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Biochemical composition, tissue origin and functional properties of egg perivitellins from *Pomacea canaliculata*

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Introduction

Protein, lipids and carbohydrates are stored in the yolk of developing oocytes as a source of nutrients during embryogenesis. In many invertebrates, the major egg yolk proteins usually form a water-soluble glycolipoprotein complex, called vitellins. They may have different origins in different groups. In most insects and some crustaceans they are usually synthesized either in the fat body or digestive gland, and are released to the circulation as precursors particles called vitellogenins, which are taken up by the ovary and incorporated into the developing oocyte (heterosynthetic mechanism) (Chen *et al*., 1999). In other arthropods, both the ovary and other tissues are involved in the process, thus showing either autosynthetic or heterosynthetic mechanisms of vitellogenesis (Wallace *et al*., 1967; Kanost *et al*., 1990; Lee, 1991; Fainzilber *et al*., 1992). In comparison, little is known about this process in mollusks. In cephalopods and some gastropods (de Jong-Brink *et al*., 1983; Barre *et al*., 1991; Bride *et al*., 1992) vitellogenesis occurring outside the ovary seems to be the main yolk forming process. Gastropod vitellins have been found in the oocytes of *Helix* (Barre *et al*., 1991), *Helisoma* (Miksys and Saleuddin, 1986), *Lymnaea* and *Planorbis* (Bottke, 1986). However, most gastropods have a perivitellin fluid (PVF) that surrounds the fertilized eggs, and which is mainly synthesized by an accessory gland of the female reproductive tract, the albumen gland. PVF represents the major supply of nutrients during embryogenesis, being consumed by the developing embryo as a source of energetic and structural materials (Heras *et al*., 1998). Therefore, proteinaceous yolk granules found in these snails' developing eggs do not serve the purpose of nutrient storage, but they function as primary lysosomes in charge of perivitelline fluid digestion instead (de Jong-Brink *et al*., 1983; Bretting *et al*., 1991).

Our studies were made in the caenogastropod snail *Pomacea canaliculata* (Lamarck 1822) which belongs to this latter group, where yolk is a minor source of nutrients to the embryo. The present review deals with glycolipocarotenoproteins present in the perivitelline fluid of the snail *P. canaliculata*, their compositions, synthesis and functions during embryo development.

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Biochemical composition

Several studies on gastropod PVF composition (Hortsmann, 1956; Raven, 1972; de Jong-Brink *et al*., 1983) reported the presence of both proteins and polysacharides, but no lipids. On the other hand, Cheesman (1958) found a carotene-glycoprotein complex in the eggs of *P. canaliculata*, with no ester lipids associated, that he called ovorubin. Since then, the study of the proteins in *P. canaliculata*'s PVF was neglected until the late 90's when our laboratory focused on the PVF biochemistry during embryogenesis (Heras and Pollero, 2002).

Lipids

Using a combination of sequential centrifugations of egg homogenates, followed by purification steps using gradient ultracentrifugation and HPLC, we were able to isolate three lipoprotein fractions from the PVF of *P. canaliculata*, that we identified as perilipovitellins 1, 2 and 3 (PV1, PV2 and PV3). They were the first lipoproteins reported in gastropods, and their lipids were characterized by microchromatographic methods (Garin *et al*., 1996). They represent, respectively, 6.7, 10.0 and 53.2% of the egg total lipids. PV1 is a red particle of high molecular weight (approximately 300 kDa), which agrees with that of the ovorubin described by Cheesman (1958) using a different methodology. Due to this and other shared characteristics, PV1 and ovorubin may be considered as the same perivitellin. In his original work, Cheesman did not report the presence of associated lipids (except for the carotenoid moiety), but further studies by Garin *et al*. (1996) showed that this carotene-glycoprotein complex had also attached lipids. Ovorubin is actually a particle with characteristics of a very high-density lipoprotein (VHDL), whose lipid moiety is mainly composed of sterols and phospholipids, both typical membrane lipids. Therefore, this particle classifies as a lipo-glyco-carotenoprotein, similar to other invertebrate lipovitellins (Cheesman *et al*., 1967; Zagalsky, 1985). The presence of the PV2 particle in the PVF of *P. canaliculata* was unexpected, since there was no mention of any comparable particle in all previous descriptions of gastropod perivitellus proteins (Cheesman, 1958; Morril *et al*., 1964). Considering its hydration density, PV2 is a lipoprotein that also falls whithin the VHDL range. It is a high molecular weightparticle of 400 kDa. As for ovorubin, the major lipid classes of PV2 are free sterols and phospholipids, although significant quantities of energy-providing triacylglycerols and free fatty acids are also present. PV3 is a heterogeneous fraction, very rich in lipids, composed of at least two particles with characteristics of high-density lipoproteins (HDL) (Garin *et al*., 1996). Subfraction PV3-I is a carotenolipoprotein of a strong yellowish color, already mentioned by Cheesman (1958) in egg homogenates. It is the most lipid-rich particle (30% of the total PVF lipids), with high levels of a carotenoid pigment, hydrocarbons, free fatty acids, phospholipids, free sterols and triacylglycerols. The lipid moiety of subfraction PV3-II is mainly composed of free sterols, phospholipids and free fatty acids.

Proteins

The three particles isolated from the PVF represents, 57.0, 7.5 and 35.5% of the egg total proteins, respectively (measured in just-laid eggs). Garin *et al*. (1996) characterized the apoprotein subunits of ovorubin as well as those of PV2 and PV3 particles by electrophoresis. The composition study of the apoprotein moiety of ovorubin, showed that it is composed of three subunits of ca. MW 35, 32 and 28 kDa. This apoprotein composition differs from that of a perivitellin with a mass similar to that of ovorubin, isolated by Morishita *et al*. (1998) from the pulmonate snail *Helisoma*. Even more, when the three protein subunits were isolated and N-terminal amino acid sequences were determined, there was no homology in these regions compared with other related proteins (Dreon *et al*., 2003), including those from the few egg snail proteins sequenced (Mukai *et al*., 2004; Miller *et al*., 1996). The protein moiety of PV2 is composed of two subunits of 67 and 31 kD. The amino acid sequences of the N-terminal portion of both purified subunits were analyzed, but again BLASTP search showed no homology with any known sequence (Dreon *et al*., 2002). The two subfractions of PV3 have different apoprotein compositions. While subfraction PV3-I is composed by a single particle of 26 kD, the subfraction PV3-II has two particles of 100 and 64 kD, which under electrophoretic dissociating conditions show subunits of 34 and 29 kD, respectively. Details of lipid and protein compositions of the three PVF lipoprotein fractions, were summarized previously (Heras and Pollero, 2002).

Carbohydrates

As compared to egg lipids, there is much more background information regarding egg glycoproteins in gastropods (Wilson, 2002), and a number of them have been

described in the albumen gland of sea hares (Kisugi *et al*., 1989; Takamatsu *et al*., 1995) and of the pulmonate *Helisoma duryi* (Morishita *et al*., 1998). Nevertheless, their olygosaccharide moieties have not been characterized. In a recent work we have studied the carbohydrate moieties and monosaccharide compositions of ovorubin and PV2 from *P. canaliculata* (Dreon *et al*., 2004a). Both perivitellins contain carbohydrates as the major prostetic group. The quantitative analysis of total carbohydrates revealed that ovorubin is highly glycosilated (17.8% w/w) while PV2 contains less carbohydrates (2.5% w/w). Monosaccharide compositions, determined by gas-liquid chromatography, showed that in both perivitellins the predominant sugar is mannose, which accounts for about 50% and 70% of total carbohydrate mass, for ovorubin and PV2, respectively. Quantitatively, the second monosaccharide is N-acetylglucosamine in ovorubin, and galactose in PV2. Liquid chromatographic analysis after chemical deglycosilation of both particles shows that the amount of O-linked oligosaccharides is higher than that of the N-linked species. We also studied the glycosilation patterns of the two perivitellins by a set of lectins, which provided information about the possible carbohydrate structure associated with the protein backbone. Lectin affinities confirmed the presence of asparagine-linked carbohydrates, probably of hybrid and high mannose type, the presence of O-linked residues derived from the T antigen, and suggested that sugars conjugated to both glycoproteins are short and structured in their biosynthetic route.

Carotenoids

Most invertebrate species present colored eggs and pigmentary changes during development, usually as a result of the presence of carotenoids, being the most common ones astaxanthin and cathaxanthin, non-covalently bound to proteins (Zagalsky *et al*., 1990). The reddish color of the main protein in the PVF of *P. canaliculata*, inspired Cheesman's name "ovorubin". We have recently characterized the carotenoid component of the ovorubin (Dreon *et al*., 2004b), with the aim of conducting further studies on its function. Chromatographic analysis showed that astaxanthin is present in the free, mono and diester forms. The predominant acids esterifying astaxanthin are saturated and monoenoic fatty acids, i.e., a fatty acid pattern similar to that found in protists by Bidigare *et al*. (1993). The presence of esterified forms of astaxanthin, suggests a possible mechanism by which a more hydrophobic form of this carotenoid might be incorporated into the embryo cytoplasmic membrane.

Tissue origin

The metabolism of gastropod egg lipoproteins was little known up till our studies on the site of synthesis, mobilization and storage of the two major perivitellins of *P. canaliculata* (Dreon *et al*., 2002; Dreon *et al*., 2003). These studies were done by a combination of incubations with labeled amino acids, antibodies directed against purified PVs and enzymatic immune assays. Albumen gland, gut, stomach, muscle, gonad/digestive gland and hemolymph were examined. Dissected tissues or the whole animal were incubated with the radioactive precursor, and labeled proteins were analyzed by electrophoresis and radiochromatography. Results from *in vitro* experiments revealed the synthesis of significant quantities of radiolabeled proteins in all tissues, but proteins with the characteristics of ovorubin and PV2 were only found in the albumen gland. Under dissociating condition, proteins corresponding to the three protein subunits of ovorubin and the two subunits of the PV2 were detected. Polyclonal antibodies against ovorubin and PV2 were utilized to detect these lipoproteins, and immunoreactive proteins were again found only in the albumen gland. Moreover, the enzymatic immunoassays, employed to quantify protein levels in different tissues, also confirmed the presence of both perivitellines only in albumen gland and developing eggs. Gonad/digestive gland were particularly looked at because there are several reports in other invertebrates supporting its importance as an egg-lipoprotein synthesizing site (Meusy, 1980; Yano and Chizei, 1987; Fainzilber *et al*., 1992). Vitellogenins circulating in plasma of vitellogenic females have been detected in most oviparous organisms such as vertebrates, crustacean, insects, and gastropods (Lee, 1991; Barre *et al*., 1991; Bride *et al*., 1992; Yepis-Plascencia *et al*., 2000). The garden snail *Helix aspersa* synthesizes their vitellogenins in the digestive gland which surrounds the ovotestis and transport them by hemolymph to vitellogenic oocytes. In the freswater pulmonates *Lymnaea* and *Planorbis*, a vitellogenin with other characteristics, such as those of the iron-reserving ferritin, have been described. It was found that ferritin is also produced by the digestive gland and circulate in hemolymph (Bottke, 1986; Bottke *et al*., 1988). In contrast, no lipoproteins similar to ovorubin or PV2 could be found in reproductively active *P. canaliculata* hemolymph, even

though highly sensitive techniques as *in vivo* radiolabeling and immunoblot were used. The absence of circulating vitellogenins has also been suggested when plasma lipoproteins were examined (Garin and Pollero, 1995). In conclusion, the albumen gland is the only site for the ovorubin and PV2 synthesis in this apple snail, as no extra-glandular synthesis, circulation or accumulation could be demonstrated.

The results above described led us to infer that these perivitellines do not follow an heterosynthetic mechanism. Unlike other lipovitellins, they are not transported by the hemolymph to the vitellogenic oocytes, but they would be incorporated to the fertilized oocytes as a secretion of the albumen gland (Hinsch and Vermeire, 1990; Catalán *et al*., 2002) during the passage of eggs through the pallial oviduct. A similar behaviour was observed by Morishita *et al*. (1998) for an egg glycoprotein in *Helisoma duryi.*

Functional aspects

Perivitellin dynamics during embryogenesis

With the aim of determining the role of ovorubin, PV2 and PV3 stored in PVF during embryo development, compositions of the three perivitellins were analyzed along this process (Heras *et al*., 1998). First, the embryos, the egg shells and the PVF were separated by density gradients, and biochemical changes were followed in PVF as well as the embryos, from fertilization to hatching. Development was divided into five stages, from morula (stage I) to newly-hatched juveniles (stage V). Also, juvenile snails were collected manually on day 3 after hatching, and were labeled as stage VI. Apoproteins and lipid classes from the whole PVF were analyzed in each stage of development.

During embryogenesis of *P. canaliculata,* total protein levels undergo significant changes, decreasing in the PVF while increasing in embryos (Heras *et al*., 1998). Embryos took up 90% of PVF proteins with a main period of consumption between stages IV and V. As there was no net increase in embryo protein content until hatching, it was suggested that most proteins incorporated at stages IV and V must have been either consumed as an energy source or converted into other components. Some of these findings were clarified when the dynamics of individual protein behavior during development was studied by electrophoresis. It was found that embryos from stages II to IV absorb ovorubin from PVF and to a lesser extent PV2. Although no net increase in embryo protein content between stages IV and V was observed, protein bands corresponding to PV2 and PV3 perivitellins appeared inside the embryos and, of course, new embryo proteins were synthesized. This suggests that the uptake systems and the metabolic pathways of embryos are more active at these stages. The PVF at stage V is very poor in proteins and mostly retains some leftovers of ovorubin. Ongoing research has shown this perivitelline is a highly thermostable protein unaltered by the relatively high temperatures to which eggs are exposed, and its presence in PVF throughout development would serve as an osmotic regulator. Embryos at stage V displayed a pattern characterized by a higher concentration of PV2 and PV3. After hatching, ovorubin was still one of the major snail proteins recovered. On the other hand, PV2 and PV3 were almost absent, probably used as an endogenous source of energy and structural molecules during this transition to free life. Protein conversion efficiency, calculated as % of perivitellus energy transformed into embryonic tissues between stages I and V, showed relatively low values (29%) (Heras *et al*., 1998), suggesting, they are not only used for embryo structure but also as energy source.

As we have already mentioned, PVF lipids in *P. canaliculata* PVF are found associated to these perivitellins (Garin *et al*., 1996). Studies on the lipid dynamics during development showed that embryos incorporate lipids together with most other nutrients, mainly between stages IV and V (Heras *et al*., 1998). This was reflected by their significant decrease in PVF paralleled by a fivefold increase in the embryo lipid content at stage V. Considering there is an important synthesis of lipids throughout embryonic development, it might thought *a priori* that lipids do not represent an important energy source. In fact, the major lipid classes (phospholipids and free sterols) show a significant *de novo* synthesis. But as these are mainly membrane lipids, could be synthesized using carbohydrates as energy and carbon sources. Something similar occurs with esterified sterols which, having high-conversion efficiency suggest an active synthesis. On the contrary, about 98% of triacylglycerols are consumed between stages V and VI (hatchlings), where triacylglycerol concentration decreases about 400 times. This fact is also coincident with the fall of the triacylglycerol-rich PV2 perivitellin after hatching. Triacylglycerols may be a particularly useful energy source for hatchlings during short fasting periods, but they could also provide the essential fatty acid pool used during embryo nervous system development. The report of Heras *et al*. (1998) referred the dy-

namics of carbohydrates along development, measuring them in total PVF. Carbohydrates in PVF are mainly represented by soluble polysaccharide galactogen, and not an integral part of perivitellins. Their conversion efficiency of only 14%, indicates that they are the major energy source in *Pomacea* embryos.

We can therefore suggest that the absorption of perivitellins from the PVF is divided into two phases. The first one shows a moderate uptake until stage IV. It is followed by a second phase of very active uptake of all nutrients and a selective consumption of proteins (from stage IV to V), where they would be utilized for organogenesis. After hatching, there would be a specific consumption of PV2 lipoprotein and its associated triacylglycerols.

Carbohydrate moiety of perivitellins

Ovorubin and PV2 from *P. canaliculata* contain carbohydrates as the major prostetic group, being the ovorubin characterized by larger amount of carbohydrates in comparison with those of other invertebrate or vertebrate vitellins. During development, *P. canaliculata* egg masses, cemented to plants and other substrates above water level, are exposed for about 15 days to air desiccation. It is interesting to note that even under such severe conditions, no significant changes in water content were observed. It is probable that the saccharide moiety of ovorubin, together with the high amount of galactogen present in the PVF, are efficient in avoiding water loss, keeping the adequate environment for the embryos as regards to water content.

Glycoproteins are involved in important physiological processes like cell-cell communications, interaction with extracellular receptors, tissue morphogenesis, immune reactions, etc. The carbohydrate moiety of glycoproteins may also be involved in the proper folding and tertiary and quaternary structure of the molecule. In addition, glycosilation increases protein solubility, and it is important for proper transport processes and for protection from intracellular proteases (Gibson *et al*., 1979; Berman and Lasky, 1985). In this regard, it has been shown that ovorubin is partially protected from trypsin proteolysis (Norden, 1972). On the other hand, we have previously shown that there is a selective uptake of the different perivitellins during the apple snail embryogenesis. Although both ovorubin and PV2 are endocyted by the developing embryo, the latter is completely consumed before hatching, while ovorubin is not immediately digested and some of it remains even in the newborn snails (Heras *et al*., 1998). As it was reported for *Xenopus* oocytes (Wall and Patel, 1987), and during insect vitellogenesis Raykhel and Dhadialla (1992), the carbohydrate moiety could be important for receptor-mediated uptake during endocytosis of these glycoproteins from PVF. A similar function might be attributed for carbohydrates in *P. canaliculata* perivitellins. Moreover, perivitellin glycosilation could also be a signal inside the albumen gland for fertilized-egg PVF endocytosis during perivitellogenesis.

Protective function of ovorubin

As eggs are cemented above the water lever, when newborn snails hatch, they fall down into the water. This strategy protects eggs from water predators, but exposes them for about two weeks to direct sunlight, air, and high temperatures. Even under this adverse environmental setting, eggs manage to develop fully, suggesting they may 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. Some studies have pointed to ovorubin as a potential candidate for these functions (Cheesman *et al*., 1967; Heras *et al*., 1998; Dreon *et al*., 2003). Recently, the capacity of ovorubin as a protection system against oxidative damage in eggs from *P. canaliculata* was investigated (Dreon *et al*., 2004b), using chemiluminescence to measure the inhibition of enzymatic and non-enzymatic membrane oxidation and so evaluate the antioxidant property of ovorubin. It was mentioned above that ovorubin is a glyco-lipo-carotene-protein, with its carotenoid component constituted by free and esterified forms of astaxanthin. It is generally accepted that carotenoids can act as antioxidants (Terao, 1989); in particular, astaxanthin is a potent antioxidant in membrane models (Palozza and Krinsky, 1992). Dreon *et al*. (2004b) showed ovorubin carotenoids have a strong antioxidant activity, which was dose-dependent with an IC50 of 3.9nmol/mg protein. Nevertheless ovorubin alone do not have antioxidant activity suggesting that astaxanthin is not easily released from ovorubin, or that when located inside ovorubin, astaxanthin is prevented from its antioxidant action having other functions (see below). The performance of astaxanthin as antioxidant in *P. canaliculata* eggs may be calculated. If we consider that the carotenoid content in eggs is approximately 72 nmol/g (calculated from Garin *et al*., 1996), and that 1 egg weighs 20 mg and contains 0.23 mg embryo protein (Heras *et al*., 1998), it therefore carries 1.5 nmol astaxanthin/egg. Hence, embryos are supplied with ad-

equate amounts of antioxidant molecules, being beneficial for the harsh conditions of their development. It must be noted that ovorubin, though the most abundant, is not the only carotenoprotein in *P. canaliculata* eggs. In fact, it has about 23% of the total carotenoid present in the PVF, whereas the rest is associated with PV2 and PV3 carotenoproteins (Garin *et al*., 1996). These three carotenoproteins are probably involved in the transport to the developing embryo of membrane antioxidants such as astaxanthin in its free and esterified forms. These last forms, more hydrophobic than the free form or the astaxanthin bound to other molecules that diminish its polarity**,** may be adequate ways for this pigment to pass through the membranes and incorporate into the embryos.

Ovorubin solubilized in PVF absorbs light throughout most of the visible range, 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 before hatching , embryos have already taken up most of PVF (with their antioxidant and light-protecting compounds, Heras *et al*., 1998). Then, the melanin pigmented embryos would no longer need to be surrounded by a light-protecting fluid.

The presence of mutual stabilization between the carotenoid component and the ovorubin apoprotein was also demonstrated (Dreon *et al*., 2004b). As it had been reported that carotenoids stabilize the native configuration of most carotenoproteins (Mantiri *et al*., 1996), we studied this possible carotenoid function in ovorubin stability. It was found that, unlike for most other invertebrate carotenoproteins, astaxanthin is not essential to maintain ovorubin structure, since apo-ovorubin maintains the same characteristics as native ovorubin (Dreon *et al*., 2004b). On the contrary, ovorubin not only transports carotenoids to the eggs, but also protect or stabilizes carotenoids from damage in the PVF before their incorporation 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; in fact, free astaxanthin is significantly more sensitive to photo-oxidation than their combined forms, such as native or *in vitro*-reconstituted ovorubin (Dreon *et al*., 2004b). 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. *In vitro* experiments showed that under severe oxidizing conditions, most of the soluble astaxanthin is damaged, while only a small part of the ovorubin-protected astaxanthin is altered. As physiological conditions are not so rigorous, virtually all carotenoids would remain undamaged and functional throughout the snail embryogenesis. In brief, ovorubin no 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.

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