Glycoconjugate Histochemistry in the Fundic Stomach and Small Intestine of the Frog (*Rana ridibunda*)

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Abstract

Mucins in the fundic stomach and small intestine of *Rana ridibunda* were studied by using the techniques such as lectins as a specific probe binding terminal sugar residues and standard histochemistry in light microscopic level. In this study, we focused on morphofunctional diversities of different regions of the digestive tract and their feasible physiological and evolutionary implications. We used the following standard histochemical techniques: contained periodic acid-Schiff (PAS), Alcian blue (AB) pH 1.0 and 2.5, toluidin blue, aldehyde fucsin and bromfenol blue. For lectin histochemistry, five different lectins were used namely, DBA, WGA, PNA, ConA and UEA-I. The glycoconjugate produced in the fundic part of the stomach is composed of mainly neutral mucins and strongly sulphated acid mucins with α-*N*-acetyl-D-galactosamine and *N*-acetyl-β-D-glucosamine residues. Besides, the glycoconjugate secreted from the small intestine consists of mostly sulphated sialo and neutral mucins with *N*-acetyl-β-D-glucosamine moieties. It can be concluded that the differences in glycoconjugates types and the sugar residues in two digestive tract regions of *Rana ridibunda* may be related to special functions and rheological characteristics of the mucins.

Keywords: Lectin histochemistry, Mucins, Fundic stomach, Small intestine, *Rana ridibunda*
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Introduction

Several histochemical studies on the digestive tract of vertebrates have shown that mucins vary in different regions of the tract in terms of proportional expression of neutral, sulphated and non-sulphated and lectin binding pattern (Madrid et al. 1989; Ferri and Liquori, 1992; Ferri et al. 1999; Domeneghini et al. 2005). Mucins in the digestive tract may have several functions such as the protection of the underlying epithelium from proteolytic damage, mechanical injuries and bacteria, lubrication of the tract and increasing digestive efficiency (Gupta 1989; Domeneghini et al. 2005) and these functions may be related to their different structures in the various parts of the digestive tract (Loo and Wong 1975; Ferri et al. 2001) and to phylogenetic relationships (Suganuma et al. 1981).

It is generally accepted that in non-mammalian vertebrates, two main gastric juice components, pepsinogen and hydrochloric acid have secreted from one type of cell, called oxynticopeptic cells, arranged in the gastric glands. However, this condition changes depending on the species in amphibians (Ferri et al. 2001). It has been reported that pepsinogen is produced by the peptic cells in the oesophageal glands and hydrochloric acid produced by oxyntic cells in the gastric glands in ranid frogs such as Rana esculenta (Suganuma et al. 1981; Bani et al. 1992; Gallego-Huidobro and Pastor 1996; Ferri et al. 2001). On the other hand, in some ranid frogs such as *Rana perezi* both pepsin producing peptic cells in oesophageal glands and

oxynticopeptic cells in the gastric glands are present (Gallego-Huidobro et al. 1992; Gallego-Huidobro and Pastor 1996). In non-ranid frogs such as *Bufo viridis* (Liquori et al. 2002) and in *Bombina variegata* (Bani et al. 1992) and in Caudata, oesophageal glands are absent, so both pepsinogen and hydrochloric acid are produced by oxynticopeptic cells in the gastric glands (Liquori et al. 2005, 2007). In Amphibia, mucins are produced by oesophageal mucous glandular cells, if oesophageal glands are present, in addition to mucins secreted by gastric mucous glandular cells, by superficial columnar cells in gastric epithelium and by goblet cells in the oesophageal and intestinal epithelium. It has been reported that histochemical characterization of the mucins varies with the secretion region, cell type, developmental stages and their functions (Suganuma et al. 1981; Ishizuya-Oka and Shimozowa 1990a, b; Oinuma et al. 1991; Ferri et al. 2001).

Lectins are proteins or glycoproteins which have a binding capacity to specific sugar residues of complex carbohydrate moieties of glycoconjugates. They have been used as histochemical and cytochemical probes to show the location of specific sugars (Navas et al. 1987). In Ranid frogs, these studies have been limited to a few species, *Rana perezi* (Gallego-Huidobro et al. 1992, Gallego-Huidobro and Pastor, 1996), *Rana esculenta* (Bani et al. 1992) and *Rana aurora aurora* (Ferri et al. 2001). In the present study, we aimed to investigate the composition of the mucins produced in the gastric and intestinal mucosa and discuss probable functions of the mucins in the two digestive tract regions, using standard histochemical methods and lectin histochemistry in *Rana ridibunda* individuals.

Material and Methods

Five male adult frogs *Rana ridibunda* were used in this study. The animals were sacrified by capitation and the fundic part of the stomach and small intestine were removed quickly from each individual. Samples were fixed in Bouin's solution for 24 h, dehydrated through graded ethanol series, cleared in xylene and embedded in paraffin. Sections were taken at 4μm thick and rehydrated. Some of them were stained with hematoxylin-eosin for normal histology. The others were stained by following methods for the characterization of glycoconjugates.

Conventional Histochemistry

For the conventional glycoconjugate histochemistry, the following staining methods were used. Rehydrated sections were stained with periodic acid-Schiff (PAS) methods for neutral glycoconjugates (Humason 1972), AB pH 2.5 for carboxylated acidic glycoconjugates, AB pH 1.0 for weakly and strongly sulphated acid mucins, bromphenol blue (BFB) for staining proteins (Mazia et al. 1953), aldehyde fuchsin (AF) and toluidine blue (TB) for sulphated sialomucins differentiation (Humason 1972).

Lectin Histochemistry

Five lectins were used to identify various sugar residues in the oligosaccharide sequences of mucin glycoconjugates. The lectins employed, their source, their sugar specificities and their inhibitory sugars are listed in Table 1. Briefly, rehydrated sections were exposed to %3 hydrogen peroxide for 10 min. to inhibit endogenous peroxidase activity and blocked with %1 bovine serum albumine (BSA). Sections were then incubated with horseradish peroxidase- labelled five lectins at different concentration, namely 20, 30, 40, 50 μg/ml for an hour at room temperature. The horseradish peroxidase activity was visualized histochemically with 3-amino-9-ethilcarbazole (AEC) chromogen for 20 min. The sections were stained with Mayer's hematoxylin for counterstaining and mounted with GVA. After each step, except for blocking with BSA, sections were washed with phosphate buffered saline (pH 7.2) twice for two min.

Table 1. Lectin used in the present study, their origin, specific and inhibitory sugars. * Optimum lectin concentration for fundic stomach, ** optimum lectin concentration for small intestine.

Lectin	Origin	Charbohydrate specifity	Lectin concentration (µg/ml)	Inhibitory sugar
UEA-I	*Ulex europaeus*	α-L-fucose	20*-30-40-50	L-Fucose
WGA	*Triticum vulgaris*	N-acetyl-β-D-glucosamine	20*-30-40-50**	N-acetyl-neuroaminic acid
PNA	*Arachis hypoaea*	β-galactose	20-30-40-50	D-galactose
DBA	*Dolichos biflorus*	α-N-acetyl-D-galactosamine	20*-30-40-50	N-acetyl-2-deoxyl-2-amino-D-galactopyranose
Con A	*Canavalia ensiformis*	α -D-mannose	20-30-40-50	α-methyl-D-mannopyranoside

Control Staining

The control tests for lectin histochemistry included a) substitution of the respective peroxidase labelled-lectins with PBS; b) incubation of peroxidase-labelled lectins with their inhibitory sugar to confirm the specificity of lectin staining. The inhibitory sugars are listed in Table 1. For inhibitory sugar test, the sections were incubated with the solution containing lectins and inhibitory sugar in equal volume.

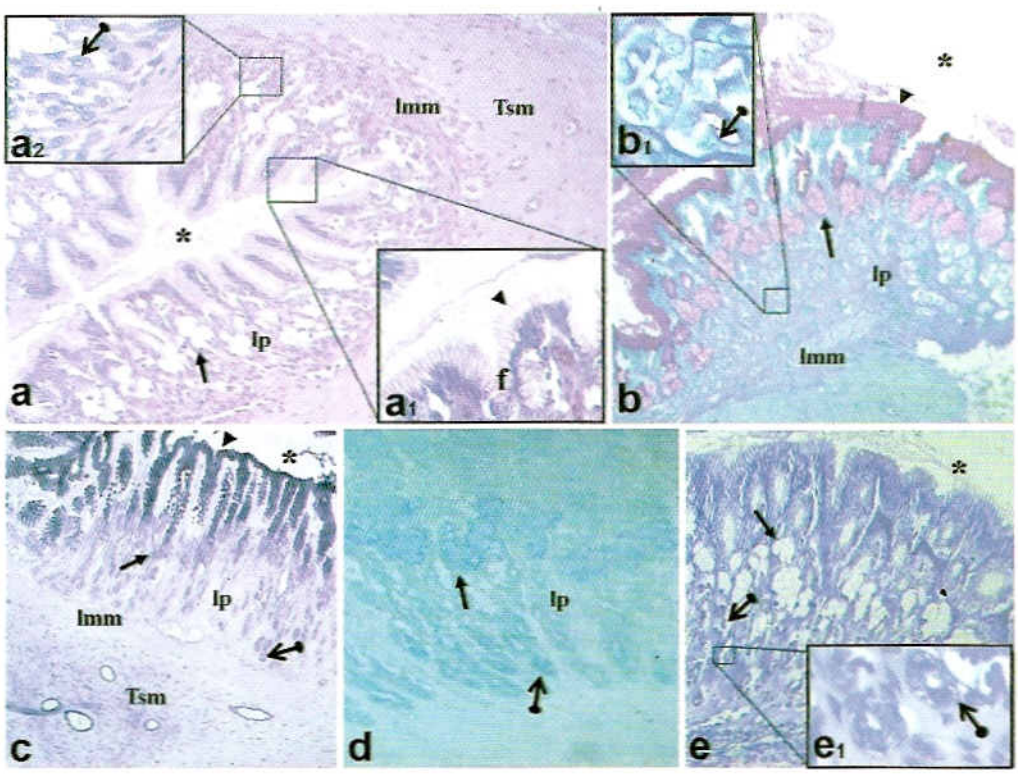

Figure 1. The general structure and histochemical properties of fundic stomach in *Rana ridibunda*. **a**) The fundic stomach histology, H-E X270 **a_1**) superficial cells and foveolar cells, H-E X540 **a_2**) Intensely eosinophilic cells H-E X640. **b**) PAS X270, **b_1**) PAS positive material in apical surface of the gland cells X1350, **c**) AF X270, **d**) AB pH 1.0 X270, **e**) Bromfenol blue X270, **e_1**) Bromfenolblue positive granules X1350. **Imm**: lamina muscularis mucosa; **lp:** lamina propria; **Tsm**: tunica submucosa; ▶superficial cells; **f**:foveolar cells; → : foamy mucous neck cells; * : lumina of the stomach; ⟵▶ : intensely eosinophilic cells.

Results

Stomach

The fundic part of the stomach in *Rana ridibunda* had a folded mucosa and luminal surface and gastric pits were lined by a simple columnar mucous secreting cells, called as superficial and foveolar cells, respectively (Fig. 1a, a_1). The luminal epithelium formed the fundic gastric glands that emptied into the base of gastric pits and penetrated deeply into the lamina propria (Fig. 1a). The fundic glands were mostly of a simple tubular type and composed of foamy mucous neck cells and intensely eosinophilic cells (Fig. 1a a_2). The mucous foamy neck cells were clustered in the upper part of the glands (Fig. 1a).

Mucins were observed mainly in the superficial cells, foveolar cells and foamy mucous neck cells. The apical cytoplasma of the superficial and foveolar cells gave strong PAS (Fig. 1b, b_1) and AF (Fig. 1c) positive reaction, but did not react with AB pH 2.5, pH 1.0 and TB. These cells reacted with WGA (Fig 2a), particularly in their apical surface (Fig. $2a_2$)

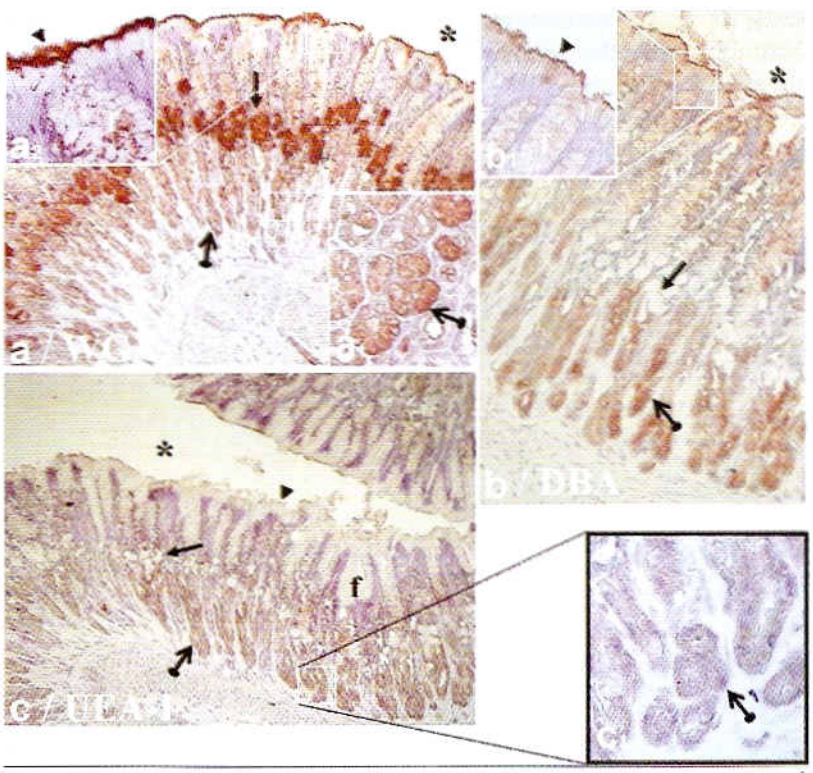

Figure 2. The lecting binding pattern of fundic stomach in *Rana ridibunda*. **a**) WGA lectin staining X270, **a_1**) WGA staining in the gland cells X 540, **a_2**) superficial and foveolar cells X540, **b**) DBA lecting staining, **b_1**) superficial and foveolar cells X540: **c**) UEA-I lecting staining X270, **c_1**) UEA-I staining in the gland cells. Abbreviations and symbols same as figure 1.

and weakly with DBA (Fig. 2b, b_1). No staining was observed with the other lectins. We observed eosinophilic cells in the lower part of the glands and these cells stained with weakly AF (Fig. 1c), AB pH 1.0 (Fig. 1d) and had PAS positive material in their apical surface (Fig. $1b_1$). These cells showed strong affinity for WGA, DBA and lower affinity for UEA-I (Fig. 2a, b, c, c_1). No staining was observed with PNA and Con A.

Mucous neck cells were positive with PAS strongly (Fig. 1b) and AF weakly (Fig 1c) but

these reactions were not as strong as superficial or foveolar cells. But these cells stained with AB at pH 1.0 (Fig. 1d). Mucous neck cells showed an intense reactivity with WGA (Fig. 2a), but did not stain with other lectins and TB.

These reactions were summarized in Table 2 and 3. Only the cells of the eosinophilic fundic gland exhibited specific reaction for protein (Fig. 1c) and contained bromfenol blue positive granules in their cytoplasm (Fig. 1e, e_1).

Table 2. Histochemical reactivity of the stomach and intestine with standard staining

Gastrointestinal region	Histochemical staining					
Stomach (Fundus)	**PAS**	**AB pH 2.5**	**AB pH 1.0**	**AF**	**TB**	**BFB**
Superficial cell	*****	-	-	*****	-	-
Foveolar cell	*****	-	-	*****	-	-
Mucous neck cell	****	-	***	**	-	-
Intensely eosinophilic cell	**	-	***	**	-	*****
Small intestine						
Goblet cell	*****	***	***	*****	-	-
Enterocyte	-	-		-		
Brush border	*****	-	***	*****	-	-

Table 3. Histochemical reactivity of the stomach and intestine with different lectins.

Gastrointestinal region	Lectins				
Stomach (Fundus)	**WGA**	**DBA**	**UEA-I**	**PNA**	**ConA**
Superficial cell	*****	****	-	-	-
Foveolar cell	***	***	-	-	-
Mucous neck cell	*****	-	-	-	-
Intensely eosinophilic cell	*****	*****	***	-	-
Small intestine					
Goblet cell	*****	-	-	-	-
Enterocyte	-	-	-	-	-
Brush border	*****	-	-	-	-

Small Intestine

The small intestine of *Rana ridibunda* was lined by simple columnar epithelium. This epithelium was composed of mainly absorptive cells (enterocytes) which possessed brush border and scattered goblet cells (Fig. $3a_1$). The lamina propria did not have any glands. Mucosa was arranged in longitudinal folds without lamina muscularis mucosa (Fig. 3a).

Mucus secreted by goblet cells was strongly PAS positive (Fig. 3b, b_1), stained with AB both at pH 2.5 and moderately at pH 1.0 (Fig. 3c, d) and gave strong AF positive reaction (Fig. 3e). No histochemical staining was observed with TB. The brush border corresponding to glycocalyx also reacted with PAS (Fig. $3b_1$) and stained intensely with AF (Fig. 3e). The enterocytes did not have any histochemically reactive granules. The goblet cells and brush border only stained with WGA lectin (Fig. 4). Any staining was observed with the other lectins. These reactions were summarized in Table 2 and 3.

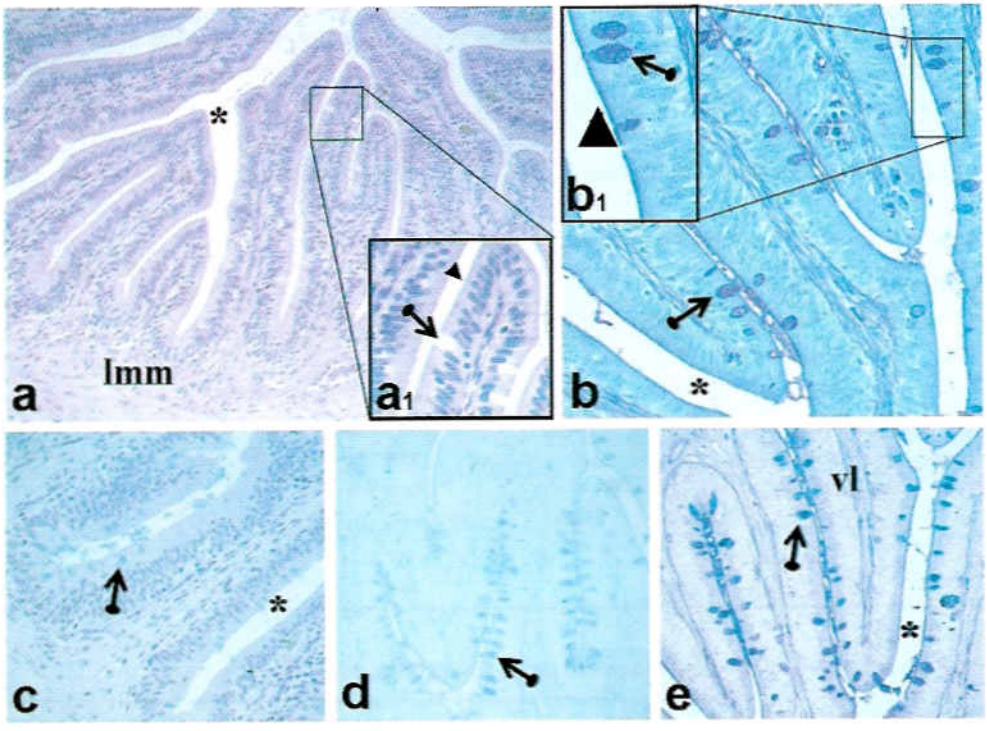

Figure 3. The general structure and histochemical properties of small intestine in *Rana ridibunda*, **a**) General histology H-E, X 270 **a$_1$**) goblet cell and enterecytes **b**) PAS **b$_1$**) goblet cell and brash border **c**) AB pH 2.5 **d**) AB pH 1.0 **e**) AF X540. * : lumina of the small intestine, ⟷ : goblet cells, ▶: brush border, vl: villus, 1mm: lamina muscularis mucosa.

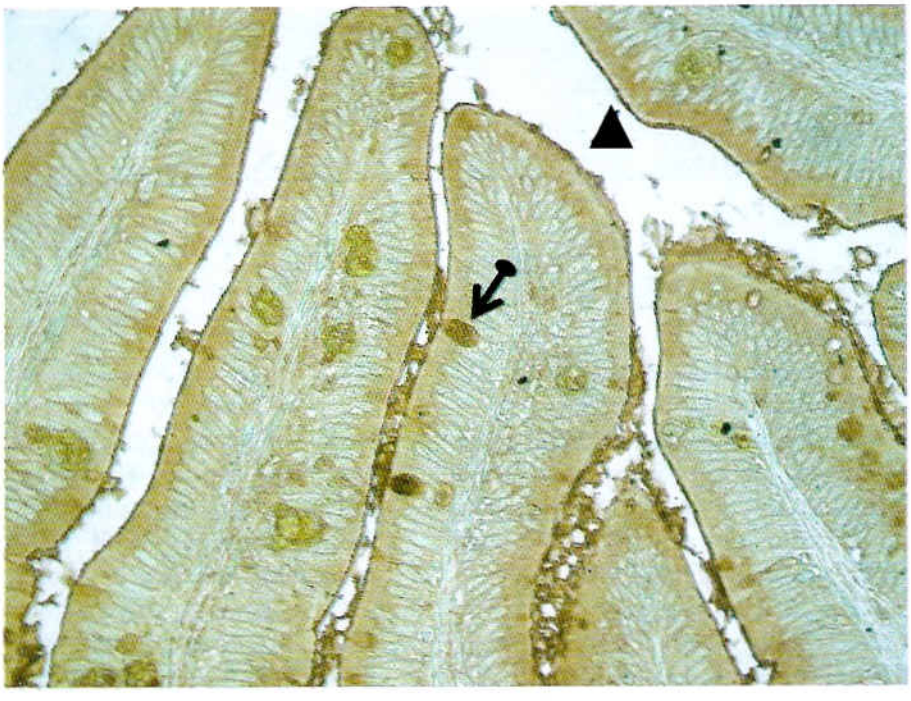

Figure 4. WGA lecting binding pattern of small intestine in *Rana ridibunda* X540. Abbreviations and symbols same as figure 3.

Discussion

The studies on the glycoconjugates in the digestive tract have been done in all vertebrate groups (Suganuma et al. 1981; Madrid et al. 1989; Ferri and Liquori, 1992; Ferri et al. 1999, 2001; Mali and Bulog, 2004; Domeneghini *et al.* 2005; Liquori et al. 2007). In amphibia, these studies in Ranid frogs have been limited with *Rana perezi* (Gallego-Huidobro *et al.* 1992; Gallego-Huidobro and Pastor, 1996), *Rana esculenta* (Bani et al. 1992) and *Rana aurora aurora* (Ferri et al. 2001). The present study has been focused on the detection of the types of the mucins and sugar residues in the glycoconjugates secreted from the two digestive tract regions, fundic stomach and small intestine in *Rana ridibunda*.

Epithelial mucins have been classified into neutral and acidic types, while the latter has three subgroups, namely, sulphated mucin, sulphated sialomucin and carboxylated (Bancroft and Cook 1984). As previously reported, the type of mucus produced in the gastrointestinal system varies depending on the different portion of the tract, the species, cell types and their function (Liquori et al. 2007)

In the fundic part of the stomach, mucus was secreted by three types of cells, superficial, foveolar and mucous neck cells. The superficial and foveolar cells gave positive reaction strongly with PAS and AF and reacted with DBA and WGA lectins, thus revealing that these cells secrete mainly neutral mucins and strongly sulphated acid mucins with α-*N*-acetyl-D-galactosamine and *N*-acetyl-β-D-glucosamine residues. Mucous neck cells in the upper part of the gastric glands contained mostly neutral mucins and sulphated acid mucins in addition to a small amount of weakly sulphated mucins as revealed by our histochemical results. Binding strongly with WGA demonstrated the presence of N-acetyl-β-D-glucosamine residues in the secretory product. As can be seen, in *Rana ridibunda,* mucous cells in the stomach mainly secreted both neutral and sulphated acid glycoconjugates. Neutral glycoconjugates have been found in some species in amphibians such as *Bufo viridis* (Liquori et al. 2002), *B. melanostictus* (Loo and Wong, 1975), *Triturus carnifex* (Liquori et al. 2007), in ruin lizard *Podarcis sicula* (Ferri and Liquori 1992), in the green anole *Anolis carolinensis* (Lehman and Smith, 1975). On the other hand, in *Rana aurora aurora* (Ferri et al. 2001), in European eel *Anguilla anguilla* (Domeghini et al. 2005), in the snake *Elaphe climacophora* and turtle *Clemmys japonica* (Suganuma et al. 1981) and in many mammals (Spicer and Sun, 1967), neutral and acidic mucins were observed in the stomach as seen in *Rana ridibunda*. These findings suggest that the nature of the glyconjugates in the stomach may change depending on the species.

In addition, glycosidic residues in the secretion vary in the different species. We detected α-*N*-acetyl-D-galactosamine and *N*-acetyl-β-D-glucosamine in superficial and foveolar cells. However, galactoseβ1,3-*N*-acetylgalactosamine and *N*-acetylgalactosamine were found in superficial and foveolar cells, respectively in *Rana aurora aurora* (Ferri et al. 2001). In *Bufo viridis* only galactoseβ1,3-*N*-acetylgalactosamine were detected in the secretion (Liquori et al. 2002). In *Triturus carnifex*, neutral glycoproteins in the surface cells contained galactoseβ-1,3-*N*-acetylgalactosamine, *N*-acetylβ1-4 glucosamine, galactose-α galactose, fucose linked α1, 6 to *N*-acetylglucosamine and terminal fucose residues (Liquori et al. 2007). In *Anguilla anguilla*, D-*N*-acetylglucosamine, D-*N*-acetylgalactosamine, α-mannose and β-D-galactose were present in surface columnar cells (Domeneghini et al. 2005). Ferri and Liquori (1992) detected D-glucose, D-mannose, D-galactose in ruin lizard *Podarcis sicula*.

In the lower part of the fundic glands, we observed strongly WGA and DBA positivity and lower affinity for UEA-I in PAS and AB pH 1.0 positive areas in the eosinophilic cells. We also detected bromphenol blue positive granules in their cytoplasm. On the other hand, in oxynticopeptic cells of *Triturus carnifex*,

which produced both HCl and pepsinogen in the gastric glands, labelling with WGA and PNA, but not UEA-I, were detected (Liquori et al. 2007). However, labelling with DBA lectin was only observed in oxynticopeptic cells of *Bufo viridis* and ruin lizard *P. sicula campestris* (Liquori *et al.* 2000, 2002). It was found that DBA, UEA-I and PNA specifically bound in the intracellular canalicular membranes, the extension of which seems to be related to the production of HCI in the parietal cells of mammals (Ito et al. 1985; Kessimian et al. 1986). In addition, the beta-subunit of the gastric H,K-ATPase is the most abundant glycoprotein in the canalicular membranes of the acid-secreting parietal cells in rabbits. The oligosaccaharides of the beta-subunit had been shown to contain fucose, N-acetylglucosamine, mannose, galactose, and N-acetylgalactosamine (Tyagarajan et al. 1996). Besides, Liquori et al. (2005) showed positive immunolabelling against C-terminus of β-subunit of porcine H^+K^+-ATPase in PAS positive apical cytoplasm of oxynticopeptic cells in *T. carnifex*. So, the lecting binding affinity in parietal and oxynticopeptic cells may be a result of presence of this subunit. Although we have not examined the presence of oesophagial glands in *Rana ridibunda,* we thought that the eosinophilic cells in the lower gland part could be oxynticopeptic cells according to PAS positive staining, presence of bromphenol blue positive granules which could be the zymogen granules and lectin binding affinity. But further studies should confirm the presence of oxynticopeptic cells in *Rana ridibunda*.

Mucous neck cells first appeared in amphibians and were detected in several species such as *Rana esculenta* (Bani et al. 1992), *Rana aurora aurora* (Ferri et al. 2001), *Bufo viridis* (Liquori et al. 2002) and *Triturus carnifex* (Liquori et al. 2007). These cells have also been found in the reptiles and mammals (Suganuma et al. 1981, 1984; Katani-Matsumoto and Kataoka, 1989) but not found in fishes and birds (Suganuma et al. 1981, Domeneghini et al. 1998; 2005). There is a debate about the gastrointestinal cell lineage of mucous neck cells. According to some authors these cells are the precursor of the chief (zymogenic) cells and intermediate between gastric stem cells and chief cells in the adult rat stomach (Suzuki et al. 1983; Fujita and Kaneko, 1994). However, Hanby et al. (1999) suggested that mucous neck cells had a defined phenotype and were a separate and distinct cell lineage.

In our specimen, we detected only *N*-acetyl-*β*-D-glucosamine in mucous neck cells. In addition to this sugar residue, mucous neck cells also contained α-L-fucose, α-D-glucose and α-D-mannose and *β*-D-galactose in humans (Ito et al. 1985); L-fucose and D-galactose in ruin lizard (Ferri and Liquori 1992); D-glucose, D-mannose, galactose*β*1,3 *N*-acetylgalactosamine in *R. aurora aurora* (Ferri et al. 2001) and in *T. carnifex* (Liquori et al. 2007). According to these findings, the differences in distribution of glycosidic residues in glycoproteins produced in mucous neck cells may occur depending on the various species of vertebrates.

The role of gastric mucus composed of neutral glycoconjugates in *Rana ridibunda* individuals could be related to physical protection against mechanical damage by ingested particles and preventing contact between digestive enzymes such as pepsin and the underlying mucosa as suggested by Kaunitz (1999) and Liquori et al. (2007). On the other hand, the sulphated acid mucin in the gastric secretion may provide a resistance againts bacteria in the stomach. Because, heavy sulphation rate limits the degredation of mucin by bacterial mucin-degrading glycosidases (Robertson and Wright 1997).

In the intestine, the secretion was only produced by scattered goblet cells in the epithelium. We did not observe either Lieberkühn glands or Brunner's glands in the small intestine. Brunner's glands are specific to mammals and have been observed in all mammals examined to date (Krause 1988). Lieberkühn glands were only observed in birds (Mendes et al. 2009) and mammals.

According to our histochemical results, the intestinal mucins are composed of mainly acidic

sulphated sialomucin and a small amounts of weakly and strongly sulphated acidic mucins. These histochemical results were consistent with other vertebrates such as *R. aurora aurora* (Ferri et al. 2001), *T. carnifex* (Liquori et al. 2007), ruin lizard *Podarcis sicula* (Ferri and Liquori 1992), all of them had acidic mainly sulphated glycoconjugates. Different from these species, the secretion contained sialomucinsin in specimen, as seen in European eel *Anguilla anguilla* (Domeneghini et al. 2005), sea bream Sparus aurata (Domeneghini et al. 1998), *Bufo melanostictus* (Loo and Wong, 1975), *Rana nigromaculata* and the turtle *Clemmys japonica* (Suganuma et al. 1981).

In *Rana ridibunda*, the goblet cells and brush border showed binding affinity only with WGA lectin, revealing the presence of *N*-acetyl-β-D-glucosamine. In additon to this glycosidic residue, in *Rana aurora aurora* (Ferri et al. 2001), and in ruin lizard (Ferri and Liquori, 1992), galactoseβ1,3 *N*-acetylgalactosamine and β D-galaktoz residues were also detected in goblet cell secretion, respectively. In *T. carnifex* (Liquori et al. 2007), much more sugar residues were detected, namely, *N*-acetlyβ1-4 glucosamine, *N*-acetylgalactosamine, mannose and/or glucose, D-galactose and sialic acid. In contrast, only *N*-acetylgalactosamine was detected in the goblet cells in the intestine in *Anguilla anguilla* (Domeneghini *et al.* 2005). Madrid et al. (1989) found that *N*-acetylglucosamine WGA binding sites revealing were the most abundant throughout the intestinal mucins of the fishes (*Sparus auratus*), amphibians (*Rana perezi*), reptiles (*Testudo graeca*), birds (*Gallus gallus*) and mammals (*Mesocricetus auratus*). These studies showed that the glycosidic residues in the goblet cell secretion may change depending on the species.

Although we did not observe any granules in the enterocytes in *Rana ridibunda*, as not seen in neotenic cave salamander *Proteus anguinus* (Mali and Bulog 2004), Loo and Wong (1975) observed AB-PAS positive granules in the enterocytes in *Bufo melanostictus*.

Neutral mucins were predominant in the stomach, and we detected acidic, particularly sulphated sialomucins were abundant in the intestine. The possible functions of intestinal mucus are probably to lubricate and protect the epithelium from pathogenic organisms. The sulpated mucins may provide resistance of the mucus against bacterial degradation, as we suggested in the stomach. In additon to this function, it has been noted that the high density of sulphate groups seems to be a regulatory factor of mucus viscosity (Slomiany et al. 1991). On the other hand, the production of sulphated mucin may be related to the fluid balance between external and internal enviroments as suggested by Smith (1989). As for sialic acid in the secretion, this group has an ability to establish a crosslink between glycoprotein molecules (Mandal and Mandal, 1990) and could be important in terms of rheology of the mucus (Yasui et al. 2003). Rhodes et al. (1985) reported that besides sulphation, sialylation generally contributed to the resistance of mucus to bacterial degradation.

We concluded that, the differences in the glycoconjugates produced in the two digestive tract regions may reflect special functions such as protection, lubrication, preventing bacterial colonisation and may be related to rheological characters of the mucins.

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