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# Exoglycosidases and lectins as sequencing approaches of salivary gland oligosaccharides

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**Summary.** This review was focused on the salivary gland oligosaccharide chains studied by lectin histochemistry combined with exoglycosidase digestion. Glycoconjugates play an important role in many biofunctions and, generally, salivary mucins, which consist of numerous oligosaccharide chains attached at closely spaced intervals to a peptide backbone, serve as lubricants and protective agents, but in many instances we are ignorant about the role of biochemically identified oligosaccharides. Lectin histochemistry represents the greatest analytic tool to study carbohydrates *in situ*; in addition, there is availability of selective enzymes, so glycosidase degradation is useful to both investigate the structure of a given oligosaccharide and verify the influence of neighbouring sugars on the affinity towards the respective specific lectins. Using stepwise digestion of samples, followed by lectin labelling, the structure of terminal short oligosaccharides with blood-group activity was also elucidated. Additional histochemical methodologies were developed to establish the presence of acetylated groups in sialic acid residues, and the position of the linkage to the underlying monosaccharide. Sequencing approaches by exoglycosidases and lectins were also seen to be particularly useful when substantial differences did not emerge in lectin affinity, glycoconjugate composition and complex carbohydrate cytochemistry.

**Key words:** Lectins, Exoglycosidases, Glycoconjugates, Salivary glands

## Introduction

Lectins are proteins or glycoproteins that bind non-covalently carbohydrate residues without induction of chemical changes. All lectins have more than one specific sugar-combining site that allows them to serve as cross-linking agents; indeed, lectins (from legere= to choose, p.p. lectus) have been firstly identified in extracts of plant seeds containing soluble factors agglutinating red blood cells (Brown and Hunt, 1978).

A number of reviews on plant, invertebrate and vertebrate lectin purification have been referred in literature (Sharon and Lis, 1972, 1989; Bird, 1974; Nicolson, 1974; Goldstein and Hayes, 1978; Barondes, 1981; Yeaton, 1981a,b; Etzler, 1985; Goldstein and Poretz, 1986; Liener et al., 1986; Lis and Sharon, 1986; Mandal and Mandal, 1990).

The nature of the carbohydrate-lectin interaction is of considerable interest and shows some controversy (Goldstein and Poretz, 1986); however, the help of these substances in structural studies of oligosaccharides is undeniable.

Biological components consisting of carbohydrates and additional substances such as lipids and proteins allow the formation of glycoconjugates, i.e. glycoproteins, proteoglycans and glycolipids. Eucaryotic cell plasma membrane exhibits glycoproteins as well as glycolipids on the outside surface. Secretory cells contain glycoconjugates that can be accordingly identified as glycoproteins and glycolipids. Proteoglycans only occasionally occur in the secretory cell cytoplasm (Storch and Kleinfeld, 1982; Bretscher, 1985; Hakomori, 1986; Cutler et al., 1987; Harrison and Auger, 1991).

Since the carbohydrate moieties of glycoconjugates are presumed to account for some of the biological functions, a great deal of effort has been exerted to determine the structure of the oligosaccharide chains. In particular, the structures of the oligosaccharide chains of many glycoproteins are very similar and fall into a few broad categories, i.e. the O-glycosidically and the N-glycosidically linked oligosaccharides. In addition, fine structural differences distinguish one member of such a group from another member (Kornfeld and Kornfeld, 1980).

The composition of secretory glycoconjugate

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carbohydrate portion largely varies within the organ tissues. The salivary gland glycocomponents generally contain N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, D-galactose, L-fucose, and sialic acid. Also D-mannose and D-glucose seem to occur in some glycoproteins (Spiro, 1969; Gottschalk and Bhargawa, 1972; Gallangher and Corfield, 1978). Nevertheless, an enormous variety of oligosaccharide structures can be obtained by arranging different combinations of the monosaccharides available in nature in a number of linkages producing both linear and highly branched glycan chains. In addition, since every hexose has four hydroxyl groups and each hexosamine has three hydroxyl radicals available for substitution there are a number of linkage positions possible for a given sequence and many opportunities for branching to occur in oligosaccharide chains (Montreuil, 1980).

It is normally possible to obtain the precise qualitative and quantitative composition of glycoconjugates using chemical procedures because the analyses can be performed *extra situm* on isolated and purified molecules. In these studies, different methods can be used for the determination of oligosaccharide structure and firstly it has been tempted to discriminate between glycopeptides. Indeed, oligosaccharides linked N-glycosidically from N-acetylglucosamine to asparagine may be distinguished from chains linked O-glycosidically to the hydroxyl groups of serine or threonine by treating the glycopeptide with mild alkali in presence of sodium borohydride. Removal of N-linked oligosaccharide chains from the peptide requires much stronger conditions and can result in degradation (Lennarz, 1980).

In our studies it was tempted to obtain information about the salivary glycoconjugates *in situ* by means of lectins recognizing different monosaccharides and specific exoglycosidases. Indeed, the discussion of lectin histochemistry, with and without prior glycosidase digestion, here concerns to a large extent experience from our laboratory. The potential opportunity of the histochemical approach offered by the introduction in glycohistochemistry of lectins accomplished by exoglycosidase degradation was chiefly utilized for localizing and characterizing the saccharide portion of carbohydrate-containing macromolecules. The greatest part of investigations was devoted to the study of salivary glands in mammals and birds where techniques of sequentiation *in situ* of oligosaccharide chains, techniques of immunolocalization of endogenous lectins and ultracytochemical stainings in order to characterize and localize surface and cytoplasmic glycoconjugates were pointed out and experimented. Most attention was focused on the investigation of heterogeneity in these secretory organs and on the evolution of the complexity of glycoconjugate macromolecules during differentiation of secretory areas and ductal segments (Bondi et al., 1983, 1984, 1985, 1987, 1991; Menghi et al., 1983a,b, 1984, 1992b). Also the aqueous medium that interacts with secretory and structural glandular components was quantified and monitored (Menghi et al., 1988b, 1989b, 1990b, 1991a; Curini et al., 1989).

Sometimes classical carbohydrate histochemistry and sequencing approaches were unsuccessful due to the large quantity of protein structures as well as to the scarcity and heterogeneity of material available for deglycosilation. Moreover, surface and cytoplasmic glycoconjugate structure of salivary glands still remain undetermined and currently there is a rapidly growing interest in the deglycosilation techniques and lectin binding in view of the possible sequentiation *in situ* of salivary carbohydrate chains.

## Materials and methods

A variety of histochemical approaches were experimented with to reconstruct the sequence of the salivary oligosaccharides. Selective and sequential degradation of the sugar chains from their non-reducing ends with different specific exoglycosidases (neuraminidase, α-mannosidase, α-fucosidase, α- and ß-galactosidase, ß-N-acetylglucosaminidase, α-N-acetylgalactosaminidase, α- and ß-glucosidase) revealed not only the sequence of sugars but also their anomeric configuration based on the specificity of the glycosidases for α or ß linkage.

Starting from chemical knowledge about carbohydrate components it was tempted to translate them into morphological terms by localizing glycosidic residues previously determined by means of biochemical methodologies (Gottschalk and Bhargawa, 1972; Keryer et al., 1973; Lombart and Winzler, 1974; Moshera and Pigman, 1975; Roukema et al., 1976; Slomiany and Slomiany, 1978; Herp et al., 1979; Denny et al., 1980; Menghi et al., 1981, 1986a,b; Fleming et al., 1982; Nieuw Amerongen et al., 1983; Menghi, 1984a; Tabak et al., 1985; Accili et al., 1989). Some sugars were visualized by direct binding between lectin and sugar, other residues were located by induction of labelling as a consequence of the removal of masking residues, other ones were identified by competition of binding and/or selective pretreatment of samples before incubation with lectins.

The first step was usually represented by the investigation on the influence of fixative components as well as fixative mixtures (Menghi, 1984b, Menghi et al., 1985a) . It was demonstrated that comparable results can be obtained on frozen sections of fresh tissues and formalin-fixed tissues (Ishiyama, 1979; Faraggiana et al., 1982; Menghi, 1984b; Menghi et al., 1985a). The use of formalin-fixed paraffin-embedded tissues makes it much easier and practical to use, above all, larger amounts of autopsy and surgical materials to investigate pathologic changes in the expression of blood group antigens (Ito and Hirota, 1992) as well as surface and secretory glycoconjugates. It was also observed that the staining of fixed paraffin-embedded sections provided more sensitive and precise localization of labelling. Salivary glands were preferably fixed by us with

Carnoy's fluid and postfixed with a mixture of 2% calcium acetate - 4% paraformaldehyde (1:1). Other authors experimented with different fixatives (Schulte and Spicer, 1983, 1984; Takai et al., 1986; Allison, 1987). However, as maintained by Spicer and Schulte (1988), Carnoy-fixed specimens offer an advantage, permitting comparative lectin and immunohistochemical studies and this fact was particularly important in research concerned with the localization of endogenous lectins by antilectin antibody (Menghi et al., 1989c, 1992c).

Recently, an extensive review has been published by Spicer and Schulte (1992) on both pre-lectin and lectin histochemistry of glycoconjugates. Thus, as concerns lectin technique and applications of lectin histochemistry, the authors refer the reader to the above mentioned review.

## Results

It has been repeatedly assumed that lectins are powerful histochemical tools for studying carbohydrates, above all as support for biochemical methodologies. Indeed, findings from lectin histochemistry contributed to discriminate between contrasting data originating from biochemical procedures in the mouse submandibular gland (Menghi et al., 1986b) about the presence or absence of fucose in isolated glycoprotein fraction (Roukema et al., 1976; Denny et al., 1980). Studying the lectin affinity patterns at electron microscopic level a certain staining polarization in convoluted granular tubule cells was also found that exhibit a codistribution of glycosidic residues on junctional complexes and a peculiar localization of fucose and ß-galactose residues at duct cell level (Menghi and Bondi, 1987). Lectin histochemistry was equally useful to investigate the effects of secretagogues on lectin affinity patterns of the convoluted granular tubules as well as secretory acini (Tsukitani and Mori, 1986; Menghi et al., 1991c).

Binding of labelled lectins for differentiating sugar residues within complex carbohydrates also offered a valid approach to further understand and classify the salivary gland secretion type (Schulte and Spicer, 1983, 1984; Accili et al., 1992). The combination of glycosidase degradation and lectin labelling experimented for the first time by us (Menghi et al., 1985b) represented a valid tool for studying carbohydrates of the rabbit submandibular and sublingual glands. In particular, it was demonstrated that sialic acid is present in the mucous acini with the terminal dimers sialic acid-ß-galactose and sialic acid-N-acetylgalactosamine within the submandibular gland, whereas in the sublingual gland it only seems present

**Table 1.** Mean absorbance of lectin labelling, before and after exoglycosidase digestion in adult cat submandibular gland[a].

| | DEMILUNES | ACINI | STRIATED DUCTS |
|---|---|---|---|
| LTA (*Lotus tetragonolobus*)[c] | 0.049±0.008 | 0.053±0.006 | 0.353±0.033 |
| Sialidase/LTA | 0.390±0.074 | 0.216±0.027 | 0.503±0.043 |
| UEA I (*Ulex europaeus*) | 0.046±0.005[b] | 0.126±0.017 | 0.032±0.001 |
| ß-N-Acetylglucosaminidase/UEA I | 0.049±0.008 | 0.465±0.013 | 0.159±0.015 |
| WGA (*Triticum vulgaris*) | 0.046±0.030[b] | 0.635±0.066 | 0.262±0.012 |
| α-fucosidase/WGA | 0.042±0.011 | 0.618±0.017 | 0.395±0.063 |
| ß-N-Acetylglucosaminidase/WGA | 0.035±0.009 | 0.519±0.029 | 0.231±0.018 |
| SBA (*Glycine max*) | 0.068±0.011 | 0.173±0.032 | 0.055±0.016 |
| Sialidase/SBA | 0.549±0.040 | 0.167±0.038 | 0.122±0.019 |
| α-Fucosidase/SBA | 0.144±0.021 | 0.346±0.029 | 0.046±0.026 |
| ß-Galactosidase/SBA | 0.045±0.011 | 0.395±0.036 | 0.093±0.011 |
| DBA (*Dolichos biflorus*) | 0.181±0.054 | 0.361±0.019 | 0.295±0.031[b] |
| Sialidase/DBA | 0.522±0.056 | 0.349±0.032 | 0.283±0.046 |
| α-Fucosidase/DBA | 0.233±0.016 | 0.450±0.027 | 0.298±0.036 |
| ß-Galactosidase/DBA | 0.166±0.020 | 0.488±0.049 | 0.264±0.032 |
| Con A (*Canavalia ensiformis*) | 0.208±0.015 | 0.232±0.019 | 0.699±0.049 |
| α-Mannosidase/Con A | 0.075±0.006 | 0.116±0.011 | 0.488±0.006 |
| ß-N-Acetylglucosaminidase/Con A | 0.209±0.030 | 0.313±0.013 | 0.601±0,038 |
| PNA (*Arachis hypogaea*) | 0.042±0.007 | 0.106±0.022 | 0.034±0.006 |
| Sialidase/PNA | 0.544±0.016 | 0.121±0.014 | 0.103±0.014 |
| α-Fucosidase/PNA | 0.217±0.035 | 0.523±0.045 | 0.040±0.010 |
| RCA I (*Ricinus communis*) | 0.066±0.017 | 0.252±0.024 | 0.062±0.010 |
| Sialidase/RCA I | 0.598±0.016 | 0.234±0.019 | 0.172±0.037 |
| α-Fucosidase/RCA I | 0.161±0.010 | 0.664±0.037 | 0.058±0.011 |

[a]: The mean absorbance under 0.049 for demilunes, 0.053 for acini and 0.055 for duct cells were due to the extinction of unreactive sites; [b]: not significant, all the other data resulted statistically significant ($p < 0.01$) by ANOVA test; [c]: for lectin specificity the authors refer the reader to Goldstein and Poretz (1986).

with the sequence sialic acid-ß-galactose. Fucose was seen to have as subterminal sugar N-acetylglucosamine in both glands (Menghi et al., 1988a).

As markers of growth processes, lectins furnished interesting information above all in the cat submandibular gland. In fact, analytical, biosynthetic, and structural studies have confirmed that the expression of secretory glycoconjugates of the cat salivary glands undergoes qualitative and quantitative changes in correlation with modifications occurring during development (Menghi et al., 1986a, 1987; Bondi et al., 1987, 1991). Structural changes in glycan chains from glycoconjugates have been investigated by comparing the lectin labelling patterns, with and without prior glycosidase digestion, in adult and sucking animals. Indeed, by stepwise digestion and sequential cleavage of sugar residues, each time visualized by appropriate specific lectins, it was observed that secretory glycoconjugates stored in acinar cells of adult subjects were characterized by the terminal sequence ß-galactose-(1-3)-N-acetyl-D-galactosamine established on the basis of increased DBA labelling after ß-galactosidase pretreatment (Table 1). Conversely, the terminal disaccharides sialic acid-N-acetylgalactosamine and sialic acid-ß-galactose were found in both demilunar and ductal cells. Glycosidase digestion revealed that N-acetylglucosamine residues occurred in terminal, subterminal and internal position. Fucoglycoconjugates showed noticeable heterogeneity; indeed, fucose was seen to form the disaccharide fucose-galactose in demilunar and acinar cells, whereas it was present with the sequence fucose-N-acetyl-D-glucosamine in the striated ducts (Menghi et al., 1989a). Also in the sucking cat submandibular gland (Table 2), lectins with and without prior enzyme degradation showed selective binding patterns towards sialo-fuco- and galacto-glycoconjugates of secretory areas. Indeed, sialoglyco-conjugates having as terminal sequences sialic acid-ß-galactose and sialic acid-N-acetylgalactosamine were visualized on demilunar cells. Fucoglycoconjugates with the terminal disaccharide fucose-galactose were detected in acinar cells (Menghi et al., 1990a). In both adult and growing subjects the histophotometrical evaluation of lectin labelling appeared to be very useful to estimate

**Table 2.** Mean absorbance values of lectin labelling, with and without prior exoglycosidase digestion, in the sucking cat submandibular gland[a].

| | DEMILUNES | ANOVA (Values of F[b]) | ACINI | ANOVA (Values of F[b]) | STRIATED DUCTS | ANOVA (Values of F[b]) |
|---|---|---|---|---|---|---|
| LTA (*Lotus tetragonolobus*)[d] | < 0.01 | | 0.437±0.026 | | < 0.01 | |
| UEA I (*Ulex europaeus*) | < 0.01 | | 0.415±0.028 | | < 0.01 | |
| DBA (*Dolichos biflorus*) | 0.305±0.018 | c.s.[c] | 0.144±0.019 | c.s. | < 0.01 | c.s. |
| Sialidase/DBA | 0.458±0.013 | 553.61 | 0.149±0.018 | 0.50 | 0.094±0.025 | 165.82 |
| α-fucosidase/DBA | 0.302±0.028 | 0.10 | 0.356±0.024 | 576.03 | < 0.01 | |
| ß-Galactosidase/DBA | 0.299±0.030 | 0.36 | 0.213±0.017 | 87.55 | < 0.01 | |
| SBA (*Glycine max*) | 0.252±0.021 | c.s. | 0.098±0.021 | c.s. | < 0.01 | c.s. |
| Sialidase/SBA | 0.428±0.018 | 498.90 | 0.095±0.016 | 0.14 | 0.108±0.015 | 632.26 |
| α-Fucosidase/SBA | 0.249±0.015 | 0.15 | 0.186±0.022 | 99.40 | < 0.01 | |
| ß-Galactosidase/SBA | 0.248±0.026 | 0.20 | 0.126±0.011 | 16.65 | < 0.01 | |
| GSA II (*Griffonia simplicifolia*) | < 0.01 | | 0.106±0.015 | | < 0.01 | |
| WGA (*Triticum vulgaris*) | < 0.01 | | 0.674±0.031 | c.s. | 0.096±0.010 | c.s. |
| ß-Glucosaminidase/WGA | < 0.01 | | 0.347±0.035 | 579.79 | 0.098±0.013 | 0.16 |
| Con A (*Canavalia ensiformis*) | 0.310±0.026 | c.s. | 0.240±0.036 | c.s. | 0.344±0.018 | c.s. |
| α-Mannosidase/Con A | 0.208±0.020 | 112.10 | 0.167±0.028 | 30.58 | 0.339±0.021 | 0.35 |
| ß-Glucosaminidase/Con A | 0.307±0.031 | 0.07 | 0.209±0.024 | 6.18 | 0.340±.0.024 | 0.21 |
| LCA (*Lens culinaris*) | < 0.01 | | 0.124±0.028 | c.s. | 0.161±0.018 | c.s. |
| α-Mannosidase/LCA | < 0.01 | | 0.092±0.014 | 12.69 | 0.153±0.011 | 1.81 |
| ß-Glucosaminidase/LCA | < 0.01 | | 0.104±0.029 | 3.00 | 0.158±0.016 | 0.18 |
| GSA $IB_4$ (*Griffonia simplicifolia*) | < 0.01 | | 0.093±0.020 | c.s. | < 0.01 | |
| α-Fucosidase/GSA $IB_4$ | < 0.01 | | 0.120±0.020 | 11.32 | < 0.01 | |
| PNA (*Arachis hypogaea*) | 0.144±0.011 | c.s. | 0.126±0.024 | c.s. | < 0.01 | |
| Sialidase/PNA | 0.456±0.022 | 1901.94 | 0.121±0.016 | 0.33 | < 0.01 | |
| α-Fucosidase/PNA | 0.146±0.011 | 0.21 | 0.326±0.047 | 172.99 | < 0.01 | |
| RCA I (*Ricinus communis*) | 0.122±0.017 | c.s. | 0.117±0.024 | c.s. | < 0.01 | |
| Sialidase/RCA I | 0.518±0.015 | 3574.77 | 0.129±0.018 | 1.81 | < 0.01 | |
| α-Fucosidase/RCA I | 0.119±0.020 | 0.15 | 0.298±0.031 | 257.01 | < 0.01 | |

[a]: Enzyme digestions which did not promote lectin affinity to change were not reported and quantified. Also, enzyme procedures performed to test the efficacy of enzymatic treatments were not summarized. [b]: (1.34 d.f., $P_{(0.01)}$= 7.45). [c]: c.s.= control sample. [d]: For lectin specificity the authors refer to the reader to Goldstein and Poretz (1986).

and quantify increased and/or decreased glycosidase-induced labelling. In particular, image analysis focused the occurrence of reactive sites on cell surface and/or cytoplasm. The cat submandibular gland has also been seen to be a suitable organ to study the differential release of glycoconjugates in response to selective stimulation of the autonomic nerve supply (Winston et al., 1992).

From a biochemical point of view ungulate submaxillary mucins have also been throughly studied and the structures of the major oligosaccharides present in these mucins have been determined (Carlson, 1968; Bertolini and Pigman, 1970a,b; McGuire, 1970; Gottschalk et al., 1971; Baig and Aminoff, 1972). Accordingly, it was found that sialic acid-(α2-6)-N-acetylgalactosamine linked O-glycosidically to serine and threonine residues is the major disaccharide chain present in ovine and bovine submandibular mucins even if a considerable degree of variability occurs in both length and sugar content. The histochemical analysis performed *in situ* by the use of sialidase and lectins accomplished by histochemical treatments provided information about the chemical structure of ovine submandibular glycoconjugates. It has been found that stored secretion in all mucous acinar cells contains disaccharide side chains consisting of sialic acid linked to penultimate α-N-acetylgalactosamine. A previously biochemically-unrecognized disaccharide formed by sialic acid linked to ß-galactose was also located in 20-30% of mucous acinar cells. In addition, occasional clusters containing oligosaccharides which terminate with sialic acid characterized by O-acetylated polyhydroxyl side chains and penultimate galactose, were visualized. Finally, fucose (assayed biochemically as a minor component) occurred in abundance in glycoconjugates at the apical surface of intercalated and striated duct cells. Similarly, terminal α-galactose, not previously detected biochemically, was localized at the apex of all duct cells (Schulte et al., 1985).

The bovine submandibular gland is generally considered to be a secretory organ producing large quantities of highly glycosylated components (Bertolini and Pigman, 1970a,b; Corfield et al., 1991). Accordingly, a significant amount of sialic acids have been found in the fractions isolated from this gland (Gottschalk, 1957; Nisizawa and Pigman, 1959; Pigman and Hashimoto, 1963; Tettamanti and Pigman, 1968) and the occurrence and position of O-acyl groups in these compounds have been established (Leeden and Yu, 1976; Pigman, 1977; Corfield and Schauer, 1982; Schauer, 1982, 1987; Reuter et al., 1983). Indeed, the enzymatic procedures of carbohydrate degradation combined with lectin labelling were integrated with histochemical methods including the use of oxidizing and deacetylating agents, and these methodological approaches were employed especially to visualize the occurrence and distribution of acyl groups in addition to sequentiate the sialoglycoconjugates of the bovine submandibular and sublingual glands. In this way it was demonstrated that O-acetylated sialic acid at $C_1$ or $C_4$ moieties occurred only in the sublingual gland (manuscript in preparation). In the submandibular gland the reduction of sialidase/PNA positivity with 1mM periodic acid (PO) pretreatment and the abolition of induced sialidase-PNA staining with 44mM PO suggested the codistribution in acinar cells of terminal periodate-labile sialic acid devoid of acyl substituents on the polyhydroxyl side chain in addition to terminal $C_7$ and/or $C_8$ acetylated sialic acid linked (α2-3,6)-galactose and/or $C_9$ acetylated sialic acid linked (α2-6)-galactose (Table 3). Furthermore, support for the interpretation that sialic acid residues with O-acetylated substituent groups in side chain were responsible for the 1mM PO-sialidase-PNA staining was the abolished reactivity of this sequence with prior KOH saponification. Conversely, the pretreatment with 1mM PO slightly modified the subsequent staining with sialidase-DBA sequence, indicating that almost all sialic acid residues linked to penultimate N-acetylgalactosamine contained O-acyl substituents. This reactivity was decreased in numerous sites after treatment with 44mM PO. Therefore, it was considered that secretory and ductal cells of submandibular gland contain $C_7$ and/or $C_8$ and/or $C_{7,8}$ acetylated sialic acids, not oxidized with 1mM PO but susceptible to 44mM PO, and sialic acids

**Table 3.** Histochemical treatments and lectin labelling in the bovine submandibular gland.

| | DEMILUNES | ACINI | STRIATED DUCTS |
|---|---|---|---|
| PNA (*Arachis hypogaea*)* | 0 | 0 | 1-2 |
| 1mM PO-PNA | 0 | 0 | 1-2 |
| 44mM PO-PNA | 0 | 0 | 0 |
| Sialidase-PNA | 0 | 1-3 | 2-3 |
| KOH-Sialidase-PNA | 0 | 1-3 | 2-3 |
| 1mM PO-Sialidase-PNA | 0 | 0-2 | 2-3 |
| 44mM PO-Sialidase-PNA | 0 | 0 | 0-1 |
| KOH-1mM PO-Sialidase-PNA | 0 | 0 | 1-2 |
| KOH-44mM PO-Sialidase-PNA | 0 | 0 | 0 |
| RCA I (*Ricinus communis*) | 0 | 0 | 1-2 |
| Sialidase-RCA I | 0 | 0-1 | 1-2 |
| ECA (*Erythrina cristagalli*) | 0 | 0 | 1-2 |
| Sialidase-ECA | 0 | 0-1 | 1-2 |
| DBA (*Dolichos biflorus*) | 0-1 | 0-2 | 0-1 |
| 1mM PO-DBA | 0-1 | 0-2 | 0-1 |
| 44mM PO-DBA | 0 | 0-1 | 0 |
| Sialidase-DBA | 0-1 | 2-4 | 1-3 |
| KOH-Sialidase-DBA | 0-1 | 2-4 | 1-3 |
| 1mM PO-Sialidase-DBA | 0-1 | 2-3 | 1-3 |
| 44mM PO-Sialidase-DBA | 0 | 1-2 | 0 |
| KOH-1mM PO-Sialidase-DBA | 0-1 | 0-2 | 0-1 |
| KOH-44mM PO-Sialidase-DBA | 0 | 0-1 | 0 |
| SBA (*Glycine max*) | 0 | 1-2 | 1-2 |
| Sialidase-SBA | 0-1 | 2-3 | 1-3 |
| GSA II (*Griffonia simplicifolia*) | 0 | 0-1 | 0 |
| WGA (*Triticum vulgaris*) | 2 | 1-2 | 1 |

Lectin affinities are expressed in arbitrary units ranging from 0 to 4 for negative to intense staining respectively. *: For lectin specificity the authors refer the reader to Goldstein and Poretz (1986).

possessing $C_9$ and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$ acetyl groups resistent to 44mM PO (Menghi et al., 1992a).

The sequencing approaches utilizing lectins and exoglycosidases also helped to visualize and localize *in situ* the neutral and acidic fucose-containing oligosaccharides which were previously identified *extra situm* by biochemical methodologies (Tsuji and Osawa, 1986; D'Arcy et al., 1989). The following oligosaccharides were investigated in the bovine submandibular gland: α-Fuc(1-2)-ß-Gal-(1-3)-[ß-GlcNAc-(1-6)]-GalNAc-ol (N-5) and α-Fuc(1-2)-ß-Gal-(1-4)-ß-GlcNAc-(1-3)-[α-NeuAc-(2-6]-GalNAc-ol (A-1a).

To this purpose many selected sequences were performed on adjacent tissue sections, since in these structural studies the choice of appropriate enzymatic sequences and subsequent lectin stainings was determinant to discriminate not only between N-5 and A-1a but also between all the other biochemically defined oligo-saccharides. Attention was focused on the elimination of the fucose-free oligosaccharides referred to by Tsuji and Osawa (1986), i.e.: (NeuGc)NeuAc-(2-6)-GalNAc-ol (A-1); ß-Gal-(1-3)-[NeuAc-(2-6)]-GalNAc-ol (A-2); ß-GlcNAc-(1-3)-[NeuAc-(2-6)]-GalNAc-ol (A-3); GalNAc-ol (N-1); ß-Gal-(1-3)-GalNAc-ol (N-2); ß-GlcNAc-(1-3)-GalNac-ol (N-3); and ß-Gal-(1-3)-[ß-GlcNAc-(1-6)]-GalNAc-ol (N-4).

Cross interferences of these structures during the visualization of fucose-containing oligosaccharides were ruled out by adequately pretreating tissues (Table 4). Results indicated that the A-1a oligosaccharide with blood-group activity is localized in acinar cells, whereas the N-5 oligosaccharide was not visualized *in situ* (Menghi et al., 1993a).

The histochemical and cytochemical localization of blood group antigens in salivary glands was also performed and included the analysis of carbohydrate chains in human submandibular gland (Riva et al., 1977; Laden et al., 1984; Ito et al., 1987; Ito and Hirota, 1992). Results revealed that in the serous cells, H and Le Y antigens are expressed irrespective of the blood group ABO of the donors and their secretion is quite strictly dependent on the secretor status of tissue donors. Histochemical analysis of carbohydrate chains in submandibular glands further suggested that the induction or derepression of α-2-L-fucosyltransferase coded by Se gene is intimately associated with maturation or differentiation of the serous cells (Ito et al., 1989a). It is well known that often the fucosylation process is associated with maturation and differentiation of salivary gland serous and ductal cells (Hand, 1979; Lima and Haddad, 1981).

Finally, the experimental approach of studying carbohydrate macromolecules and sequentiating them *in situ*, was employed to identify and locate endogenous lectins. On the basis of investigations concerned with the extraction, localization and function of plant and animal lectins, occurrence of endogenous lectins within tissues largely rich in glycosidic residues was hypothesized. Accordingly, an immunohistochemical approach based on the use of commercially available antilectin antibodies conjugated with horseradish peroxidase was attempted. In the first instance, a cross-reactivity between plant-derived antibodies and animal endogenous lectins was tested but negative findings were obtained. So, an indirect method, based on the fact that the endogenous lectin can immobilize an exogenous appropriate sugar present in the incubation medium, was experimented. Indeed, antilectin antibodies selectively bound only to the respective specific lectins which have

**Table 4.** Lectin labelling, consequent to sequential exoglycosidase degradation, occurring in the bovine submandibular gland.

| | DEMILUNES | ACINI | STRIATED DUCTS |
|---|---|---|---|
| UEA I (*Ulex europaeus*)[c] | 0 | 1-2 | 0-1[b] |
| LTA (*Lotus tetragonolobus*) | 0 | 1-2 | 0-1[b] |
| PNA (*Arachis hypogaea*) | 0 | 0 | 1-2[a] |
| Sequence 1 | 0 | 0 | 1-2[a] |
| Sequence 5 | 0 | 2-3 | 1-2[a] |
| RCA I (*Ricinus communis*) | 0 | 0 | 1-2[a] |
| Sequence 1 | 0 | 0-2 | 1-2[a] |
| ECA (*Erythrina cristagalli*) | 0 | 0 | 1-2[a] |
| Sequence 1 | 0 | 0-2 | 1-2[a] |
| GSA II (*Griffonia simplicifolia*) | 0 | 0-1 | 0 |
| Sequence 1 | 0 | 0-1 | 0 |
| Sequence 2 | 0 | 1-2 | 0 |
| Sequence 5 | 0 | 0 | 0 |
| WGA (*Triticum vulgaris*) | 2 | 0-1 | 1[b] |
| Sequence 1 | 2 | 0-1 | 1[b] |
| Sequence 2 | 2 | 1-2 | 1[b] |
| Sequence 5 | 2 | 0-1 | 1[b] |
| DBA (*Dolichos biflorus*) | 0-1 | 0-2 | 0-2[a] |
| Sequence 2 | 0-1 | 0-2 | 0-2[a] |
| Sequence 3 | 0 | 0 | 0 |
| Sequence 4 | 1 | 3-4 | 0-2[a,b] |
| SBA (*Glycine max*) | 0-1 | 0-2 | 0-2[a] |
| Sequence 2 | 0-1 | 0-2 | 0-2[a] |
| Sequence 3 | 0 | 0 | 0 |
| Sequence 4 | 1 | 3-4 | 0-2[a,b] |

Numbers indicate staining intensity on a subjectively estimated scale from 0, no staining, to 4, strong staining. [a]: Staining present to the luminal border. [b]: Staining present in the lumnial material. [c]: For lectin specificity the authors refer the reader to Goldstein and Poretz (1986).
Sequence1:
a) α-Fucosidase; b) GSA II, WGA, PNA, RCA I, ECA.
Sequence 2:
a) α-Fucosidase; b) ß-Galactosidase; c) GSA II, WGA, DBA, SBA.
Sequence 3:
a) ß-N-Acetylglucosaminidase; b) ß-Galactosidase; c) α-N-Acetylgalactosaminidase; d) α-Fucosidase; e) ß-Galactosidase; f) DBA, SBA.
Sequence 4:
a) ß-N-Acetylglucosaminidase; b) Sialidase; c) α-Fucosidase; d) ß-Galactosidase; e) α-N-Acetylgalactosaminidase; f) ß-N-Acetylglucosaminidase; g) DBA, SBA.
Sequence 5:
a) ß-N-Acetylglucosaminidase; b) Sialidase; c) α-Fucosidase; d) ß-Galactosidase; e) α-N-Acetylgalactosaminidase; f) ß-N-Acetylglucosaminidase; g) α-N-Acetylgalactosaminidase; h) PNA, GSA II.

formed a sandwich with the appropriate hapten sugars and endogenous lectins. To destroy the binding between endogenous sugars and respective exogenous lectins, samples were subjected to a pretreatment with the appropriate exoglycosidase (Menghi et al., 1989c, 1992c).

## Concluding remarks

Enzyme digestion is useful to sequentiate *in situ* oligosaccharide chains as well as to verify the occurrence of competition between different sugars towards the same lectin. It should be pointed out that interpreting selective stainings in chemical terms requires first of all knowledge of the influence of neighbouring sugars on the affinity of lectins for the respective acceptor sugars. Sequential application of glycosidases and lectins was also seen to be extremely effective in attempting to verify if the presence of certain sugars can affect the affinity of individual lectins towards their respective hapten sugars.

It is possible to discriminate between a simple steric hindrance and an effective exposition of a given residue. In some organ tissues (Menghi et al., 1993b) the cleavage of sialic acid enhances WGA binding, in contrast to the commonly accepted idea that WGA can also recognize sialic acid (Monsigny et al., 1980). In this case it was rather easy to test the subterminal sugar for sialic acid by incubating sample with an appropriate lectin recognizing terminal N-acetylglucosamine, i.e. GSA II. Sometimes sialidase treatment can also influence affinity of lectins recognizing terminal residues such as α-L-fucose (Menghi et al., 1985b, 1989a). Lectin reactivity may be affected by the presence of sialic acid residues which, when present at the same sites together with fucose, can show reciprocal rearrangements in different chains of the same molecule, which may condition the affinity of specific lectins; sulphates also often participate in these rearrangements. In some cases also the cleavage of fucose facilitated the reactivity of some lectins recognizing sugar residues which usually do not act as acceptors for terminal fucose (Menghi et al., 1989a, 1990a). Further complicating the evaluation of findings from sequential deglycosylation techniques is the fact that a lectin which recognizes a given monosaccharide may do so with even greater avidity when that sugar is part of a disaccharide, oligosaccharide or other more complex glycoconjugates.

So, all the information available for each specimen studied can be taken into account for correctly evaluating native and induced lectin binding sites.

In some cases differences in the glycoconjugate structure and composition in morphologically comparable cell types could be due to intrinsic differences in the cellular processing enzymes, to differences in the duration of intracellular transit of glycoproteins, or to differences in the physical accessibility of oligosaccharide residues during their biosynthesis and storage. It is well known that interaction of protein signals with certain glycosyltransferases which mediate the terminal steps in oligosaccharide assembly determines the structure of the mature carbohydrate chains. Alternatively, differences in the interactions of the oligosaccharide with the underlying sugar may determine the nature of final structure (Kornfeld and Kornfeld, 1985). In many cases the accessibility rather than the presence of a specific acceptor sugar might be the critical factor. Indeed, lectins recognize and bind appropriate sugar residues in measure to their accessibility within glycomolecules. In addition, some lectins can protect their receptors from degradation when cells are treated with enzymes. Due to the fact that lectins bind to the carbohydrate portion of their receptors, it is clear that receptor molecules for the same lectin on different cell types need not have the same biofunction. The same or similar sugar residues may exist on different glycoproteins; in addition, the same glycoprotein species may contain receptor sites for more than one lectin (Brown and Hunt, 1978).

It is unquestionable that lectin staining methods combined with glycosidase digestion procedures may pave the way for analyzing the chemical structures of carbohydrate chains in tissue sections. However, at present, only a limited number of suitable and reliable enzymes are available and applications of exoglycosidases for analysis of carbohydrate chains has been restricted to certain human (Ito et al., 1987, 1988a,b, 1989a,b, 1990, 1993; Bolognani Fantin et al., 1989) and animal tissues (Zieske and Bernstein, 1982; Menghi et al., 1985b, 1988a, 1989a, 1990a, 1991b,c, 1992a, 1993a; Prime et al., 1985; Hennigar et al., 1986; Hosaka et al., 1986; Herrera and Rodríguez, 1990; Meyer et al., 1993).

Histochemical studies using endoglycosidase digestion procedures have been much more restricted (Yamada et al., 1983; Murata et al., 1984; Ito et al., 1989a,b; Castells et al., 1992; Martínez-Menárguez et al., 1992). Introduction of endoglycosidases such as endo-ß-galactosidase, endo-ß-glycosidase F and N-glycanase is indispensable for analyzing the inner core structures and glycoconjugate species.

At present, also reverse lectin histochemistry concerned with the use of synthetic neoglycoproteins offers considerable potential for detection of cells and tissue lectins (Gabius and Bardosi, 1991; Akif et al., 1993; Gabius et al., 1993).

The research concerned with carbohydrates has been greatly stimulated by the employ of lectins even if it should be noted that researchers who study carbohydrates have been repeatedly discouraged owing to the complexity of the arrangements and to the consequent difficulty in the understanding and correct interpretation of data obtained.

Indeed, as maintained by Spicer and Schulte (1992), the capacity to characterize carbohydrate *in situ* was strongly enhanced by the use of carbohydrate-degrading enzymes to localize moieties having a specific linkage labile to a given enzyme, and of immunostaining to

identify specific epitopes. It can be added that the use of labelled lectins is at the moment a valid tool for glycohistochemistry studies and when combined with exo- and/or endo-glycosidase sequential degradation can provide information about the structure of oligosaccharides. The complete elucidation of sugar sequences is very difficult to reach; differently from nucleic acids and proteins whose monomer can be bound only in one way, the monosaccharide units of oligosaccharides can be linked in more than one way. For example, two monosaccharides can form eleven different disaccharides, while two aminoacids can form only one peptide. This enormous versatility of carbohydrates makes possible very different events in life but sends the researchers interested in the study of them mad.

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