

Characterization of glycoconjugates in the pharyngeal cavity and the oesophagus of *Odontesthes bonariensis* by lectins

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Submitted in 04/11/2011

Accepted for publishing in 02/08/2012

Resumo

Caracterização de glicoconjugados da cavidade faríngea e esôfago de *Odontesthes bonariensis* por lectinas. Sete tipos de lectinas biotinizadas foram usados para examinar padrões de expressão de glicoconjugados (GCs) da mucosa da cavidade faríngea e esôfago do *Odontesthes bonariensis*. Sítios de ligação para Con-A, RCA-I e WGA foram positivos para o glicocálix, células epiteliais e células mucosas em ambos os órgãos com padrões de reação similar. PNA apenas marcou o glicocálix e as células epiteliais da mucosa em ambos os órgãos. DBA, SBA e UEA-I marcaram o esôfago exclusivamente e uma maior afinidade com o glicocálix e as células epiteliais foi observada. Os resultados deste estudo foram discutidos e suas possíveis implicações funcionais foram comparadas.

Palavras-chave: Cavidade da faringe; Esôfago; Glicoconjugados; Histoquímica com lectinas; ‘SilverSide’

Abstract

Seven types of biotinylated lectins were used to examine glycoconjugates (GCs) expression patterns in the mucosa of the pharyngeal cavity and the oesophagus of *Odontesthes bonariensis*. Con-A, RCA-I, and WGA binding sites were positive for glycocalyx, epithelial cells, and mucous cells in both organs with similar reaction patterns. PNA just stained glycocalyx and epithelial cells in the mucosa in both organs. DBA, SBA, and UEA-I stained only the oesophagus, and a greater affinity to glycocalyx and the epithelial cells was observed. The results of this study were discussed and their possible functional implications were compared.

Key words: Glycoconjugates; Lectin histochemistry; Oesophagus; Pharyngeal cavity; ‘SilverSide’

Introduction

'Silverside' *Odontesthes bonariensis* (Pisces, Atherinopsidae) is a commercially important fish species from South America which is intensively cultivated in France, Israel, Italy, and Japan (STRÜSSMANN; TAKASHIMA, 1990). Even though *O. bonariensis* is considered a freshwater species, there are studies indicating that freshwater may not be its optimal environment (TSUZUKI et al., 2001). This species is perhaps among the most captured ones for commerce and sport fishing in the Argentinean "pampasic" shallow lakes. Almost all studies previously carried out involve the estimation of 'silverside' stocks of eggs and larvae, as well as juvenile and adult individuals (SOMOZA et al., 2008) and reproduction in captivity (MIRANDA et al., 2006). In spite of the considerable interest in this species, little progress has been made with regard to morphological studies on 'silverside' in general (VIGLIANO et al., 2006; DÍAZ et al., 2010) and its digestive tract in particular (DÍAZ et al., 2006; VIGLIANO et al., 2011).

We have previously reported on the histology and histochemistry of the pharyngeal cavity and the oesophagus of 'silverside' (DÍAZ et al., 2006). The mucosa has longitudinal folds with a stratified epithelium, abundant mucous cells, and taste buds, which are absent in the oesophagus. Most of our histochemical research has been carried out through conventional or classical histochemical methods, which allow the recognition of carbohydrate-containing macromolecules from other components of body tissues (DÍAZ et al., 2005a; 2005b; 2006; 2008; 2010). In fishes, the digestive tract and the structures intimately related to it present changes with regard to taxonomy and feeding habits (DOMENEGHINI et al., 1998).

It's generally assumed that the digestive tract produces glycoconjugates (GCs), which are the main components of mucosubstances in vertebrates. It's known they have a variety of functions, such as lubrication, protection against microorganisms, and

proteolytic degradation (MITTAL et al., 1994; DÍAZ et al., 2008). In fishes, mucosubstances also have an osmotic function, in the form of binding and transport of water and various ions (LORETZ, 1995).

Lectins, which are naturally occurring carbohydrate-binding proteins other than enzymes or antibodies, are a valuable tool to characterize and localize GCs in tissues. Histochemical studies have shown the usefulness of lectins as biomarkers for specific secretory functions, structural components, and changes in cells and tissues (CHAN; HO, 1999).

Since no research has used lectin histochemistry in the mucosa of the pharyngeal cavity and the oesophagus of 'silverside', and as lectins can provide some understanding of the GCs characteristics in these organs, this study has lectin histochemistry as its basis, in order to gather information on the specific functions of mucus production.

Material and Methods

The female and male specimens of *O. bonariensis* used in this research had 24.0 ± 2.0 cm in length, 180.0 ± 5.0 g in weight; they were collected in Los Padres Lagoon (Province of Buenos Aires, Argentina), $37^{\circ}56'30''$ S, $57^{\circ}44'30''$ W, and immediately transported to the laboratory in containers filled with water. Fishes were anaesthetized with Tricaine (MS-222) before being measured, weighed, and sexed. The digestive tract was rapidly removed and samples from the pharyngeal cavity and oesophagus were fixed by immersion in 10% buffered formalin before dehydration in an ethanol series, and embedded in paraffin. The care and use of experimental animals complied with the guidelines of the Safety Committee of Universidad Nacional de Mar del Plata.

Lectins staining methodologies were performed according to Gimeno et al. (1995). Seven different specific lectins were used (Table 1). All of them were purchased at Vector Laboratories, Inc. (Burlingame, CA, USA). Paraffin-embedded sections mounted on

slides coated with Poly-L-Lisine (Sigma Diagnostics, St. Louis, MO, USA) were deparaffinized with xylene. They were next incubated in 0.3% H₂O₂ in methanol for 30min at room temperature, in order to block endogenous peroxidase activity. They were then hydrated, washed in 0.01M phosphate-buffered saline (PBS), pH 7.2, and treated with 0.1% bovine serum albumin in PBS for 15min, to inhibit non-specific binding. Sections were then incubated with biotinylated lectins for 30min at room temperature and rinsed in PBS for 15min. After that, sections were treated with avidin biotin-peroxidase complex (ABC) (Vector Laboratories, Inc, Burlingame, CA, USA) for 45min. The horseradish peroxidase was activated by incubation for 4-10min with a buffered 0.05M Tris-HCl pH 7.6 solution containing 0.02% diaminobenzidine (DAB) and 0.05% H₂O₂. Each lectin was used at a 30µg/ml dilution in PBS, except PNA, which was applied at a 10µg/ml concentration. Two types of control were performed: (1) lectin solution was replaced by PBS; and (2) lectin labeling was performed as described above, after lectins were preincubated for 1h in the presence of appropriate hapten sugars (0.2M in PBS), as listed in Table 1, at room temperature.

Evaluation of labeling intensities was based on subjective estimates by all authors after examination of two sections per sample of all animals tested.

TABLE 1: Lectin used and their carbohydrate specificities.

Lectin	Acronym	Specificity/hapten sugar ^{a,b}
<i>Canavalia ensiformis</i> agglutinin	Con-A	α-D-Man; α-D-Glc
<i>Triticum vulgare</i> agglutinin	WGA	β-D-GlcNAc; NeuNAc
<i>Dolichos biflorus</i> agglutinin	DBA	α-D-GalNAc
<i>Glycine max</i> agglutinin	SBA	α-D-GalNAc; β-D-GalNAc
<i>Arachis hypogaea</i> agglutinin	PNA	β-D-Gal (β1->3) D-GalNAc
<i>Ulex europaeus</i> agglutinin-I	UEA-I	α-L-Fuc
<i>Ricinus communis</i> agglutinin-I	RCA-I	β-Gal; β-GalNAc

^aGoldstein; Hayes (1978). ^bFuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; NeuNAc, acetylneuraminic acid (sialic acid).

Results

The results of lectin histochemistry used to reveal GCs in the mucosa of the pharyngeal cavity and the oesophagus of *O. bonariensis* are summarized in Table 2.

Con-A binding sites were mainly observed at a glycocalyx level and in epithelial cells, and less importantly in mucous cells from the pharyngeal cavity and oesophagus (Figure 1A, C). WGA showed a moderate affinity to the glycocalyx, epithelial cells and mucous cells in both organs (Figure 1E, e). Binding sites

TABLE 2: Lectin binding in *Odontesthes bonariensis* pharyngeal cavity and oesophagus.

	Pharyngeal cavity			Oesophagus		
	Mucous cells	Epithelial cells	Glycocalyx	Mucous cells	Epithelial cells	Glycocalyx
Con-A	1	2	2-3	1	2	2-3
WGA	2	1-2	2	2	1-2	1-2
DBA	0	0	0	0	0-1	1
SBA	0	0	0	0-1	2	2
PNA	0-1	0	0-1	0-1	0	1
UEA-I	0	0	0	0	1	2
RCA-I	2	1	2	2-3	1	2-3

Numbers indicate staining intensity on a subjective scale: 0 unstained; 1 weak; 2 moderate; 3 strong.

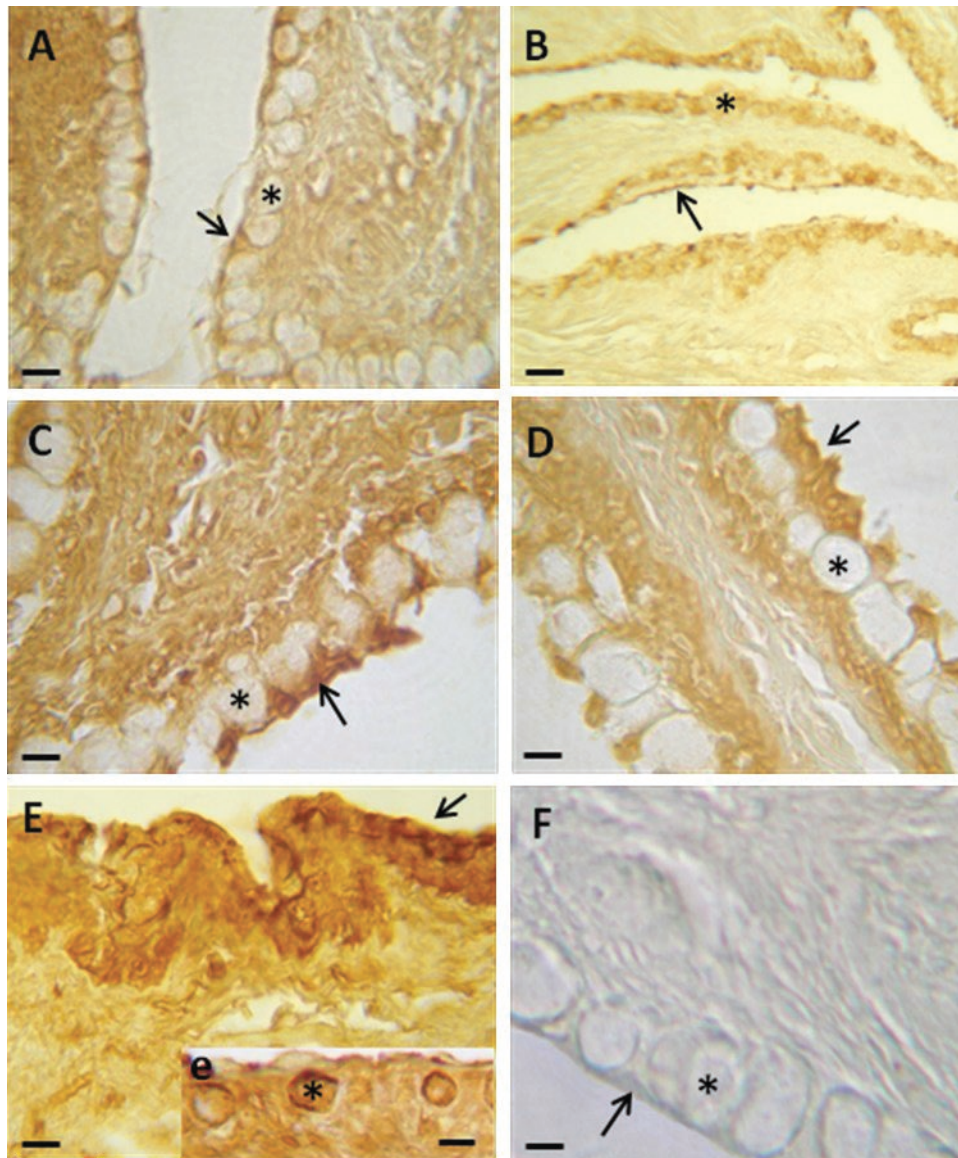
for DBA were only observed in the glycocalyx and the epithelial cells of oesophagus. SBA stained only the oesophagus, where a greater affinity was evidenced in the glycocalyx and epithelial cells. Mucous cells were labeled weakly with SBA lectin (Figure 1D). PNA scarcely stained the glycocalyx and mucous cells of both organs. Epithelial cells were unstained. Binding sites for UEA-I were visualized in the glycocalyx and the epithelial cells of oesophagus, although not in the pharyngeal cavity. RCA-I showed a similar positive

reaction pattern in both organs, albeit mucous cells and glycocalyx of oesophagus showed a higher labeling (Figure 1B). No labeling was detected in control sections (Figure 1F).

Discussion

Using lectin histochemistry, it was possible to establish the heterogeneity of GCs present in cells of the pharyngeal cavity and the oesophagus of *O. bonariensis*.

FIGURE 1: Lectin histochemistry of the pharyngeal cavity and oesophagus of *O. bonariensis*. Pharyngeal cavity: (A) Con-A. Scale bar = 28 μ m. (B) RCA-I. Scale bar = 78 μ m. Oesophagus: (C) Con-A. Scale bar = 34 μ m. (D) SBA. Scale bar = 39 μ m. (E) WGA. Scale bar = 25 μ m. (e) WGA. Scale bar = 30 μ m. (F) Control. Scale bar = 40 μ m. Adherent mucus gel (arrow) and mucus cells (asterisk).



The oligosaccharide chains of GCs have been categorized into two families: N- and O-linked oligosaccharides. The first group classically contains a reducing terminal N-acetylglucosamine (GlcNAc) N-glycosidically bound to asparagine. The O-linked oligosaccharide (mucin type) is characterized by a reducing N-acetylgalactosamine (GalNAc) terminal O-glycosidically linked to serine or threonine. These amino acid residues can also link fucose (Fuc), glucose (Glc), mannose (Man), and N-acetylglucosamine (GlcNAc) to form other classes of O-linked glycans (DESANTIS et al., 2006; PARRILLO et al., 2009). The oligosaccharides of GCs N- and O-chains are correlated to different functions, such as lubrication, absorption, and transport of molecules through the membranes, protection against proteolytic degradation, and inhibition of microorganisms. The study of carbohydrate chains composition and their location in a specific alimentary canal could help understanding their functions in digestive processes.

Whereas most lectins reacted in like manner both in the pharyngeal cavity and the oesophagus, differences arose with DBA, SBA, and UEA-I, which were positive only in epithelial cells from the oesophagus. The presence of a large variety of carbohydrates indicates not only a simple function in protecting or lubricating the mucosa of the alimentary canal against chemical or mechanical injuries, but also other functions, including cooperation in pre-gastric digestion (MARCHETTI et al., 2006).

This corroborates other researches indicating that in some fish species digestion starts in the oesophagus and continues in the stomach (DOMENEGHINI et al., 1998). Likewise, the high level of histochemical complexity in the oesophagus could be related to the absence of salivary glands (SCOCCO et al., 1996). It's important to notice that digestion in mammals starts in the mouth, due to the presence of secretion from the salivary glands. Therefore, the oesophageal mucous cells can replace the secretory function of salivary glands in lubricating the wall of the oesophagus (PEDINI et al., 2004; MARCHETTI et al., 2006; DÍAZ et al., 2008).

The results with DBA and SBA for *O. bonariensis* corroborate the existence of GalNAc. DBA binds

especially to α -D-GalNAc, indicating the presence, although scarce, of this glucidic residue in the glycocalyx and epithelial cells of oesophagus, whereas SBA doesn't have anomeric specificity.

Con-A, like other Man-binding lectins, has been used to identify GCs containing N-linked oligosaccharides, since O-linked oligosaccharides contain less than 1% Man (SPICER; SCHULTE, 1992). In accordance with other fish species, the surface cells of both organs have mannose residues, with a probable protective role against bacterial infections (LEMAITRE et al., 1996; BURKHARDT-HOLM, 1997; DÍAZ et al., 2005b). In addition, a protective role of mannose residues was also described by Lemaitre et al. (1996) in the skin of carp.

WGA exhibits a strong affinity to sialic acid at its terminal position, indicating the location of this carbohydrate in the mucous cells, in the epithelial cells and in the glycocalyx in both organs. As in this study, sialic acid has been found in several fish species through histochemical methods and chemical analyses (GENTEN; DANGUY, 1990; DÍAZ et al., 2008; 2010). Glycoconjugates in mucus are major determinants of mucus viscosity, and the acidic glycoproteins of the sialylated type are indicative of a rather fluid mucosal secretion which improves prey sliding within the oesophageal lumen by reducing viscous drag (DOMENEGHINI et al., 1999; MEYER et al., 2009). Moreover, sialic acids are essential components of receptors, e.g. the capacity of viruses and toxins of infecting cells depends on the presence of these acids in the cell membranes (PAJAK; DANGUY, 1993). On the contrary, the weakly or non-lectin binding with UEA-I, indicative of lack of fucose, corroborates previous studies reporting on gills and epidermis of fish (ALBANAW et al., 2009; DÍAZ et al., 2010).

Although in an earlier study (DÍAZ et al., 2006) traditional histochemistry failed to show different types of mucosubstances in the pharyngeal cavity and the oesophagus, here it was proven to be true. It's worth underlining that a discrepancy between traditional and lectin histochemistry had already been found (DESANTIS et al., 2007).

Differences in the mucus composition of both organs are probably related to different functions. Mucus in the pharyngeal cavity is likely to be involved in lubricating and protecting epithelium from mechanical injuries and bacterial invasion. Though 'silverside' is considered a freshwater fish, environments with intermediate salinities might be most favorable (TSUZUKI et al., 2000). Considering the plasticity of 'silverside', it's possible that the presence of added mucosubstances in the oesophagus might enable a response to changes in the environmental conditions early on.

Acknowledgements

This study was partly supported by the grant EXA 491/10 from Universidad Nacional de Mar del Plata (UNMdP).

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