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Lectin histochemistry and identification of O-acetylated sialoderivatives in the horse sublingual gland

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SUMMARY

This study was aimed at characterizing the glycoconjugates produced by the horse sublingual gland and, in particular, at discriminating between the sialoderivatives by means of differential oxidation and saponification combined with lectin histochemistry and enzymatic degradation. The results showed a predominance of sialoglycoconjugates with β-galactose as acceptor sugar in the salivary mucins produced by the sublingual gland. Besides being the most represented terminal residue, sialic acid was also expressed in a great variety of derivatives distinguishable on the basis of acceptor sugars to the penultimate β-galactose as well as linkage and acetylation degree of the pyranose ring and the polyhydroxyl side chain. A role in the protection of mucous membranes from physical, chemical and pathogenic agents can be hypothesized for the horse sublingual mucins.

INTRODUCTION

In animal tissues, sialic acids represent common terminal residues in the oligosaccharide chains pertaining to glycoproteins and glycolipids. Salivary glycoproteins are an important source of sialic acids with different properties depending on the linkage of sialic acid to the acceptor sugars and on the degree of O-acetylation (Reutter *et al.*, 1982; Schauer, 1982). In addition, O-acetylated sialoderivatives seem to perform a key role in processes of both physiological and pathological nature (Devine *et al.*, 1991; Mancini *et al.*, 1991; Werner *et al.*, 1991). In particular, in the bovine sublingual gland, acinar cells express glycoconjugates characterized by the disaccharides sialic acid-galactose and sialic acid-N-acetylgalactosamine. The majority of sialic acid residues were found to be C_7, C_8 and C_9 acetylated and some residues showed C_4 hydroxyl substituted by an acetyl group (Accili *et al.*, 1994).

This work aimed to characterize *in situ* glycoconjugates occurring in the sublingual gland of horse and, in particular, to identify the sialoderivatives and their acceptor sugars by means of differential oxidation and saponification combined with lectin histochemistry and enzymatic degradation.

MATERIALS AND METHODS

Tissue preparation

Sublingual glands were removed from adult horses of both sexes, fixed at room temperature in

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Carnoy's fluid for 24 h and postfixed in 2% calcium acetate-4% paraformaldehyde solution (1:1) for 3 h. Specimens were then dehydrated, embedded in paraffin wax and cut in 5m thick serial sections.

Histochemical procedures

Mild and strong periodate oxidation with periodic acid (PO) were performed by immersing specimens in 1mM or 44mM periodic acid solution respectively for 15 min at room temperature to reveal the presence of terminal sialic acid residues with O-acetyl substituents (Reid *et al.*, 1978; Schauer, 1978).

Saponification was carried out by 0.5% potassium hydroxide in 70% ethanol for 30 min at room temperature (Reid *et al.*, 1978).

Lectin staining was performed as previously described by Menghi (1984). Briefly, after inhibition of endogenous peroxidase activity by 0.3% H_2O_2/methanol for 30 min, tissue sections were immersed in lectin horseradish peroxidase (HRP) conjugates (0.02-0.2 mg/ml) in 0.05M phosphate-buffered saline (PBS), pH 7.2 for 30 min at room temperature. After rinsing in PBS and 0.5M Tris/HCl buffer, pH 7.4, sections were treated with diaminobenzidine-H_2O_2 medium (Graham and Karnovsky, 1966) for 10 min at room temperature before dehydration and mounting in Permount. The following lectins were used: PNA from *Arachis hypogaea* [terminal β-D-galactose(1-3)-N-acetylgalactosamine], DBA from *Dolichos biflorus* (terminal α-N-acetylgalactosamine), SBA from *Glycine max* (terminal α- and β-N-acetylgalactosamine), RCA I from *Ricinus communis* [terminal β-D-galactose(1-4)-N-acetylglucosamine], GSA IB_4 from *Griffonia simplicifolia* (terminal α-D-galactose), WGA from *Triticum vulgaris* (terminal and internal β-N-acetylglucosamine>>sialic acid), GSA II from *Griffonia simplicifolia* (terminal N-acetylglucosamine), Con A from *Canavalia ensiformis* (terminal and internal α-D-mannose>α-D-glucose), UEA I from *Ulex europaeus* and LTA from *Lotus tetragonolobus* (terminal α-L-fucose), SNA from *Sambucus nigra* N-acetylneuraminic acid(α2-6)-galactose/N-acetylgalactosamine, MAA from *Maackia amurensis* [α-sialil(2-3)lactose]. All the lectins employed were peroxidase conjugated with the exception of MAA biotinylated lectin. This was used at a concentration of 20μg ml^{-1} and, after rinsing with PBS, the sections were incubated in streptavidin/peroxidase complex (VECTOR Lab. Inc.).

Before lectin histochemical staining, some sections were incubated, at 37°C for 16 hours, in 0.86U/mg protein of sialidase (Type V, from *Clostridium perfringens*) dissolved in 0.1M sodium acetate buffer, pH 5.5, containing 10 mM $CaCl_2$. Other sections were incubated at 37°C for 14 hours with α-fucosidase from bovine epididymis (2.3U/mg protein) dissolved in 0.1M sodium citrate buffer, pH 6.0, containing 25mM EDTA (Menghi *et al.*, 1989).

Lectins and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO, USA) with the exception of SNA obtained from USB (Cleveland, OH, USA), and MAA obtained from VECTOR Laboratoires Inc. (Burlingame, CA, USA).

The respective peroxidase-conjugated lectins were omitted and/or their hapten sugars were added in control sections. As controls for enzyme digestion, sections were incubated with the respective enzyme-free buffers under the same experimental conditions, or with the lectins specific for sugar detached by the enzyme pretreatment. Additional controls for SNA and MAA were performed by means of sialidase treatment preceded or not by saponification before incubating sections with lectin solutions.

The following histochemical procedures were performed as sequential treatments:

a) PNA - RCA I - DBA
b) 1mM - 44mM PO/PNA - RCA I - DBA
c) Sialidase/PNA - RCA I - DBA
d) KOH/Sialidase/PNA - RCA I - DBA
e) 1mM - 44mM PO/Sialidase/PNA - RCA I - DBA
f) 1mM - 44mM PO/KOH/Sialidase/PNA - RCA I - DBA
g) KOH/ 1mM - 44mM PO/Sialidase/PNA - RCA I - DBA

Immunohistochemical procedures

The immunohistochemical investigation was performed according to the procedure of Bancroft and Stevens (1996).

De-waxed sections were incubated for 30 min in a solution of 0.3% H_2O_2, in order to inhibit endogenous peroxidase activity. Then the sections were incubated in normal horse serum for 30 min

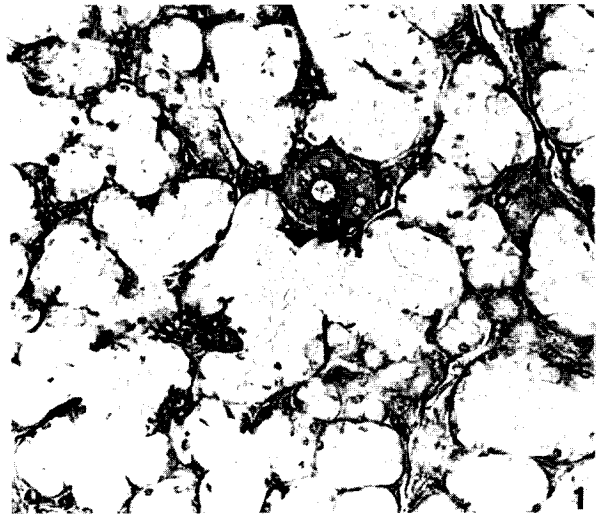


Fig. 1 - Con A-HRP staining. Note the reactivity localized at the striated duct (SD) level and in the connective tissue.220x.

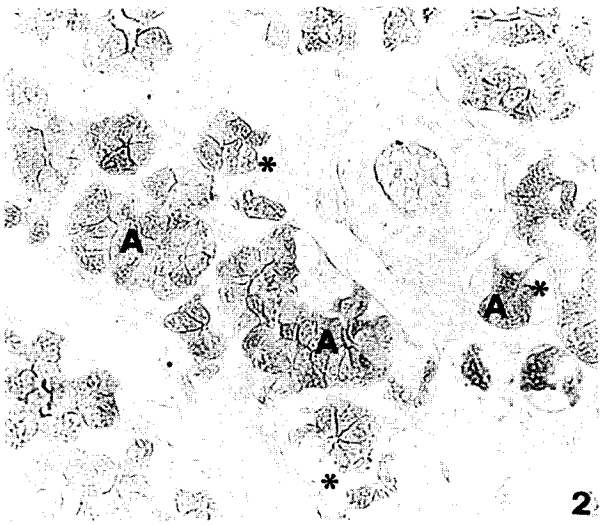


Fig. 2 - DBA-HRP staining. Binding patterns are present in acinar cells (A). Demilunes (*).220x.

and in the rabbit anti-human lysozyme antibody (Dako Corporation, Carpinteria, CA) overnight at room temperature in a moist chamber. After washing in PBS, sections were treated with the biotinylated universal secondary antibody for 30 min and, finally, with the streptavidin-peroxidase preformed complex for 30 min (Universal Quick Kit-Vector-Vector Lab., Burlingame, CA). Sections were washed in PBS and the antigenic sites were revealed using DAB medium as a chromogen.

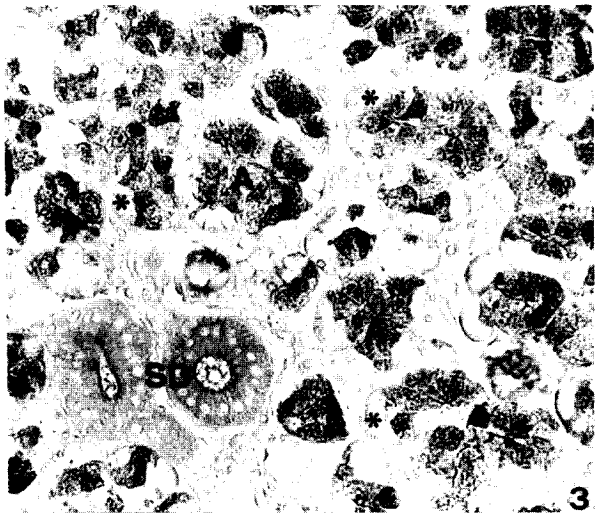


Fig. 3 - WGA-HRP staining. Reaction intensity range from moderate to strong in acinar cells (A), while in striated ducts (SD) is moderate. Demilunes (*) are negative. 220x.

Controls were prepared by omitting the anti-lysozyme antibody or substituting it with preimmune rabbit serum.

RESULTS

No appreciable differences emerged between male and female sublingual glands.

Con A binding patterns were chiefly present in the striated ducts (Fig. 1).

SBA marked both acinar and ductal cells, while DBA showed reactivity in the acinar cells (Fig. 2). DBA reactivity was not affected by sialidase and KOH/sialidase treatments.

WGA strongly reacted in the acini and also in ductal cells (Fig. 3), while GSA II weakly marked ducts only; after sialidase treatment GSA II binding patterns appeared in some acinar cells (Fig. 4).

Ductal cells reacted to LTA and UEA I that showed strong affinity also for the acinar cells (Fig. 5).

GSA IB_4 and SNA did not show binding patterns, while MAA showed aspecific background staining that was not abolished by sialidase and KOH/sialidase treatment.

Fucosidase slightly induced PNA reactivity in some acinar cells (Fig. 6).

Native PNA and RCA I binding sites and modifications consequent to the treatments detailed in the Materials and Methods section, were illustrated in

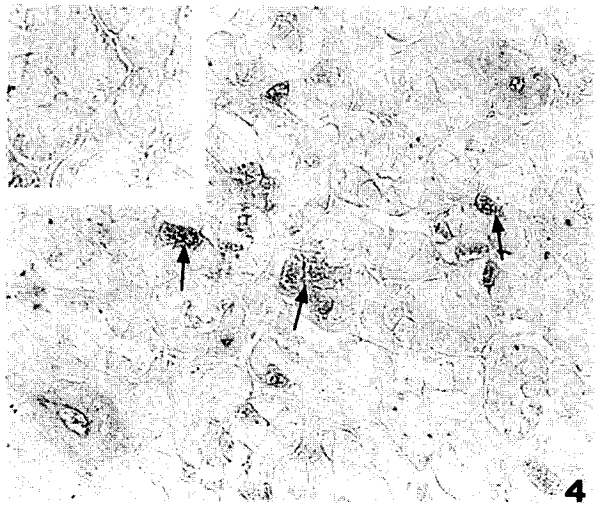


Fig. 4 - Sialidase/GSA II-HRP staining. Note the modest staining induced by sialidase digestion in some acinar cells (↑) in comparison with GSA II negative reaction showed in the inset. 220x.

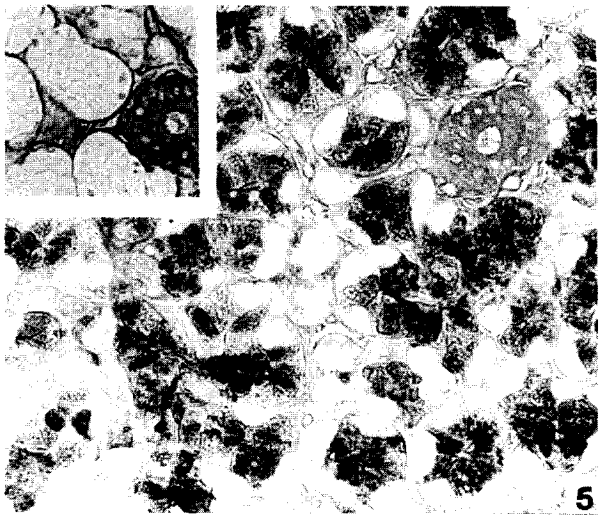


Fig. 5 - UEA I-HRP staining. Most UEA I binding is present in acinar cells (A). Inset: LTA binding patterns are appreciable only in striated ducts (SD) 220x.

Figs. 7-12 and summarized in Table I in which an indication of carbohydrate moieties visualized by effective treatments performed was also provided.

Lysozyme was evidenced at demilune and striated duct level (Fig. 13).

No staining occurred in sections treated with unconjugated lectins or after incubation with hapten sugars in lectin-HRP solutions.

The immersion of sections in enzyme-free buffer solution resulted in unmodified binding.

1mM PO treatment did not affect PNA and RCA I binding, while 44mM PO treatment abolished the reactivity of both lectins.

Saponification with KOH abolished the reactivity promoted by periodate/sialidase/PNA - RCA I treatments.

Controls for the immunohistochemical technique showed no reaction.

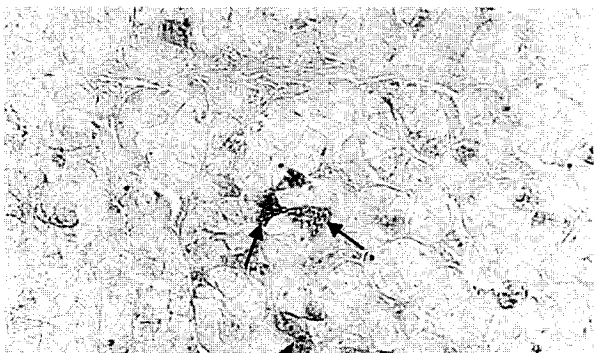


Fig. 6 - Fucosidase/PNA-HRP staining. Sequence shows the presence of fucose-galactose dimer in some acinar cells (↑).220x.

DISCUSSION

The horse sublingual gland was found to be an organ producing heterogeneous glycoconjugates, particularly rich in fucose and sialoderivatives.

α-L-Fucose, a typically terminal sugar, was evidenced only by UEA I in the acini and by UEA I and LTA in the striated ducts. Although some authors noticed mutually exclusive binding patterns between these two lectins (Laden *et al.*, 1984; Schulte and Spicer, 1983, 1984), others reported overlapping binding sites (Menghi *et al.*, 1989). The different behaviour of fucose-recognizing lectins can be explained by the observation that UEA I seems to preferentially evidence fucose bound to galactose (Menghi *et al.*, 1989); accordingly, the sequence fucosidase/PNA demonstrated a minimal presence of the disaccharide α-L-

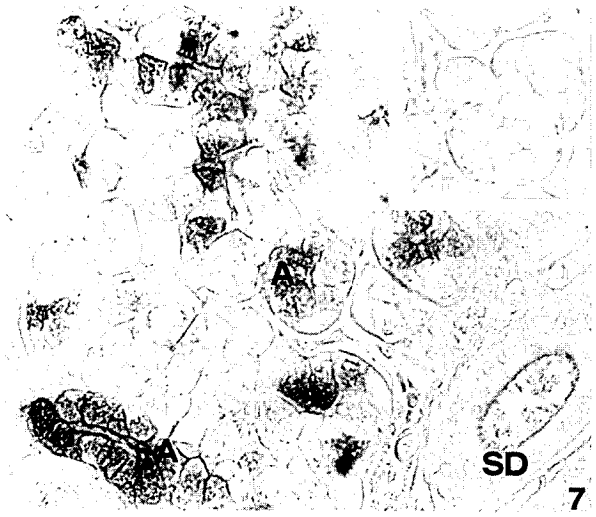
Table I
PNA and RCA I reactivities in horse SL gland

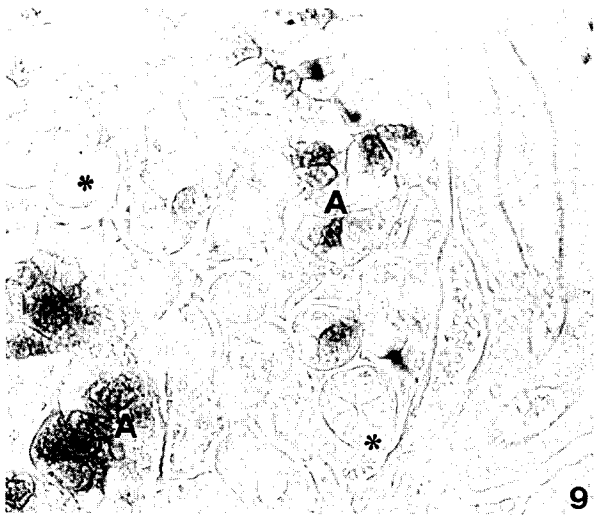
	Demilunes	Acini	Striated Ducts	Carbohydrate moieties visualized
PNA	–	–	–	β-Gal residues in terminal non-reducing position linked (1-3) GalNAc did not occur
Sialidase/PNA	–	–/+	±[a,b]	Detection of terminal disaccharide sialic acid-β-Gal with sialic acid not acetylated at C_4 level
KOH/Sialidase/PNA	–	±/++	+[a,b]	Visualization of sialic acid with and without C_4 acetyl groups linked to β-Gal(1-3)GalNAc
1mM PO/Sialidase/PNA	–	–/+	±[a]	Presence of sialic acid substituted on the polyhydroxyl side chain with acetyl groups
44mM PO/Sialidase/PNA	–	–/±	–	Reactivity of C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$ O-acetylated sialic acid linked (α2-3)-Gal(1-3)GalNAc
1mM PO/KOH/Sialidase/PNA	–	±/++	±[a]	Occurrence of sialic acid with and without C_4 groups, in addition to O-acetylated side chain
44mM PO/KOH/Sialidase/PNA	–	–/+	–	Terminal sequence sialic acid (α2-3)-Gal(1-3)GalNAc with sialic acid acetylated in C_4 and in C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$
RCA I	–	–	–	β-Gal residues in terminal non-reducing position linked (1-4) GlcNAc did not occur
Sialidase/RCA I	–	±/+	–	Detection of terminal disaccharide sialic acid-βGal with sialic residues not acetylated in C_4
KOH/Sialidase/RCA I	–	±/+	–	Presence of sialic acid not C_4O-acetylated linked to β-Gal(1-4)GlcNAc
1mM PO/Sialidase/RCA I	–	±/+	–	Reactivity of sialic acid with acyl substituents on the polyhydroxyl side chain
44mM PO/Sialidase/RCA I	–	±/+	–	Visualization of C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$ O-acetylated sialic acid linked (α2-3)-Gal(1-4)GlcNAc

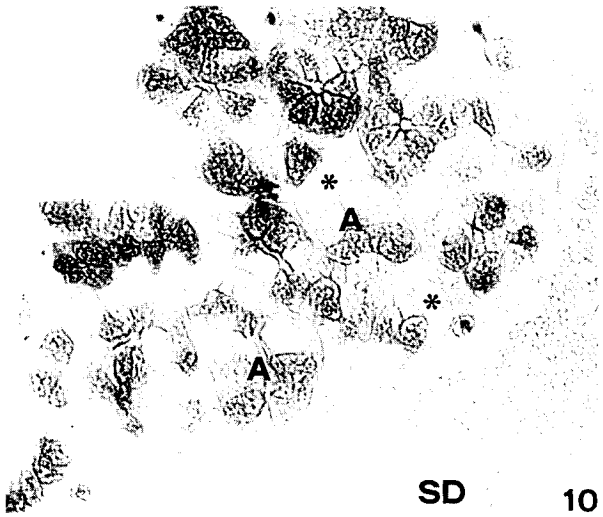
Results are given in arbitrary units as follows: –, negative; ±, weak; +, moderate; ++, strong staining. [a]luminal border; [b]luminal material.

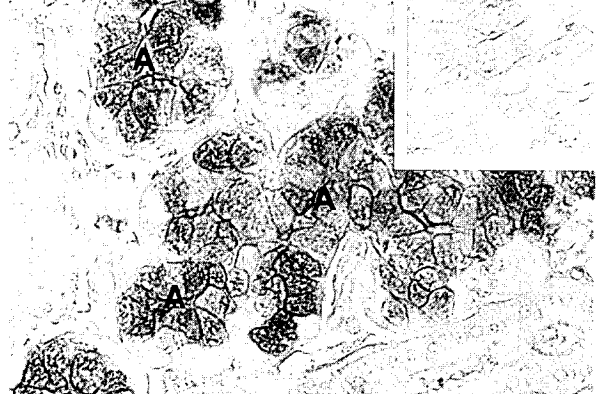
fucose-galactose at the acinous level. The reactivity of these two lectins should not be affected by the difference between their molecular weights since LTA did not show internalization difficulty at the duct level. We are unable to detect the acceptor sugar for the great part of UEA I recognized α-L-fucose that may form very short side chains and link internal β-galactose and β-N-acetylglucosamine residues as observed in other organ tissues (Kornfeld and Kornfeld, 1985; Menghi *et al.*, 1993, 1995; Wu *et al.*, 1994).

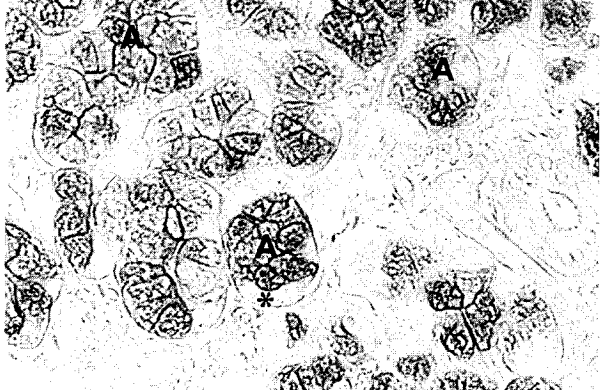
In acinar cells, α-N-acetylgalactosamine was present in the terminal position of glucidic chains, as evidenced by DBA reactivity; conversely, N-











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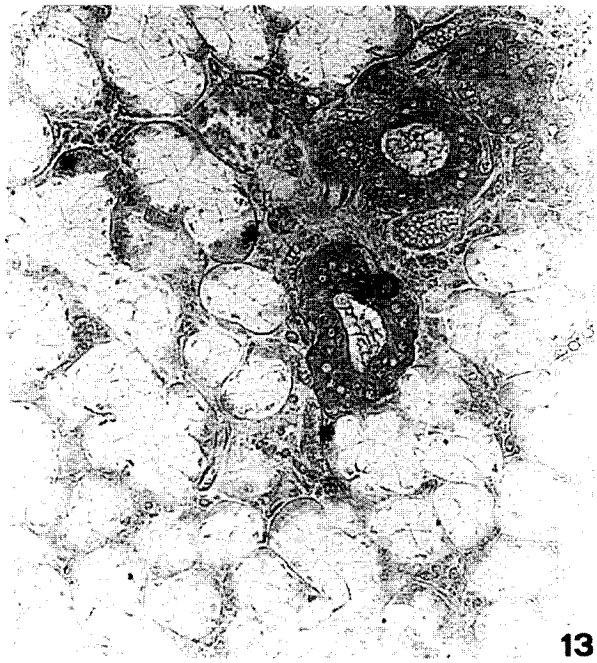

Fig. 13 - Immunohistochemical localization of lysozyme. Note the weak staining of striated ducts (SD) and demilunes (*) 220x.

acetylglucosamine was scarcely present as a terminal residue as demonstrated by the faint reactivity of GSA II in ductal cells.

Although lectins with nominal specificity towards sialic acid, such as MAA and SNA, gave negative results, sialic acid was the most represented terminal sugar in the horse sublingual gland. Indeed, sialic acid residues were indirectly evidenced using sialidase digestion, preceded or not by saponification, and lectins specific for their usually acceptor sugars.

Sialidase treatment only modified PNA and RCA I reactivities, showing the presence, in acini and ducts, of oligosaccharide chains with the terminal sequences sialic acid-β-galactose(1-3)N-acetylgalactosamine and sialic acid-β-galactose(1-4)N-acetylglucosamine, respectively, and the lack of glucidic chains with the terminal disaccharide sialic acid-α-N-acetylgalactosamine.

KOH/sialidase treatment increased PNA reactivity more than sialidase digestion alone, indicating that a quota of sialic acids linked to β-galactose(1-3)N-acetylgalactosamine was acetylated at C_4. Mild oxidation did not modify sialidase/PNA reactivity preceded or not by saponification, showing the absence of periodate labile sialic acid. Strong oxidation caused a decrease of PNA reactivity previously induced by both sialidase and KOH/sialidase treatment, indicating the presence of sialic acid residues with acyl groups at C_7 and/or C_8 and/or C_9 (α2-6)galactose that were partly acetylated also at C_4. Residual staining was due to the presence of sialic acid bound via (α2-3) to β-galactose and acetylated at C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$, and, also in this case, partly acetylated at C_4. On the basis of the above treatments followed by RCA I staining, it was found that also sialic acid residues bound via (α2-3) to the disaccharide β-galactose(1-4)N-acetylglucosamine contained acetyl groups at C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$.

In summary, the characterization of sialoglycoconjugates of the salivary mucins produced by the horse sublingual gland indicated that, besides being the most represented terminal residue, sialic acid is also present in a great variety of derivatives distinguishable on the basis of acceptor sugars to the penultimate β-galactose as well as linkage and acetylation degree.

The lack of periodate labile sialic acids could make the salivary mucins resistant to mild oxidizing agents; in addition, the presence of C_9 acetylated sialic acids contributes to hinder the action of strong oxidizing agents. Moreover, the acetylation at C_4 inhibits the effect of sialidase, an enzyme also produced by many bacteria. In addition, it was demonstrated that acetylation at the C_4 and C_9 level prevents influenza C virus from recognizing its receptor determinant (Hanaoka *et al.*, 1989; Zimmer *et al.*, 1992).

Lysozyme hampers the attack of bacteria destroying the cell wall (Jolles and Jolles, 1984).

Figs. 7-12 - **(7)** Sialidase/PNA-HRP staining. Sialidase treatment imparts affinity for PNA to some acinar cells (A) and to luminal border of striated ducts (SD). Inset: Negative PNA-HRP staining. 260x. **(8)** KOH/Sialidase/PNA-HRP staining. Saponification enhances PNA sialidase-induced reactivity in acinar cells (A) and at the striated duct (SD) level. Demilunes (*)260x. **(9)** 44mM PO/Sialidase/PNA-HRP staining. Strong oxidation reduces sialidase/PNA reactivity of some acinar cells (A). Demilunes (*)260x. **(10)** 44mM PO/KOH/Sialidase/PNA-HRP staining. A population of PNA positive acinar cells (A) persists after 44 mM PO/KOH/Sialidase/PNA sequence. Reactivity of ductal (SD) luminal border desappears. Demilunes (*) 260x. **(11)** Sialidase/RCA I-HRP staining. Sialidase digestion causes the appearence of RCA I positive sites at the acinar (A) level. Inset: Negative RCA I-HRP staining 260x. **(12)** 44mM PO/Sialidase/RCA I-HRP staining. Strong oxidation does not modify the RCA I sialidase-induced reactivity. Acini (A). Demilunes (*) 260x.

The resistance to oxidizing agents and to enzymatic degradation confers many properties to the salivary mucins that, besides lubricating and hydrating the chewed food, play an important role in the protection of mucous membranes from physical, chemical and pathogenic agents.

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