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Differences in the glycosylation of rat submandibular kallikreins

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The glycosylations of five different rat submandibular kallikreins, rK1, rK2, rK7, rK9 and rK10, vacuum-blotted onto nitrocellulose membranes, have been studied by means of labelled lectins using enhanced chemiluminescence detection. The results demonstrated that individual submandibular kallikreins are not heavily glycosylated in rats, but consistently show different patterns of glycosylation. Following digestion of slot-blotted enzymes with peptide-N-glycosidase F (PNGase): binding by lectin from *Lens culinaris* (αMan-directed) was abolished, whilst that of lectin from *Maclura pomifera* (Galβ1,3GalNAc-directed) persisted (but could be abolished by periodate oxidation and endo-α-*N*-acetylgalactosaminidase digestion), revealing that there are O- as well as N-linked sugar chains on the kallikreins; a novel observation for this family of enzymes. The presence of GalNAc in addition to GlcNAc, Fuc, Gal and Man, in sugar chains of rK1 was confirmed by high pH anion exchange chromatography following acid hydrolysis. Different intensities of binding by lectin from *Limax flavus* (NeuNAc-directed) suggest that sialylation of individual kallikreins differs, whilst sialidase and PNGase digestions suggest that sialic acid is the terminal residue of some N-linked but not O-linked structures.

Keywords: rat, salivary Glands, kallikreins, glycosylation, lectin, glycosidases, slot-blot, chemiluminescence, HPAEC/PAD

Introduction

Tissue kallikreins represent a gene family of closely related proteinases [1]. In rat there are a large number of tissue kallikrein gene products (rK1–rK10) and many of these proteinases have been identified in the granular duct cells of the submandibular gland [1, 2]. Despite the high sequence homology of the mRNAs of these proteinases (on average 88% [3]), the possibility arose from lectin probing of blots after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of glandular extracts that the kallikreins may be differently glycosylated in rat submandibular glands [4]. In this paper we describe a more precise lectin-based investigation of glycosylational diversity amongst five of the most abundant kallikreins from rat submandibular glands; these were rK1 (true tissue kallikrein), rK2 (tonin), rK7 (esterase A), rK9 (S3) and rK10 (T-kininogenase). Preparations of different purified kallikreins [5] were slot-blotted onto nitrocellulose and their glycosylations were assessed by means of labelled lectins using an enhanced chemiluminescence (ECL) detection system [4, 6]. The slot-blot protocol described in the present study is ideally suited for the study of the glycosylations of submandibular kallikreins as these enzymes are particularly well resolved by isoelectric focusing in a wide pH gradient [7] from which they can be conveniently isolated. The use of eluates from IEF gels as opposed to direct blotting of the gel allows equal loads of the kallikreins to be vacuum-blotted onto nitrocellulose to enable semi-quantitative comparison of the glycosylation of the different enzymes. The use of ECL detection with the slot-blot protocol offers the advantages of greater sensitivity than colorimetric development, and easy

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semiquantitative assessment of the results on photographic film.

Materials and methods

Preparation of submandibular gland extracts

Five adult male Wistar rats (King's strain) weighing 355–458 g were fasted overnight and submandibular glands taken under ketamine/xylazine (75 and 15 mg kg^{-1} respectively, ip) anaesthesia. Ten percent glandular homogenates were prepared at 4 °C in 100 mM phosphate buffer, pH 6.0, containing 2 mM EDTA and 0.02% Triton X-100. The homogenates were centrifuged at 13 000 × g for 20 min and the supernatants aliquoted and stored at −70 °C. Total protein concentration of the glandular extract was determined with Folin-phenol reagent [8] using a mixture of human serum albumin and gamma globulin (Sigma Ltd, UK) as a standard.

Isolation of submandibular kallikreins from isoelectric focusing (IEF) gels

This was performed as described previously [9, 10]. Fifty microlitres of rat submandibular gland extracts (about 200 μg of proteins) were focused at 10 °C on 1 mm thick, pH 3.5–9.5 or 4.0–6.5, precast IEF gels (Ampholine PAG plates, Pharmacia Ltd, UK) according to the manufacturer's instructions. Immediately after focusing bands representing different kallikreins were visualized by overlaying the gels with cellulose diacetate membranes impregnated with 100 μM solutions of the fluorogenic oligopeptide proteinase substrates D-Val-Leu-Arg-7-amino-4-trifluoromethyl coumarin (DVLR-AFC) or Z-Val-Lys-Lys-Arg-AFC (ZVKKR-AFC) (both from Enzyme Systems Products, USA) and viewing under long wavelength UV light. Selected major kallikrein bands were carefully excised by scalpel and the protein in the underlying gel piece eluted into 0.5–1 ml of 20 mM Tris buffer containing 150 mM sodium chloride (TBS), pH 8.5, by agitating overnight at 4 °C. The eluates were filtered to remove gel pieces and aliquoted for storage at −70 °C.

Gold staining of slot-blots

Eluates of the previously identified kallikreins (10): rK1 (isoenzyme 1), rK1 (isoenzymes 2 and 3), rK2, rK7, rK9 and rK10, were slot blotted in duplicate onto nitrocellulose membrane (pore size = 0.2 μm; Bio-Rad Ltd, UK) under vacuum using a Bio-Dot SF slot format microfiltration apparatus (Bio-Rad Ltd) according to the manufacturer's instructions. In initial experiments native blots were compared with blots which were subsequently either fixed or boiled. For fixation, 1 ml of an aqueous solution of 1% glutaraldehyde was allowed to drain through each slot by gravity over 4 h at room temperature and then blots were washed in TBS for 6 h. Other blots were boiled in TBS for 15 min [11]. Following these steps the blots were blocked in TBS containing 0.1% Tween 20 (TTBS), pH 7.5, for 1 h. The protein contents of these eluates were determined from nitrocellulose blots which had been stained with colloidal gold (Protogold; Bio-Cell Ltd, UK) for 3 h, washed in distilled water and air dried. Stain intensity was quantified using an integrating scanning laser densitometer (Pharmacia Ltd, UK) after the nitrocellulose had been made transparent by liquid paraffin. Standard curves for this procedure were created using a variety of standard proteins and glycoproteins including bovine serum albumin (BSA; BDH Ltd, UK), protein assay standard {containing human serum albumin (HSA)/γ-globulin}, fetuin and asialofetuin (Sigma Ltd) at amounts ranging from 0.01 to 3.5 μg per slot. In addition the binding and staining of different amounts of kallikreins in eluates were compared.

Table 1. Lectins used in the present study.

Lectin (from)	*Working concentration* ($\mu g\, ml^{-1}$)	*Sugar specificity*
Ulex europaeus (UEA-I)	10	αFuc (α-L-fucose) (α1,2 linkage preferred)
Lotus tetragonolobus (LTA)	10	αFuc (α1,6 linkage preferred)
Limax flavus (LFA)	10	NeuNAc (*N*-acetylneuraminic acid)
Maclura pomifera (MPA)	5	Terminal Galβ1,3GalNAc[1] (β-D(+)galactose1,3N-acetylα-D-galactosamine)
Arachis hypogaea (PNA)	10	Terminal Galβ1,3GalNAc[1]
Lens culinaris (LCA)	5	αMan (αmannose) linked to fucosylated GlcNAc (*N*-acetylβ-D-glucosamine)[2]
Succinyl *Triticum vulgare* (sWGA)	10	GlcNAcβ1,4GlcNAc[2]
Phytolacca americana (PWM)	10	Terminal or internal GlcNAcβ1,4GlcNAc or Galβ1,4GlcNAc[2]
Ricinis communis (RCA I)	10	Terminal Galβ1,4GlcNAc[2]

[1]Sequences indicating O-linked glycosylation.
[2]Sequences indicating N-linked glycosylation.

Lectin binding studies on slot-blots

Following the TTBS blocking procedure the nitrocellulose blots were placed in solutions of different labelled lectins as indicated in Table 1. Most lectins were obtained as biotinylated conjugates (Vector Laboratories Ltd, UK or Sigma Ltd). LFA (NovaBiochem Ltd, UK) was obtained unlabelled and conjugated with HRP (Type VI, Sigma Ltd) as described previously [12]. Labelled lectins were stored at −70 °C in aliquots (2–5 $mg\,ml^{-1}$) and diluted in TTBS on the day of use. Blots were incubated with lectins for either 1 h (biotinylated) or 2 h (HRP conjugated) then washed with three changes for 5 min in TTBS, followed, in the case of biotinylated lectins, by incubation with avidin-biotin-HRP complex (Vector labs Ltd) diluted 1:100 with TTBS, for 30 min. After a 15 min wash in TTBS, lectin binding was detected by ECL Western blotting reagents (Amersham Ltd, UK) according to the manufacturer's instructions, being visualized by photographic development on Hyperfilm (Amersham Ltd). Photographic film was exposed to the slot-blot membrane for 4 min except for LCA which was exposed for 2 min after the addition of the ECL reagents. Whole submandibular glandular extract or TBS buffer were used as positive or negative staining controls respectively. The specificity of lectin binding was determined by adding inhibitory monosaccharides (0.1 M) to lectin solutions 1 h before incubation with the nitrocellulose membrane. Galactose was used as the inhibitory sugar for MPA, PNA and RCA I; L-fucose for UEA I and LTA; sialic acid for LFA; mannose for LCA and *N*-acetylglucosamine for sWGA and PWM. The sensitivity of the ECL detection technique was compared with conventional detection using the peroxidase substrate diaminobenzidine (DAB) at a concentration of 0.05% by loading different amounts (0.001–2 μg per slot) of asialofetuin (Sigma Ltd). Semi-quantitative assessments of lectin binding were provided by scanning densitometry, particularly when marginal differences were being compared.

Glycosidase digestion and periodic acid oxidation of slot-blots

For sialidase (from *Clostridium perfringens*; Oxford Glycosystems Ltd, UK) digestion blots were immersed in a working buffer consisting of 50 mM sodium acetate, pH 5, containing 0.1% Tween 20, 25 mM EDTA and 0.02% sodium azide for 30 min at room temperature. Blots were then incubated with sialidase at a concentration of 0.01 U per 100 μl of working buffer for 18–20 h at 37 °C.

Endo-α-*N*-acetylgalactosaminidase (from *Streptococcus pneumoniae*; Oxford GlycoSystems Ltd, UK) digestion was performed as described for sialidase in a buffer of pH 6 which contained 1 mM EDTA. The concentration of the enzyme was 0.3 mU per 100 μl of buffer.

For peptide-N-glycosidase F (PNGase; from *Flavobacterium meningoseptium*; New England Biolabs Ltd, UK) digestion the working buffer was 20 mM sodium phosphate, pH 7.5, containing 0.1% Tween 20, 25 mM EDTA and 0.02% sodium azide. Following a 30 min incubation in this buffer the blot was digested with PNGase at a concentration of 0.016–0.04 mU per 100 μl buffer for 18–20 h at 25 °C.

Periodic acid oxidation was performed by incubating slot-blots with 0.5% periodic acid for 1 h at room temperature.

Following glycosidase digestion and periodic acid oxidation, the blots were blocked in TTBS followed by lectin staining as described above.

SDS gel electrophoresis of IEF eluates

Eluates of the kallikreins from IEF gels were also run on SDS gels under non-reducing conditions as described previously [13] then Western blotted onto nitrocellulose membrane [4] and gold stained or incubated with lectins as decribed above in order to check on the purity of the preparations.

Compositional analysis of neutral and amino monosaccharides of rK1

The composition of neutral and amino monosaccharides of rK1 was determined using high pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) [14], after acid hydrolysis of the stained protein band on a polyvinylidene fluoride (PVDF) membrane [15]. Eluates containing rK1 isoenzymes were electrophoresed on SDS gels, then electro-blotted onto a PVDF membrane (Immobilon-P; Millipore Co, UK). The blot was stained with 0.1% Coomassie Brilliant Blue R-250 in water:methanol:acetic acid (5:4:1) for 10 min and then destained with 50% methanol:10% acetic acid and finally rinsed in distilled water. The blot was left at room temperature for about 30 min for drying. The membrane with stained rK1 band was carefully excised and wetted with 90% methanol, then transferred into a 1.5 ml polypropylene microcentrifuge tube with a cap and an O-ring (Sarstedt Ltd, Germany). The membrane band was submerged in 400 μl of fresh 2 M trifluoroacetic acid (BDH Ltd). The tube was capped and incubated at 100 °C in a heated block (Grant Instrument Ltd, UK) for 5 h. The membrane was removed and the supernatant evaporated to dryness in the presence of the drying agent diphosphorous pentaoxide (BDH Ltd) in a desiccator. The dried sample was reconstituted with 100 μl of deionized water and filtered. Twenty five microlitres of the filtered sample was injected onto a CarboPac PA-1 analytical column equipped with a PA1 guard column in a DX 500 HPLC System (Dionex Ltd, UK) for analysis of monosaccharides present in rK1. Neutral and amino monosaccharides were separated isocratically (16 mM NaOH) at a flow rate of 1 $ml\,min^{-1}$ for 30 min. The column was regenerated with

200 mM NaOH for 10 min between each run and followed by 6 min re-equilibration with 16 mM NaOH. All the NaOH eluents were prepared from a commercial 50% NaOH solution (Fisons Sci Equip, UK). The monosaccharides were detected using PAD (ED40 electrochemical gold detector, Dionex Ltd). The chromatographic data for monosaccharide analyses were collected and the peak areas and retention times determined using PeakNet software (Dionex Ltd). The neutral and amino sugars in rK1 were identified by comparing retention times with those of standard sugars. A mixture of six monosaccharides (fucose, galactosamine, glucosamine, galactose, glucose, mannose, 100 nmol each; Dionex Ltd) was used as an external sugar standard. Fetuin (Sigma Ltd) was used as a control glycoprotein.

Results

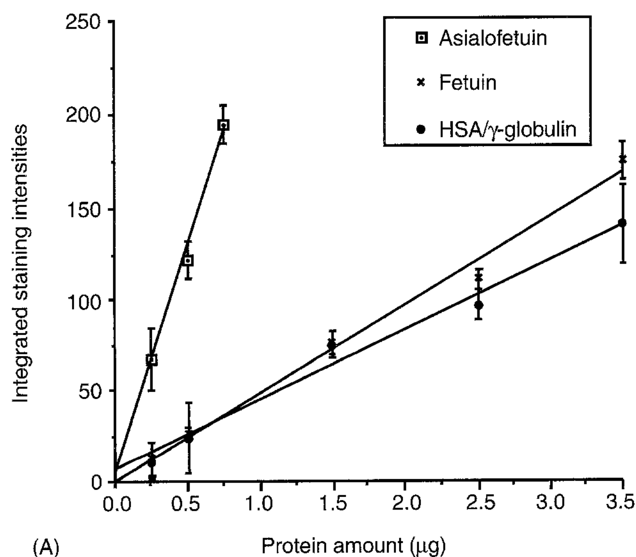
Gold staining of slot-blots

The slopes of the curves obtained using standard proteins are shown in Fig. 1A. Asialofetuin was stained more intensely by gold compared with either fetuin or HSA/γ-globulin, and also BSA (data not shown). Eluates of different rat kallikreins loaded onto nitrocellulose in increasing amounts produced similar slopes to those shown for fetuin and HSA/γ-globulin. That there was no contamination of the kallikreins in different eluates by other glycoproteins was confirmed by gold staining and by lectin binding studies on nitrocellulose electroblots following SDS gel electrophoresis of the different IEF gel eluates. Thus, using the fetuin standard curve, it was possible to assay the kallikrein protein content of eluates and then to adjust the volumes of different eluates loaded onto slot-blots so as to obtain equal protein loads of the different kallikreins (Fig. 2B). For the purposes of the lectin binding studies on slot-blots, described below, the optimal load of kallikrein was found to be equivalent to 2.5 μg of fetuin per slot.

Lectin staining of slot-blots

The sensitivity of ECL detection was assessed using asialofetuin and was compared with that of conventional detection with the peroxidase substrate diaminobenzidine (DAB; BDH Ltd). Using the ECL system 0.05 μg asialofetuin per slot could easily be detected compared to a requirement of 0.3 μg per slot by DAB (Fig. 1B). Scanning densitometry of the photographic film (Fig. 1B) was performed and a standard curve of photographic density against the amount of asialofetuin loaded onto slots was linear (data not shown).

Binding of most lectins to kallikreins on untreated slot-blots showed variations between different blots. These inconsistencies were overcome either by chemical fixa-



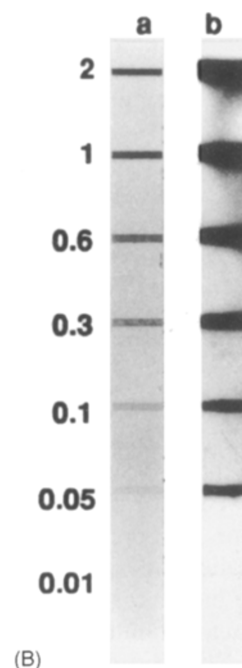


Figure 1. (A) Standard curves for asialofetuin, fetuin and human serum albumin (HSA)/γ-globulin which were slot-blotted onto a nitrocellulose membrane, stained with gold and scanned by a laser densitometer. (B) Comparison of two detection systems for visualising lectin binding. *Arachis hypogaea* (PNA) binds asialofetuin (μg) on slot blots, a) detected by diaminobenzidine; b) detected by enhanced chemiluminescence.

tion or by boiling the membrane following blotting. Binding of certain lectins, UEA I, PNA, MPA, sWGA and PWM to some of the slot-blotted kallikreins was enhanced by boiling which was therefore incorporated into the protocol in subsequent studies (Fig. 2A). The lectin bindings obtained with different kallikreins are shown in Fig. 2B. LCA bound to all of the kallikreins although binding to rK9 was less than the others. Likewise PWM bound strongly to most of the kallikreins but bound weakly to rK1 (isoenzymes 2 and 3). However sWGA bound to rK1 (isoenzymes 2 and 3). PNA and MPA bound particularly strongly to rK1 (isoenzyme 1), rK1 (isoenzymes 2 and 3) and rK10. In addition MPA bound strongly to rK7. UEA I bound to all of the kallikreins although to differing extents, the strongest binding was to the rK1 isoenzymes and to rK2. In contrast LTA bound weakly to the rK1 isoenzymes, particularly isoenzymes 2 and 3. LFA binding was greatest with rK10, less with rK1 and rK9 whilst rK2 and rK7 bound LFA poorly. In all cases lectin binding was inhibited by the appropriate sugar.

Glycosidase digestion and periodic acid oxidation of blots

To investigate the structures of the sugar chains on the kallikreins further, blots were digested with sialidase or PNGase. LFA binding was completely abolished following sialidase digestion (Fig. 3A, a and b). Likewise PNGase digestion abolished LCA binding whilst PNA and MPA binding remained unaffected (Fig. 3B). Periodic acid oxidation or endo-α-N-acetylgalactosaminidase digestion greatly reduced PNA binding to rK1 isoenzymes and rK10 (Fig. 3C, b and c). The penultimate sugar residues of the sialylated sugar chains of the different kallikreins were determined by comparing PNA, MPA and RCA I binding with and without sialidase digestion. PNA and MPA binding showed little change (data not shown) whilst RCA I binding was increased (Fig. 3A, c and d) after sialidase treatment compared with a control. Scanning densitometry revealed increased binding of rK10 (320%), rK1 (iso 1) (80%), rK9 (70%), rK2 (50%) whilst rK1 (iso 2, 3) and rK7 showed little change. Major features of sugar chains of individual kallikreins as indicated by lectin bindings and glycosidase digestions are summarized in Table 2.

Monosaccharide composition of rK1

Figure 4 shows the profile of peaks obtained when a hydrolysate of rK1 was chromatographed on a CarboPac PA-1 column at high pH. The peaks were identified as fucose, galactosamine, glucosamine, galactose and mannose by comparison with a mixture of sugar standards.

Discussion

Analysis of different submandibular tissue kallikreins on slot-blots has established that the enzymes are differently glycosylated and so has greatly extended our earlier work on electroblots of SDS gels [4]. Rat submandibular kallikreins are not well resolved by SDS gel electrophoresis as they show very similar mobilities under such conditions. However lectin binding on SDS electroblots

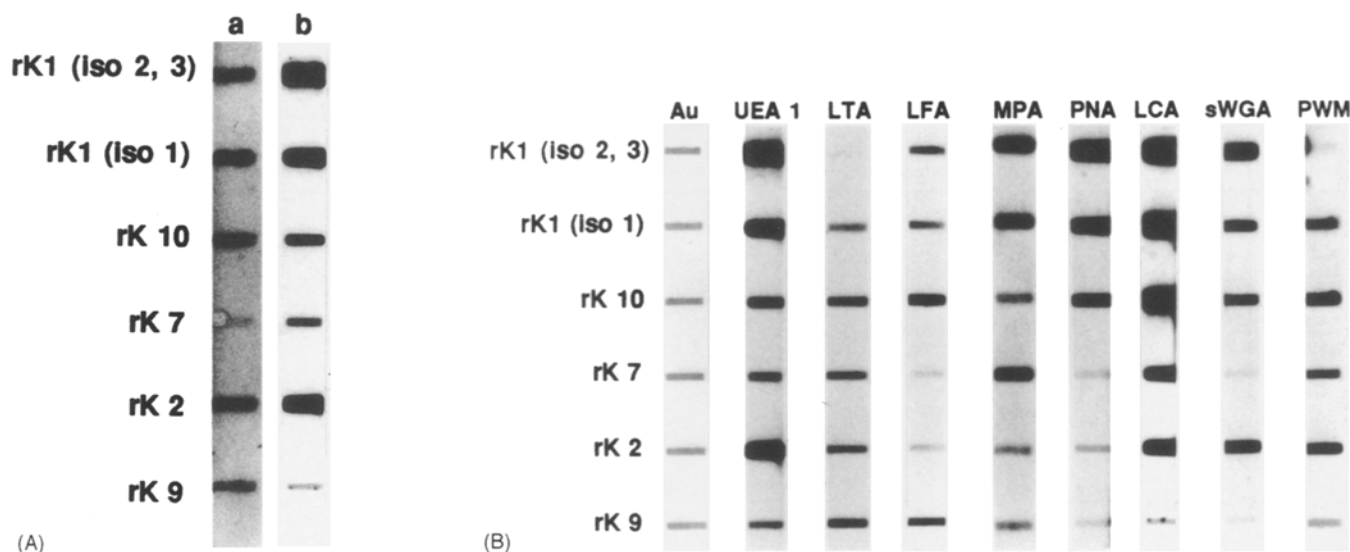


Figure 2. (A) Effects of different pre-treatments of selected kallikreins on slot blots. Binding with lectin from *Ulex europaeus* (UEA I), a) glutaraldehyde fixed blot; b) boiled blot on which rK1 and rK2 show greater staining. (B) Binding by different lectins (UEA I, LTA, LFA, MPA, PNA, LCA, sWGA and PWM) to a series of kallikreins from isoelectric focussing gel eluates (rK1, rK10, rK7, rK2, rK9, in order of increasing isoelectric points) on slot blots. Gold (Au) staining of the different kallikreins on slot blots shows that an equal amount of protein was loaded onto the nitrocellulose membrane for each kallikrein.

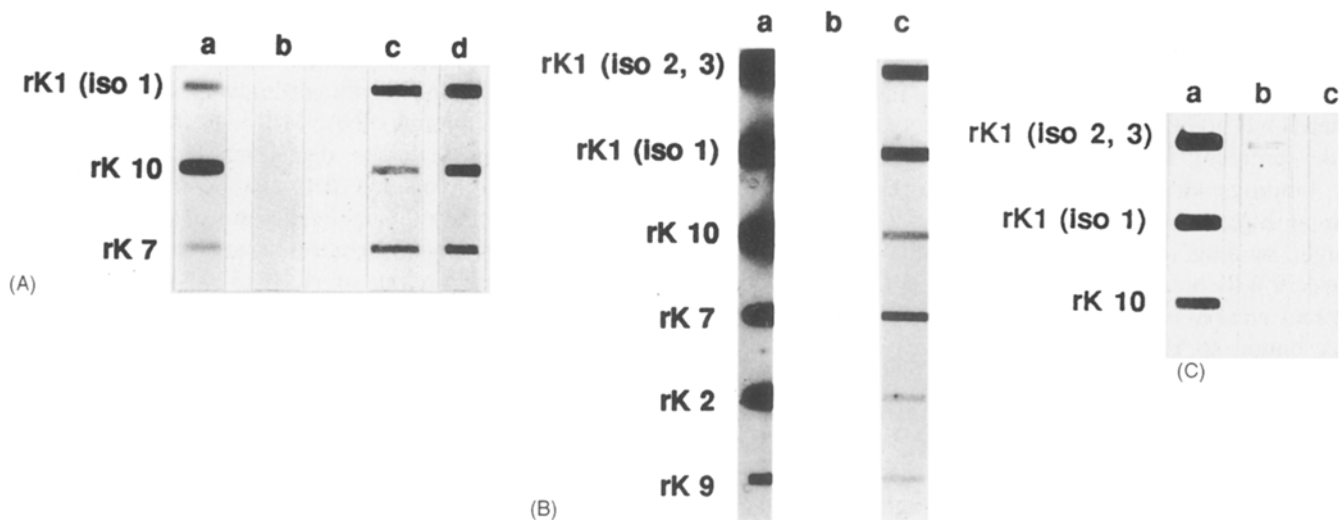


Figure 3. (A) The effects of sialidase digestion on lectin binding of slot blots of kallikreins rK1, rK7 and rK10. a) *Limax flavus* agglutinin (LFA) binds all three kallikreins; b) LFA binding is abolished after sialidase digestion; c) *Ricinis communis* agglutinin (RCA I) binds the three kallikreins; d) RCA I binding to rK1 and rK10 has been increased to different extents after sialidase digestion. (B) The effects of peptide-N-glycosydase F (PNGase) digestion on lectin bindings of slot blots of selected kallikreins. a) *Lens culinaris* (LCA) binds all selected kallikreins; b) LCA binding is abolished after PNGase digestion. c) MPA binding to the kallikreins remains after PNGase digestion. (C) The effects of periodic acid oxidation on lectin bindings of slot-blots of kallikreins rK1 isoenzymes and rK10. *Arachis hypogaea* (PNA) binding following incubation in the presence of (b) 0.5% periodic acid or (c) 0.3 mU per 100 μl of endo-α-*N*-acetylgalactosaminidase is greatly reduced compared to the blot incubated in their absence (a).

Table 2. Major features of sugar chains of individual kallikreins indicated by lectin bindings.

Kallikrein	Features
rK1 (iso 2,3)	GalNAc-containing O-linked sugar chain in addition to N-linked sugar chain; α1,2-linked fucose but little α1,6-linked fucose. Some N-linked sialylation.
rK1 (iso 1)	As iso 2,3 but with more α1,6-linked fucose and slightly less sialylation.
rK2	Mostly N-linked sugar chains with few GalNAc-containing O-linked sugar chains; little sialylation; more α1,2-linked fucose.
rK7	Both O- and N-linked sugar chains; α1,2 & α1,6-linked fucosylation but little sialylation.
rK9	The least glycosylation; few N-linked sugar chains; some GalNAc-containing O-linked sugar chains; fucosylated and sialylated.
rK10	As rK1 but much greater sialylation (mostly N-linked) and both α1,2 & α1,6-linked fucosylation.

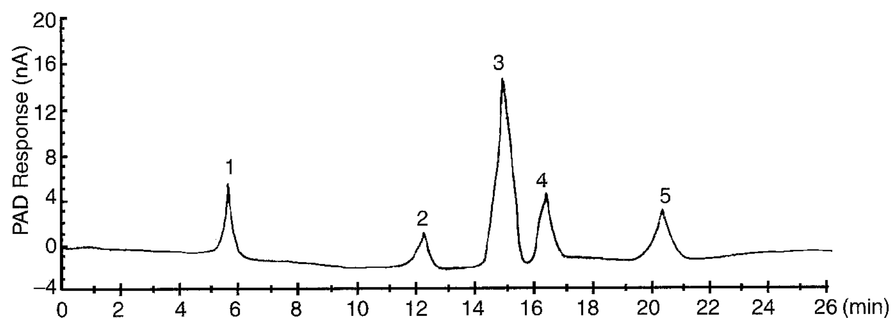


Figure 4. Profile of the neutral and amino sugars of rK1 obtained by high pH anion exchange chromatography on a CarboPac PA1 column eluted isocratically with 16 mM NaOH. The peaks were identified as (1) fucose, (2) galactosamine, (3) glucosamine, (4) galactose and (5) mannose, by comparison with the elution of sugar standards.

did suggest that certain of the kallikreins might be both O- and N-glycosylated [4]. The slot-blot protocol used in the present study was particularly suited for analysis of submandibular kallikrein glycosylations as the enzymes are well-resolved in IEF gradients and easily localized using oligopeptide substrates impregnated into overlay membranes [5, 10]. Furthermore, SDS gel electrophoresis of the eluates in the present and previous [10] studies and showed that there is little or no contamination from non-kallikrein components in the extracts and for each eluate there is one kallikrein except for the rK10 eluate which may contain two.

LCA, which binds preferentially to fucosylated core regions of N-linked sugar chains [16], bound to all the kallikreins. Similarly PWM which binds to sequences commonly found in N-linked sugar chains (*N*-acetylglucosamineβ1,4 *N*-acetylglucosamine and galactoseβ1,4 *N*-acetylglucosamine [17]), showed widespread binding amongst the different kallikreins. N-glycosylation was confirmed by digestion of the blots with PNGase which abolished all LCA binding. PNA and MPA, which bind to galactoseβ1,3 *N*-acetylgalactosamine sequences commonly found in O-linked sugar chains [18, 19], bound strongly to the isoenzymes of rK1 and to rK10. MPA also bound strongly to rK7 and this may be a feature of its slightly different sugar specificity compared to PNA [20]. The binding of these lectins was maintained following removal of N-linked sugar chains by PNGase treatment but was greatly reduced by periodic acid oxidation. Periodic acid abolishes specific lectin binding by oxidizing neutral sugars, such as galactose, which contain vicinol diols [21, 22]. The presence of O-linked galactoseβ1,3 *N*-acetylgalactosamine on rK1 and rK10 was further confirmed by the reduction in binding of PNA resulting from endo-α-*N*-acetylgalactosaminidase treatment of blots. The result indicates that this enzyme can be used to digest glycoproteins immobilized on slot-blots as well as glycoproteins in solution [23]. HPAEC confirmed the presence of *N*-acetylgalactosamine in addition to fucose, *N*-acetylglucosamine, galactose and mannose on rK1. Overall these observations indicate that O-linked oligosaccharide chains are present. To our knowledge, O-glycosylation of rat tissue kallikreins has not previously been described. Possible O-glycosylation sites have been described on prostate specific antigen [24], a member of the human tissue kallikrein gene family, at serine residues in positions 69 and 71, as the yields of these amino acids obtained by Edman degradation were very low compared with other serine residues. Serines in positions 69 and 71 are also present in some of the rat submandibular kallikreins, including rK1 [25] which may therefore be O-glycosylated at these sites. As the bindings of PNA and MPA were not greatly increased following sialidase treatment of slot-blots most of the O-linked chains appear not to be sialylated. Binding of LFA, a sialic acid directed lectin [26], to the different proteinases was largely abolished by treatment with PNGase (data not shown), establishing that in these kallikreins sialylation occurs mostly on N-linked sugar chains. This deduction was confirmed for rK10 by the increased binding of RCA I following sialidase treatment, as this lectin binds to the penultimate galactoseβ1,4 *N*-acetylglucosamine sequences frequently found in N-linked carbohydrates [27].

The presence of both O- and N-linked oligosaccharide chains on the tissue kallikreins represents a novel finding as it is generally considered that the secretory proteins of serous-type exocrine cells such as those of salivary glands, are not O-glycosylated [28]. Other differences were found in the glycosylation of the kallikreins, for example in the degree and position of fucosylation, as indicated by LTA and UEA I binding [29]. UEA I shows a great specificity for α1,2-linked fucose residues, which is the basis of its specificity for the H-antigen in studies of human blood group antigens [30]. In contrast LTA shows a greater specificity for fucose linked α1,6- or α-1,3- to unsubstituted *N*-acetylglucosamine [27, 31]. It would appear therefore that differing numbers of these linkages are present in different kallikreins. The degree of sialylation of the kallikreins differed as was indicated by LFA binding. These differences in fucosylation and sialylation occurred even though, as mentioned earlier, the many different kallikrein mRNAs found in submandibular granular duct cells are on average 88% homologous in nucleotide sequence. It is likely that each kallikrein contains at least one N-glycosylation site at asparagine number 84, and in some enzymes there may be another potential N-glycosylation site, for example at asparagine 169 as has been described for rK2 [32]. The submandibular kallikreins therefore represent an interesting model system upon which to study the factors controlling glycosylation and secretion of proteins by salivary and other exocrine cells.

Comparison of the lectin binding of IEF gel eluates with that of asialofetuin indicated that the submandibular kallikreins are relatively sparsely glycosylated. Thus for optimal signals 2.5 μg of eluate protein was loaded onto the nitrocellulose. Similar signals were obtained with asialofetuin at loads of approximately 0.1–0.3 μg per slot. This sparse glycosylation of the rat tissue kallikreins was confirmed by HPAEC. The amount of sugar eluted from the PA-1 column suggested that approximately 3% of rK1 was sugar. This required a large amount of hydrolysate (equivalent to 125 μg of rK1) to be loaded onto the PA-1 column in order to obtain significant signals with PAD, making further studies with HPAEC/PAD impractical.

An indication of the sensitivity of lectin-ECL detection on slot-blots was obtained using asialofetuin, which was easily detected at 0.05 μg per slot and could be detected down to a minimum of 0.01 μg per slot and this proved

to be many times more sensitive than DAB detection. This was not unexpected since we have found previously, on electroblots of SDS gels, that quantities of glycoprotein undetectable with DAB were conveniently visualized using the ECL system [4]. The present slot-blot protocol showed similar sensitivity for asialofetuin detection to that previously obtained using digoxigenin-labelled lectins with alkaline phosphatase-labelled anti-digoxigenin antibody [33]. Unlike the latter study in which samples were applied at uniform concentrations in 1 μl volumes, to a small area of nitrocellulose membrane, we were able to apply samples in larger volumes which enabled us to use very dilute glycoprotein preparations.

Binding of certain lectins to some of the kallikreins was increased by boiling the nitrocellulose blots before applying the lectins. A likely explanation for this increase is that some 'hidden' sugar sequences became accessible to the lectins, a phenomenon noted in a previous study of immunoglobulin glycosylation [11].

In conclusion, lectin binding on slot-blots of IEF gel eluates shows that submandibular tissue kallikreins rK1, rK2, rK7, rK9 and rK10 are differently glycosylated and all contain different levels of O- and N-linked sugar chains. The present study also indicates that isoenzymes 2 & 3 of rK1 are the most glycosylated. The mechanisms controlling the differential glycosylation of these structurally similar secretory glycoproteins are currently being investigated.

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References

1. Clements JA (1989) *Endocr Rev* **10**: 393–419.
2. Berg T, Bradshaw RA, Carretero OA, Chao J, Chao L, Clements JA, Fahnestock M, Fritz H, Gauthier F, MacDonald RJ, Margolius HS, Morris BJ, Richards RI, Scicli AG (1992) *Agents and Actions* **38**: 19–25.
3. Murray SR, Chao J, Lin F-K, Chao L (1990) *J Cardiovasc Pharmacol* **15** (suppl): S7–S16.
4. Zhang XS, Proctor GB, Garrett JR, Schulte BA, Shori DK (1994) *J Histochem Cytochem* **42**: 1261–69.
5. Shori DK, Proctor GB, Garrett JR (1993) *Anal Biochem* **211**: 123–30.
6. Jadach J, Turner GA (1993) *Anal Biochem* **212**: 293–95.
7. Proctor GB, Shori DK, Smith RE, Garrett JR (1991) In *2D PAGE 191*: Proceedings of the International Meeting on Two-dimensional electrophoresis (Dunn MJ, ed.) pp. 40–44. Department of Cardiothoracic Surgery, National Heart Lung Institute, UK.
8. Petersen GL (1977) *Anal Biochem* **83**: 346–56.
9. Clarke GA, Proctor GB, Garrett JR, Smith RE (1988) In *Electrophoresis 88*, (Schafer-Nielson C, ed.) pp. 257–62. Copenhagen: The Protein Lab, University of Copenhagen.
10. Shori DK, Proctor GB, Chao J, Chan K-M, Garrett JR (1992) *Biochem Pharmacol* **43**: 1209–17.
11. Sumar N, Bodman KB, Rademacher TW, Dwek RA, Williams P, Parekh RB, Edge J, Rook GAW, Isenberg DA, Hay FC, Roitt IM (1990) *J Immunol Meth* **131**: 127–36.
12. Avrameas S (1969) *Immunochemistry* **6**: 43–52.
13. Laemmli UK (1970) *Nature* **227**: 680–85.
14. Hardy MR, Townsend RR, Lee YC (1988) *Anal Biochem* **170**: 54–62.
15. Weitzhandler M, Kadlecek D, Avdalovic N, Forte JG, Chow D, Townsend RR (1993) *J Biol Chem* **268**: 5121–30.
16. Yamamoto K, Tsuji T, Osawa T (1982) *Carbohyd Res* **110**: 283–89.
17. Spicer SS, Schulte BA (1992) *J Histochem Cytochem* **40**: 1–38.
18. Pereira MEA, Kabat EA, Lotan R, Sharon N (1976) *Carbohyd Res* **51**: 107–18.
19. Sarkar M, Wu AM, Kabat EA (1981) *Arch Biochem Biophys* **209**: 204–18.
20. Laden SA, Schulte BA, Spicer SS (1984) *J Histochem Cytochem* **32**: 965–72.
21. Pearse AGE (1985) In *Histochemistry Theoretical and Applied*, Vol. 2, 4th ed., pp. 686–92. London: Churchill Livingstone.
22. Schulte BA, Spicer SS (1983) *J Histochem Cytochem* **31**: 19–24.
23. Keating MT, Harryman CC, Williams LT (1989) *J Biol Chem* **264**: 9129–32.
24. Watt KWK, Lee PJ, Timkulu TM, Chan WP, Loor R (1986) *Proc Natl Acad Sci USA* **83**: 3160–70.
25. Wines DR, Brady JM, Southard EM, MacDonald RJ (1991) *J Mol Evol* **32**: 476–92.
26. Miller RL, Collawn jr JF, Fisk WW (1982) *J Biol Chem* **257**: 7574–80.
27. Debray H, Decout D, Strecker G, Spik G, Montreuil J (1981) *Eur J Biochem* **117**: 41–55.
28. Levine MJ, Reddy MS, Tabak LA, Loomis RE, Bergey EJ, Jones PC, Cohen RE, Stinson MW, Al-Hashimi I (1987) *J Dent Res* **66**: 436–41.
29. Sugii S, Kabat EA, Baer HH (1982) *Carbohyd Res* **99**: 99–101.
30. Pereira MEA, Kisailus EC, Gruezo F, Kabat EA (1978) *Arch Biochem Biophys* **185**: 108–15.
31. Srivatsan J, Smith DF, Cummings RD (1992) *J Biol Chem* **267**: 20196–203.
32. Lazure C, Leduc R, Seidah NG, Thibault G, Genest J, Chrétien M (1987) *Biochem Cell Biol* **65**: 320–37.
33. Haselbeck A, Schickaneder E, von der Eltz H, Hösel W (1990) *Anal Biochem* **191**: 25–30.