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Glycoproteins histochemistry of the gills of *Odontesthes bonariensis* (Teleostei, Atherinopsidae)

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The histochemistry of glycoproteins (GP) in the mucous cells of the gills of the silverside *Odontesthes bonariensis* was identified with: (1) oxidizable vicinal diols; (2) sialic acid and some of their chain variants, carbon 7 (^{7}C), carbon 8 (^{8}C) or carbon 9 (^{9}C); (3) sialic acid residues without O-acyl substitution and with O-acyl substitution at ^{7}C, ^{8}C or ^{9}C; (4) carboxyl groups and (5) sulphate groups. A battery of seven biotinylated lectins allowed GPs sugar residues to be distinguished. Mucous cells showed the presence of neutral, sulphated and sialylated GPs. *Dolichos biflorus* agglutinin (DBA) and *Glycine max* agglutinin (SBA) showed strong positive staining; *Arachis hypogaea* agglutinin (PNA), *Ricinus communis* agglutinin-I (RCA-I) and *Triticum vulgaris* agglutinin (WGA) showed moderate staining, while *Ulex europaeus* agglutinin-I (UEA-I) was completely negative.
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Key words: histochemical reactions; mucous cells; silverside; teleosts.

INTRODUCTION

The silverside *Odontesthes bonariensis* (Valenciennes) is a commercially important fish species in South America intensively cultivated in France, Israel, Italy and Japan (Strüssmann & Takashima, 1990). This species is probably one of the most captured, not only for commercial purposes but also for sport fishing in pampean ponds and reservoirs in Argentina. Bacteria, viruses, parasites, nutrients and water pollution can produce severe mortality or reduce growth of the fish. In determining fish diseases caused by different agents, it is necessary to know *a priori* the fish's normal morphology. Most studies previously carried out include the estimation of *O. bonariensis* stocks of eggs, larvae, juveniles and adults (Somoza *et al*., 2008), and the histology (De Carlo & López, 1957) and histochemistry of the gut (Díaz *et al*., 2006).

The general morphology of gill cells (Wilson & Laurent, 2002) and the histochemistry of the mucosubstances from fish gills and the different types of mucous cells in the gill epithelium have been described by Sabóia-Moraes *et al*. (1996),

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Díaz *et al*. (2005*a*, *b*) and Çinar *et al*. (2008). The glycoproteins (GP), as components of the mucosubstances, are known to have a large variety of functions, from merely mechanical, through antimicrobial and antiviral, to osmotic (Allen, 1981). In *O. bonariensis*, however, no information is available on either mucous cells or their secretions.

The purpose of this work was to describe the morphology of the mucous cells paying special attention to the glycoproteins secreted by the gills of *O. bonariensis*. It was intended to give a deeper insight into the morphological and functional aspects of the gills and to provide a basis for the species culture.

This study was performed with a battery of seven lectins and histochemical traditional treatments. This is part of a series of investigations of glycoproteins in the gills of several fish species of high commercial value that are being carried out (Díaz *et al*., 2001, 2005*a*, *b*, 2009).

MATERIALS AND METHODS

Five female and five male *O. bonariensis* [mean ± S.D. standard length (L_S) 24·0 ±2·0 cm; mean ± S.D. mass (M) 180·0 ± 5·0 g] were collected from Los Padres Lake, Province of Buenos Aires, Argentina (37° 56′ 30″ S; 57° 44′ 30″ W), and immediately transported to the laboratory. The handling, collection and killing of all individuals followed the guidelines of the American Fisheries Society (AFS, 2004). Fish were sacrificed by cervical dislocation. The gills were rapidly removed from the fish and samples were fixed by immersion in 10% buffered formalin before dehydration in an ethanol series, and then embedded in paraffin.

Sections (4–5 μm thick) were stained with routine haematoxylin and eosin (H-E), Masson's trichrome stain for morphology and Mayer's mucicarmine for mucin identification. Also, sections of tissue were subjected to histochemical techniques for the localization and differentiation of carbohydrate moieties (Table I). Micrographs were taken with an Olympus microscope, CH 30 (Olympus; www.olympus.com).

Sections were stained with: (1) periodic acid Schiff reagent (PAS) to demonstrate periodate-reactive vicinal diols; (2) the acetylation before PAS technique to block the oxidation of the 1,2 glycol groups by the periodic acid; (3) the acetylation, saponification, PAS sequence to restore the 1,2 glycol groups which react with the periodic acid; (4) α-amylase digestion before PAS reaction for a control of the presence of GPs with oxidizable vicinal diols; (5) periodic acid oxidation-phenylhydrazine-Schiff (PAPS). Following oxidation with 1% periodic acid for 2 h at room temperature, sections were treated with 0·5% phenylhydrazine hydrochloride for 2 h. They were then incubated with pararosaniline Schiff for 4 h. This method demonstrates the presence of GPs with sialic acids without O-acyl substitution and with O-acyl substitution at carbon 7 (^{7}C); (6) PA–Bh–KOH–PAS (periodic acid–borohydride reduction-saponification–periodic acid Schiff reaction): this method was carried out using a 2 h oxidation at room temperature with 1% periodic acid. The aldehydes generated by the initial oxidation were reduced to Schiff-unreactive primary alcohols with sodium borohydride (PA-Bh). Following saponification (KOH), sialic acids with O-acyl substituents at ^{7}C, carbon 8 (^{8}C) or carbon 9 (^{9}C) (or two or three side-chains O-acyl substituents) and O-acyl sugars are PAS positive; (7) KOH–PA*–Bh–PAS (saponification-selective periodic acid-borohydride reduction-periodic acid Schiff reaction) for the characterization of neutral sugars; (8) AB pH 2·5 (alcian blue 8GX pH 2·5): to demonstrate GPs with carboxyl groups (sialic acid or uronic acid) and with O-sulphate esters; (9) AB pH 1·0 (alcian blue 8GX pH 1·0) to demonstrate GPs with O-sulphate esters; (10) AB pH 0·5 (alcian blue 8GX pH 0·5) to demonstrate highly sulphated GPs; (11) AB pH 2·5/PAS (alcian blue 8GX pH 2·5 PAS staining): to demonstrate GPs with carboxyl groups and GPs with O-sulphate esters (turquoise), periodate-reactive vicinal diols (magenta), and presence of GPs with carboxyl groups and with GPs with O-sulphate esters together with periodate-reactive vicinal diols (purple); (12) TB

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TABLE I. Histochemical procedures for visualizing and identifying glycoproteins (GP) in the mucous cells of the gills of *Odontesthes bonariensis*

Procedures	Interpretation of staining reactions	References	Reactions
1. PAS	GPs with oxidizable vicinal diols and glycogen	McManus (1948)	+/++M
2. Acetylation–PAS	GPs with oxidizable vicinal diols and glycogen	Lillie & Fullmer (1976)	–
3. Acetylation–KOH–PAS	GPs with oxidizable vicinal diols and glycogen	Lillie & Fullmer (1976)	+/++M
4. α-amylase–PAS	GPs with oxidizable vicinal diols	Pearse (1985)	+/++M
5. PAPS	GPs with sialic acid residues without O-acyl substitution and with O-acyl substitution at ^{7}C	Reid & Park (1990)	+/++M
6. PA–Bh–KOH–PAS	Sialic acid residues with O-acyl substitution at ^{7}C, ^{8}C or ^{9}C and O-acyl sugars	Reid *et al*. (1973)	++/+++M
7. KOH–PA*–Bh–PAS	GPs with oxidizable vicinal diols and with O-acyl sugars	Volz *et al*. (1987)	++/+++M
8. AB pH 2·5	GPs with carboxyl groups and with O-sulphate esters	Lev & Spicer (1964)	+++T
9. AB pH 1·0	GPs with O-sulphate esters	Lev & Spicer (1964)	++T
10. AB pH 0·5	Highly sulphated GPs	Lev & Spicer (1964)	++T
11. AB pH 2·5/PAS	Same as 8 and 1	Mowry (1963)	+++P
12. TB pH 4·2	GPs with O-sulphate esters	Lison (1953)	++m
13. TB pH 5·6	GPs with O-sulphate esters and carboxyl groups	Lison (1953)	++m

AB, alcian blue; Bh, borohydride; M, magenta; m, metachromasia; PAS, periodic acid Schiff reagent; PAPS, periodic acid oxidation-phenylhydrazine-Schiff; PA, periodic acid; TB, toluidine blue; PA*, selective periodic acid oxidation; T, turquoise; P, purple. Staining intensity: –, negative reaction; +/++, feeble to moderate reactions; ++/+++, moderate to very strong reactions.

pH 4·2 (toluidine blue) to demonstrate GPs with O-sulphate esters; (13) TB pH 5·6 (toluidine blue) to demonstrate GPs with O-sulphate esters and carboxyl groups.

Labelling with biotinylated lectins was used to identify specific sugar residues of GPs. Lectin labelling was performed according to the method described by Gimeno *et al*. (1995). Briefly, paraffin wax sections were mounted on slides coated with poly-L-lysine (Sigma Diagnostics; www.sigma-aldrich.com). These were deparaffinized with xylene, then incubated in 0·3% H_2O_2 in methanol for 30 min at room temperature in order to block endogenous peroxidase activity. They were then hydrated through a graded ethanol series, washed in a phosphate-buffered saline (PBS) 0·01 M, pH 7·2 and incubated for 30 min with biotinylated lectins (Con-A, WGA, DBA, SBA, PNA, UEA-I and RCA-I). Table II lists the seven lectins used in this study, their sources and their major sugar specificities. All lectins were employed at a dilution of 30 mg ml^{-1} in PBS, except for PNA which was applied at a concentration of 10 mg ml^{-1}. Biotinylated lectins were purchased from Vector Laboratories Inc.

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TABLE II. Lectins used and their carbohydrate specificities

Lectin	Acronym	Specificity[a,b]
Canavalia ensiformis agglutinin	Con-A	α-D-Man; α-D-Glc
Triticum vulgaris agglutinin	WGA	β-D-GlcNAc; NeuNAc
Dolichos biflorus agglutinin	DBA	α-D-GalNAc
Glycine max agglutinin	SBA	α-D-GalNAc; β-D-GalNAc
Arachis hypogaea agglutinin	PNA	β-D-Gal (β1->3) D-GalNAc
Ulex europaeus agglutinin-I	UEA-I	α-L-Fuc
Ricinus communis agglutinin-I	RCA-I	β-Gal

[a]Goldstein & Hayes (1978).

[b]Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; NeuNAc, acetyl neuraminic acid (sialic acid).

(www.vectorlabs.com). Afterwards, sections were incubated for 30 min with ABC reagent, prepared according to manufacturer's instructions (Vectastain Elite PK 6200, Vector Laboratories Inc.). Lectin binding was revealed by incubation with 0·5 mg mol^{-1} diaminobenzidine tetrahydrochloride (DAB) (DAKO) in tris buffer 0·1 M, pH 7·2, plus 0·02% H_2O_2. All dilutions and thorough washes between steps were carried out using PBS unless otherwise stated. Two types of controls were performed: (1) lectin solution was replaced by PBS and (2) lectin labelling was performed as described above after 1 h pre-incubation of the lectins in the presence of the appropriate hapten sugars (0·2 M in PBS), as listed in Table II, at room temperature. No labelling was detected in control sections. Evaluation of labelling intensities was based on subjective estimates by all authors after examination of two sections per sample of all the animals tested.

RESULTS

HISTOLOGY

The gill arch structure of *O. bonariensis* is essentially similar to the majority of teleosts. It is composed of four holobranchs, each formed by two hemibranchs with gill filaments and secondary lamellae. The gill filaments receive vascular branches supported by fibroelastic tissue and are covered by a multilayered epithelium with mitochondria-rich cells and mucous cells. The secondary lamellae have an external layer of squamous epithelium with mucous cells that is supported by a thin non-cellular basement membrane under which blood spaces pass. Mucous cells have eccentrically placed nuclei and large cytoplasmic mucous vacuoles whose mucous discharge is performed by exocytosis.

HISTOCHEMISTRY

Histochemical reactions of the GPs from the epithelium of the gill filaments and secondary lamellae of *O. bonariensis* are summarized in Table I. Although the number of mucous cells was fewer in the secondary lamellae, no histochemical differences were detected between the mucous cells of the gill filaments and the secondary lamellae. Mucous cells with PAS showed a positive response; the colouration disappeared

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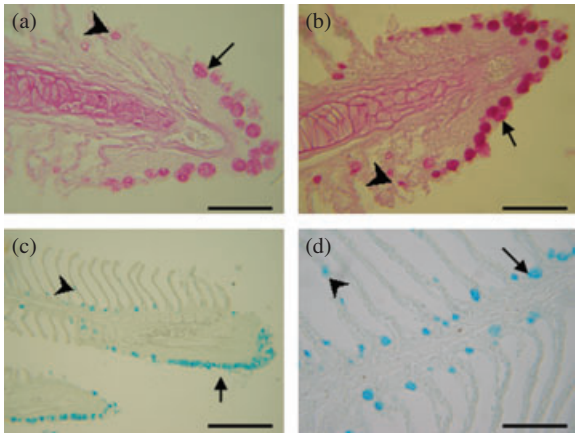

FIG. 1. Sections of *Odontesthes bonariensis* gills showing reactions to GPs in the mucous cells from gill filaments (➞) and secondary lamellae (➤). (a) Periodic acid Schiff's reaction; scale bar: 83 μm. (b) Periodic acid–borohydride reduction-saponification–periodic acid Schiff reaction; scale bar: 69 μm. (c) Alcian blue pH 2·5, scale bar: 226 μm. (d) Alcian blue pH 1·0; scale bar: 60 μm.

after acetylation and recovered after saponification [Fig. 1(a)]. Control sections subjected to α-amylase were positive to the PAS reaction. These reactions with the combination of the methods involving PAS indicate the presence of GPs with oxidizable vicinal diols. Staining with PA–P–S techniques revealed the presence of GPs with sialic acid residues without O-acyl substitution and with O-acyl substitution at ^{7}C. The reaction with the PA–Bh–KOH–PAS method indicated sialic acid residues with O-acyl substitution at ^{7}C, ^{8}C or ^{9}C [Fig. 1(b)]. Neutral GPs with oxidizable vicinal diols and GPs were demonstrated by the KOH–PA*–Bh–PAS. A procedure sequence using alcian blue at different pHs showed the presence of strong and weak sulphated GPs and GPs bearing carboxyl groups [Fig. 1(c), (d)]. Alcian blue pH 2·.5–PAS demonstrated the presence of neutral and acid mucopolysaccharide groups. With toluidine blue at pH 4·2 and pH 5·6, the mucous cells show metachromasia, indicating the presence of GPs with carboxyl groups and GPs with O-sulphate esters.

Seven kinds of biotinylated lectins were used in order to examine glycoprotein expression patterns in the mucous cells from the gills of *O. bonariensis*. The lectin histochemical results are presented in Fig. 2. Gill filaments and secondary lamellae showed the same distribution pattern as lectins of the mucous cells. With regard to the whole spectrum, DBA gave the strongest positive reaction; WGA, SBA, PNA and RCA-I gave moderate reactions; Con-A gave a weak reaction and UEA-I gave a negative reaction.

DISCUSSION

The general gill morphology of *O. bonariensis* is similar to those of teleosteans (Hughes, 1984; Hossler *et al*., 1986; Wilson & Laurent, 2002; Díaz *et al*., 2009). The presence of mucous secretory cells on the gill filaments and secondary lamellae is a common characteristic of fishes, although their distribution may vary among species

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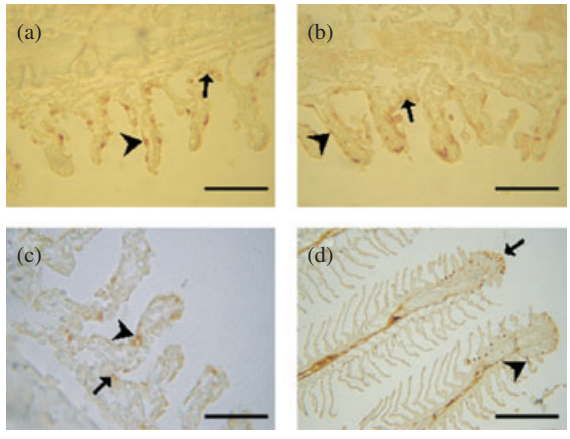

FIG. 2. Lectin histochemistry (see Table II) of the mucous cells from gill filaments (➞) and secondary lamellae (➤) of *Odontesthes bonariensis*. (a) *Glycine max* agglutinin (SBA); scale bar: 73 µm. (b) *Triticum vulgaris* agglutinin (WGA); scale bar: 77 µm. (c) *Canavalia ensiformis* agglutinin (Con-A); scale bar: 53 µm. (d) *Ricinus communis* agglutinin-I (RCA-I); scale bar: 258 µm.

(Ojha *et al*., 1987). In *O. bonariensis*, the gill filaments showed a greater number of mucous cells than the secondary or respiratory lamellae. It is possible that this distribution, on one hand reduces the thickness of the blood–water diffusion barrier, and on the other, avoids the particle retention by the mucus produced by the mucous cells (Eiras-Stofella *et al*., 2001).

The gill filaments and the secondary lamellae of *O. bonariensis* exhibited both neutral and acidic glycoproteins in the mucous cells, as demonstrated with conventional histochemistry. It emphasizes the predominance of GPs with oxidizable vicinal diols and sialoglycoproteins in the mucous cells. The results were comparable to *Cynoscion guatucupa* (Cuvier) (Díaz *et al*., 2005*a*) and *Scomber australasicus* Cuvier (Perera, 1993). In the gills of *O. bonariensis*, the presence of GPs with O-sulphate esters agrees with previous observations on the chemical composition of mucus in the gills of fishes such as *Poecilia vivipara* Bloch & Schneider (Sabóia-Moraes *et al*., 1996).

Acidic mucins, mainly sulphated mucins, have also been found in other freshwater fishes such as in the epidermal mucous cells of *Monopterus cuchia* (Hamilton) (Mittal *et al*., 1980, 1994*a*) and *Blennius pholis* L. (Whitear & Mittal, 1984).

A mixture of neutral and acidic glycoproteins, both sulphated and sialylated, has been found in the gut of the euryhaline fish species *Sparus aurata* L. (Domeneghini *et al*., 1998). Similar studies on lower vertebrates showed that the production of acidic GPs, mainly sulphated GPs, predominates in their mucous cells (Liquori *et al*., 2007). According to the environment, the mucous compounds differ in various teleost species (Reifel & Travill, 1978; Ostos-Garrido *et al*., 1993); identical species under different conditions have shown differences in the type of GPs produced (Solanki & Benjamin, 1982). Thus, Vigliano *et al*. (2006) have reported the presence of sulphated acid mucins in mucous cells from juvenile *O. bonariensis.* It has been postulated that sulphated GPs deter the proliferation of pathogenic micro-organisms in freshwater fishes which are more likely to become infected in this type of environment (Mittal *et al*., 1994*b*).

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It is well known that lectin histochemistry represents a sensitive method for detecting glycoproteins (Danguy *et al*., 1994). The method showed no marked differences between gill filaments and secondary lamellae. Lectin histochemistry differentiation of the GPs present in the mucous cells of the gills corroborates, to a certain extent, the view developed above. The intensity of the reaction varied with the use of the different lectins and, in particular, the strong DBA reactivity showed that the most abundant sugar residue present in the lamellae is N-acetylgalactosamine. In addition, the considerable presence of glycoproteins with N-acetylglucosamine and acetylneuraminic acid (WGA labelling), β-galactose residues (RCA-I labelling) and α-β-N-acetylgalactosamine (SBA labelling) was demonstrated, whereas scarce quantities of mannose (Con-A labelling) and no fucose (UEA-I) residues were evident. The residues of D-N-acetyl galactosamine were in the α and β-anomeric form, supporting the overlapping results of the respective lectins (Damjanov, 1987).

In some fish species, mucous cells exhibited different histochemical properties according to gill location (Sabóia-Moraes *et al*., 1996). In *O. bonariensis*, the histochemical techniques revealed that mucous cells from gill filaments or secondary lamellae have the same histochemical properties. These results agree with Vigliano *et al*. (2006) for gills of juvenile *O. bonariensis*.

In vertebrates, mucous GPs have been related to various functions, like lubrication, control of infections and prevention of dehydration. Also, a relationship could be ascribed between different GPs and their possible functions. Acid GPs are engaged in the prevention of epithelium damage: in particular GPs with sulphate esters are associated with a lubricant role (Mittal *et al*., 1994*a*, *b*, 2002; Park *et al*., 2003), GPs, with sialic acid residues are associated with protection against bacterial and viral invasion (Suprasert *et al*., 1987), and neutral GPs are associated with the absorption and transport of molecules through the membranes (Sarasquete *et al*., 2001). As found in the gills of *C. guatucupa* (Díaz *et al*., 2005*a*), in *O. bonariensis* a single type of mucous cell secretes the different acid and neutral GPs. Acid GPs could be engaged in the prevention of epithelium damages. In a freshwater fish, such as *O. bonariensis*, there is a continuous loss of salts and water gain; the active branchial uptake of Na^+ and Cl^- becomes crucial for ion homeostasis, and then the presence of neutral GPs could be related to the transport of ions across the gill.

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