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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*.

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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:

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