Comparative Biochemistry and Physiology, Part B 157 (2010) 66–72

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

# Carbohydrates and glycoforms of the major egg perivitellins from *Pomacea* apple snails (Architaenioglossa: Ampullariidae)

S. Ituarte [a], M.S. Dreon [a], M.Y. Pasquevich [a], P.E. Fernández [b], H. Heras [a,*]

[a] *Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CONICET CCT La Plata — Universidad Nacional de La Plata (UNLP), 60 y 120, (1900) La Plata, Argentina*
[b] *Instituto de Patología B. Epstein, Cátedra de Patología General Veterinaria, Facultad Cs. Veterinarias, UNLP, 60 y 118, (1900) La Plata, Argentina*

ARTICLE INFO

*Article history:*
Received 3 February 2010
Received in revised form 7 May 2010
Accepted 8 May 2010
Available online 13 May 2010

*Keywords:*
Carotenoprotein
Fucose
Gastropod
Ovorubin
Scalarin
Sialic acid

ABSTRACT

To better understand how glycans contribute to the multiple roles of perivitellins in embryo development, the carbohydrate moieties and glycoforms of the carotenoglycoproteins ovorubin and scalarin from the eggs of *Pomacea canaliculata* (Lamarck, 1822) and *Pomacea scalaris* (d'Orbigny, 1835) were studied. All subunits of both proteins are glycosylated and appear to be glycoforms with isoelectric points ranging from ~5.3 to ~9.1. Complete deglycosylation reduced subunit heterogeneity to spots of similar molecular weight (~27 and ~25 kDa in scalarin and ovorubin, respectively) but with varying IP. Serine phosphorylation, present in both perivitellins, explains part of the isoforms. Glycosylation patterns of scalarin were determined using biotinylated lectins, PNGaseF treatment and selective chemical deglycosylation, which revealed the presence of hybrid and oligomannose N-linked glycans in all subunits. Scalarin has terminal sialic acid residues possibly resistant to neuraminidase and O-linked residues derived from the T- and Tn antigens. Ovorubin displayed predominantly the same glycans, though in different amounts. Capillary gas chromatography (GC) showed galactose and mannose as the major monosaccharides followed by GlcNAc and fucose. An interesting feature was the important amount of sialylated and fucosylated structures found in both perivitellins determined by GC, spectroscopy and lectins. This is the first report of these structures in gastropods other than heterobranchs.

© 2010 Elsevier Inc. All rights reserved.

## 1. Introduction

Freshwater snails in the family Ampullariidae have got a wide distribution (probably following the fragmentation of Gondwana (Berthold, 1991)) encompassing tropical, subtropical and temperate habitats, being the genus *Pomacea* (Perry, 1810) native of South and Central America, the Caribbean and southern North America. Some species of this group have been introduced in Southeast Asia, Hawaii and mainland U.S. where they spread and became a risk for agriculture and native wetland ecosystems (Carlsson et al., 2004; Rawlings et al., 2007). Also, in Southeast Asia they became opportunistic hosts of *Angiostrogylus cantonensis*, a nematode which is the etiologic agent of human eosinophilic meningoencephalitis (Lv et al., 2009).

The ampullariids exhibit two oviposition strategies: the most basal clades of the family lay their egg masses at or below the water line, while the most derived groups, mostly from the genus *Pomacea*, present aerial egg-laying, cementing colored clutches to vegetation or other substrata outside the water. The shift to aerial egg-laying was probably a key innovation in the family, allowing diversification and spread (Hayes et al., 2009), but required physiological innovations in the eggs which are exposed for weeks to adverse environmental conditions such as direct sunlight, high temperature, desiccation and predators (Estebenet and Cazzaniga, 1993; Albrecht et al., 1999; Estebenet and Martín, 2002; Albrecht et al., 2004). The eggs of intertidal snails which are also exposed to the aerial environment overcome the deleterious effects of these factors by protecting mechanisms which can be mechanical, concerning egg membranes or capsules (Pechenik, 1986; Przeslawski, 2004), or biochemical, involving the egg components (Przeslawski, 2005).

The eggs of these gastropods are coated by a perivitelline fluid (PVF), which is secreted by the female albumen gland, that is mainly composed of polysaccharides and glycoproteins (Jong-Brink et al., 1983). The PVF glycoproteins, named perivitellins, were studied in only a few species and in Ampullariidae only the perivitellins of *Pomacea canaliculata*

*Abbreviations:* BSL 1, *Bandeiraea simplicifolia* lectin 1; Con A, Concanavalin A; DBA, *Dolichos biflorus* agglutinin; DSL, *Datura stramonium* lectin; ECL, *Erythrina cristagalli* lectin; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; IP, isoelectric point; JAC, Artocarpus integrifolia; LCA, *Lens culinaris* agglutinin; Man, mannose; MW, molecular weight; Neu5Ac, N-acetyleuraminic acid; PBST, phosphate buffered saline Tween; PNA, peanut agglutinin, *Arachis hypogaea*; PNGaseF, N-Glycosidase F; PSA, *Pisum sativum* agglutinin; PVF, perivitelline fluid; PVL, *Psathyrella velutina* lectin; RCA 1, *Ricinus comunis* agglutinin; SBA, soybean agglutinin *Glycine max*; Sias, sialic acids; UEA1, *Ulex europaeus* agglutinin 1; TFMS, trifluoromethanesulfonic acid; WGA, wheat germ agglutinin *Triticum vulgaris*; Xyl, xylose.

* Corresponding author. INIBIOLP — Fac. Cs. Médicas, 60 y 120, La Plata (1900), Argentina. Tel.: +54 221 4824894; fax: +54 221 4258988.
*E-mail address:* h-heras@med.unlp.edu.ar (H. Heras).

1096-4959/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2010.05.004

(Lamarck, 1822) and *Pomacea scalaris* (d'Orbigny, 1835) are known. The biochemical and structural characteristics of the perivitellins of these species suggest that they are involved in the adaptation of the eggs to the aerial environment (Heras et al., 2007). The eggs of *P. canaliculata* have a multifunctional perivitellin called ovorubin, a reddish carotene-glycoprotein which plays critical roles in embryo development such as nutrient provision, photo protection, antioxidant stabilization and trypsin inhibition (Norden, 1972; Heras et al., 2007). Some of these varied functions have recently been related to remarkable structural features, ovorubin being structurally stable in a wide pH range (pH 4.0–12.0) and thermo stable up to 95 °C (Dreon et al., 2007; Heras et al., 2007; Dreon et al., 2008). Ovorubin functions are complemented by another perivitellin, PV2, recently reported as a neurotoxin involved in the chemical defense of the embryos (Heras et al., 2008).

A protein similar to ovorubin was found in the eggs of the congeneric species, *P. scalaris* (Ituarte et al., 2008). This perivitellin, named scalarin, is comparable to ovorubin, both sharing similar MW, subunit composition, dimensions, and ligands. Regarding function, even though scalarin has not been as thoroughly characterized as ovorubin, it also seems to play similar roles during embryo development (Ituarte et al., 2008). Thus, the presence of multifunctional perivitellins is probably characteristic of aerial egg-laying *Pomacea*. Another shared characteristic between scalarin and ovorubin is their remarkably high glycosylation, with a carbohydrate content of 17% (w/w) in ovorubin and 21% (w/w) in scalarin. However, differences in their glycan composition have been suggested based on the lack of polyclonal antibodies cross-reactivity (Ituarte et al., 2008).

Glycosylation is a very common post and/or co-translational modification of proteins in which glycans are covalently linked to the protein. This modification is involved in proper folding, structural stabilization, and trafficking of the glycoprotein and is also important in cell and tissue signaling (Varki and Lowe, 2009). Gastropod glycoproteins have recently gained attention as a source of new and interesting glycosylation patterns with potential biotechnological applications (Gutternigg et al., 2007) but most studies focus on the characterization of the glycans, the glycome, without establishing to which proteins these oligosaccharides are attached, or which could be their functions. Regarding egg-glycoproteins, only a few have been described, from pulmonates (Mukai et al., 2004; Sanchez et al., 2006), nudibranchs (Kisugi et al., 1989; Iijima et al., 1995; Kamiya et al., 2006) and ampullarids (Garín et al., 1996; Ituarte et al., 2008). The carbohydrate moiety was only studied in *P. canaliculata* perivitellins (Dreon et al., 2004), and in *Helix pomatia* agglutinin, although in this case the protein was isolated from the albumen gland without confirmation of its presence in the egg.

In this work we further study the perivitellins of aerial egg-laying ampullariids. In particular, we focused on the glycan characterization of scalarin and ovorubin, and provide evidence on the occurrence of glycoforms in both egg-glycoproteins. Finally we discuss potential roles of glycans in perivitellins.

## 2. Materials and methods

### 2.1. Perivitellin purification

Scalarin was purified from *P. scalaris* eggs by ultracentrifugation and exclusion chromatography as previously described (Ituarte et al., 2008). Ovorubin was purified from *P. canaliculata* eggs according to Garín et al. (1996). Protein concentration was measured by the method of Lowry et al. (1951). Native and dissociating PAGE were performed in a Mini-Protean III System (Bio Rad, Hercules, CA, USA) following manufacturer directions, molecular weight standards were obtained from GE Healthcare (Uppsala, Sweden).

### 2.2. Monosaccharide analysis

Glycoprotein samples containing 5–50 µg of total sugar were incubated in methanolic HCl 0.5 M to yield methyl glycosides, which were then treated with hexamethyldisilazane/trimethylchlorosilane/pyridine 2:1:10, (v/v/v) to prepare volatile sialylated derivatives (Manzi, 1995a). The sialylated samples were analyzed by gas chromatography coupled with a flame ionization detector (GC) using an HP6890 capillary GC (Hewlett Packard, Palo Alto, CA, USA) fitted with a HP-5 column 30 m × 0.32 mm, 0.25 µm phase (Hewlett Packard) Conditions and temperatures were: FID 280 °C; injector 280 °C. The column temperature was programmed as follows: initial temperature 50 °C held for 3 min; increased at a rate of 20 °C $min^{-1}$ to a temperature of 170 °C, a second ramp of 6 °C $min^{-1}$ to a final temperature of 250 °C. The carrier gas was helium at a pressure of 20 psig and hydrogen and air pressures were 13 and 23 psig, respectively. Standard monosaccharides (Sigma-Aldrich, St. Louis, MO, USA) were silylated and analyzed under the same conditions.

### 2.3. Sialic acid determination

Sialic acid was released from the glycoproteins by mild acid hydrolysis (Manzi, 1995b): lyophilized glycoproteins were incubated in 2 M acetic acid at 80 °C for 4 h and samples were dialysed twice against 10 vol. MilliQ water overnight. The dialysates were pooled and lyophilized for sialic acid determination by the ferric orcinol assay (Manzi and Esko, 1995). N-acetylneuraminic acid (MP Biomedicals, Santa Ana, CA, USA) was used as standard.

### 2.4. N- and O-linked oligosaccharides analysis

O-linked oligosaccharides were released from the glycoprotein through β-elimination with 1 M $NaBH_4$/0.05 M NaOH (Fukuda, 1995). The supernatant of this reaction was passed through a 1 × 30 cm Sephadex G-25 column, and 1 mL fractions were collected. These fractions were spotted onto a silica gel-coated HPTLC plate (Merck, Darmstadt, Germany), and visualized by spraying with orcinol/$H_2SO_4$ and heating at 150 °C for 5 min. Fractions containing carbohydrates displayed a brown colour, the plates were analyzed densitometrically using Sigmagel v.1.0 (Jandel Sci., USA).

The protein precipitate from the O-linked release was digested with 1 M $NaBH_4$/1 M NaOH at 100 °C for 6 h to release N-linked glycans. The supernatant was analyzed as described above.

### 2.5. Lectin dot blot

A set of 14 biotinylated lectins were used for the analysis, namely PVL, BSL 1, DSL, JAC, SBA, Con A, WGA, DBA, UEA 1, PNA, RCA I, ECL, PSA and LCA (Vector Labs, Burlingame, CA, USA). Glycoproteins (10 µg) were adsorbed onto nitrocellulose strips (Hybond, GE Healthcare, Uppsala, Sweden) and incubated for 1.5 h at 37 °C in a humid environment (Harlow and Lane, 1988, ch. 6). The strips were then blocked for 1 h at 37 °C with 5% (w/v) non-fat dry milk in PBST, and incubated overnight at 4 °C with the lectin dilutions in 1% (w/v) non-fat dry milk in PBST. Lectins were used at limiting concentrations to avoid nonspecific binding. Control strips were incubated in the same conditions but without lectin. After three washes in PBST binding was detected using a horseradish peorxidase–streptavidin conjugate (Vector Labs) and visualized by chemiluminescence using the coumaric-luminol reaction (Sigma-Aldrich).

### 2.6. Anti-phosphoserine dot-blot assay

The presence of phosphorylated serine residues in scalarin and ovorubin was determined by a dot-blot analysis using rabbit anti-phosphoserine antibody (Ab1603 — Chemicon Intl., Temecula, CA,

USA). Spots of 250 ng of each protein were adsorbed onto a nitrocellulose membrane (GE Healthcare, Uppsala, Sweden). After blocking for 1 h at 37 °C with 5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) in PBST, the membrane was incubated for 2 h at room temperature with the anti-phosphoserine antibody, diluted 1:250 with 3% (w/v) BSA in PBST. The presence of specific reactivity was visualized by chemiluminiscence as described above.

### 2.7. Chemical deglycosylation

Proteins were deglycosylated using trifluoromethanesulfonic acid (TFMS) (Sigma-Aldrich) following manufacturer directions. Briefly: 1 mg of each lyophilized protein was incubated for 3 h on ice with 150 µL of pre-cooled 10% anhydrous anisole in TFMS; after incubation the acid was neutralized by adding 60% pyridine drop wise, keeping the samples at about −15 °C using chilled 96% ethanol.

Addition of water to the neutralized mix caused protein precipitation which was transferred and washed few times with $ddH_2O$ and finally redissolved in 1.5 M Tris/HCl pH 8.5, 0.2% SDS, 0.2 mM DTT (PAGE sample buffer). Results were analyzed by SDS-PAGE and 2-DE. Complete deglycosylation was checked by staining blotted samples with Amersham ECL glycoprotein detection system (GE Healthcare).

### 2.8. Enzymatic deglycosylation

Enzymatic N-deglycosylation was assayed under native and denatured conditions, in the latter the protein was incubated for 5 min at 95 °C, in the supplied protein denaturation buffer (NE Biolabs, Beverly, MA, #P0745) [0.04 M DTT, 0.5% SDS]. In these experiments, PNGaseF (NE Biolabs, #P0745) was added to native or previously denatured glycoprotein (100 UE/µg glycoprotein) and incubated for 20 h at 37 °C in the supplied buffers; RNAse B (NE Biolabs) was treated similarly as a positive control. Sialic acid digestion was assayed in native and denaturing conditions by adding neuraminidase from *Clostridium perfringens* (NE Biolabs, #P07205) to a solution of glycoprotein in the supplied buffer (50 UE/µg of glycoprotein). The mixture was incubated for 20 h at 37 °C using fetuin (Sigma-Aldrich) as a positive control. Results were analyzed by native or SDS-PAGE and by 2-DE.

### 2.9. Two-dimensional electrophoresis (2-DE)

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

### 2.10. Statistical analysis

All experiments were performed in triplicate, unless otherwise stated. Results are expressed as mean ± standard deviation.

## 3. Results

### 3.1. Monosaccharide composition

Native scalarin, isolated by ultracentrifugation and purified by FPLC, showed a single band of 380 kDa in native PAGE.

GC analysis of monosaccharides released from scalarin showed that monosaccharides composition was dominated by Gal (33.86%) and Man (28.86%) followed by GlcNAc (16.78%) and Fuc (7.09%) (Table 1). Fuc content was also determined for ovorubin and accounts for 7.81%.

Sialic acid content, as determined by the ferric orcinol assay after mild acid hydrolysis, is 0.10% in scalarin and 0.12% in ovorubin.

### 3.2. Analysis of N- and O-linked oligosaccharides

O- and N-linked glycans were released from scalarin by β-elimination and NaOH treatment, respectively and separated by size exclusion chromatography.

The elution profile of both treatments is depicted in Fig. 1, which shows the presence of 4 N-linked (Fig. 1A) and 2 O-linked (Fig. 1B) glycans.

### 3.3. Lectin analysis

Scalarin glycosylation patterns were investigated by lectin dot-blot analysis. Table 2 lists the lectins employed and their reactivity to scalarin oligosaccharides; data on lectin binding to ovorubin are shown for comparison (taken from Dreon et al., 2004).

Reinforcing β-elimination results, N-glycosylation was also demonstrated by the binding of ConA and WGA lectins, which recognize oligo- or high mannose and hybrid-type structures. Complex-type N-glycans binding lectins (PSA and LCA) did not bind indicating these structures are not present. Poly N-acetyllactosaminyl elongation was suggested by DSL, ECL and RCA binding, the latter indicating terminal β-galactosyl groups. The presence of sialylated N-glycans was suggested by the binding of PVL.

As observed by β-elimination and exhaustive enzymatic N-linked deglycosylation results (see below), extensive O-linked glycosylation was shown by the strong JAC binding. This lectin, together with PNA indicates the presence of the T-antigen (Gal β1,3-GalNAc), although JAC also binds the sialylated form of the disaccharide. Tn antigen (GalNAc α-O-Ser/Thr) was detected by SBA binding. UEA1 binding revealed the presence of α1,2-L-fucosyl residues. Nonreducing terminal α-GalNAc was shown by DBA and BSL-1 binding.

### 3.4. Enzymatic deglycosylation

Native scalarin and ovorubin were resistant to PNGase F digestion, a result usually due to steric hindrance. When both proteins were

**Table 1**
Monosaccharide composition of scalarin and ovorubin egg perivitellins.

| | SC | OR[a] |
|---|---|---|
| Gal | 33.86 | 16.33 |
| Man | 28.86 | 44.62 |
| Glc | 5.39 | 1.01 |
| Xyl | 2.60 | 4.87 |
| Fuc | 7.09 | 7.81 |
| GalNAc | 2.50 | 3.45 |
| GlcNAc | 16.78 | 19.57 |
| Sias[b] | 0.12 | 0.10 |
| Others | 2.80 | 2.23 |

Values expressed as % by wt.
[a] Dreon et al., 2004.
[b] Determined by ferric orcinol assay after release by mild acid hydrolysis (Manzi and Esko, 1995).

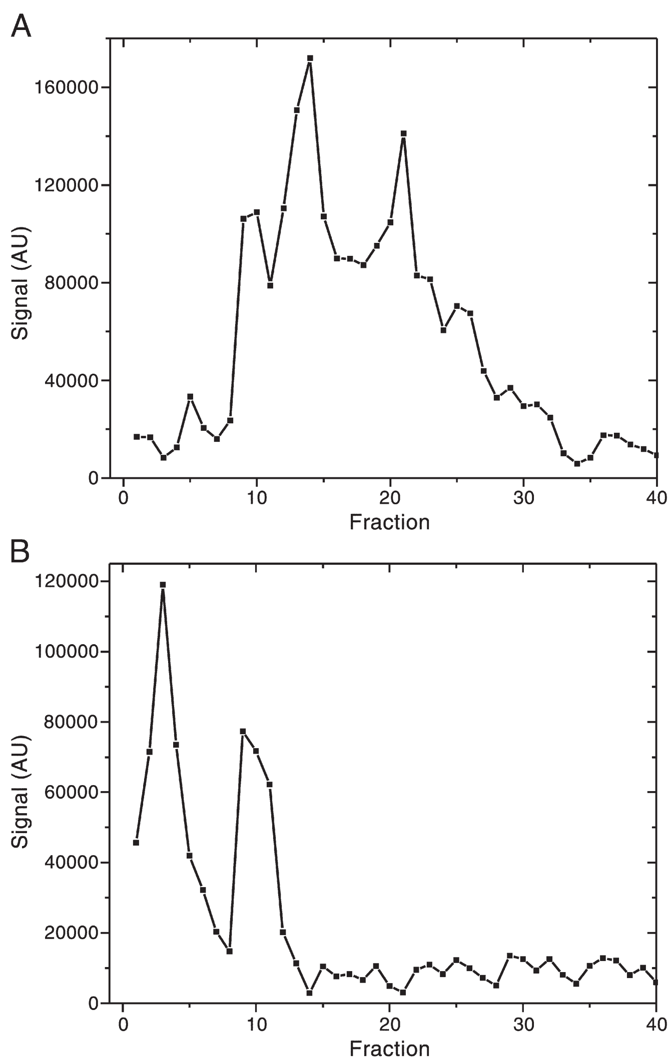


**Fig. 1.** Oligosaccharides released from scalarin and separated by gel filtration. (A) N-linked and (B) O-linked. Aliquots of 0.25 mL from each fraction were spotted on a silica gel-coated TLC plate. Void volume fractions were not plated. Fractions containing carbohydrates were detected using the orcinol/sulfuric acid reagent. The quantitation was done by densitometric analysis, using image analysis software (Sigmagel v. 1.0, Jendel).

denatured before the enzymatic treatment, a shift to lower MW in the electrophoretic pattern was observed in all their subunits indicating that they all contain N-glycans (Fig. 2). Deglycosylated scalarin showed three bands of ~32 kDa, ~27 kDa and ~25 kDa; deglycosylated ovorubin showed three bands of ~34 kDa, ~29 kDa, ~27 kDa (electrophoresis resolution was not enough to determine the exact MW shift of subunits).

Neuraminidase treatment of both perivitellins was assayed under both native and denatured conditions, but, unlike PNGase F treatment, no shifts were observed in SDS-PAGE (not shown). Moreover, 2-D electrophoresis of the neuraminidase treated samples showed no isoelectric point (IP) shifts (Appendix). A possible explanation for this negative result could be that the sialic structures present in these perivitellins (which were revealed by lectins and monosaccharide analysis) were resistant to neuraminidase. The lack of IP shift after the treatment could also be due to the presence of other posttranslational modifications (see Section 3.5).

### *3.5. Presence of glycoforms in scalarin and ovorubin subunits*

The high carbohydrate content of scalarin and ovorubin led us to consider that the 3 subunits previously described for both proteins could in fact be glycoforms (*i.e.* isoforms of the same polypeptide varying only in the amount and composition of glycans). In addition, the presence/absence of sialic acids often produces glycoforms with similar MW but different IP (Gravel, 2002). In order to investigate these hypotheses, the IP and MW of ovorubin and scalarin subunits were analyzed by 2-DE.

**Table 2**
Lectin binding of scalarin and ovorubin glycoproteins from *Pomacea* eggs.

| Lectin | Binding specificity[a] | Scalarin | Ovorubin[b] |
|---|---|---|---|
| Con A | Man α3[Man α6] man, terminal αMan; oligomannose-type N-glycan; hybrid-type N-glycan | + | + |
| WGA | Neu5Acα3/6/8 R; | + | + |
| PSA | Terminal α-D-mannosyl residues; *N*-acetylchitobiose-linked Fuc | – | – |
| LCA | Bi- and triantennary complex-type N-glycan | – | – |
| DSL | Terminal LacNAc | + | + |
| ECL | Terminal LacNAc | + | – |
| RCA 1 | Terminal βGal | + | – |
| PVL | GlcNAc Neu5Ac α26-Gal | + | – |
| JAC | Neu5Ac α 3 Gal β13-GalNAc; Gal β13-GalNAc | + | + |
| PNA | Terminal Gal β13-GalNAc | + | – |
| SBA | Tn antigen, terminal α GalNAc | + | – |
| UEA1 | α(1,2) L-Fuc | + | + |
| DBA | GalNAc α1,3 GalNAc/Gal | + | + |
| BSL 1 | αGalNAc>αGal | + | – |

[a] Taken from Cummings and Etzler (2009), Mao et al. (2004) and Matsumura et al., (1993).
[b] Taken from Dreon et al. (2004).

In both proteins a complex pattern of multiple spots was found (Fig. 3A, B). The spots are arranged in at least three groups of similar MW, which are coincident with the MW of the three subunits reported in 1D-PAGE. This would indicate that each of the previously identified subunits possesses several IP isoforms.

Ovorubin subunits show several IP isoforms with IP values ranging from ~5.0 to ~8.7 (Fig. 3A). Namely, the 35 kDa subunit has about 12 IP isoforms (IPs between 5.0 and 8.7); the 32 kDa subunit 1 predominant form (IP = 5.1) and the 28 kDa subunit displayed 5 isoforms (IPs between 5.5 and 8.2).

Scalarin subunits also show several isoforms with IP values in a range similar as that of ovorubin, from ~5.4 to ~9.1 (Fig. 3B). Scalarin 35 kDa subunit shows about 4 isoforms (IPs between 7.4 and 9.0); the 28 kDa subunit has 5 isoforms (IPs between 5.9 and 9.1) while the 24 kDa subunit has about 6 isoforms (IPs between 5.4 and 9.1).

To further confirm the presence of glycoforms, complete deglycosylation of scalarin and ovorubin was performed by TFMS treatment. This treatment reduced subunit heterogeneity to a single 25 kDa band for ovorubin and 27 kDa for scalarin in SDS-PAGE (Fig. 2B.). These protein bands did not react with the ECL glycoprotein detection system, indicating a complete loss of glycans.

When the deglycosylated proteins were analyzed by 2-DE a striking simplification of the spot pattern was also found (Fig. 3C, D), although part of the IP heterogeneity persisted. Both deglycosylated proteins showed at least 4 spots, all within a MW of ~25 kDa. Ovorubin showed 4 spots with an acidic IP (between 5.5 and 6.5), and one large spot of basic IP (~9.7) (Fig. 3C). Similarly, scalarin showed 3 acidic spots (IPs between 5.7 and 6.9) and a large basic spot (IP = 9.2) (Fig. 3D). If we attribute some of the multiple IP isoforms to the charged Sia residues, then the large basic spots which are seen after deglycosylation may represent those isoforms which lost Sia, being the shift due to the loss of negative charges. The more acidic spots, which persist after deglycosylation, may be due to some other charged posttranslational modification (see below).

Both proteins were phosphorylated, as revealed by the specific binding of polyclonal anti-phosphoserine antibody, being the scalarin signal higher than that of ovorubin (results not shown). Serine phosphorylation may be responsible for part of the subunit IP heterogeneity of these proteins, explaining the acidic spots observed after complete deglycosylation.

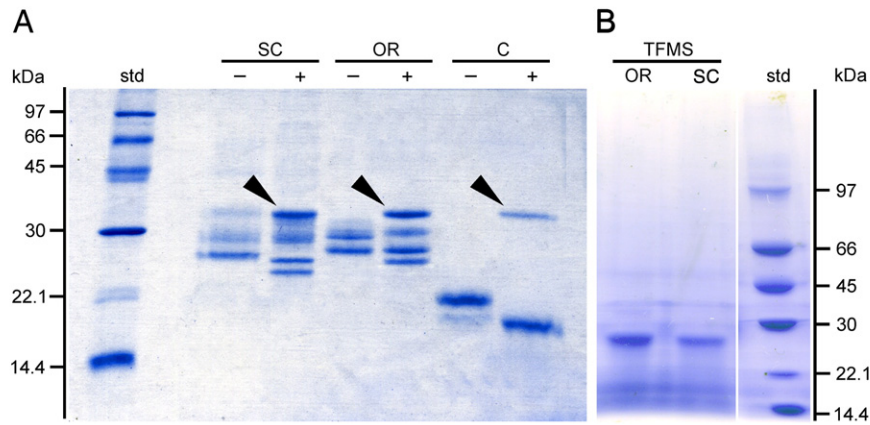


**Fig. 2.** (A) Effect of PNGase F treatment on previously denatured ovorubin and scalarin, analyzed on 15–20% SDS-PAGE. Std: molecular mass markers; SC: scalarin; OR: ovorubin; C: control (RNAse); arrowheads indicate the PNGase F band; − and + indicate absence/presence of enzyme respectively. (B) Effect of chemical deglycosylation on ovorubin and scalarin, analyzed on 4–20% SDS-PAGE. Std: molecular mass markers; SC: scalarin; OR: ovorubin.

## 4. Discussion

Whereas the amino acid sequence of a protein is encoded in the genome, posttranslational modifications depend on which enzymes and substrates are present in the cell. Amongst such modifications glycosylation contributes not only to physical properties, such as conformational stability, protease resistance, charge or hydrophilicity, but glycans may also function as recognition determinants in host–pathogen relationships, protein targeting and cell–cell interactions (Elbein, 1987; Rademacher et al., 1988).

In order to better understand how glycans contribute to the multiple roles of perivitellins in embryo development, the carbohydrates and

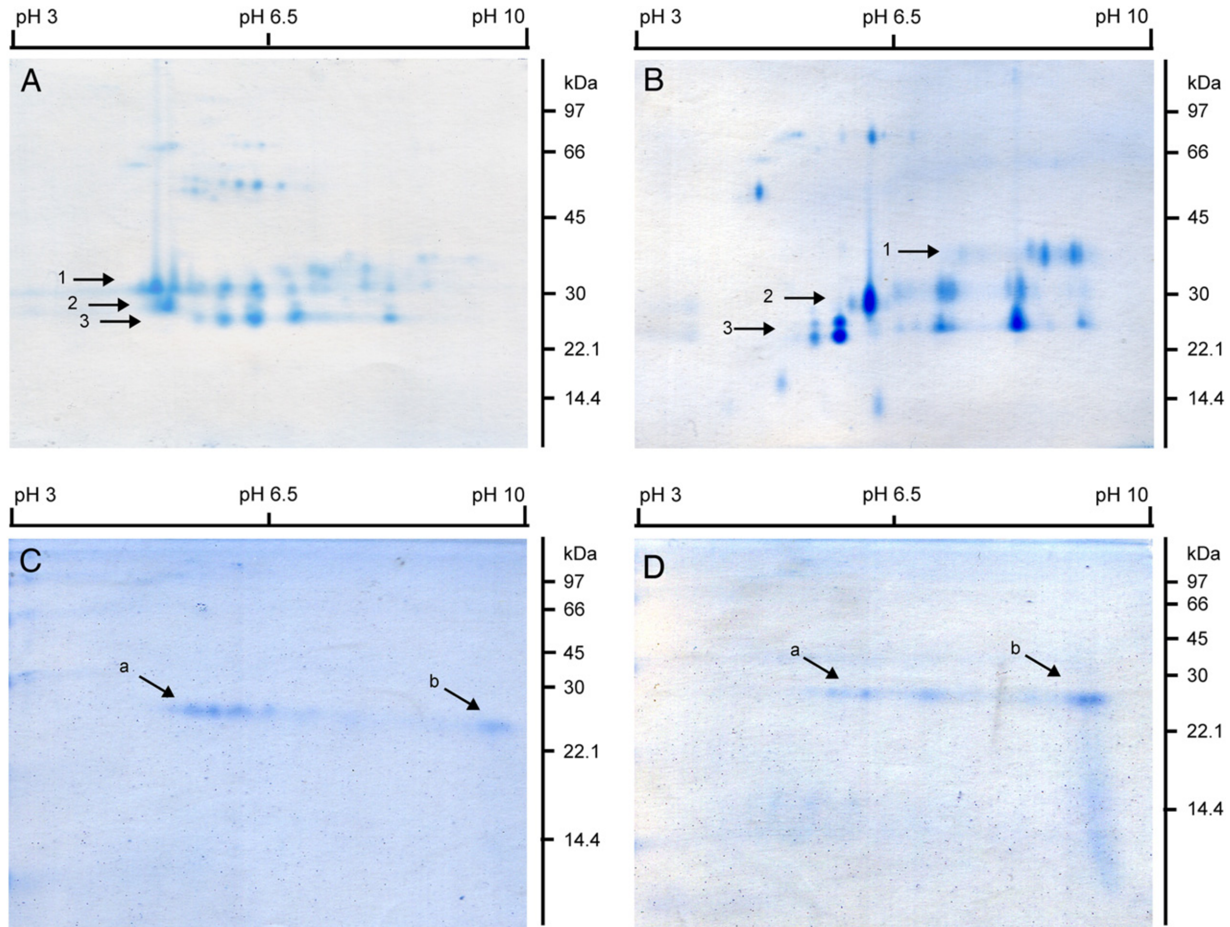


**Fig. 3.** 2-DE analysis of scalarin and ovorubin egg perivitellins. (A) Ovorubin subunits; 1: IP isoforms of the 35 kDa subunit, 2: IP isoforms of the 28 kDa subunit, and 3: IP isoforms of the 28 kDa subunit. (B) Scalarin subunits; 1: IP isoforms of the 35 kDa subunit, 2: IP isoforms of the 28 kDa subunit, and 3: IP isoforms of the 28 kDa subunit. (C) Chemically deglycosylated ovorubin; a: acidic IP spots, and b: basic IP spots. (D) Chemically deglycosylated scalarin; a: acidic IP spots, and b: basic IP spots.

glycoforms of scalarin from *P. scalaris* were characterized and compared with those of ovorubin from the eggs of the related species *P. canaliculata*.

Lectin binding analysis of scalarin showed the presence of oligomannose and hybrid-type N-glycans, as previously described in ovorubin, but with lactosamine elongation, which is absent in ovorubin. Another interesting shared feature of scalarin and ovorubin is the presence of N-glycans in all of their subunits, as shown with PNGase F digestion. O-glycans also appear to display similar structures in both proteins, with the presence of T and Tn antigen, the former probably sialylated.

Monosaccharide composition showed that the predominant monosaccharides of scalarin are Gal and Man, coincident with the lectin and chemical deglycosylation analysis, showing abundant N-glycans, whereas in ovorubin Man and GlcNAc are the predominant carbohydrates (Dreon et al., 2004). Fucose was found in both perivitellins in agreement with UEA-1 binding to fucosylated structures. This sugar is important in recognition processes and may be involved in perivitellin uptake during embryogenesis, as it has been reported in other glycoproteins (Varki and Lowe, 2009). In this regard, it has been reported that *P. canaliculata* embryos take up egg proteins differentially according to developing stage (Heras et al., 1998). Among the minor components determined is Xyl, not previously reported in Caenogastropoda. This pentose, classically regarded as characteristic of plants, was reported in some pulmonate snails and it might be a characteristic of other gastropods N-glycans too (Staudacher et al., 1999; Gutternigg et al., 2007).

Sialic acids in gastropods have been detected only in pulmonates, where sialylation is similar to mammals, and so far no polysialic acid has been found in snails (Burgmayr et al., 2001). The presence of Sias in both ovorubin and scalarin thus extends the occurrence of these charged glycans to gastropods other than Pulmonata. The varied biological functions attributed to Sias include conformational stabilization and protection of the glycoprotein against protease attack, and the enhancement of the viscosity of glycoproteins like mucins (Varki et al., 2009). These functions have been reported in ovorubin (Heras et al., 2007; Dreon et al., 2008). Thus, the presence of Sias might contribute to the high chemical and thermal stability reported for ovorubin (Heras et al., 2007; Dreon et al., 2008) and scalarin, which has a thermal stability measured up to 80 °C (Ituarte, unpublished results). On the other hand, the high viscosity of the PVF resulting from highly-concentrated perivitellins containing Sias (up to 13% protein, dry wt in *P. canaliculata* (Heras et al., 1998)) may represent a barrier against microbial invasion, similarly to the role suggested for ovomucin in hen egg (Stevens, 1996). More comparative studies will reveal if sialylation is also a characteristic of other snail perivitellins.

Our attempt to remove the Sias with *C. perfringens* sialidase was unsuccessful, even though this enzyme has a broad specificity. Natural sialidase-resistant derivatives of Sias have been reported in glycoproteins from horse and pig saliva (Nohle et al., 1985), this could also be the case of apple snail perivitellins. Moreover, Gutternigg et al. (2007) reported the presence of exoglycosydase-resistant glycans (containing 3-O-methylation of terminal hexoses) in pulmonate snails, and interpreted them as a possible way to shield the glycoprotein and protect it from digestion. This could explain the high *in vitro* resistance to pepsin reported in ovorubin, and observed in scalarin (unpublished results), and would reinforce the current interpretation of this resistance as an antinutritive defense of the egg (Dreon et al., 2008). Thus, glycosylation would shield ovorubin from the action of proteases allowing it to exert its potent trypsin inhibition (Norden, 1972) rendering egg proteins indigestible to the predator. This and other mechanisms (Heras et al., 2008) may explain why the eggs of *Pomacea* snails are almost devoid of natural predators worldwide (Snyder and Snyder, 1971; Yusa, 2001).

Chemical deglycosylation indicates that the previously described subunits of both perivitellins are glycoforms apparently of the same polypeptide, with MW and IP differences due to variations in the glycan moiety. Since the attachment of a particular glycan to a glycosylation site in the protein depends on the presence of a precise set of glycosyltransferases and glycans, this always generates some degree of heterogeneity in glycoproteins. In the case of scalarin and ovorubin, this variability is quite extensive — probably due to the unusually high content of glycans and involves IP isoforms, which could be attributed to the presence of Sias since most of these isoforms are not present in the completely deglycosylated samples. In addition, the fact that both proteins are phosphorylated may also contribute to the IP heterogeneneity. The presence of potential phosphorylation sites was reported in the sequence of a perivitellin from the pulmonate *Helysoma duryi*, although phosphorylation was not confirmed (Mukai et al., 2004). The presence of phosphates in perivitellins could represent a phosphorous reserve for the embryo, as it has been established in other egg proteins such as phosvitins (Anton et al., 2006).

The differences in glycosylation patterns found between the main perivitellins of *P. scalaris* and *P. canaliculata* agree with the previously reported lack of cross-reactivity (Ituarte et al., 2008). However these different patterns are not paralleled by functional differences, probably because they reside in the fine structure of the glycans, which is known are often not as important for protein function as the presence or absence of the bulk glycan itself (Taylor and Drickamer, 2003).

As a whole, it seems that the high glycosylation of these perivitellins is tightly related to their functions. The sugars may also have a function *per se*, as a source of energy and structural precursors for the developing embryo, like it was demonstrated for the protein moiety (Heras et al., 1998).

Molluscan glycobiology is at an early stage of development, and only few egg-glycoproteins have been well studied to date. The present results are coincident with previous works reporting a mixture of structural elements from both vertebrates and invertebrates in snails (Burgmayr et al., 2001; Gutternigg et al., 2004; Gutternigg et al., 2007). Although we are far from understanding the role of perivitellin glycans in *Pomacea* embryo development, it is evident that a number of interesting processes are at play. Ongoing research is looking at the primary structure of these perivitellins to better understand the structure–function relationships of these particles essential in embryogenesis.

## Acknowledgements

This work was partially supported by grants from CONICET PIP no. 5888 and ANPCyT no. 2166. MSD is member of Carrera del Investigador CICBA, Argentina. HH is member of Carrera del Investigador CONICET, Argentina. SI and MYP are doctoral fellows of CONICET, Argentina. HH is professor at Universidad Nacional de La Plata, Argentina. We are grateful to Dr. E.J. Gimeno and Dr. C. Mundiña for kindly supplying the biotynilated lectins and anti-phosphoserine antibodies, respectively. We also wish to thank the anonymous reviewers for their valuable comments on the manuscript.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbpb.2010.05.004.

## References

Albrecht, E.A., Carreño, N.B., Castro-Vazquez, A., 1999. A quantitative study of environmental factors influencing the seasonal onset of reproductive behaviour in the south American apple-snail *Pomacea canaliculata* (Gastropoda: Ampullariidae). J. Mollusc. Stud. 65, 241–250.

Albrecht, E.A., Koch, E., Carreño, N.B., Castro-Vazquez, A., 2004. Control of the seasonal arrest of copulation and spawning in the apple snail *Pomacea canaliculata* (Prosobranchia: Ampullariidae): differential effects of food availability, water temperature, and day length. Veliger 47, 169–174.

Anton, M., Castellani, O., Guérin-Dubiard, C., 2006. Phosvitin. In: Huopalahti, R., López-Fandiño, R., Anton, M., Schade, R. (Eds.), Bioactive Egg Compounds. Springer-Verlag, Heidelberg, pp. 17–24.

Berthold, T., 1991. Vergleichende Anatomie, Phylogenie und historische Biogeographie der Ampullariidae (Mollusca: Gastropoda). Abhand. Naturwiss. Ver. Hamburg (NF) 29, 1–256.

Burgmayr, S., Grabher-Meier, H., Staudacher, E., 2001. Sialic acids in gastropods. FEBS Letts. 508, 95–98.

Carlsson, N.O.L., Brönmark, C., Hansson, L.A., 2004. Invading herbivory: the golden apple snail alters ecosystem functioning in Asian wetlands. Ecology 85, 1575–1580.

Cummings, R.D., Etzler, M.E., 2009. Antibodies and lectins in glycan analysis. In: Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.S., Hart, G.W., Etzler, M.E. (Eds.), Essentials of Glycobiology. Cold Spring Harbor Laboratory Press, New York, pp. 633–648.

Dreon, M.S., Heras, H., Pollero, R.J., 2004. Characterization of the major egg glycolipoproteins from the perivitellin fluid of the apple snail *Pomacea canaliculata*. Mol. Reprod. Dev. 68, 359–364.

Dreon, M.S., Ceolín, M., Heras, H., 2007. Astaxanthin binding and structural stability of the apple snail carotenoprotein ovorubin. Arch. Biochem. Biophys. 460, 107–112.

Dreon, M.S., Ituarte, S., Ceolin, M., Heras, H., 2008. Global shape and pH stability of ovorubin, an oligomeric protein from the eggs of *Pomacea canaliculata*. FEBS J. 275, 4522–4530.

Elbein, A.D., 1987. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. Annu. Rev. Biochem. 56, 497–534.

Estebenet, A.L., Cazzaniga, N.J., 1993. Egg variability and the reproductive strategy of *Pomacea canaliculata* (Gastropoda: Ampullariidae). Apex 8, 129–138.

Estebenet, A.L., Martín, P.R., 2002. *Pomacea canaliculata* (Gastropoda: Ampullariidae): life-history traits and their plasticity. Biocell 26, 83–89.

Fukuda, M., 1995. β-elimination for release of o-GalNAc-linked oligosaccharides from glycoproteins and glycopeptides. Current Protocols in Molecular Biology. John Wiley & Sons, Inc, New Jersey. 17.15.4–17.15.9.

Garín, C.F., Heras, H., Pollero, R.J., 1996. Lipoproteins of the egg perivitellin fluid of *Pomacea canaliculata* snails (Mollusca: Gastropoda). J. Exp. Zool. 276, 307–314.

Görg, A., Postel, W., Günther, S., 1988. The current state of two dimensional electrophoresis with immobilized pH gradients. Electrophoresis 9, 531–546.

Gravel, P., 2002. Identification of glycoproteins on nitrocellulose membranes using lectin blotting. In: Walker, J.M. (Ed.), The Protein Protocols Handbook. Humana Press Inc., New Jersey, pp. 779–793.

Gutternigg, M., Ahrer, K., Grabher-Meier, H., Burgmayr, S., Staudacher, E., 2004. Neutral N-glycans of the gastropod Arion lusitanicus. Eur. J. Biochem. 271, 1348–1356.

Gutternigg, M., Burgmayr, S., Poltl, G., Rudolf, J., Staudacher, E., 2007. Neutral N-glycan patterns of the gastropods *Limax maximus*, *Cepaea hortensis*, *Planorbarius corneus*, *Arianta arbustorum* and *Achatina fulica*. Glycoconj. J. 24, 475–489.

Harlow, E., Lane, D., 1988. Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

Hayes, K.A., Cowie, R.H., Thiengo, S.C., 2009. A global phylogeny of apple snails: Gondwanan origin, generic relationships, and the influence of outgroup choice (Caenogastropoda: Ampullariidae). Biol. J. Linn. Soc. 98, 61–76.

Heras, H., Garín, C.F., Pollero, R.J., 1998. Biochemical composition and energy sources during embryo development and in early juveniles of the snail *Pomacea canaliculata* (Mollusca: Gastropoda). J. Exp. Zool. 280, 375–383.

Heras, H., Dreon, M.S., Ituarte, S., Pollero, R.J., 2007. Egg carotenoproteins in neotropical Ampullariidae (Gastropoda: Arquitaenioglossa). Comp. Biochem. Physiol. C 146, 158–167.

Heras, H., Frassa, M.V., Fernández, P.E., Galosi, C.M., Gimeno, E.J., Dreon, M.S., 2008. First egg protein with a neurotoxic effect on mice. Toxicon 52, 481–488.

Iijima, R., Kisugi, J., Yamazaki, M., 1995. Antifungal activity of aplysianin E, a cytotoxic protein of sea hare (*Aplysia kurodai*) eggs. Dev. Comp. Immunol. 19, 13–19.

Ituarte, S., Dreon, M.S., Pollero, R.J., Heras, H., 2008. Isolation and partial characterization of a new lipo-glyco-carotenoprotein from *Pomacea scalaris* (Gastropoda: Ampullariidae). Mol. Reprod. Dev. 75, 1441–1448.

Jong-Brink, M., Boer, H.H., Joosse, J., 1983. Mollusca. In: Adiyodi, K.G., Adiyodi, R.G. (Eds.), Reproductive Biology of Invertebrates. : Oogenesis Oviposition and Oosorption, 1. John Wiley & Sons, New York, pp. 297–355.

Kamiya, H., Sakai, R., Jimbo, M., 2006. Bioactive molecules from sea hares. In: Cimino, G., Gavagnin, M. (Eds.), Molluscs. From Chemo-ecological Study to Biotechnological Application, Vol. 43. Springer-Verlag, Berlin, pp. 215–239.

Kisugi, J., Yamazaki, M., Ishii, Y., Tansho, S., Muramoto, K., Kamiya, H., 1989. Purification of a novel cytolytic protein from the albumen gland of the sea hare, *Dolabella auricularia*. Chem. Pharm. Bull. 37, 2773–2776.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lv, S., Zhang, Y., Liu, H.X., Hu, L., Yang, K., Steinmann, P., Chen, Z., Wang, L.Y., Utzinger, J.+., Zhou, X.N., 2009. Invasive snails and an emerging infectious disease: Results from the first national survey on *Angiostrongylus cantonensis* in China. PLoS Negl. Trop. Dis. 3, e368.

Manzi, A.E., 1995a. Total Compositional Analysis by High-Performance Liquid Chromatography or Gas–Liquid Chromatography. Current Protocols in Molecular Biology. John Wiley & Sons, Inc, New Jersey. 17.19.1–17.19.25.

Manzi, A.E., 1995b. Acid hydrolysis for release of monosaccharides. Current Protocols in Molecular Biology. John Wiley & Sons, Inc, New Jersey. 17.16.1–17.16.11.

Manzi, A.E., Esko, J.D., 1995. Direct chemical analysis of glycoconjugates for carbohydrates. Current Protocols in Molecular Biology. John Wiley & Sons, Inc. 17.9.1–17.9.11.

Mao, X., Luo, Y., Dai, Z., Wang, K., Du, Y., Lin, B., 2004. Integrated lectin affinity microfluidic chip for glycoform separation. Anal. Chemy 76, 6941–6947.

Matsumura, K., Nakasu, S., Nioka, H., Handa, J., 1993. Lectin histochemistry of normal and neoplastic peripheral nerve sheath. 1. Lectin binding pattern of normal peripheral nerve in man. Acta Neuropathol. 86, 554–558.

Mukai, S.T., Hoque, T., Morishita, F., Saleuddin, A.S., 2004. Cloning and characterization of a candidate nutritive glycoprotein from the albumen gland of the freshwater snail, *Helisoma duryi* (Mollusca: Pulmonata). Invertebr. Biol. 123, 83–92.

Nohle, U., Shukla, A.K., Schroder, C., Reuter, G., Schauer, R., Kamerling, J.P., Vliegenthart, J.F., 1985. Structural parameters and natural occurrence of 2-deoxy-2, 3-didehydro-N-glycoloylneuraminic acid. Eur. J. Biochem. 152, 459–463.

Norden, D.A., 1972. The inhibition of trypsin and some other proteases by ovorubin, a protein from the eggs of *Pomacea canaliculata*. Comp. Biochem. Physiol. B 42, 569–576.

Pechenik, J.A., 1986. The encapsulation of eggs and embryos by Molluscs: an overview. Am. Malacol. Bull. 4, 165–172.

Przeslawski, R., 2004. A review of the effects of environmental stress on embryonic development within intertidal gastropod egg masses. Moll. Res. 24, 43–63.

Przeslawski, R., 2005. Combined effects of solar radiation and desiccation on the mortality and development of encapsulated embryos of rocky shore gastropods. Mar. Ecol. Prog. Ser. 298, 169–177.

Rademacher, T.W., Parekh, R.B., Dwek, R.A., 1988. Glycobiology. Annu. Rev. Biochem. 57, 785–838.

Rawlings, T.A., Hayes, K.A., Cowie, R.H., Collins, T.M., 2007. The identity, distribution, and impacts of non-native apple snails in the continental United States. BMC Evol. Biol. 7, 97.

Sanchez, J.F., Lescar, J., Chazalet, V., Audfray, A., Gagnon, J., Alvarez, R., Breton, C., Imberty, A., Mitchell, E.P., 2006. Biochemical and structural analysis of *Helix pomatia* agglutinin. A hexameric lectin with a novel fold. J. Biol. Chem. 281, 20,171–20,180.

Snyder, N.F.R., Snyder, H.A., 1971. Defenses of the Florida apple snail *Pomacea paludosa*. Behaviour 40, 175–215.

Staudacher, E., Altmann, F., Wilson, I.B., Marz, L., 1999. Fucose in N-glycans: from plant to man. Biochim. Biophys. Acta 1473, 216–236.

Stevens, L., 1996. Egg proteins: what are their functions? Sci. Prog. 79, 65–87.

Taylor, M.E., Drickamer, K., 2003. Effects of glycosylation on protein structure and function. Introduction to Glycobiology. Oxford University Press Inc, New York, pp. 97–110.

Varki, A., Lowe, J.B., 2009. Biological roles of glycans. In: Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.S., Hart, G.W., Etzler, M.E. (Eds.), Essentials of Glycobiology. Cold Spring Harbor Laboratory Press, New York, pp. 75–89.

Varki, A., Freeze, H.H., Gagneux, P., 2009. Evolution of glycan diversity. In: Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.S., Hart, G.W., Etzler, M.E. (Eds.), Essentials of Glycobiology. Cold Spring Harbor Laboratory Press, New York, pp. 281–292.

Yusa, Y., 2001. Predation on eggs of the apple snail *Pomacea canaliculata* (Gastropoda: Ampullariidae) by the fire ant *Solenopsis geminata*. J. Mollus. Stud. 67, 275–279.