmPV1

Fig. 3a shows the relative percent of protein, glucid and lipid in the PmPV1 particle. The particle is composed mainly by protein (85.8%). The electrophoretic behavior of purified PmPV1, under native conditions (Fig. 3b) showed only one protein band with an approximate MW of 294 kDa. Under dissociating and reducing conditions (Fig. 3c) this particle disaggregates in a ladder of five bands ranging between 25 and 35 kDa.

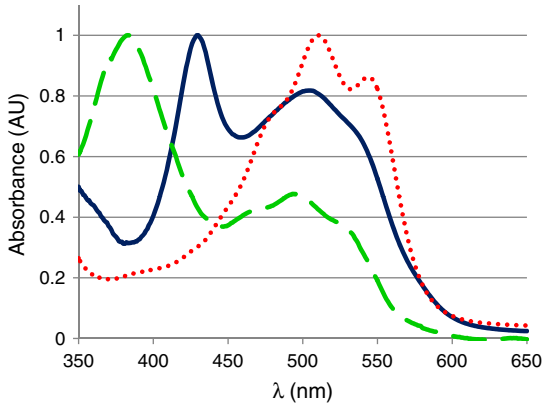

Fig. 2. Comparison of absorption spectra of purified egg carotenoproteins from apple snails. Absorption spectra of *P. maculata* (full line), *P. canaliculata* (dotted line) and *P. scalaris* (dashed line), (i.e. PmPV1, PcOvo, and PsSC respectively). PsSC data was taken from Ituarte *et al.* (2008).

The lipid content of PmPV1 was 0.8 ± 0.2% (w/w) mainly composed by free fatty acids, free sterols-astaxanthin esters and phospholipids (Fig. 3d).

The particle is highly glycosylated with a total of 13.4 ± 2.7% (w/w) hexoses. The GC analysis of monosaccharide released from PmPV1 showed that carbohydrate composition was dominated by mannose (34.44%) and galactose (24.16%), followed by N-acetyl-glucosamine (6.81%), xylose (5.61%), fucose (5.25%) and glucose (3.93%) (Fig. 3e).

3.4. Presence of glycoforms in PmPV1 subunits

We knew from the proximal analysis that the particle was heavily glycosylated, which was probably causing the ladder pattern observed for the apparent MW of the subunits when analyzed by SDS-PAGE. To evaluate this possibility PmPV1 was chemically deglycosylated and analyzed again by SDS-PAGE. The ladder pattern was eliminated and a single subunit with a MW of approximately 24 kDa was observed (Fig. 4a).

As glycosylation can modify both the molecular weight and the isoelectric point (pI) of a protein (Barrabes et al., 2010), the electrophoresis

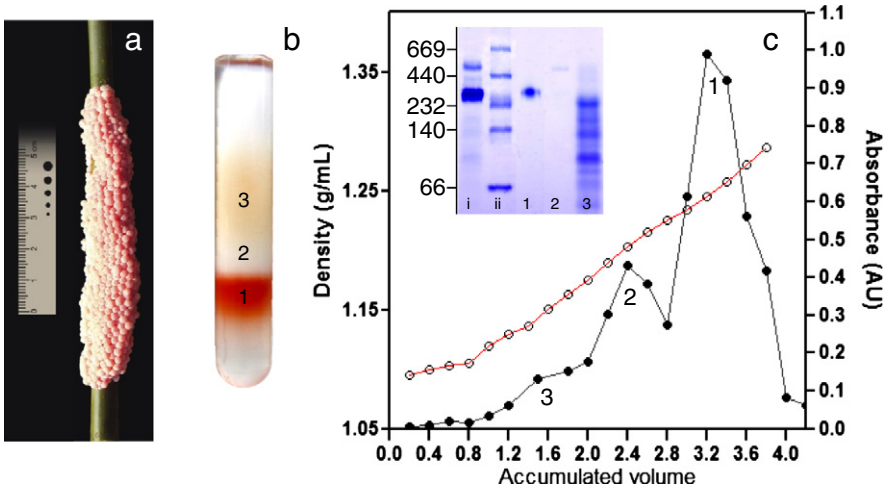

Fig. 1. Purification of PmPV1. a. *Pomacea maculata* egg clutch. b. NaBr ultracentrifugation of perivitellin fluid indicating the 3 fractions observed in c. c. Hydration density (empty circles) and protein profiles (filled circles) of the egg soluble fraction. Inset: native gel electrophoresis showing protein component of peaks 1, 2 and 3. Lane i, total egg protein soluble fraction; lane ii, high molecular weight marker; lanes 1, 2 and 3: major absorbance peaks 1, 2 and 3 respectively (corresponding to PmPV1, PmPV2, and PmPV3 fractions, respectively).

Table 1
Main features of Ampullariidae egg glicolipo-carotenoproteins.

Carotenoprotein (species)	λ_{max} (nm)	kDa (native)	Subunits (kDa range)	Hydration density (g/mL)	Lipids (wt.%)	Carbohydrate (wt.%)/major monosaccharides	Glycoforms pI range	Carotenoid
PcOVO[1,2,3,4,6] (*P. canaliculata*)	480[a], 510[a], 545[a]	300	3 (28–35)	1.26–1.28	0.3	17 Man > GlcNAc	~5.0–~8.7	ASX–ASX ester
PsSC[5,6] (*P. scalaris*)	465[a], 495[a],532[a]	380	3 (24–35)	1.26	0.7	21 Gal > Man	~5.4–9.1	ASX > unknown
PmPV1[8] (*P. maculata*)	506[b, 7]/430[a], 504	294	5 (26–36)	1.25	0.7	13 Man > Gal	~4.7–~9.8	ASX–ASX ester

[1] (Garín et al., 1996).
[2] (Cheesman, D. F. 1958).
[3] (Heras and Pollero, 2002).
[4] (Dreon et al., 2004a).
[5] (Ituarte et al., 2008).
[6] (Ituarte et al., 2010).
[7] (Clark et al., 1980).
[8] Present study.
[a] Dissolved in 0.05 M Tris/HCl, pH 7.4.
[b] Dissolved in 0.05 M Tris/HCl, pH 6.5.

pattern was also evaluated by 2DE (Fig. 4b). A complex pattern of multiple spots was found, but all spots were arranged in 5 groups corresponding to the MW of the 5 subunits observed in 1D-PAGE (Fig. 4, inset), indicating that each of the previously identified subunits possess several pI isoforms. The pI values of PmPV1 isoforms range from ~4.7 to ~9.8; the 36 kDa subunit shows about 3 isoforms (pI between 8.0 and 9.2); the 32 kDa subunit has 5 isoforms (pI between 7.4 and 9.8); the 28 kDa subunit has about 6 isoforms (pI between 4.7 and 6.4); and the 26 kDa subunit has about 4 subunits (pI between 5.1 and 7.2). The lowest MW subunit is represented by a unique spot of pI 8.7.

3.5. Carotenoid analysis

The absorption spectrum of PmPV1 in buffered aqueous solution showed a maximum at 430 nm (Fig. 2). The extraction of the pigment

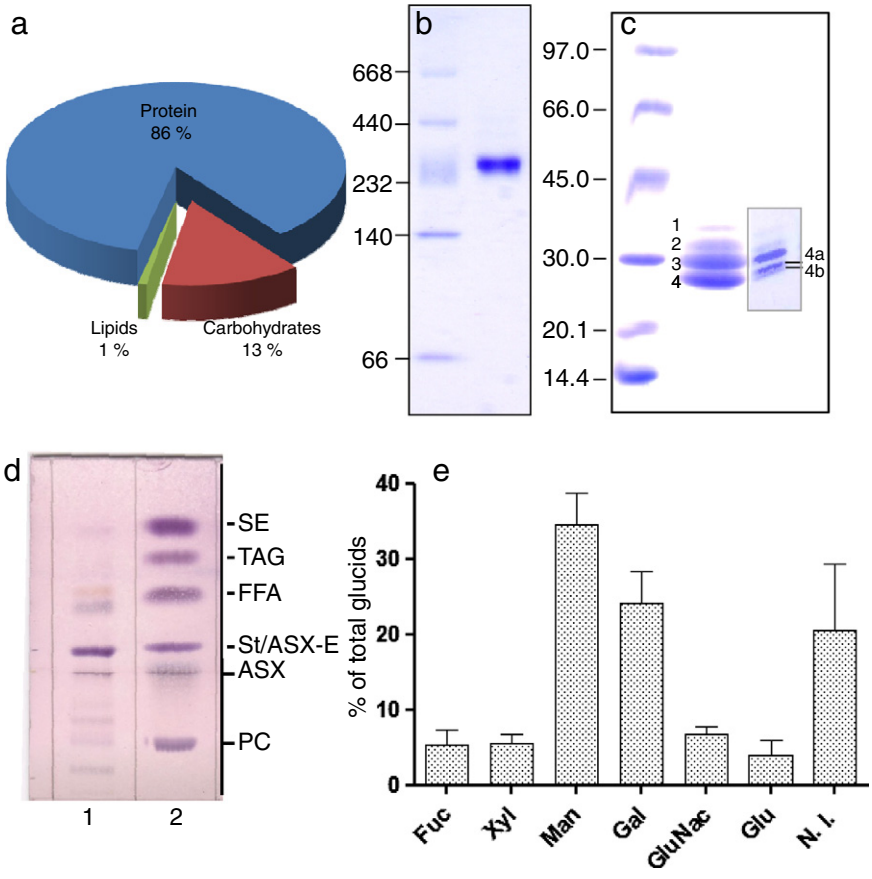

Fig. 3. a. Biochemical composition of PmPV1 particle. A. Percent distribution of proteins, lipids and glucids in PmPV1. B. Non dissociating PAGE. C. Dissociating PAGE. Inset: PmPV1 at lower concentration (1.2 μg) to show the split of band number four into two subunits. PmPV1 subunits were numbered according their decreasing molecular weight. D. Lipid composition analyzed by thin layer chromatography. E. Monosaccharides determined by GC. ASX, astaxanthin; FFA, free fatty acid; PC, phosphatidylcholine; SE, sterol esters; St/ASX-E, Sterol/astaxanthin-ester; TAG, triacylglyecrol. Glc, Glucose; Gal, Galactose; Xyl, Xylose; GlcNAc, N-acetyl-glucosamine; Fuc, Fucose; Man, Mannose.

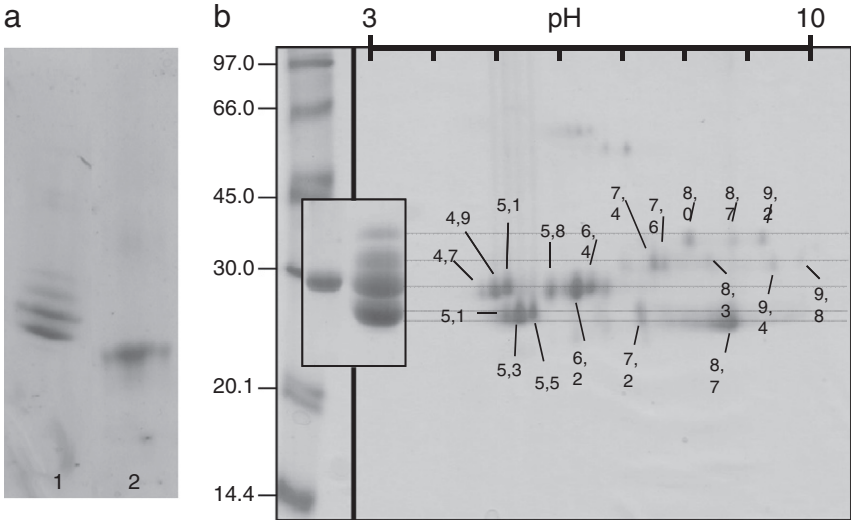

Fig. 4. Electrophoretic analysis of PmPV1. a. SDS-PAGE. Line 1, non-deglycosylated PmPV1; line 2, deglycosylated PmPV1. b. Two-dimensional electrophoresis. Spots represent isoforms of PmPV1 subunits with different pI. Inset: SDS-PAGE of PmPV1.

and its analysis in acetone caused a bathochromic shift to 475 nm (Fig. 5a).

Analysis of PmPV1 carotenoids by HPTLC shows 3 conspicuous spots (Fig. 5b). By comparison with known carotenoids they were identified as ASX, astaxanthin monoester (ASX-Me) and astaxanthin diester (ASX-De) representing 25.3 ± 4.3%, 27.6 ± 0.6%, and 43.4 ± 4.0%, respectively (Fig. 5b). The remaining 3.8 ± 1.1% corresponds to unidentified carotenoids.

No changes in the electrophoretic pattern were caused by the carotenoid extraction, that is, native (holo) and apo-PmPV1 show the same apparent MW indicating that quaternary structure is not modified by the lack of carotenoids (Fig. 5c).

3.6. N-terminal sequence

Table 2 shows the N-terminal amino acid sequences of PmPV1 subunits. A search against NCBI nr database revealed homology with sequences of PcOvo, but no with other sequences. Table 3 shows the alignment between one PmPV1 N-terminal subunit sequence (4a) and, PsSC and PcOvo known N-terminal sequences. This comparison reveals similitude of sequence among all of them.

3.7. Cross immunological reactivity between carotenoproteins

Polyclonal antibodies (PAbs) anti-PcOvo immunoreact with PmPV1 in a Western blot assay as shown (Fig. 6). Moreover, among all egg soluble proteins, only PmPV1 is immunodetected by this anti PcOvo PAb indicating the presence of conserved cryptic epitopes in both carotenoproteins.

3.8. Protective effect of PmPV1 on its carotenoids

In this experiment the capacity of PmPV1 to protect carotenoids from environmental damage was analyzed. Under the assayed conditions (35 °C, aerial exposition, fluorescent light) free ASX was significantly less stable than PmPV1-bound carotenoids ($P < 0.01$ and $P < 0.001$ from 50 min on) (Fig. 7). Reconstituted PmPV1 bound carotenoids were more stable than free ASX ($P < 0.001$ from 110 min on).

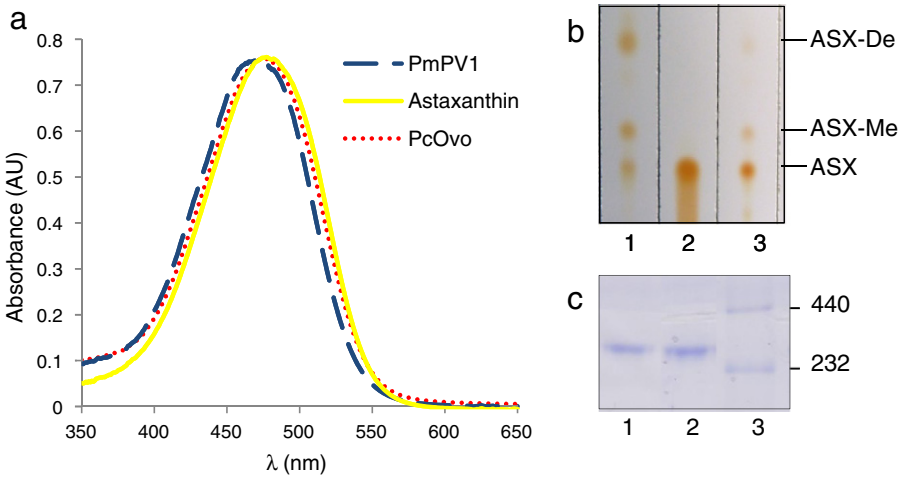

Fig. 5. Analysis of PmPV1 carotenoids. a. Absorption spectra of PmPV1 carotenoids (dashed line), PcOvo carotenoids (dotted line) and free astaxanthin (full line) in acetone. b. HPTLC analysis of carotenoids: lane 1, PmPV1 carotenoids; lane 2, standard of free astaxanthin; lane 3, PcOvo carotenoids. c. Effect of carotenoid removal on PmPV1 analyzed by non-dissociating PAGE. Lane 1, holo-PmPV1; lane 2, apo-PmPV1; and lane 3, molecular weight markers.

Table 2
N-terminal sequences of PmPV1 subunits. Comparison with NCBI nr sequences.

	N-terminal sequence	Expect		GenBank
1	(D)(K)HDLVFXEVD(S)	0.25	perivitellin protein [Pomacea canaliculata]	AFQ23945.1
2	VEDVQDLVFAEXD(K)G	0.00004	perivitellin protein [Pomacea canaliculata]	AFQ23937.1
3	Y(G/D)QD(Q/I)(I/Y)XXLP			
4a	NKEYLLLDIRDA	0.001	perivitellin ovorubin-3 [Pomacea canaliculata]	AFQ23939.1
4b	SPHNYLIMDIEP	0.0001	perivitellin ovorubin-1 [Pomacea canaliculata]	AFQ23940.1

4. Discussion

Carotenoids are widespread pigments among invertebrates (Matsuno, 2001), and many of their biological functions are possible due to their interactions with proteins forming carotenoprotein complexes. In mollusk only two carotenoproteins, PcOvo and PsSC, have been studied so far (Heras et al., 2007). Here we characterized a novel carotenoprotein, PmPV1, which shows shared and different characteristics with PcOvo and PsSC. A comparison among the cartenoproteins from *Pomacea* eggs from these specie's is summarized in Table 1 and discussed below.

PmPV1 absorbs light throughout most of the visible range (λ_{max} 430 and 504 nm), and may exert a photoprotective effect on the embryo cells against harmful sunlight radiation at the beginning of development. The light absorption of ASX interacting with lipids and proteins in carotenoprotein complexes differs from the spectrum of the free carotenoids in an organic solvent. In fact, the spectral shift of protein-bound ASX in invertebrates can cover nearly the complete visible absorption spectrum by forming complexes with different proteins (Zagalsky, 2003). Not only different protein environment, but also variations on the amounts of the different forms of astaxanthin (non-esterified or esterified) could be responsible of color changes, and indeed changes in both protein and astaxanthin forms were observed among the *Pomacea* carotenoproteins (Table 1).

Differentiating *P. maculata* from *P. canaliculata* egg clutches in the field is not always easy and mostly rely on the fact that *P. canaliculata* lays fewer but larger eggs than *P. maculata* (Hayes et al., 2012). The absorption spectra of carotenoproteins would be a way to avoid misidentification. This novel species-specific character extend the characters currently employed to differentiate *P. canaliculata* from *P. maculata*.

Apple snail egg sugars are a source of energy and structural precursors for the developing embryo (Heras et al., 1998). Besides, glycans may also be recognition determinants in protein targeting and cell–cell interactions (Elbein, 1987; Rademacher et al., 1988). Structurally, protein glycosylation favors conformational stability, solubility, protease resistance, and modify protein charge. In order to better understand how glycans contribute to the roles of perivitellins in embryo development, the monosaccharide composition and glycoforms from PmPV1 were characterized and compared with those from the egg carotenoproteins of *P. canaliculata* and *P. scalaris*. All subunits of PmPV1 contain covalently attached carbohydrates as the principal prosthetic group and appear to be glycoforms with a very wide pI range, as reported for PcOvo and PsSC (Table 1). Although PmPV1 is highly glycosylated, carbohydrate content is not as high as in the other two *Pomacea* egg carotenoproteins (Table 1) (Dreon et al., 2004a; Ituarte et al., 2010). High glycosylation was related to the properties and functions in PcOvo, probably increasing protease resistance and enhancing its antinutritional role (Dreon et al., 2010).

Sugar analysis showed that mannose and galactose are the predominant sugars, as in PcOvo and PsSC (Table 1). However, sugar composition showed differences with the other *Pomacea* carotenoproteins (Dreon et al., 2004a; Ituarte et al., 2010). Fucose, an important sugar in recognition processes, was consistently found in PmPV1 and all the other perivitellins so far analyzed. It has been proposed that fucose may be involved in protein uptake during embryogenesis, as it has been reported in other glycoproteins (Varki et al., 2009) and in agreement with the selective perivitellin uptake observed in *P. canaliculata* embryos (Heras et al., 1998).

Low lipid content (<1% w/w) also seems to be a characteristic of apple snail carotenoproteins (Garín et al., 1996; Ituarte et al., 2008), and this is also shared by PmPV1; the major lipid classes in PmPV1

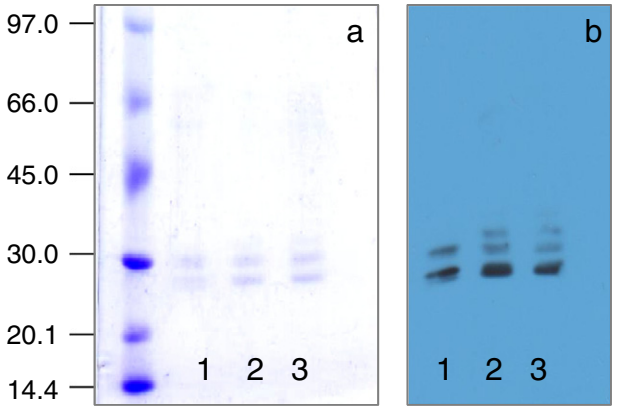

Fig. 6. Cross reactivity between *P. canaliculata* anti PcOvo PAb and *P. maculata* egg proteins. a. Coomassie stained SDS-PAGE. Lane 1: PcOvo. Lane 2: PmPV1. Lane 3: *P. maculata* egg soluble fraction. b. Western blot assay of proteins shown in a, blotted onto nitrocellulose and revealed using anti-PcOvo PAb. Two μg of each protein were loaded.

Table 3
N-terminal sequence of 4a subunit of PmPV1[a]. Comparison with N-terminal sequences of PcOvo (from *P. canaliculata*) and PsSC (from *P. scalaris*).

Sequence ID	N-terminal sequences	Aminoacids quantity	Pairwise Score[d]
SEQUENCE_PcOvo[b]	NKEXLLLDIIDATTS---	15	83.33
SEQUENCE_PmPV1	NKEYLLLDIRDA------	12	
SEQUENCE_PsSC[c]	-DEXLLLDIIDASTEEIN	17	66.67
Conserved residues	* ***** **		

* Positions which have a single, fully conserved residue.
[a] See Fig 3b inset.
[b] Taken from *Dreon et al.* (2010).
[c] Taken from *Ituarte et al.* (2012).
[d] Pairwise score indicates the number of identities between PmPV1 and the other two sequences (PcOvo and PsSC), divided by the length of the alignment, expressed as a percentage.

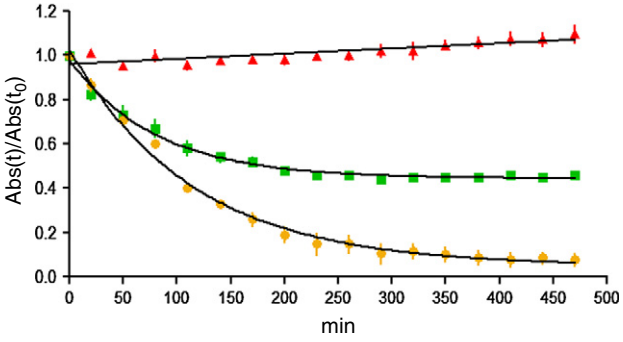

Fig. 7. Protective effect of PmPV1 against astaxanthin damage as a function of time. Samples were exposed to air, fluorescent light and 35 °C for 470 min. Free astaxanthin was more sensitive to photooxidation than combined astaxanthin. The absorbance was recorded as Abs(t) / Abs(t_0). λ = 492 nm. ▲: PmPV1; ■: reconstituted PmPV1; ●: free astaxanthin. Data represents the mean of three determinations ± SD.

were free fatty acids, phospholipids and astaxanthin ester/sterols, suggesting that lipid reserve or lipid transport is not the principal role of these protein complexes (Ituarte et al., 2008).

Structural similarities between PmPV1 and PcOvo are evidenced by the reactivity of antibodies generated against PcOvo that cross react with PmPV1 but not with PsSC (Ituarte et al., 2008). Further, there is a high similarity among the N-terminal amino acid sequences of PmPV1 subunits with those of PcOvo and PsSC (Dreon et al., 2010; Ituarte et al., 2012). These immunological and amino acid sequence similarities, together with the carotenoid composition of the three carotenoproteins are in agreement with the current hypothesis about the phylogenetic relationship among *Pomacea*. Hayes et al. (2009b) describe *P. canaliculata* and *P. maculata* forming part of a clade named *P. canaliculata* group and *P. scalaris* (also a sympatric species in southern South America) belonging to the *Pomacea bridgesii* group.

PmPV1 may be a major carotenoid storage in the eggs and also clearly protects or stabilizes them up to the moment they are taken up by embryos. This is supported by the fact that under the oxidizing conditions employed in vitro, most of the soluble ASX was damaged, while in the same period the PmPV1-protected ASX was significantly less altered. As previously suggested for PcOvo, after PmPV1 uptake by the embryo, the hydrophobic carotenoid molecules it carries, would be freely available as a lipid-phase antioxidant in membranes, protecting lipids from free oxygen radicals (Dreon et al., 2004b; Dreon et al., 2007).

The study shows that PmPV1 is the most abundant protein of the perivitelline fluid of *P. maculata* suggesting a nutritional role. Several studies have demonstrated that *Pomacea* egg carotenoproteins are the most abundant perivitellins and are also multifunctional proteins involved in the nutrition (Heras et al., 1998) and the defense of embryos against both predators (Dreon et al., 2010) and environmental factors (Dreon et al., 2004b). Regarding protection against abiotic factors, as eggs are laid in late spring and summer, clutches are exposed to high temperatures, solar radiation and desiccation. PmPV1 would protect embryos against these factors and against oxidation as it was suggested for other snail carotenoproteins (Dreon et al., 2007). Thus, while as part of PVF carotenoproteins, the major role of carotenoids would be the embryo photoprotection. A potential role against predators (at least as an aposematic or warning coloration) can also be suggested in light of its similarity with PcOvo and the role ascribed to this protein in the unusual defenses found in *P. canaliculata* (Dreon et al., 2010).

As a whole, these results allowed the first generalization about the composition of the major egg proteins of *Pomacea* eggs as well as comparisons that increase the knowledge on the reproductive strategies of these invasive mollusks. We also suggest PmPV1 has protective functions similar to those of PcOvo. In addition, the substantially different spectral properties of egg carotenoproteins would help to distinguish *P. canaliculata* from *P. maculata*. Further work is needed to better understand the functions that carotenoproteins have acquired along evolution especially their role in the successful reproductive strategy of *Pomacea*.

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