Isolation and Characterization of a Novel Perivitellin From the Eggs of *Pomacea scalaris* (Mollusca, Ampullariidae)

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ABSTRACT Perivitellins are important components of the perivitelline fluid (PVF) that surrounds gastropod embryos. The glyco–lipo–carotenoprotein ovorubin (OR) from eggs of the snail *Pomacea canaliculata* has been the most studied to date. Here we report the characterization of scalarin (SC), a glyco–lipo–carotenoprotein from the PVF of *P. scalaris*. SC was purified by ultracentrifugation and exclusion chromatography. It is the major egg protein, representing 64% of the total soluble protein. The particle has a hydration density of 1.26 g/ml, an apparent molecular mass of 380 kDa and it is an elongated compact protein as estimated by small angle X-ray scattering (SAXS). It is composed of three subunits of *ca*. 35, 28, and 24 kDa noncovalently bonded. SC is highly glycosylated (carbohydrate content 20.1%, by wt.), with a low lipid content (0.7%), being esterified sterols, pigments and polar lipids the most abundant lipid classes. HPTLC and spectrophotometric analysis of the carotenoid fraction revealed the presence of free astaxanthin (ASX; 62.0%), and an unidentified carotenoid (38.0%). The carotenoid–apoprotein interaction was studied by spectrophotometry. Carotenoids do not seem to affect the structural characteristics of the oligomer. However, the carotenoid–protein association protected ASX against oxidation. The cross-reactivity between SC and perivitellins of *P. canaliculata* was tested using polyclonal antibodies (PAb) against SC, OR, and perivitellin PV2. The PAbs failed to cross-react with any egg proteins of either the same or other species. SC, among other functional similarities with OR, would be an antioxidant carrier, protecting at the same time carotenoids from oxidation in the perivitellin fluid of the egg. *Mol. Reprod. Dev.*

Key Words: apple snails; carotenoprotein; glycoprotein; carotenoid; embryogenesis; antibodies; mollusc; gastropod

INTRODUCTION

Invertebrate vitellogenesis has been extensively studied in insects and crustaceans. In these groups, the synthesis of the vitellins, major components of the proteinaceous yolk, follows mainly a heterosynthetic mechanism in which precursor vitellogenins are usually synthesized outside the oocyte, released to circulation and incorporated into the maturing oocyte in the ovary. In molluscs, cephalopods and some gastropods seem to follow a similar heterosynthetic pathway. Unlike this general mechanism, most gastropods have a perivitelline fluid (PVF) that represents the major source of nutrients for the embryo. This fluid is synthesized by a female accessory reproductive gland, the albumen gland (AG), and is secreted onto the fertilized egg as it passes through the oviduct (de Jong-Brink et al., 1983). The PVF contains polysaccharides, proteins and lipo–glycoprotein complexes called perivitellins. Research has been focused on PVF synthesis and secretion (Morishita et al., 1998), however, perivitellin biochemistry has advanced at a surprisingly slow pace, considering the central role these molecules play in reproduction and embryo nutrition in this economically and ecologically important group.

Although perivitellins have been thought to be primarily nutritive, several novel functions for these proteins have been described in recent years. A candidate nutritive glycoprotein (HdAGP) with potential bactericidal function has been cloned from the AG of *Helisoma duryi* (Mukai et al., 2004). Also, in the Nudibranchia, some proteins with varied functions have been isolated from the egg PVF. Two such proteins, Aplysianin E and Dollabelanin E, from *Aplysia kurodai* and *Dolabella auricularia* possess antimicrobial and antineoplasic properties, respectively (Kisugi et al., 1989; Iijima et al., 1995).

In the ampullariids, a widespread family of freshwater snails which includes Asian, African, and American

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species, three perivitellins from the snail *Pomacea canaliculata* have been characterized, namely, ovorubin (OR), PV2, and PV3 (Cheesman, 1958; Garín et al., 1996). These perivitellins can also be considered carotenoproteins given their pigment composition.

The reproductive strategy of many species of the genus *Pomacea* involves cementing brightly colored egg clutches on different substrata above the water line, thus exposing the embryos for several days to sunlight, high temperatures, and predators. It has been proposed that their perivitellins, besides the usual role as reserve proteins, are also involved in the adaptation of the embryo to these extreme conditions. In particular, several studies on OR suggest that some of the unusual characteristics of this perivitellin might be involved in this adaptation [for review see Heras et al. (2007) and references therein]. Among them, OR was found to be essential in stabilizing its prosthetic carotenoid (astaxanthin, ASX), which in turn plays a role in photoprotection and is probably used by the embryo as a lipid-phase antioxidant (Dreon et al., 2004b). In addition, Norden (1972) has shown that OR also has the capacity to inhibit trypsin as well as some bacterial proteases.

In an immunological study comparing carotenoproteins from different invertebrate phyla, Zagalsky et al. (1995) concluded that OR is unlikely to share domain homology with known carotenoproteins. However, perivitellins of similar characteristics could be present in other *Pomacea* species having aerial egg laying strategies. In order to test this hypothesis, and to better understand the role of carotenoproteins in the reproductive biology of the Ampullariidae, we have studied the PVF protein composition of a related species, *P. scalaris*. This endemic South American species deposit eggs above the water level, in the same fashion as *P. canaliculata* does, but clutches have a different coloration.

In the present work, the isolation and characterization of a new perivitellin from *P. scalaris* are reported, and its compositional and immunological characteristics are compared with those of other perivitellins.

MATERIALS AND METHODS

Eggs

Adult females of *P. scalaris* were collected in Palermo Lake (Buenos Aires city, Argentina) during the reproductive season, and raised in aquaria. Animals were checked daily and egg masses collected immediately after laying, weighed and kept at −70°C until processed. Voucher specimens were deposited in the Museo Argentino de Ciencias Naturales Collection (MACN-In 37268).

Isolation and Purification of *P. scalaris* Perivitellins

Egg masses were homogenized in ice-cold 20 mM Tris/HCl, pH 6.8, containing a protease inhibitor cocktail (Sigma Chemicals, St. Louis, MO) using a Potter type homogenizer (Thomas Sci., Swedesvoro, NJ). The buffer: sample ratio was kept at 4:1 v/w. The crude homogenate was centrifuged sequentially at 10,000*g* for 30 min and at 100,000*g* for 50 min. The pellet was discarded and the supernatant was layered on NaBr $\delta = 1.28$ g/ml and ultracentrifuged at 207,000*g* for 19 hr, at 10°C on a Beckman L8M with a swinging bucket rotor SW 60.Ti (Beckman, Palo Alto, CA). A tube layered with NaCl $\delta = 1.07$ g/ml in lieu of the sample was used as a blank for density calculations. After ultracentrifugation, 19 aliquots of 200 μL were collected from the top of the tubes. Absorbance at 280 nm was measured on each aliquot to obtain the protein profile. Refractive index for the blank tube aliquots was determined with a refractometer (Bausch & Lomb, Inc., Rochester, NY), and converted to density using tabulated values (Orr et al., 1991). Aliquots with visible color were pooled and desalted on a Sephadex G25 column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM NaH_2PO_4, pH 7.4, 150 mM NaCl. A final purification step was performed by size exclusion chromatography (SEC), using a Superdex 200 HR 10/20 column (Pharmacia) equilibrated with 50 mM NaH_2PO_4, pH 7.4, 150 mM NaCl on a Merck-Hitachi high performance liquid chromatograph (HPLC; Hitachi Ltd., Tokyo, Japan) with L-6200 Intelligent Pump and a L-4200 UV Detector set at 280 nm. Purity of the particle was checked by PAGE as described below.

Perivitellins From *P. canaliculata*

P. canaliculata egg soluble fraction and purified perivitellins were prepared as previously described (Garín et al., 1996).

Gel Electrophoresis

Protein content was determined by the method of Lowry et al. (1951) using BSA as standard. Samples were concentrated using Centricon membrane concentrators with an MW 50,000 cut off (Amicon, Beverly, MA). Native PAGE was performed in 4–20% gradient polyacrylamide gels in a Mini-Protean III System (Bio Rad Laboratories, Inc., Hercules, CA), high molecular weight standards (Pharmacia) were run in the same gels. Samples were analyzed by SDS–PAGE in 4–20% gradient polyacrylamide gels containing 0.1% SDS; samples were denatured at 95°C, with and without dithiotreitol treatment (Laemmli, 1970). Low molecular weight standards (Pharmacia) were used and gels were stained with Coomassie Brilliant Blue R-250 (Sigma Chemicals).

Carbohydrate Determination

Total hexose content was determined by the phenol/sulfuric method using D-glucose (Sigma Chemicals) as standard. Purified carotenoprotein samples were treated with a 5% solution of phenol in concentrated H_2SO_4. After 30 min at 37°C absorbance was read at 485 nm (Dubois et al., 1956).

Lipid Analysis

Lipids were extracted with a chloroform–methanol mixture according to Bligh and Dyer (1959). Total lipid

content was determined gravimetrically. Lipid classes were analyzed by thin layer chromatography (TLC) on silica gel (Chromarods type S-III) with quantitation by flame ionization detector (FID) with an Iatroscan TH 10 apparatus (Iatron Laboratories, Inc., Tokyo, Japan). The separation was conducted with a series of three solvent systems by a modification of the procedure described by Ackman and Heras (1997). The first development was carried out in hexane/diethyl ether/ethyl acetate/formic acid (91:6:3:1 v/v/v/v). Chromarods were dried, partially scanned to determine neutral lipids, and then developed in acetone to quantify the ASX. Finally the Chromarods were developed in 100% methanol and completely scanned to quantify phospholipids. Tetracosanol was used as internal standard, and quantitation was performed by obtaining calibration curves of authentic standards run under the same conditions (Parrish and Ackman, 1985).

Carotenoid Analysis

A solution of purified carotenoprotein was extracted with ice cold acetone, keeping the sample/solvent ratio at 1:4 (v/v), and the supernatant was then extracted twice with hexane (Dreon et al., 2004b). The extracts were pooled, dried under N_2, and the carotenoid residue was redissolved in hexane. 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. Carotenoid relative amounts (%) were determined by densitometric analysis of the plates, using an image analysis software (Sigmagel v.1.0, Jandel Sci., San Rafael, CA.). The nature of the carotenoids was also determined according to the spectral characteristics of the pigment recorded in different solvents (Britton, 1995), and co-chromatographic comparisons with authentic carotenoid standards (Sigma Chemicals) run in the same conditions.

Protective Effect of Scalarin on its Carotenoids

A 1.5 mg/ml solution of reconstituted scalarin (SC) using a commercial ASX standard (Sigma Chemicals) was prepared as previously described (Dreon et al., 2004b), and its integrity checked by PAGE.

In order to assay the protective effect of SC on carotenoids, solutions containing either SC, reconstituted SC, or free ASX were exposed to fluorescent light and oxygen at 27°C for 440 min. Sample absorbance was monitored at 490 nm (λmax of ASX in DMSO). DMSO (10 μl) was used as ASX carrier; therefore, this DMSO volume was added to every tube (final volume 500 μl).

Global Shape and Molecular Weight Estimation

Analytical SEC was carried out with a Superdex 200 HR 10/20 column (Pharmacia) equilibrated and eluted with 50 mM NaH_2PO_4, pH 7.4, 150 mM NaCl on a Merck-Hitachi HPLC with L-6200 Intelligent Pump and a L-4200 UV Detector set at 280 nm. Apparent molecular weight was calculated from a calibration curve of standard proteins using thyroglobulin, ferritin, catalase, and alcohol dehydrogenase (Amersham, Princeton, NJ).

Small angle X-ray scattering (SAXS) experiments were performed at the D02A-SAXS2 line, in the Laboratório Nacional de Luz Sincrotron, Campinas (Brazil). The experiments were performed using a wavelength of 1.448 Å for the incident X-ray beam to minimize carbon absorption, with a 100 μl sample holder. The distance between the sample and the detector was kept to 1,065.6 mm. The temperature was controlled using a circulating-water bath kept at 15°C. Each individual run was corrected for sample absorption, photon flux and buffer scattering. Five independent curves were averaged to calculate global shape parameters and the distance distribution function P(r) by Fourier inversion of the scattering intensity I(q) using the program GNOM (Svergun, 1992). The scattering pattern was detected using a MARCCD bidimensional detector and the corresponding image analyzed using the software FIT-2D (http://www.esrf.fr/computing/scientific/fit2d).

Polyclonal Antibodies Preparation

Anti-SC serum was prepared in Balb-c mice. Animals were immunized by intraperitoneal injection of 50 μg of purified SC in Freund's complete adjuvant (Sigma Chemicals), with a boost dose of 50 μg in PBS after 19 days. Serum was harvested 4 days later, and kept at −70°C until used.

The Anti-OR and anti-PV_2 polyclonal antibodies (PAb) were prepared in previous studies (Dreon et al., 2002, 2003).

Immunoblotting

Proteins were transferred from native PAGE gels onto nitrocellulose membranes (Amersham) in a Mini Transblot Cell (Bio Rad Laboratories, Inc.), using 25 mM Tris–HCl, 192 mM glycine, 20% (v/v) methanol, pH 8.3 buffer. After blocking for 1 hr 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 in 1% (w/v) nonfat dry milk in PBS–Tween. Specific antigens were detected by goat anti-rabbit or anti-mouse IgG horseradish peroxidase conjugate (Bio Rad Laboratories, Inc.). Immunoreactivity was visualized by electro-chemiluminiscence.

RESULTS

Isolation and Purification

After ultracentrifugation in a NaBr gradient of the soluble proteins of the eggs, two lipoprotein fractions were obtained (Fig. 1): An orange fraction with a relative density of 1.26 g/ml, and a colorless fraction with a relative density of 1.22 g/ml. When the colored fraction was subjected to SEC one major peak was collected, although a minor high MW peak was also observed (Fig. 2). Native PAGE of the main SEC peak showed that the colored fraction corresponded to a *ca*. 380 kDa protein (henceforth called scalarin, SC), but the presence of two or sometimes three additional high MW bands was also detected (Fig. 3A). To test if those minor

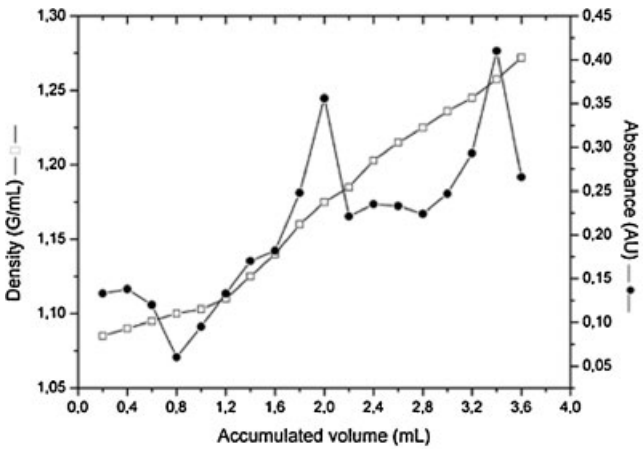

Fig. 1. Protein and density profiles of fractions obtained from the ultracentrifugation gradient of the soluble fraction of *Pomacea scalaris* eggs. Sample was layered on NaBr $\delta = 1.26$ g/ml and centrifuged at 207,000*g* for 22 hr. AU, arbitrary units.

bands were artifacts due to nonnative aggregation upon freezing, samples of SC were re-chromatographed after the following treatments: (a) immediately after the first chromatography; (b) after 5 days at 4°C, and (c) after a freezing at −20°C and thawing cycle; the apoprotein composition of each sample was analyzed by SDS–PAGE. The samples that were not frozen (a and b) showed a single peak when re-chromatographed, that corresponded to SC. On the other hand, the frozen-and-thawed sample (c) showed the additional high MW peaks. Dissociating electrophoresis of the sample containing the high MW bands showed the same banding pattern, confirming they were SC aggregates caused by freezing (results not shown).

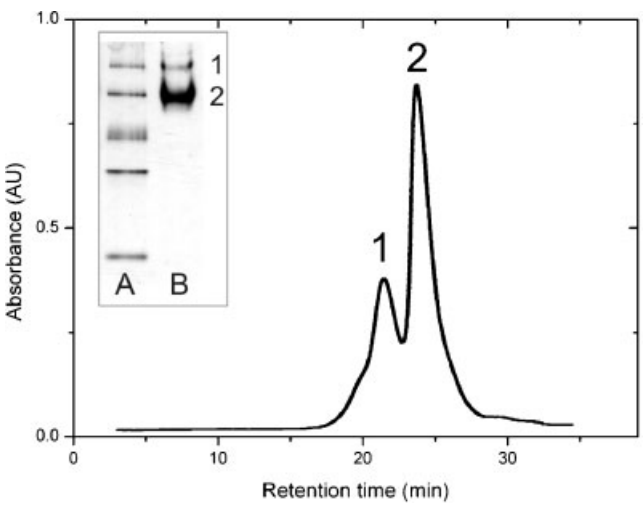

Fig. 2. HPLC elution profile of the protein fractions isolated by density gradient ultracentrifugation. Purification was done using an isocratic gradient of sodium phosphate buffer 50 mM, pH 7.4 on a Superdex 200 HR10/20 column, and visualized by monitoring elution at 280 nm. AU, arbitrary units; 1, aggregate; 2, Scalarin (SC). Inset: Native PAGE profile of the combined fractions.

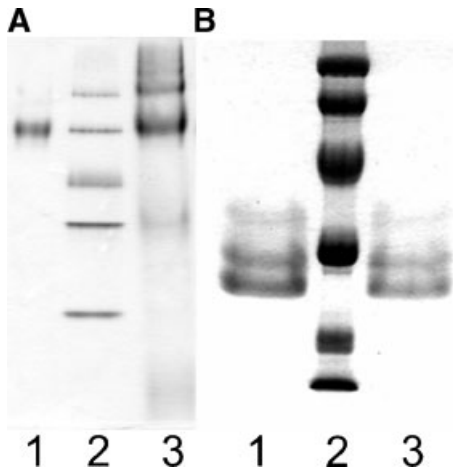

Fig. 3. Native (**A**) and dissociating gel electrophoresis (**B**) of SC particle. Panel A: Native PAGE was done using 4–20% w/v polyacrylamide gradient. **Lane 1**: purified SC (3 μg); **Lane 2**: High molecular weight standards; **Lane 3**: egg cytosol (10 μg). Panel B: SDS–PAGE was performed in 12% polyacrylamide. **Lane 1**: DTT-treated SC (5 μg): **Lane 2** low molecular weight standards; **Lane 3**: SC (5 μg) without DTT. High molecular weight standards: Native proteins: Thyroglobulin (MW 669,000), Ferritin (MW 440,000), Catalase (MW 232,000), Lactate Dehydrogenase (MW 140,000), and Albumin (MW 67,000). Low molecular weight standards: Phosphorylase b (MW 97,000), Albumin (MW 66,000), Ovalbumin (MW 45,000), Carbonic anhydrase (MW 30,000), Trypsin inhibitor (MW 22,100), α-Lactalbumin (MW 14,400).

Scalarin Subunits

To investigate SC subunit composition and to determine the nature of the interactions among the subunits, SC was denatured and subjected to SDS–PAGE under reducing and nonreducing conditions. This experiment showed no differences between the electrophoretic profiles, indicating the absence of intermolecular disulfide bridges in the perivitellin particle (Fig. 3B), and the presence of three subunits of *ca* 35.2 (±3.7), 28.2 (±1.7), and 24.3 (±1.3) kDa. The relative amount of each subunit was determined densitometrically as 14.5%, 37.3%, and 48.2%, respectively.

Lipid and Carbohydrate Analysis

Total hexoses, as determined by the phenol/sulfuric reagent method, accounted for $20.3 \pm 1.6\%$ (w/w) of SC.

Lipid extracts were identified and quantified by TLC-FID, and accounted for 0.7% (w/w) of the particle. Table 1 summarizes the qualitative and quantitative analysis of the lipid classes found in SC. Sterified sterols accounted for 9.6% of the total lipids of SC, while carotenoid pigments were approximately 40%. Phospholipids accounted for approximately 31%. The remaining lipid classes were free fatty acids and free sterols (Table 1).

Carotenoid Analysis

The absorption spectrum of an aqueous solution of SC showed two absorption maxima at 383 and 494 nm, with two inflexions at 467 and 525 nm (Fig. 4B). In contrast,

TABLE 1. Lipid Class Composition of the Major Carotenoprotein From the Perivitelline Fluid of *P. scalaris*

Lipid classes	(% w/w)
Esterified sterols	9.6 ± 1.4
Triacylglycerols	6.7 ± 1.0
Free fatty acids + carotenoid pigment[a]	14.3 ± 2.0
Free sterols + diacylglycerols	11.2 ± 3.7
Astaxanthin	27.2 ± 2.9
Polar lipids	31.1 ± 6.0

[a]Mostly carotenoid pigment as determined by HPTLC analysis. Values are expressed as the mean of triplicate analysis ± 1 SD.

the spectrum of the acetone-extracted carotenoids showed a single band, with fine structure, and blue shifted with respect to the SC spectrum (Fig. 4B). The chromatographic analysis of this acetonic extract showed two major carotenoid fractions (Fig. 4A): a low Rf orange band, which co-migrated with the ASX standard (band a), and a yellowish band, with a higher Rf (band B). Both bands were scraped from the plates, and their absorption spectra were recorded. Band A gave a coincident spectrum with standard ASX (not shown), while band b showed a more complex spectrum (Fig. 4B). When chromatographic behavior was compared with the carotenoids extracted from OR, it was observed that ASX mono and di esters were not present in SC (Fig. 4A).

Free ASX was the major carotenoid of SC (62%), followed by the unidentified carotenoid (38%).

Protective Effect of Scalarin on its Carotenoids

In this experiment the capacity of SC to protect carotenoids from oxidative damage was investigated. Under the assayed conditions, ASX sensitivity to oxidation was evident in SC, reconstituted SC, and free ASX (Fig. 5). SC-bound ASX was found to be significantly more stable than the free form.

Global Shape and Molecular Weight Estimation

Due to the high degree of glycosylation, MW estimation by PAGE gives biased results. In fact the ca MW by this method was 380 kDa (see above). Therefore, as the particle was too big to use mass spectrometry techniques, the molecular weight of SC was estimated by SEC analysis, which gave a molecular weight of 264 ± 3 kDa.

SAXS data for SC and the corresponding Kratky-plots ($I(Q).Q^2$ vs. Q) are depicted in Figure 6A. The bell-shaped Kratky-plot is typical for globular proteins. The inset depicts a linearized representation of the Guinier region, and the straight line corresponds to a fit with a gyration radius for SC of 43.4 ± 0.7 Å.

The pair-distance distribution P(R) drawn from the SAXS experimental data shown in Figure 6B, is typical for an elongated and compact protein and shows a maximum around 75.6 Å. The lack of a well-defined D_{max} value (defined from the condition $P(R > D_{max}) = 0$) is a clear indication of the presence of molecular aggregates. Moreover, the departure of the Guinier plot (Fig. 6A, inset) from the linear behavior is another clear indication of the presence of high order aggregates. This aggregation behavior of Sc observed by SEC and PAGE was therefore confirmed.

Cross-Reactivity of Carotenoproteins

A summary of the results of cross-reaction of OR, PV2 and SC PAbs with soluble proteins from eggs of *P. scalaris* and *P. canaliculata* are shown in Figure 7. The PAbs failed to cross-react with the other carotenoproteins assayed (either using native or denatured proteins), a remarkable result considering that tested carotenoproteins are from organisms of the same genus.

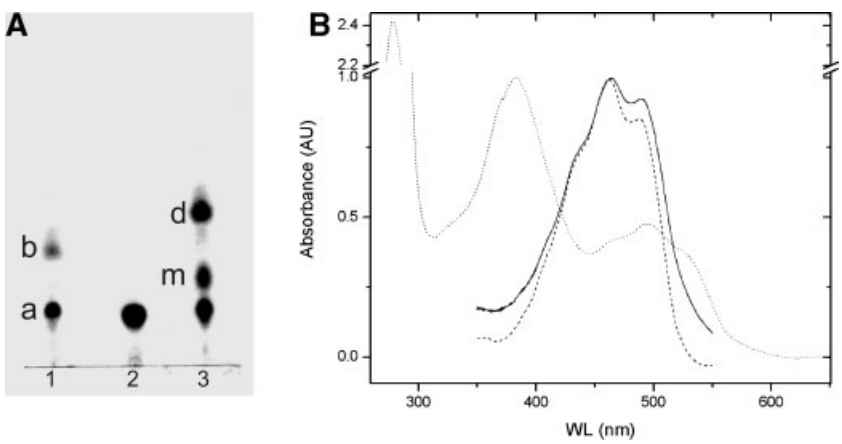

Fig. 4. Analysis of SC carotenoids by HPTLC (**A**), and comparison of the absorption spectra of native SC with its acetone extract and the unknown carotenoid extracted from HPTLC (**B**). Panel A: HPTLC was developed with hexane: acetone 80:20 (v/v). **Lane 1**: Carotenoids extracted from *P. scalaris* SC, **Lane 2**: Astaxanthin (ASX) standard. **Lane 3**: Carotenoids extracted from *P. canaliculata* ovorubin. a, Free ASX; b, Unknown; m, ASX monoester; d, ASX diester. Panel B: Native SC (dotted line); acetone extract (full line); unknown carotenoid (dashed line).

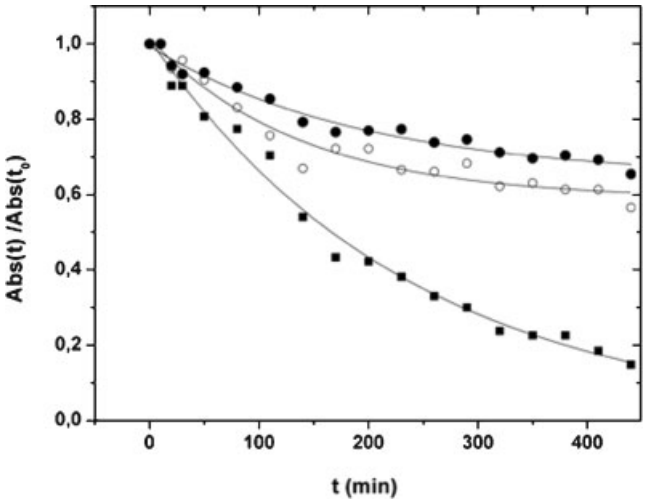

Fig. 5. Protective effect of apo-SC against ASX damage as a function of time. Samples were exposed to fluorescent light and air at 27°C for 440 min. Free ASX was more sensitive to photo-oxidation than combined ASX. Absorbance was recorded as Abs (t)/Abs(t_0). $\lambda = 490$ nm. SC (full circles), reconstituted SC (empty circles), free ASX (squares).

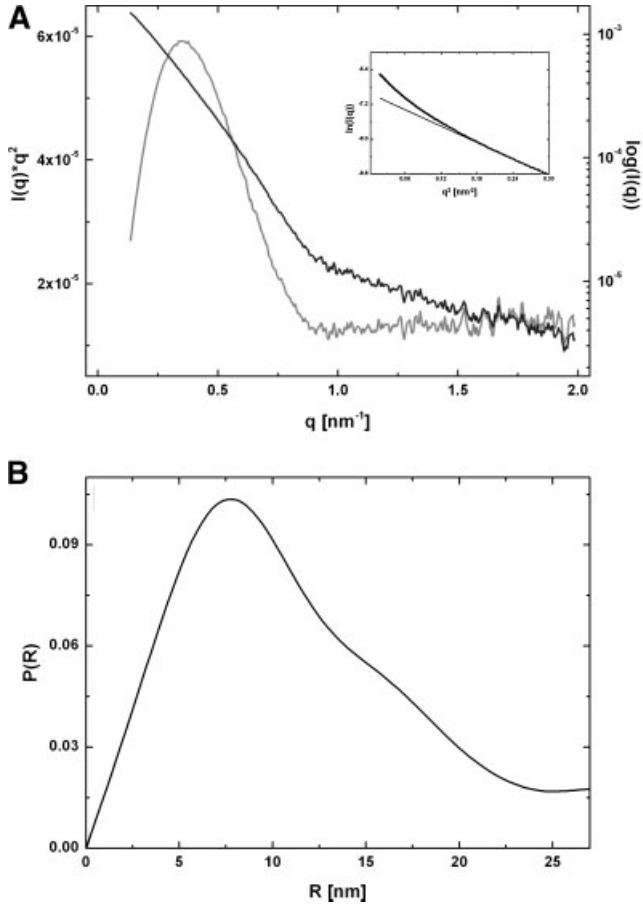

Fig. 6. SAXS spectra and pair-distance distribution function of SC in phosphate buffer. **Panel A**: SAXS spectra (black curve, right axis), Kratky plot (gray curve, left axis) and Guinier plot and the corresponding linear fit (inset; full circles and full line respectively). **Panel B**: Pair-distance distribution function obtained using the indirect transform method implemented in GNOM 4.5 (see text for details).

DISCUSSION

Eggs from the freshwater snail *P. scalaris* are cemented in aerial clutches over plants and other substrates, so that hatchlings fall down into the water course. This strategy protects them from water predators, but it exposes the eggs for about 2 weeks to severe desiccating conditions, direct sunlight, air and high temperatures. Even under this adverse environmental setting, eggs manage to fully develop, suggesting they must possess metabolic adaptations to cope with these harsh conditions. One such adaptation would deal with the provision of adequate amounts of antioxidant and photoprotective molecules to the embryo and also the capability of the PVF to avoid water loss. Perivitellins seem to be involved in these adaptations, as has recently been reviewed (Heras et al., 2007).

The egg-laying strategy of *P. scalaris* is similar to that of *P. canaliculata*. We were able to isolate and purify SC, a new carotenoprotein which showed many similarities and some remarkable differences with OR. These features are discussed below in a comparative way as they are the only egg carotenoproteins of Gastropoda so far studied.

SC presents the characteristic density of a VHDL (1.26 g/ml), although the lipid content is relatively low (0.7% w/w). This flotation density is probably due to the oligosaccharide moieties as SC is a highly glycosylated protein (21% w/w). Though we are far from understanding the function these oligosaccharides play in the molecule, it is known that glycans associated to proteins are important trafficking signals, and are also involved in the folding and structural stability (Taylor and Drickamer, 2003). The high degree of glycosylation of SC is equivalent to that of OR that contains 17% carbohydrates by wt (Dreon et al., 2004a). In OR, it has been suggested that glycosylation is related with the extremely high thermostability of the complex, showing only minor structural perturbations even at temperatures as high as 95°C (Dreon et al., 2007). SC thermostability has not been tested yet, but it was observed that after boiling SC for 15 min its electrophoretic behavior was not altered (unpublished results).

The comparison of shape and size of SC with OR indicated they share several similarities. Both are compact, globular, anisometric carotenoproteins, with a gyration radius value within the same range. Their shape contrasts with the cylinder-like structure of crustacyanin, the only invertebrate carotenoproteins whose shape has been reported (Dellisanti et al., 2003). In addition, the MW of native and apoprotein subunits of SC is also similar to that of OR.

The presence of intersubunit disulfide bridges could be discarded in SC according to the results of SDS–PAGE analysis, a feature also similar to OR (Dreon M.S. unpublished work). The lack of intersubunit

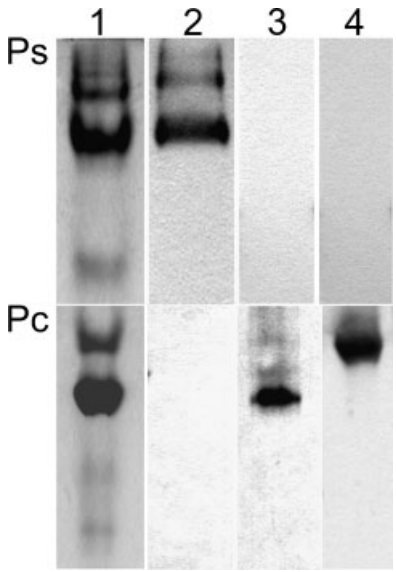

Fig. 7. Immunoblotting of SC, OR and PV2 PAbs. No Cross-reactivity can be seen among them. **Lane 1**: Coomassie blue-stained soluble proteins, **Lane 2**: SC PAb, **Lane 3**: OR PAb, **Lane 4**: PV2 PAb. SC PAb diluted 1:4,000; OR PAb diluted 1:8,000; PV2 PAb diluted 1:1,000. Ps, *P. scalaris* cytosol; Pc, *Pomacea canaliculata* cytosol. Proteins were blotted onto nitrocellulose from 4% to 20% polyacrylamide gels.

disulfide bridges in these oligomers makes their high thermostability even more notable.

On the other hand, the tendency of SC to form aggregates upon freezing has been observed in other carotenoproteins (Zagalsky, 1985). In contrast OR is an extremely stable protein able to withstand repeated freeze–thawing cycles.

The low lipid content, similar to that of OR (Garín et al., 1996), would indicate that lipid reserve or lipid transport are not the main roles of these perivitellins. This is also supported by the lipid class composition, as the main lipid classes of SC and OR are carotenoid pigments and structural lipids.

When SC carotenoid composition was analyzed, differences were found compared to that of OR. This would explain the different coloration of the eggs of *P. scalaris* as compared with *P. canaliculata*, but different ligand–protein interactions could also be involved (Zagalsky et al., 1990). Although ASX is present in both proteins, ASX esters are absent in SC. HPTLC analysis of SC also revealed the presence of an unidentified carotenoid. The presence of cantaxanthin, a carotenoid frequently found in invertebrate carotenoproteins (Zagalsky et al., 1990; Britton, 1995) could be discarded due to the lack of fine structure on the absorption spectrum.

Regarding the functions these carotenoids would play, it has been reported that in *P. canaliculata* they act as a molecular sunscreen while bound to OR (Dreon et al., 2007), and are likely lipid-phase antioxidants when delivered to the embryos (Dreon et al., 2004b). Thus, SC carotenoids would play a similar role. In fact, SC absorbs light throughout the visible range, and may exert a photoprotective effect on the embryo cells at the beginning of development. Moreover, the amount of carotenoid in *P. scalaris* is in the same order as that of *P. canaliculata*, supplying the embryos with adequate amounts of antioxidant molecules. It is interesting to note that carotenoids are distributed in the PVF of these two species in a different fashion; in *P. scalaris* they are all concentrated in SC, while in *P. canaliculata* they are distributed among a few carotenoproteins (Garín et al., 1996).

SC not only transports carotenoids to the eggs, but also protects or stabilizes them up to the moment they are taken up by embryos. Under the severe oxidizing conditions employed in vitro, most of the soluble ASX was damaged, while in the same period the SC-protected ASX was significantly less altered.

A comparison of OR with other carotenoproteins was performed by Zagalsky et al. (1995). Based on antibody affinity assays he concluded that it was unlikely that OR may share domain homology with the carotenoproteins known at that time. We tested if this was valid for OR and the perivitellins SC and PV2, and found no cross-reactivity using highly specific PAbs. Since SC and OR are highly glycosylated, PAb are raised against not only peptide epitopes but also carbohydrate moieties, therefore cross-reactivity between them would be expected. The results indicated that even if there were some similarities between OR and SC oligosaccharides, they were not enough to produce cross-reactivity in the experimental conditions assayed. This is an interesting result considering that SC and OR share many biochemical characteristics.

In conclusion, *P. scalaris* eggs, as shown in this work, present a protein which is very similar to OR. Their common features, a high glycosylation and the presence of carotenoids, are probably associated with the snail adaptation to aerial egg laying, although further comparative work is needed. In fact, perivitellins, previously regarded only as nourishing sources for the developing embryos in this taxon, are now emerging as a new group of multifunctional glycoproteins, reinforcing the importance of studying their biochemistry.

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