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Invited Review

Use of lectin-probes for correlative histochemical and biochemical assessments of the glycosylation patterns of secretory proteins, including kallikreins, in salivary glands and saliva

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Summary. Labelled lectins were used as probes to study the glycosylation and secretion of submandibular glycoproteins not only in sections of fixed glands but also in glandular extracts and in nerve-induced saliva, after electrophoretic separations and immobilization in nitrocellulose membranes.

In cats the glycoproteins in sympathetic saliva differed considerably from those in parasympathetic saliva. In sympathetic saliva they were found to originate mainly from striated ducts, to some extent from demilunar cells and to a small extent from acinar cells, whereas in parasympathetic saliva they arose mainly from acinar cells and demilunes and only to a small extent from striated ducts.

In rat submandibular glands sympathetic stimulation caused extensive depletion of lectin stainable granules from granular tubules. Corresponding strong binding occurred with the same lectins to constituents in saliva that ran between 25 and 35 kD on SDS gel electrophoresis and were shown to contain tissue kallikreins. Their binding patterns suggested that individual kallikreins from the same gland may be glycosylated in different ways. This possibility was tested on five different kallikreins after separation from submandibular extracts by isoelectric focussing. Lectin bindings on slot blot preparations of these kallikreins were tested before and after N-glycosidase F, sialidase or endo-α-N-acetylgalactosaminidase digestions. Results showed that, despite their close genetic and structural similarities, the kallikreins are in fact differently sialylated and fucosylated and the novel finding that some contain O-glycosidically linked side chains as well as the anticipated N-glycosidically linked side chains was revealed.

Thus, correlative histochemical and biochemical assessments of bindings with lectin probes has provided important new information about differences in the glycosylation patterns of individual glycoproteins stored within the same secretory granules.

Key words: Lectin, Salivary glands, Glycoproteins, Saliva, Kallikreins

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Introduction

Lectins are multivalent saccharide-binding proteins or glycoproteins of non-immune origin that bind carbohydrate residues on glycoconjugates. Individual lectins have specificities for different sugars or sugar sequences and thus afford a means to characterize oligosaccharide chains on glycoproteins (see Spicer and Schulte, 1992; Sharon and Lis, 1993). Labelled lectins have been used extensively on tissue sections of salivary glands to study the glycosylation patterns of glycoconjugates in secretory granules of parenchymal cells from a wide variety of species (see Yamada and Shimizu, 1979; Schulte and Spicer, 1983, 1984, 1985; Menghi et al., 1985, 1987, 1992; Hosaka et al., 1986; Schulte, 1987; Accili et al., 1992, 1994; Pedini et al., 1994a,b). Such studies have revealed a considerable heterogeneity in the glycosylation of proteins in salivary cells.

A recent review in this journal by Menghi and Materazzi (1994) gives clear indications of the value of lectin histochemistry for *in situ* study of the carbohydrate sequences of secretory glycoconjugates in salivary glands, especially after the sequential application of glycosidases to reveal underlying sugar residues. This approach was particularly helpful for determining the structural arrangements of the oligosaccharide side chains. Lectin histochemistry has great potential also for studying the effects of different secretory stimuli on the differential release and

resynthesis of salivary secretory glycoconjugates. For example Tsukitani and Mori (1986) showed that ß-galactose-binding lectins were localised histochemically to secretory granules in the granular tubules of mouse submandibular glands and that the lectin-positive granules were secreted during pharmacological stimulation with adrenergic agonists.

However, all methods have their limitations and, though lectin histochemistry *per se* may provide an excellent collective concept about the glycosylation of all the constituents in secretory granules, it fails to provide specific information about possible differences in the glycosylation of individual secretory components within the granules.

The purpose of the work to be included in this review was to extend the results with *in situ* lectin histochemistry of the secretory glycoproteins in sections of glandular tissue by applying similar methods to study the individual glycoconjugates actually secreted. Saliva secretion was induced by well controlled nerve stimulations rather than pharmacological manipulations in order to reflect events in life more effectively. The constituents in the saliva were separated electrophoretically and characterized in membrane preparations by their binding affinity to various labelled lectins. This was undertaken in order to gain information about the glycosylation of individual constituents entering the saliva and to ascertain whether they remain in the presecretory state found in the glands, or undergo alterations on secretion. To this end similar biochemical assessments were made on the soluble glycoproteins in homogenates of the glands. The results reveal limitations with both histochemical and biochemical approaches, when considered in isolation, but when both are undertaken in a correlative manner this helps to provide more meaningful information.

Our combined studies will be presented in the chronological order in which they were done, beginning with cat submandibular glands and nerve-induced saliva. Although new information was obtained in this study the results were relatively crude. Subsequent studies on rat submandibular glands employed refinements of the procedures as they were developed and led to the unexpected revelation that differences exist in the glycosylation patterns of different members of the closely related kallikrein family in these glands. Throughout this review attention has been given mainly to the glycosylation of salivary proteins with lower molecular weights rather than to mucous glycoproteins (for a good review of which see Wu et al., 1994).

Cat submandibular glands and saliva

This work is largely covered in the paper by Winston et al. (1992). Cats were anaesthetised with sodium pentobarbitone and in most instances one submandibular gland was cannulated for collection of the saliva formed in response to electrical stimulation of either the sympathetic or the parasympathetic nerve to the gland. After stimulation (usually for 1 hour) the stimulated and contralateral control unstimulated glands were removed for both histochemical and biochemical assessments of lectin bindings. Since some variations in lectin binding patterns were detected among different cats, a few were used to study all aspects in the same animal. After taking an initial biopsy of one lobe from the posterior part of one gland for control purposes, one submandibular gland then received sympathetic stimulation and the other parasympathetic stimulation. The saliva formed was collected for analysis and after stimulation each gland was removed. A wedge of each tissue was taken for histochemistry and the remainder was homogenized.

After exploratory work with a battery of some 20 different lectins, 5 were selected as being potentially of most value for studying the secretory glycoproteins, based on their histochemical localization in the different parenchymal cells of control glands. In this study we used lectins conjugated to horseradish peroxidase (HRP) and binding sites were visualized by diaminobenzidine (DAB)-H_2O_2 substrate medium. Fixation in a mixture of 0.2% glutaraldehyde in buffered mercuric chloride gave the most satisfactory results for light microscopical histochemistry. The lectins used were:

1) PNA (*Arachis hypogea*) - terminal Galß1, 3GalNAc directed - which stained central acinar cell mucins.

2) GSA 1-B_4 (*Griffonia simplicifolia*) - terminal αGal directed - which stained central acinar mucins.

3) LTA (*Lotus tetragonolobus*) - Fuc directed - which stained striated ducts and central acinar secretory materials.

4) LFA (*Limax flavus*) - sialic acid directed - which stained striated ducts and demilunar cells.

5) DBA (*Dolichos biflorus*) - terminal αGalNAc directed - which stained demilunar cells.

Histochemical results with 3 of the lectins are shown in Fig. 1, which also illustrates the effects of parasympathetic and sympathetic stimulations on *in situ* lectin reactivity. From this part of the study it was evident that parasympathetic stimulation caused secretion of secretory glycoconjugates not only from the central acinar cells but also from the demilunar cells. In contrast sympathetic stimulation promoted extensive depletion of secretory granules from striated ducts but only moderate secretion from the demilunar cells. Our results complemented earlier studies using enzyme and mucosubstance histochemistry and electron microscopy (Garrett and Kidd, 1975, 1977; Garrett et al., 1985, 1987), and extended them by demonstrating that secretion of demilunar glycoproteins is more of a parasympathetic function than had been realised previously.

Supernatants from homogenates and the saliva samples were subjected to SDS-polyacrylamide gel electrophoresis, using equivalent amounts of protein in each instance. The separated proteins were then electroblotted onto nitrocellulose membranes and lectin affinities with the different constituents were assessed

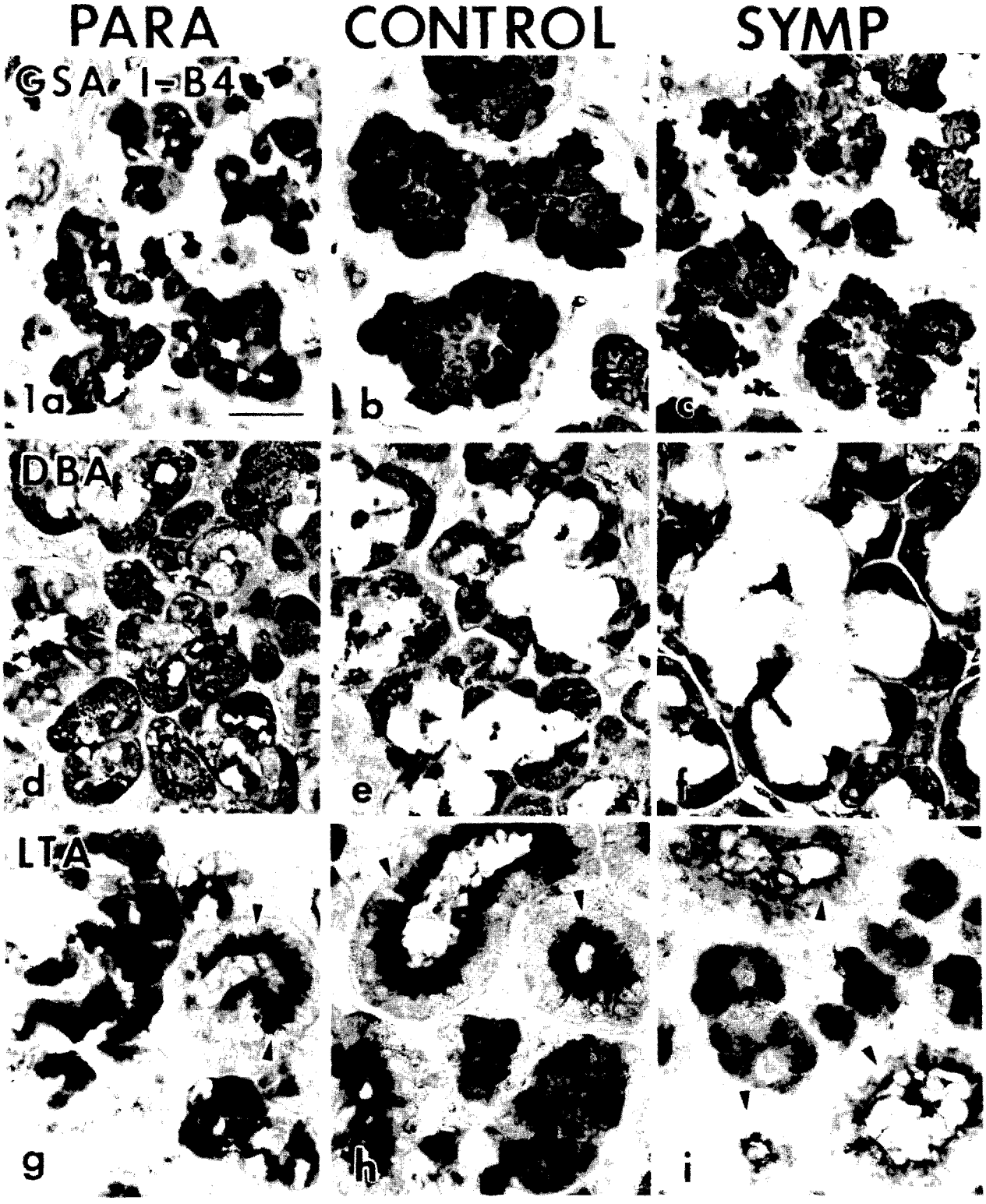

Fig. 1. Lectin-bindings by GSA I-B_4 - αGal directed (**a, b and c**); DBA - αGalNAc directed (**d, e and f**); and LTA - Fuc-directed (**g, h and i**) with sections of cat submandibular glands. All tissues were taken from the same animal. Control tissue (-initial biopsy-) (b, e and h) shows central acinar cells filled with GSA 1-B4 - and LTA - reactive material, demilunar cells react selectively with DBA and striated ducts stain selectively in the apical regions with LTA (arrowheads). After parasympathetic nerve stimulation (PARA) there is widespread depletion of central acinar staining plus vacuolation (a) and demilunar vacuolation (d) but no change in the striated ducts (g). Sympathetic stimulation (SYMP) on the other hand caused little change to central acini (c) a decrease in demilunar cell size (f) but extensive depletion of periluminal secretory material in striated ducts (i). Bar: 50 μm. (Reproduced by kind permission from Winston et al. Fig. 1. J. Histochem. Cytochem. 40, 1751-1760; 1992).

using procedures similar to those employed for histochemical detection with HRP-labelled lectins and colorimetric development with DAB. Lectin binding on the blots showed stronger staining of many bands than was apparent with conventional protein staining. The constituents detected in saliva had mobilities similar to those in glandular extracts, which is taken to indicate that they had undergone little change in the processes of secretion. Certain bands were concentrated selectively in the saliva and lectin binding patterns of the blots were more complex than was anticipated from the histochemical features. This probably relates to a number of factors including the occurrence of different constituents in the same secretory granules, the purifying effects of electrophoresis and increased access to sugar binding sites in the blots. Lectin blots of cat saliva samples are shown in Fig. 2, using the same lectins as in Fig. 1. Certain bands were common to both sympathetic and parasympathetic saliva, albeit in different relative concentrations. Other bands were distinctively different. A low molecular weight constituent was common to both sympathetic and parasympathetic saliva. It was stained by all the labelled lectins used and was the only band with affinity for PNA (galactose directed). Since PNA binding was confined histochemically to the secretory granule zone in central acinar cells, this component is likely to have arisen from these cells, but the low molecular weight precludes it from being one of the mucins that they synthesise. The presence of this constituent in sympathetic saliva suggests that sympathetic impulses do have some secretory effects on central acinar cells, as had been suggested previously from the secretion of acid phosphatase (Garrett and Kidd, 1977).

The principal constituent in sympathetic saliva

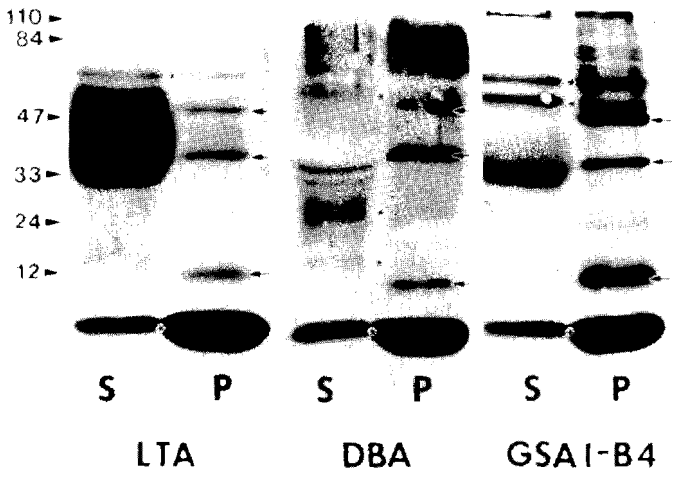

Fig. 2. Lectin blots of cat submandibular saliva (S: sympathetic saliva; P: parasympathetic saliva), showing binding-reactions with the same lectins as used in Fig. 1. Asterisks mark bands common to both types of saliva and arrows mark proteins unique to parasympathetic saliva. Numbers on the left margin are molecular weight approximations in kD. (Reproduced by kind permission from Winston et al. Fig. 4. J. Histochem. Cytochem. 40, 1751-1760; 1992).

appeared as a diffuse blob in the range of 30 to 50 kD that stained with labelled LTA (α-fucose directed). Application of an overlay membrane impregnated with the fluorogenic proteinase substrate D-Val-Leu-Arg-7-amino-4-trifluoromethylcoumarin (D-V-L-R-AFC) (a technique introduced by Smith, 1984, and Garrett et al., 1985), indicated that this diffuse band was attributtable to the presence of kallikrein from striated ducts, which is known to be heavily fucosylated in the cat (Fukuoka et al., 1979).

This initial study vindicated the use of the techniques but left many interesting questions unanswered concerning individual constituents in cat submandibular saliva. So far they have not been resolved because our work took a different direction, investigating secretion from granular tubules in rat submandibular glands, as will be described in the next two sections. Nevertheless, the rat studies evolved from our correlative histochemical and biochemical assessment of cat submandibular glycoproteins.

Rat submandibular glands and saliva

A similar study to the preceding one was next undertaken on rat submandibular glands and saliva (see Zhang et al., 1994) giving special attention to the kallikreins that occur in the granular tubules.

Improved signalling of lectin binding sites with lower background noise was achieved by the use of biotinylated lectins (when available) and detection by an avidin-biotin-HRP complex. Visualisation for histochemical purposes was still by DAB but for binding sites on blots an enhanced chemiluminescence system was used (Amersham International) and this provided a much more sensitive detection system than DAB. The system also has the great advantage that exposure times with the photographic film can be varied to optimize the signal, and the blot preparation can also be used subsequently for other staining techniques to provide further useful data about the individual bands from the same preparation.

A previous morphometric study had shown that sympathetic stimulation of rat submandibular glands induces extensive exocytosis of preformed secretory granules from both acinar and ductal cells into saliva, whereas parasympathetic stimulation did not cause any obvious exocytosis from either cell type despite evoking a large flow of saliva (Garrett et al., 1991). Therefore, for the present experiments, only sympathetic nerve stimulations were used. Male Wistar rats were anaesthetised by i.v. chloralose after induction with i.p. pentobarbitone. One submandibular duct was cannulated for the collection of saliva evoked by electrical stimulation of the cervical sympathetic trunk for 1 hour. After stimulation the test and control contralateral submandibular glands were excised for histochemical and biochemical assessments as previously. Ten lectins were used in this study, as listed in Table 1 giving their carbohydrate binding sites and their histochemical

Table 1. Lectin binding to sections of unstimulated rat submandibular glands.

LECTINS	SUGAR SPECIFICITIES	ACINI	GRANULAR TUBULES
Lotus tetragonolobus (LTA)	αFuc	0	2-3
Ulex europaeus (UEA I)	αFuc	0	2-3
Limax flavus (LFA)	NeuAc	0	2-3
Arachis hypogaea (PNA)	Gal	0-2	3-4
Maclura pomifera (MPA)	Gal	3-4	3-4
Lens culinaris (LCA)	αMan, αGlc	3	3-4
Triticum vulgaris (WGA)	GlcNAc, NeuAc	2-4	3-4
Succinyl WGA (sWGA)	GlcNAc	3	3-4
Dolichos biflorus (DBA)	GalNAc	4	0
Glycine max (SBA)	GalNAc	3	0

locations. These lectins were chosen for their ability to distinguish and differentiate histochemically the two main types of secretory cells in sections of control submandibular glands. As an example histochemical localizations of LTA (fucose directed) on sections from control and sympathetically stimulated glands are shown in Fig. 3.

In general terms, the secretory granules in both acinar and granular tubule cells were reduced conspicuously after sympathetic nerve stimulation for 1 hour and the lectin binding patterns were reduced accordingly. However, the picture tended to be obscured when the binding was not confined to the granules such as with LTA. With PNA (galactose directed) the control acini usually showed little or no binding but after sympathetic stimulation for 1 hour it was common to see staining in a Golgi-like distribution in the acinar cells, which suggests that protein resynthesis had already begun, despite the ongoing stimulation and that post translational modification included the addition of ß-galactose to the nascent oligosaccharide chain. The absence of such staining in control cells with mature secretory granules suggests that additional glycosylation subsequently obscures the affinity for PNA.

For SDS-PAGE of saliva and glandular extracts non-reducing conditions were used. Although this resolves fewer rat submandibular protein bands the conditions do not disrupt glycoproteins that consist of 2 peptide subunits - such as kallikreins - which thereby remain intact and retain their carbohydrate chains as *in vivo*. Furthermore, it allows the kallikreins to be localised by the substrate impregnated overlay membrane procedure following removal of SDS and renaturation of the gel. Typical results of lectin binding sites on blots from SDS-PAGE of saliva and extracts from stimulated and control submandibular glands are shown in Fig. 4, which also includes the localization of the kallikrein related proteinases by the overlay membrane technique using the substrate D-V-L-R-AFC. There were two main regions of lectin binding. One zone of lower molecular weight (25-35 kD) corresponded to the kallikreins that occur in granular tubules. The other zone with molecular weight >94 kD could also be visualised by staining with periodic acid-Schiff reaction or Alcian blue, especially after silver enhancement (Jay et al., 1990), thus confirming its content of acinar mucin. In general terms the reactivity of the appropriate bands was in accord with the histochemical findings but not always of comparable staining intensity, e.g. the weak staining with PNA on blots. Often it was evident that concentration of a corresponding constituent had occurred in the saliva. Sometimes a band was less evident in the saliva suggesting that not all of the histochemical localization on sections may be

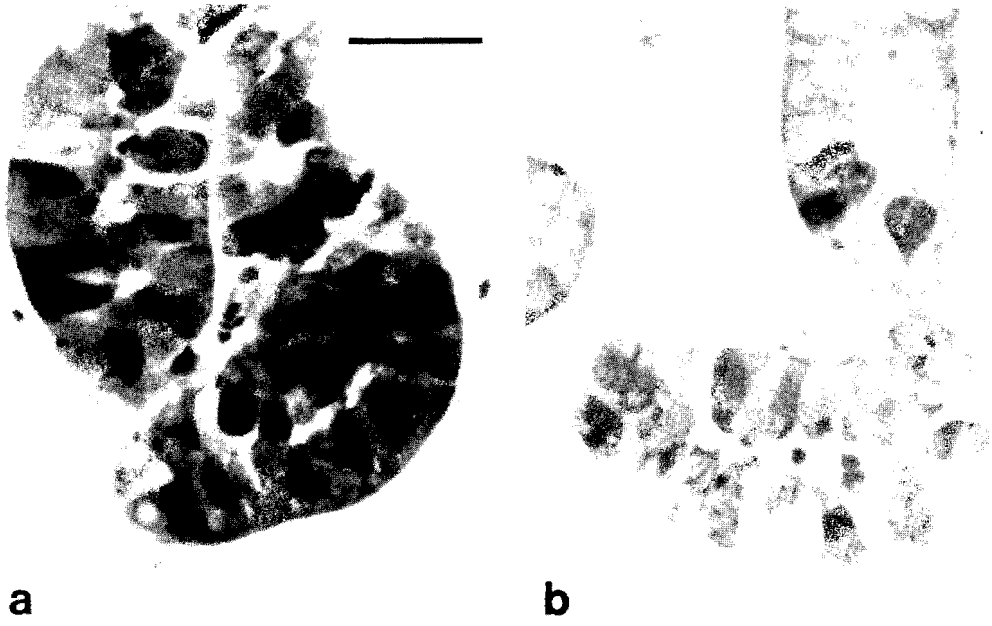

Fig. 3. LTA-binding (Fuc directed) with sections of rat submandibular glands.
a. control unstimulated gland showing strong staining of most granular tubule cells.
b. contralateral, sympathetically stimulated gland showing a big depletion of granules from most cells but a non-granular staining of other cellular components remains.
Bar: 36 µm.

attributable to secretory material or that an alteration had occurred during secretion. Similarly, bands were sometimes evident in glandular extracts that were not seen in saliva particularly in the regions between 35 kD and 94 kD.

The results show there are abundant αGalNAc residues in the high molecular weight acinar mucins that enter saliva. The disparity in labelling with DBA and SBA is likely to be due to differences with regard to the penultimate sugar and anomeric configuration. The strong binding with DBA is consistent with known O-linked glycosylation of mucins (Keryer et al., 1973; Fleming et al., 1982; Schulte and Spicer, 1984; Tabak et al., 1985; Quissell and Tabak, 1989). Large amounts of sialic acid were present in the high molecular weight mucins as indicated by staining with LFA and WGA, since sWGA (directed only against GlcNAc), did not stain.

The different kallikreins, originating from the granular tubules, are found in the major bands that separate electrophoretically between 25 and 35 kD and this was confirmed by their enzymic activities with overlay membranes impregnated with a fluorogenic proteinase substrate, as in Fig. 4, or by means of antibodies on Western blots (Shori et al., 1992b). Lectin binding showed that these bands contain αFuc, NeuAc, terminal αGalNAc with small amounts of terminal ßGal both in the glandular extracts and in the saliva. DBA (αGalNAc directed) appeared to give anomalous results in binding to the kallikrein region after electrophoresis but not to the granular tubules histochemically. This may be accounted for in several ways; it is possible that the binding site is freely available in the biochemical preparations but is masked after fixation and subsequent tissue processing, or the component may have leaked out during histological preparation. Alternatively it is possible that it may be due to an acinar constituent that comigrates with components from the granular tubules from glandular preparations and saliva. The dilemma that such a finding creates shows that neither method, histochemical or biochemical, is complete in isolation.

An interesting feature in the kallikrein region of 25-35 kD on SDS-PAGE blots is that the resolved bands differ from each other in their lectin affinities (see Fig. 4). For example the band binding to MPA appears to be of a higher molecular weight than those bands with affinity for UEA I. SDS-PAGE is not the best method for discriminating the different kallikreins found in rat submandibular glands and saliva because they have closely similar molecular weights and mobilities. Nevertheless, lectin binding features of the electrophoresed saliva, as seen in Fig. 4, evoked the interesting possibility that the different kallikreins may possess different patterns of glycosylation, despite their close genetic relationships and origin from the same cells. We have now undertaken a further study to unravel this interesting possibility, which will be described in the next section

Lectin probe studies on different rat submandibular kallikreins

Individual kallikreins are well resolved by isoelectric focusing (IEF) (Shori et al., 1992a,b, 1993) and this has provided a convenient relatively simple method for identifying and purifying them. After IEF, a cellulose diacetate membrane overlay impregnated with the suitable fluorogenic oligopeptide substrate (D-V-L-R-AFC or Z-V-K-K-R-AFC) is applied, as above, and the amidolytic release of the leaving group (AFC) causes

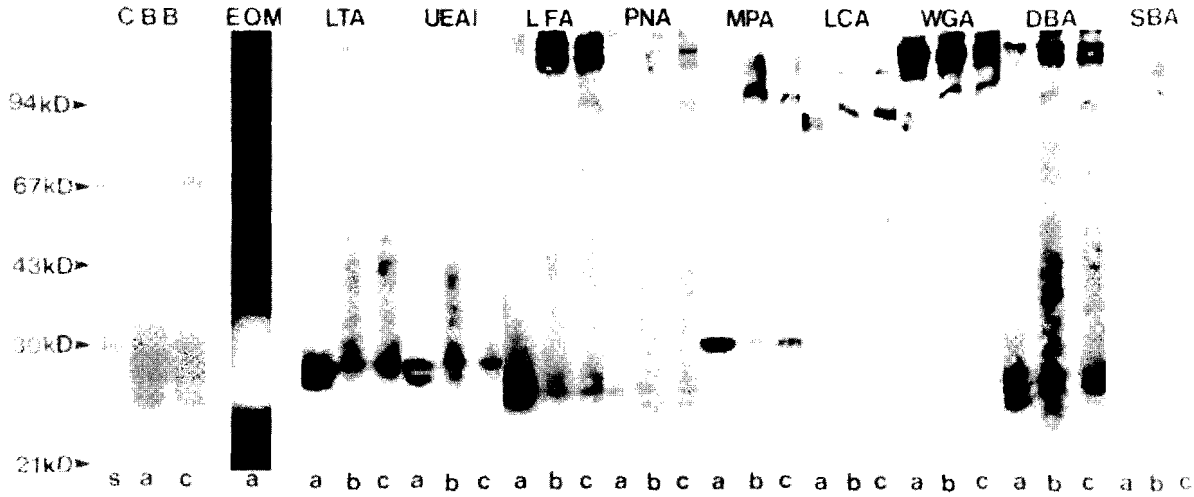

Fig. 4. Staining by different labelled lectins of Western blots from SDS-PAGE gels of rat submandibular salivary constituents. Lanes **a**: submandibular sympathetic saliva; **b**: extracts from sympathetically stimulated glands; **c**: extracts from control unstimulated glands; **s**: molecular weight markers. The lectins used are labelled above the lanes. The left column (CBB) is a gel stained with Coomassie Brilliant Blue. The next column labelled «EOM» shows kallikrein-like activities demonstrated by an overlay membrane impregnated with D-V-L-R-AFC. Note differences in the lectin bindings of saliva from those of glandular extracts and differences in the bindings by resolved bands between 25 and 35 kD. (Reproduced by kind permission from Zhang et al. Fig. 4. J. Histochem. Cytochem. 42: 1261-1269; 1994).

Table 2. Lectins used in the assessment of different rat kallikreins.

LECTIN	SUGAR SPECIFICITY
Ulex europaeus (UEA I)	αFuc (α-L-fucose) (α1,2 linkage preferred)
Lotus tetragonolobus (LTA)	αFuc (α1,6 linkage preferred)
Limax flavus (LFA)	NeuAc (N-acetylneuraminic acid)
Maclura pomifera (MPA)	Terminal Galß1,3GalNAc[1] (ß-D-(+)galactose1,3N-acetyl-D-galactosamine)
Arachis hypogaea (PNA)	Terminal Galß1,3GalNAc[1]
Lens culinaris (LCA)	αMan (αmannose) linked to fucosylated GlcNAc (N-acetyl-D-glucosamine)[2]
Succinyl *Triticum vulgaris* (sWGA)	GlcNacß1,4GlcNAc[2]
Phytolacca americana (PWM)	Terminal or internal GlcNAcß1,4GlcNAc or Galß1,4GlcNAc[2]
Ricinus communis (RCA I)	Terminal Galß1,4GlcNAc[2]

[1]: sequences indicating O-linked glycosylation; [2]: sequences indicating N-linked glycosylation.

readily identifiable fluorescent bands to form over the different separated kallikreins. The clearly demarked fluorescent bands are then used, as templates, to enable precise excision of the underlying gel with a scalpel. The protein in each excised band is carefully eluted and can be used for subsequent characterization, including identification with specific antibodies (Shori et al., 1993).

In this way a selected number of different kallikreins were isolated from extracts of rat submandibular glands for assessment of their glycosylation characteristics on slot blot preparations by means of lectin probes. Ten rat submandibular kallikreins have been described and designated rK1-rK10 (Berg et al., 1992). For the present investigation (see Zhang et al., 1996) five different kallikreins were selected rK1 (true tissue kallikrein), rK2 (tonin), rK7 (esterase-A), rK9 (kallikrein S3), and rK10 (T-kininogenase). The isoenzymes of rK1 were extracted in two parts - designated a) isoenzymes 1, and b) isoenzymes 2 and 3.

After IEF separation of glandular extracts, eluates from the excised gel bands that contained the selected kallikreins were slot blotted onto nitrocellulose membranes. A method was introduced, using gold staining of the blots, to quantify the amounts of enzyme protein present in each eluate. This made it possible to apply equal loads of the separated enzymes to subsequent slot blots prior to studying their glycosylation characteristics by means of labelled lectins. Nine lectin probes were used as listed in Table 2. The LFA was labelled directly with HRP but the other lectins were biotinylated and subsequently identified indirectly using avidin-biotin-HRP complex. The enhanced chemiluminescence system was again used for detection of HRP sites of bound lectin.

Initially untreated blots were used but inconsistencies occurred between different preparations. So fixation procedures were tested and the best results were obtained after boiling the membrane following blotting; this enhanced the detection with certain lectins and the results became consistent.

Our findings indicated that none of the different rat tissue kallikreins is heavily glycosylated, and this may have helped to resolve their different lectin-binding characteristics. Results with hydrolysates of rK1 chromatographed by high pH anion exchange chromatography (HPAEC) showed that the amount of sugar in rK1 was only about 3%. The presence of Fuc, GalNAc, GlcNAc, Gal and Man were confirmed by this method but a large amount of starting material had to be used to obtain satisfactory signals, which serves to reinforce the great sensitivity advantage of lectin probes.

Consistent differences in lectin binding patterns occurred as shown in Fig. 5. This confirms that normally the individual kallikreins from rat submandibular glands are glycosylated differently; a surprising finding in light

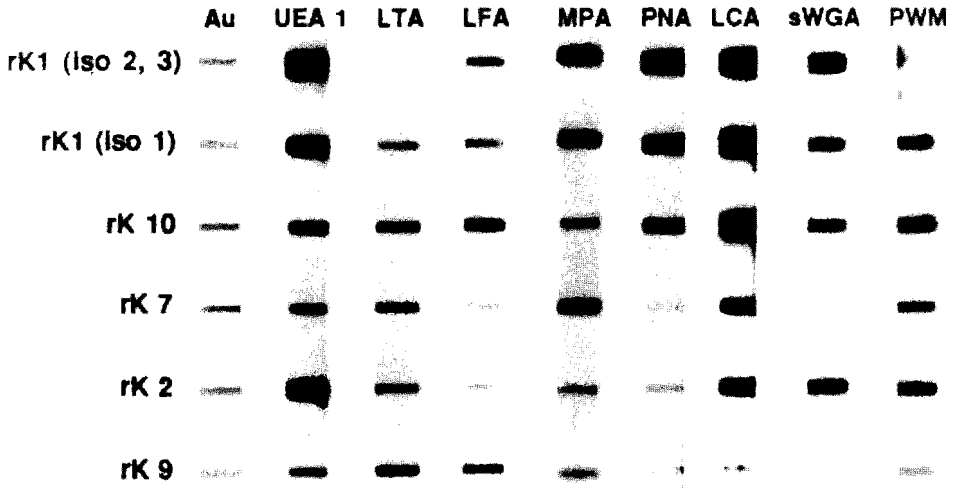

Fig. 5. Binding by different lectin probes to a series of kallikreins (rK1, rK10, rK7, rK2 and rK9 in order of increasing isoelectric point) isolated in eluates from isoelectric focused gels and slot blotted onto a nitrocellulose membrane. Gold staining (Au) of the different kallikreins on the slot blots indicates that equal amounts of protein were loaded onto the nitrocellulose membrane for each kallikrein. Differences in the lectin binding patterns with the different kallikreins are evident. (Reproduced by kind permission from Zhang et al., Fig. 2b. Glycoconjugate J. 13, 91-98; 1996).

of the high amino acid sequence homology among these proteinases (on average 88%; Murray et al., 1990).

In all cases the lectin binding was blocked by the appropriate sugar. Further information about the oligosaccharide chain structure was obtained by digesting the blotted kallikreins with sialidase or peptide-N-glycosidase F. Sialidase completely abolished LFA binding confirming the presence of sialic acid. Sialidase treatment also increased the RCA I (Galß1,4GlcNAc directed) binding of rK10, >rK1 (iso 1), >rK9, >rK2 with little change in rK1 (iso 2 and 3) or rK7. N-glycosylation, suggested by the affinity of tissue kallikreins for LCA and PWM, was confirmed by peptide-N-glycosidase F digestion which abolished all LCA binding, and is normal for secretory proteins from serous cells.

PNA and MPA binding, however, suggested the presence of O-linked side chains as well, especially on rK1 isoenzymes and rK10. This novel possibility for secretory proteins from so-called serous cells was supported by unchanged binding with PNA and MPA after removal of N-linked sugars by peptide-N-glycosidase F treatment and by the greatly reduced binding of both lectins after periodic acid oxidation. Further support came from the detection of GalNAc by means of HPAEC. Digestion of Galß1,3GalNAc on kallikreins by endo-α-N-acetylgalactosaminidase greatly reduced the PNA binding to rK1 isoenzymes and rK10, confirming the presence of O-linked Galß1,3GalNAc on rK1 and rK10. This was the first indication that O-glycosylation occurs in rat tissue kallikreins and testifies to the value of the methods used. Serines have been found in positions 69 and 71 in some rat kallikreins including rK1 (Wines et al., 1991) and so may be sites for O-glycosylation. The effects of sialidase treatment: removal of LFA binding, no change in binding with O-linked sugar directed lectins MPA and PNA, but enhancement of RCA 1 binding indicates that the sialylation of rat tissue kallikreins occurs mainly on N-linked side chains.

Thus, assessment of lectin bindings with equal protein loads of the 5 selected rat submandibular kallikreins on slot blots has clearly demonstrated that they are in fact differently glycosylated and has greatly extended our understanding of these interesting enzymes. However, the functional significance of their different glycosylation patterns is at present unclear and is likely to remain so until the functions of the different kallikreins and their natural substrates are better understood. Glycoforms of other enzymes (e.g. pancreatic ribonuclease B) have been studied and found to endow differences in both activity and susceptibility to proteolytic modification and degradation (Rudd et al., 1994) so it is possible that individual tissue kallikreins may show similar differences. Recently Brillard-Bourdet et al. (1995) have found that differences in the specificities of different rat submandibular kallikreins are influenced by the amino acid configuration in extended peptide substrates. From this they conclude that the importance of extended interaction sites on the kallikreins «suggests that differing specificities of individual kallikreins are partly due to the presence of proteinase subsites which accommodate residues remote from the scissile bond in the substrate». It now seems possible that the different glycosylations of the kallikreins may also have a role in influencing substrate binding.

Other significant features have been revealed and it is clear form our work that some serous-type salivary cells are able to O-glycosylate as well as N-glycosylate the same secretory products. Hitherto O-glycosylation of secretory proteins have been thought of as the preserve of mucin secreting salivary cells (Levine et al., 1987). Recently we have found that the granular tubules in rat submandibular glands secrete tissue kallikreins by both regulated and constitutive pathways, depending on the type of stimulation (Proctor et al., 1992; Shori et al., 1992a,b). In sympathetic saliva the kallikreins arise from the regulated exocytosis of preformed granules but in parasympathetic saliva they are secreted from a non-granular pool, that is likely to use a vesicular route. The proportions of the enzymes and their isoforms differ according to which secretory pathway is being utilised. Interestingly the isoenzymes 2 and 3 of rK1 are the most heavily glycosylated of the different kallikreins, they are synthesized most rapidly during resynthesis after cyclocytidine-induced degranulation (Shori et al., 1992b) and are found in greatest proportion in parasympathetic saliva. It is possible, therefore, that differences in the glycosylation of tissue kallikreins may influence the route by which they are secreted, but this remains to be tested.

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