

## Antioxidant defense system in the apple snail eggs, the role of ovorubin

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### Abstract

A novel role of ovorubin as a protection system against oxidative damage in eggs from *Pomacea canaliculata* was investigated. Carotenoid composition, and their antioxidant capacity, as well as the carotenoid-apoprotein interaction, were studied for this lipoglycocarotenoprotein. Carotenoid extracts from ovorubin were analysed by TLC and spectrophotometry. The major carotenoid was astaxanthin in its free (40%), monoester (24%), and diester (35%) forms, mainly esterified with 16:0 fatty acid. The antioxidant capacity of ovorubin carotenoids was studied by the inhibition of microsomal oxidation in a non-enzymatic system, showing strong protection against oxidative damage (IC<sub>50</sub> = 3.9 nmol/mg protein). The carotenoid-apoprotein interaction was studied by spectrophotometry and electrophoresis using reconstituted ovorubin. Astaxanthin does not seem to affect the structural characteristics of ovorubin, however the carotenoid-protein association significantly protected astaxanthin against oxidation. Ovorubin therefore, besides its role in providing energy and structural precursors during embryogenesis, would be an antioxidant carrier, protecting at the same time this pigment from oxidation in the perivitellin fluid environment of the egg.

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The physiology and metabolism of the freshwater prosobranch *Pomacea canaliculata* are currently under active research, particularly because this freshwater snail has spread rapidly across Asia during the last decades. The results of such an invasion have damaged rice fields and can be devastating for the food production. Moreover, the snail has become the vector of the human eosinophilic meningoencephalitis [1].

An accessory gland called albumen gland, associated with the female reproductive tract, synthesises, and secretes the perivitellin fluid (PVF)<sup>1</sup> surrounding fertilised eggs which is mainly composed of proteins called perivitellins and polysaccharides [2]. The PVF is consumed by the developing embryo serving as an energetic and

structural source [3]. We have previously detected in the PVF the presence of two lipoproteins called PV1 and PV2, plus one heterogeneous lipoprotein fraction named PV3 [4]. PV1 corresponds to a carotenoprotein first described as ovorubin by Cheesman [5]; it represents 60% of the total protein content of the egg being the major egg lipoprotein present in the PVF of the apple snail *P. canaliculata* [4]. Indeed, it is a lipoglycocarotenoprotein with an apparent molecular mass of 300 kDa composed of three subunits of 28, 32, and 35 kDa. Also it is a very high-density lipoprotein due to its hydration density of 1.27 g/ml [4]. The carotenoid component of this particle is responsible for the characteristic reddish pigmentation of the eggs.

Most invertebrate species present coloured eggs and pigmentary changes during development, usually as a result of the presence of carotenoids non-covalently bound to proteins, astaxanthin (3,3'-hydroxy-β-β-carotene-4,4'-dione) and canthaxanthin being the most common [7]. The metabolism of carotenoid pigments

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<sup>1</sup> Abbreviations used: PVF, perivitellin fluid; HPLC, high performance liquid chromatograph; PAGGE, polyacrylamide gradient; DMSO, dimethyl sulphoxide; GLC, gas-liquid chromatography.

during embryogenesis has been studied in crustaceans [8,9] but its biochemical and physiological role has not been studied in molluscs. It is generally accepted that carotenoids can act as antioxidants [10]; in particular, astaxanthin is a potent antioxidant in membrane models [11]. In the present work, the role of oворubin in gastropod embryo protection against oxidative damage was investigated. To elucidate the physiological role of these pigments in mollusc embryogenesis, the carotenoid composition of oворubin, and their antioxidant activity were determined, as well as the presence of mutual stabilisation between the carotenoid component and the apo-protein.

## Materials and methods

### *Egg collection*

Adults of *P. canaliculata* were collected in streams or ponds near La Plata, province of Buenos Aires, Argentina. Eggs were collected from females either raised in our laboratory or taken from the wild between November and April (reproductive season). Embryo development was checked in each egg mass microscopically [4], and only egg masses having embryos developed to no more than the morula stage were used.

### *Ovorubin isolation and purification*

Fertilised eggs were repeatedly rinsed with ice-cold 20 mM Tris–HCl, pH 6.8, containing 0.8  $\mu$ M aprotinin (Trasylol, Mobay Chemical, New York, USA) and homogenised in a Potter type homogenizer (Thomas Sci., Swedesboro, NJ, USA) in the dark and under N<sub>2</sub> atmosphere. The relation buffer: sample was kept at 5:1 v/w [3]. The crude homogenates were then sonicated for 15 s and centrifuged sequentially at 10,000g for 30 min, and then at 100,000g for 60 min. The pellet was discarded and the supernatant was stored at –70 °C until analysis. Protein content was determined by the method of Bradford et al. [12] using bovine serum albumin as standard.

The soluble protein fraction obtained using the above procedure was purified in a Merk–Hitachi high performance liquid chromatograph (HPLC) (Hitachi, Tokyo, Japan) with an L-6200 Intelligent Pump and an L-4200 UV detector set at 280 nm. A serial HPLC purification was performed. First, the sample was analysed 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 buffer. The oворubin peak was then purified further by size exclusion chromatography (Superdex 200 HR 10/20, Amersham–Pharmacia, Uppsala, Sweden) using an isocratic gradient of sodium phosphate buffer 50 mM, 150 mM NaCl, pH 7.6. Purity of the single peak obtained was checked by native electrophoresis.

### *Preparation of Apo-ovorubin*

A solution of oворubin (10 mg/ml) in 20 mM Tris–HCl buffer (pH 7.4) was cooled down to 0 °C and extracted with 4 volumes of ice-cold acetone, added in small aliquots with vigorous stirring. The precipitated protein was centrifuged, dissolved in ice-cold water to a volume equal to that of the original solution, and reprecipitated under the same conditions, yielding an almost colourless protein. This pellet was redissolved in water, centrifuged at 10,000g to remove any denatured material, and delipidated with Triton X-100, 0.02 g/g protein (Sigma Chemical, St. Louis, USA). The mixture was incubated at room temperature for 1 h with shaking. The detergent was eliminated using macroreticular adsorbent beads 1.0 g/g protein (Bio-Beads SM-2, Bio-Rad Laboratories, CA), keeping the system at room temperature with shaking for 1 h [13]. Beads were then separated by filtration on lipid-free glass wool. An aliquot of the delipidated fraction was extracted with organic solvents [14], and the extract was analysed by TLC-FID using an Iatroscan apparatus to ensure that total delipidation had been achieved [15]. The integrity of the apo-ovorubin was checked by PAGGE. The delipidated apo-ovorubin was stored at –70 °C until analysis.

### *Gel electrophoresis*

Total proteins were measured by the method of Bradford [12]. Non-dissociating electrophoresis was performed by a 4–20% polyacrylamide gradient (PAGGE) [16,17]. The gels were stained with Coomassie brilliant blue R-250 (Sigma Chemical, St. Louis, USA).

### *Carotenoid extraction and quantitation*

The acetonetic extracts obtained in the preparation of apo-ovorubin were pooled and extracted three times with hexane, in a ratio sample:hexane 3:1 (v/v). The extracts were pooled and dried under a stream of N<sub>2</sub>, and the carotenoid residue was redissolved in dimethyl sulphoxide (DMSO). All procedures were conducted in the dark at below 4 °C. Estimation of the carotenoid content was done spectrophotometrically at 495 nm, using an astaxanthin standard (Sigma Chemical, St. Louis, USA) dissolved in DMSO ( $\lambda$  max in DMSO).

### *Analysis of the carotenoid components*

Carotenoid components were dissolved in hexane and analysed on Silicagel G-60 HPTLC plates (Merk, Darmstadt, Germany) using hexane:acetone 80:20 (v/v) as a mobile phase. Identification was performed using appropriate standards (Sigma Chemical, St. Louis, USA). Carotenoid relative amounts (%) were deter-

mined by densitometric analysis of the plates, using an image analysis software (Sigmagel v.1.0, Jendel Sci., USA). The nature of the carotenoids was also determined according to the spectral characteristics of the pigment, its behaviour after alkaline hydrolysis in 15% KOH in methanol, and co-chromatographic comparisons with authentic carotenoid standards.

#### *Fatty acid analysis*

An aliquot of oovorubin carotenoids, extracted and purified as described above, was saponified with KOH–ethanol solution. Fatty acids were extracted with hexane after acidification and esterified with  $\text{BF}_3/\text{MeOH}$  [18]. Fatty acid methyl esters were analysed by gas–liquid chromatography (GLC) on an Omegawax 250 (30 m  $\times$  0.25 mm, 0.25  $\mu\text{m}$  film) capillary column in a Hewlett Packard HP-6890, equipped with a flame ionisation detector. The column temperature was programmed for a linear increase of 3  $^\circ\text{C}/\text{min}$  from 175 to 230  $^\circ\text{C}$ . Peaks were identified comparing the retention times with those from a mixture of standard methyl esters.

Microsome fatty acids were analysed in a similar fashion.

#### *Microsome preparation*

Male Wistar rats, weighing 200–250 g, were employed to obtain microsomes. They were sacrificed by decapitation and the liver was rapidly removed, cut into small pieces, and thoroughly washed with 0.15 M NaCl. A 30% homogenate (w/v) was prepared in 0.25 M sucrose, 10 mM Tris–HCl buffer, pH 7.4, containing 1 mM PMSF, using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 10,000g for 10 min. The supernatant was applied to a Sepharose 4B column 1.6  $\times$  12 cm (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 10 mM Tris–HCl buffer, pH 7.4, containing 0.01%  $\text{NaN}_3$ . The microsomal fraction collected in the void volume (10–16 ml) was brought to 0.25 M sucrose by adding solid sucrose. All operations were carried out at 4  $^\circ\text{C}$ . With regard to concentrations and activities of certain enzymes, these microsomal preparations have a similar composition to that obtained by ultracentrifugation [19].

#### *Inhibition of non-enzymatic microsomal oxidation*

Microsomes (1 mg protein) were incubated at 37  $^\circ\text{C}$  with 0.05 M Tris–HCl buffer (pH 7.4), 0.4 mM ascorbate, final volume 1 ml, in tubes containing different amounts of carotenoid extract in DMSO (1% of final volume). Tubes containing 10  $\mu\text{l}$  DMSO were used as control. Chemiluminescence [20] was initiated by adding ascorbate to the microsomal preparations [21]. Microsomal preparations lacking ascorbate were simulta-

neously analysed as positive control. In the same fashion, different quantities of oovorubin (up to 500  $\mu\text{g}$ ) in 0.05 M Tris–HCl buffer (pH 7.4) were assayed to determine the antioxidant activity of the carotenoprotein per se. Chemiluminescence was recorded as cpm every 10 min for 350 min in a Packard 1900 TR liquid scintillation analyser (Packard Instrument, Meriden, USA). The sum of the total chemiluminescence was used to calculate cpm/mg protein.

#### *Inhibition of enzymatic microsomal oxidation*

Microsomes (1 mg protein) were incubated at 37  $^\circ\text{C}$  with 0.05 M Tris–HCl buffer (pH 7.4), final volume 1 ml, in tubes containing different amounts of carotenoid extracts in DMSO. Tubes containing 10  $\mu\text{l}$  DMSO were used as control. Chemiluminescence was initiated by adding NADPH to the microsomal preparation (final concentration 0.1 mM). Membrane preparations lacking NADPH were simultaneously analysed [22]. Chemiluminescence was recorded for 120 min in the same fashion as with the non-enzymatic method.

#### *Vitamin E determination*

Vitamin E was extracted from oovorubin by a combination of the methods described by Malnoe et al. [23] and Castignani [24]. As much as 1.5 ml of sample, containing 5–10 mg f protein, was vigorously and intermittently shaken with 6 ml of hexane: isopropanol 3:2 (v/v) containing 0.001% BHT as antioxidant. After centrifugation at 1000g for 5 min, the upper organic phase was removed and the process was repeated again with the lower phase. The extract containing vitamin E was evaporated to dryness under nitrogen, and the residue redissolved in 150  $\mu\text{l}$  methanol:diethylether (3:1 v/v). The content of vitamin E was determined by HPLC using a Knauer pump 64 (Knauer, Berlin, Germany) and a Tosoh UV-8011 UV detector (Tosoh, Tokyo, Japan) set at 298 nm. The sample was analysed in an Eurosphere 100-C18 column (5  $\mu\text{m}$ , 250 mm  $\times$  4 mm i.d., Knauer, Berlin, Germany) using a mobile phase of methanol:water 95:5 (v/v) at a flow rate of 2 ml/min at room temperature. A standard  $\alpha$ -tocopherol curve between 0.126 and 0.756  $\mu\text{g}$  was determined.

#### *Preparation and isolation of reconstituted oovorubin*

A 500  $\mu\text{l}$  aliquot of purified apo-ooovorubin (10 mg/ml) was incubated with 10  $\mu\text{l}$  standard solution of astaxanthin (390  $\mu\text{g}/\text{ml}$ ) in DMSO. After incubation for 1 h in the dark at 25  $^\circ\text{C}$  with shaking, reconstituted oovorubin was cleaned from unbound astaxanthin on a Sepharose G-25 column (Amersham–Pharmacia, Uppsala, Sweden) (1  $\times$  25 cm), pre-equilibrated with 0.05 M Tris–HCl buffer, pH 7.4. The eluted fractions were monitored for

their absorption at 280 nm. The overubin-containing fractions were pooled and stored at 4 °C until analysis. The integrity of the reconstituted overubin was checked by PAGGE.

#### Protective effect of overubin on its carotenoid

Solutions containing either overubin (5 mg protein,  $\approx 3$  nmol carotenoid), reconstituted overubin (5 mg protein,  $\approx 3$  nmol carotenoid), or free astaxanthin (3 nmol) were exposed to fluorescent light and oxygen at 37 °C for 12 h. Sample absorbance was monitored at 495 nm ( $\lambda_{\max}$  of astaxanthin in DMSO). As DMSO (10  $\mu$ l) was used as astaxanthin carrier, this DMSO volume was added to every tube (final volume 500  $\mu$ l).

## Results

#### Apo-overubin and reconstituted overubin

The integrity of apo-overubin and reconstituted overubin was checked by native PAGGE. Fig. 1 shows a single band of 300 kDa for both proteins, which had the same electrophoretic mobility as native overubin (lane 1).

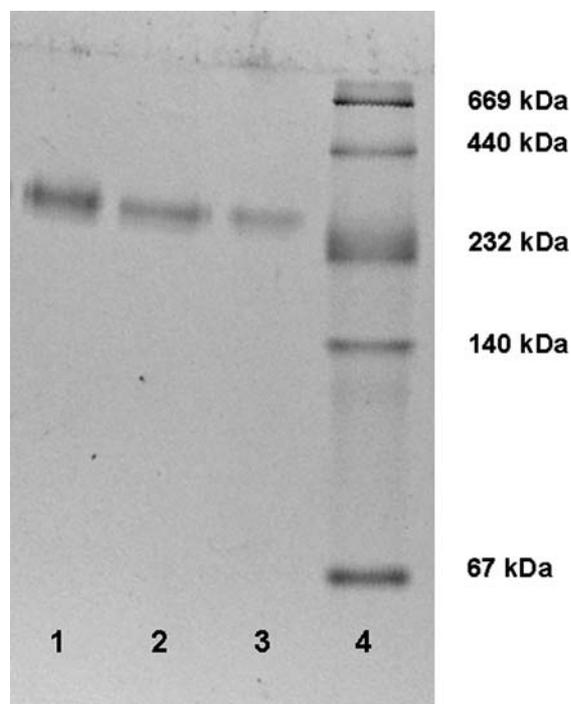


Fig. 1. Native gel electrophoresis of overubin, apo-overubin, and reconstituted overubin. The gel was prepared using an acrylamide gradient of 4–20% w/v. Lanes: 1, overubin; 2, apo-overubin; 3, reconstituted overubin; and 4, STD. Proteins were revealed by Coomassie blue staining. STD: High Molecular 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). A single band of 300 kDa for the three samples was observed.

#### Analysis of overubin carotenoids

The chromatographic analyses of the carotenoid extract from the red soluble protein overubin by HPTLC showed three fractions. The highly adsorbed red band “A” represents the main carotenoid corresponding to free astaxanthin (Fig. 2). This band and the other two red-orange spots (A, M, and D in Fig. 2) were scraped from the plates; their absorption spectra were recorded before and after saponification in KOH. The three forms of astaxanthin shifted from 467 to 472 nm after saponification, confirming the presence of free astaxanthin, and its monoester and diester forms. Free astaxanthin was the major carotenoid of overubin (40%), followed by astaxanthin diester (35%) and monoester (24%) (Table 1). An unidentified carotenoid occurring in

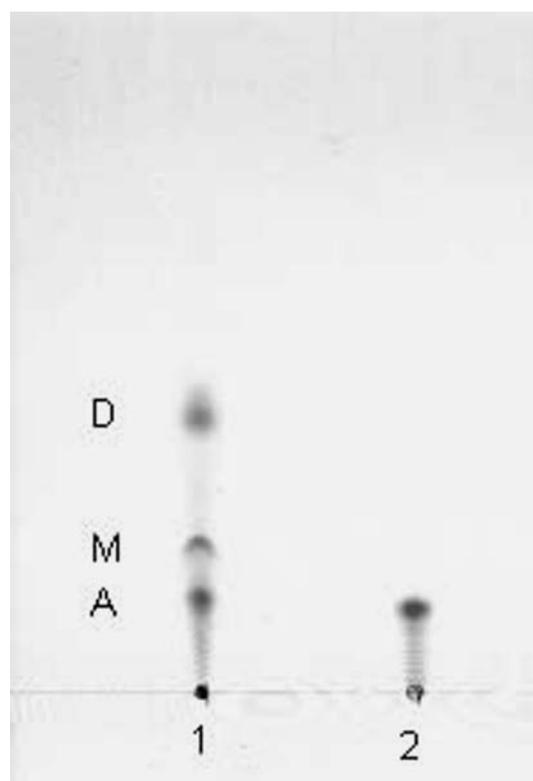


Fig. 2. Analysis of overubin carotenoids by HPTLC. The HPTLC was developed with hexane:acetone 80:20 (v/v). Lane 1, carotenoids extracted from overubin; lane 2, astaxanthin standard. A, free astaxanthin; M, astaxanthin monoester; and D, astaxanthin diester.

Table 1

Relative amounts of the carotenoids present in the overubin

Carotenoid	Relative amounts (%)
Astaxanthin monoester	24 $\pm$ 1.2
Astaxanthin diester	35 $\pm$ 2.9
Free astaxanthin	40 $\pm$ 3.4
Others	1 $\pm$ 0.08

Values are means of two independent determinations  $\pm$  SE of HPTLC image analysis.

very low amounts with an  $R_f$  higher than astaxanthin diester was also detected.

To gain knowledge about the fatty acids attached to astaxanthin derivatives, they were extracted from TLC, esterified, and analysed by GLC. Saturated and mono-unsaturated fatty acids were the major groups esterified to ovorubin carotenoids (Table 2). Palmitic acid (16:0) was the major fatty acid followed by stearic (18:0), oleic (18:1*n*–9), and eicosamonoenoic (20:1*n*–7), representing more than 60% of the fatty acids esterified to ovorubin carotenoids.

#### Inhibition of non-enzymatic microsomal oxidation

Fig. 3A shows the variation of membrane oxidation in control and carotenoid exposed microsomes as a function of time. After the addition of ascorbic acid (0.4 mM), the light emission increased markedly in the assay containing control microsomes, whereas microsomes exposed to different carotenoid extract concentrations showed a lower increase in chemiluminescence. The percentage of chemiluminescence inhibition achieved in exposed microsomes was dose-dependent with an  $IC_{50}$  of 3.9 nmol carotenoid/mg protein.

Table 3 shows the fatty acid composition from control and oxidised microsomes. There was a significant decrease of 20:4*n*–6 and 22:6*n*–3 fatty acids in ascorbic-treated membranes, evidencing the membrane oxidation process.

The antioxidant activity of ovorubin was analysed in microsomes, and no significant differences in the oxidation were found between control and ovorubin-supplemented microsomes.

#### Inhibition of enzymatic microsomal oxidation

Fig. 3B shows the effect of the addition of carotenoid extract to rat liver microsomes with respect to enzymatic oxidation as a function of time. After the addition of

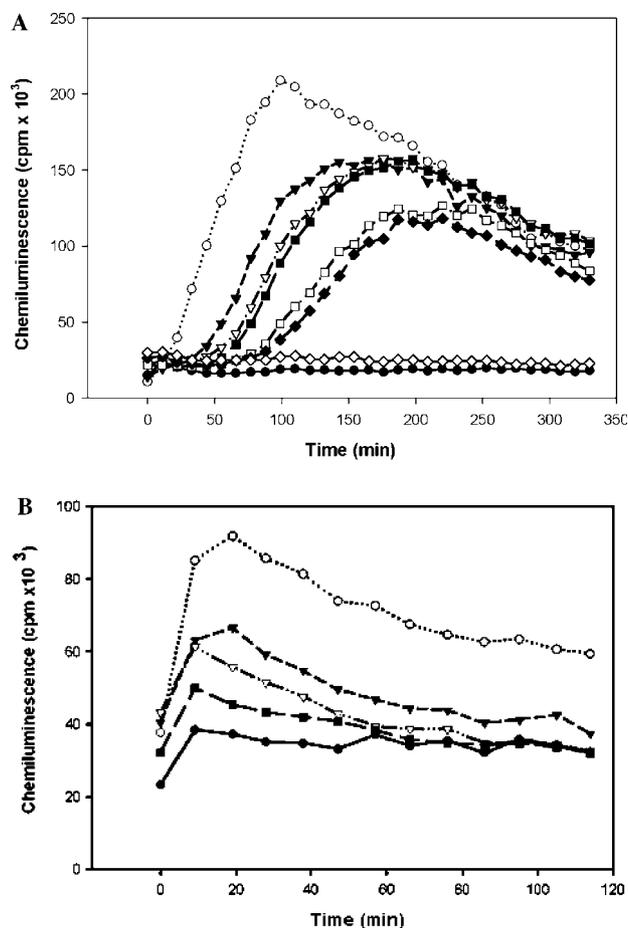


Fig. 3. (A) Effect of ovorubin carotenoids on the non-enzymatic oxidation inhibition of rat liver microsomes. Carotenoid concentrations (with 0.4 mM ascorbic acid): ○, no carotenoid added; ▼, 1.8 nmol; ▽, 2.7 nmol; ■, 3 nmol; □, 3.9 nmol; ◆, 4.2 nmol; ◇, 4.8 nmol; ●, control without ascorbic acid. The figure shows a dose-dependency reaching 86% of inhibition with 4.8 nmol/mg protein. Results are means of duplicate analyses. (B) Effect of ovorubin carotenoids on the enzymatic oxidation inhibition of rat liver microsomes. Carotenoid concentrations (with 0.1 mM NADPH): ○, no carotenoid added; ▼, 1.8 nmol; ▽, 2.7 nmol; ■, 3 nmol; ●, control without NADPH. The figure shows a dose-dependency reaching a maximum of 46% of inhibition with 3.0 nmol/mg protein. Results are means of duplicate analyses.

Table 2  
Major fatty acid composition of ovorubin carotenoids (% w/w)

Fatty acid	% Composition
14:0	5.14 ± 0.23
15:0	2.72 ± 0.19
16:0	28.26 ± 2.23
16:1 <i>n</i> –7	7.60 ± 0.51
18:0	15.17 ± 0.79
18:1 <i>n</i> –9	12.26 ± 0.84
18:2 <i>n</i> –6	2.24 ± 0.35
20:1 <i>n</i> –9	5.40 ± 0.69
20:1 <i>n</i> –7	10.36 ± 1.37
20:3 <i>n</i> –6	4.42 ± 0.61
Others	6.34 ± 1.46

Fatty acid methyl esters prepared from carotenoids scraped off from TLC plates, and analysed by GLC. Values are means of duplicate analyses ± SE. Only fatty acids with more than 2% w/w are listed.

Table 3  
Fatty acid composition (% w/w) of total lipids from rat microsomes, before and after oxidation

Fatty acid	Control	Peroxidised
14:0	2.2 ± 0.2	2.5 ± 0.2
16:0	23.5 ± 2.9	27.1 ± 3.1
18:0	25.2 ± 2.6	34.1 ± 2.8
18:1 <i>n</i> –9	5.2 ± 0.6	6.6 ± 0.5
18:1 <i>n</i> –7	1.5 ± 0.2	1.7 ± 0.3
18:2 <i>n</i> –6	11.9 ± 1.3	10.3 ± 1.0
20:4 <i>n</i> –6*	21.5 ± 1.9	12.2 ± 1.1
22:6 <i>n</i> –3*	4.2 ± 0.4	2.0 ± 0.2
Others	4.8 ± 0.4	3.5 ± 0.4

Fatty acid methyl esters prepared from total lipid microsomes were analysed by GLC. Values are means of duplicate analyses ± SE (\* $P < 0.05$ ).

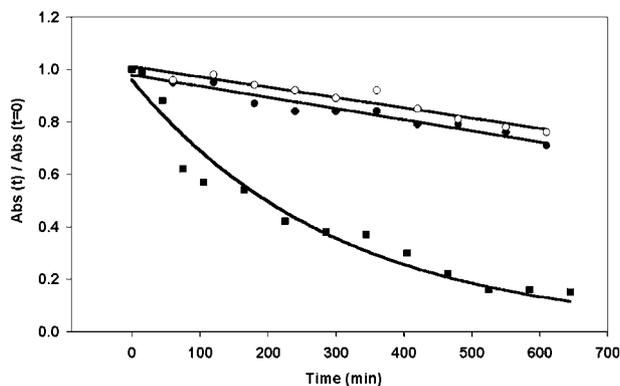


Fig. 4. Protective effect of apo-ovorubin against astaxanthin damage as a function of time. Samples were exposed to oxygen, fluorescent light, at 25 °C for 650 min. Free astaxanthin was more sensitive to photo-oxidation than combined astaxanthin. The absorbance was recorded as  $Abs(t)/Abs(t_0)$ .  $\lambda = 495$  nm.  $\circ$ , ovorubin;  $\bullet$ , reconstituted ovorubin; and  $\blacksquare$ , free astaxanthin.

NADPH, the light emission increased significantly when no carotenoid was present, whereas microsomes exposed to different carotenoid concentrations showed lesser increases in chemiluminescence. The maximum percentage of chemiluminescence inhibition achieved in the exposed microsomes was about 46% with 3.0 nmol carotenoid/mg protein.

#### Vitamin E determination

To gain more knowledge on the antioxidant molecules present in PVF, we also analysed the presence of vitamin E in ovorubin. No E vitamers could be detected by the methodology used.

#### Protective effect of ovorubin on its carotenoid component

In this experiment, the capacity of ovorubin to protect carotenoids from oxidative damage was investigated. Astaxanthin sensitivity to oxidation was evident in ovorubin, reconstituted ovorubin, and free astaxanthin (Fig. 4). The combined form of astaxanthin was significantly more stable than the free form, under the incubation conditions.

#### Discussion

Eggs from the freshwater snail *P. canaliculata* are cemented to plants and other substrates above the water level so when the newborn snails hatch, they fall down into the water course. This strategy protects them from water predators, but exposes the eggs for about 15 days 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 involve the provision of adequate amounts of antioxidant and photoprotection molecules to the embryo and also the capability of the perivitelline fluid to avoid water loss. Previous studies have pointed to the glycolipocarotenoprotein ovorubin as a potential candidate for these functions [3,6,25].

The composition analysis of the carotenoids present in ovorubin was analysed in the first developing stage (embryos developed from morula to gastrula, less than 250  $\mu\text{m}$  size), showing that three forms of astaxanthin were present, the free and diester forms being the major ones. The fatty acid composition of these carotenoids was dominated by long chain saturated (palmitic and stearic) and monounsaturated (oleic and eicosamonoenoic acids) fatty acids; a similar occurrence was found in other organisms [26].

The presence of esterified forms of astaxanthin would indicate a possible mechanism by which this carotenoid could be incorporated into the embryo cytoplasmic membrane as it is energetically easier to pass through the membrane in this more hydrophobic form. Esters are very active metabolic forms in the pigmentary physiology of various fish and crustaceans [8]. In addition, free astaxanthin must form intramolecular hydrogen bonds between the hydroxyl and carbonyl groups of the  $\beta$ -ionone rings to diminish its polarity, and get into the membrane to finally accommodate in the interior of membranes forming hydrogen bonds with the polar heads of phospholipids [27]. The above processes are probably taking place in some crustacean eggs which are first loaded with free astaxanthin that is later esterified by the embryo while postlarvae and adult stages possess a larger proportion of esterified forms [8]. In contrast, the capability of gastropod embryos to modify carotenoids remains unknown.

Ovorubin carotenoids showed a strong antioxidant activity which was dose-dependent in the enzymatic and non-enzymatic systems tested. Palozza and Krinsky [11] reported an  $IC_{50}$  for free astaxanthin of 3.5 nmol/mg protein which is in the same range as the one observed for ovorubin carotenoids in our experimental conditions (Fig. 3). Astaxanthin is probably the strongest antioxidant studied so far (sometimes termed “super vitamin E”). If we consider that the carotenoid content in golden apple snail eggs is approximately 72 nmol/g [4], and that 1 egg weighs 20 mg and contains 0.23 mg embryo protein (calculated from [3]), it therefore carries 1.5 nmol astaxanthin/egg. Hence, apple snail embryos are supplied with adequate amounts of antioxidant molecules, allowing their development under harsh conditions as above stated. The absence of ovorubin antioxidant activity would suggest first that astaxanthin is not easily released by ovorubin and second that when located inside ovorubin, astaxanthin is prevented from its antioxidant action.

Tocopherol, another quencher and oxygen radical scavenger common in animal tissues, was not detected in *P. canaliculata* just-laid eggs. In fact, astaxanthin and other xanthophylls are reported to be much better quenchers and scavengers than vitamin E [28].

It must be noted that ovorubin, though the most abundant, is not the only carotenoprotein in eggs. In fact, it has about 23% of the total carotenoid present in the egg PVF, whereas the rest was associated with the recently reported PV2 and PV3 carotenoproteins [4]. These three PVF carotenoproteins are probably involved in the transport to the developing embryo of membrane antioxidants such as astaxanthin in its free and esterified forms.

Ovorubin solubilised in the PVF absorbs light throughout most of the visible range ( $\lambda_{\max}$  480, 510, and 545 nm), and may exert a photoprotective effect on the embryo cells against harmful sunlight radiation at the beginning of development. At late developing stages, and particularly at the end of development, embryos showed to have taken up most of PVF antioxidant compounds by endocytosis of ovorubin and other PVF carotenoproteins [3]. Thus pigmented, the embryos would not need to be surrounded by a light-protecting fluid.

As carotenoids have been reported to stabilise the native configuration of most carotenoproteins [7], we studied the carotenoid function on ovorubin stability. We found that, unlike other carotenoproteins from invertebrate, astaxanthin is not essential to maintain ovorubin structure, at least we were able to obtain an apo-ovorubin with the same electrophoretic characteristics of native ovorubin, suggesting that the quaternary structure is not significantly affected by the presence of this carotenoid. In this regard, asteriarubin and linckiacyanin were the only carotenoproteins in which carotenoid is not involved in maintaining their quaternary structures so far reported [7].

Ovorubin not only transports carotenoids to the eggs, but also protects or stabilises carotenoids from damage in the PVF before their delivery to the embryo. This conclusion is based on the results about the half-life of the free pigment as compared to the carotenoid associated with the protein (Fig. 4). It is known that carotenoids are altered or partially destroyed by light especially in combination with oxygen and/or heat, resulting in discoloration and loss of activity. Under the severe oxidising conditions employed in vitro, most of the soluble astaxanthin was damaged, while in the same period only 20% of the ovorubin-protected astaxanthin was altered. As physiological conditions are not so rigorous, virtually all carotenoids would remain undamaged and functional throughout apple snail embryogenesis up to the moment they are taken up by embryos.

It has recently been reported that astaxanthin traps radicals not only at the conjugated polyene chain as other carotenoids do, but also in the terminal ring

moiety. This unique property should be associated with its potent antiperoxidative activity. This is probably another reason for its selection by so many invertebrates for the pigmentation of their eggs, including this mollusc.

Ovorubin not only seems to play a major role in the storage and transport of carotenoids, but also seems to protect the carotenoid from oxidative degradation by forming a stable complex in the PVF. Ovorubin could serve as a vehicle to carry astaxanthin to its final destination in the embryo. This function is suggested by the fact that (a) it is a highly glycosylated glycoprotein and (b) important quantities of ovorubin are retained in the visceral hump of the young snail, probably as a reserve for the first days of free life [3]. The oligosaccharide moiety would be involved in this selective uptake and is the subject of ongoing research. Further studies will shed light on the fate of carotenoids and carotenoproteins during embryogenesis in the golden apple snail, leading to other physiological roles for ovorubin.

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