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# High performance anion exchange chromatography of reduced oligosaccharides from sialomucins

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High performance anion exchange chromatography on pellicular ion exchange resins under high pH conditions with detection of sugars using a pulsed amperometric detector has been developed as a method for the separation and analysis of reduced oligosaccharides liberated from mucins by alkaline borohydride treatment. Ovine, bovine and porcine submaxillary mucins were used as models to develop the method. Although neutral reduced di–to tetraoligosaccharides were poorly retained on the column, a variety of sialylated reduced oligosaccharides could be separated efficiently. Treatment of the samples with sialidase and rechromatography identified the sialylated compounds in the elution profile. A striking finding was the greatly delayed elution times given by *N*-glycolylneuraminic acid containing compounds in comparison with the corresponding *N*-acetylneuraminic acid containing analogues. The elution profiles for the product from the mucins closely corresponded to those expected for the major oligosaccharides from these mucins. The procedures described will be useful for analysing sialomucins on a microscale without resorting to radiolabelling procedures.

*Keywords*: chromatography, ion exchange, mucins, sialomucins, *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, oligosaccharides, method

Mucins are high molecular weight glycoproteins characterized by having numerous carbohydrate chains linked to a peptide core, mainly to serine and threonine (reviewed in [1, 2]). The sugar linked directly to serine or threonine is invariably *N*-acetylgalactosamine (GalNAc), whereas the structure of the remainder of the sugar chains varies according to the source of the mucin. A convenient method for liberating the carbohydrate chains from the peptide backbone for further analysis uses alkaline elimination, usually in the presence of sodium borohydride [3], which reduces the terminal GalNAc to the alcohol and prevents 'peeling' of the carbohydrate chain [4]. Although this procedure is quite straightforward, the subsequent separation of the liberated reduced oligosaccharides is much more difficult. Among procedures that have been used are gel filtration [5], paper chromatography [6], and high performance liquid chromatography (HPLC) on ion exchange, amino bonded or reversed phase columns [7–11]. Although these methods can be quite effective, the procedures are laborious and difficult to apply to small samples.

The recent introduction of HPLC using pellicular ion exchange resins under high pH conditions and detection of the sugars with a pulsed amperometric detector (PAD) has simplified the separation and analysis of many monosaccharides and oligosaccharides (reviewed in [12]). The technique has been applied to the analysis of purified oligosaccharides and oligosaccharides derived from N-linked chains [13–16] but not oligosaccharides characteristic of O-linked chains. We now report the application of this approach to the separation and analysis of reduced oligosaccharides derived from three well-characterized mucins: ovine submaxillary mucin (OSM), porcine submaxillary mucin (PSM), and bovine submaxillary mucin (BSM).

## Materials and methods

### *Mucins and oligosaccharides*

Ovine submaxillary mucin and A-positive and A-negative porcine submaxillary mucins were purchased from BioCarb Inc. (Gaithersburg, MD, USA). Bovine submaxillary mucin and *N*-acetyl- and *N*-glycolylneuraminic acids were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Oligosaccharides **5–8** were generous gifts from Dr. E. A. Kabat. Oligosaccharides **9–15** have been reported previously [17]. Compound **12** was isolated from glycophorin by a modification of the method of Thomas and Winzler [18].

### *High performance anion exchange (HPAE) chromatography*

HPAE chromatography was carried out on a Dionex BioLc system (Dionex Corp, Sunnyvale, CA, USA) fitted with a

PAD detector, a Spectra Physics integrator/recorder and a Carbopak PA1 column (4 × 250 mm). For separating neutral sugars and hexosamines the column was eluted at 1.0 ml $min^{-1}$ with 0.015 M NaOH for 20 min, followed with 0.3 M NaOH for 15 min between runs and reequilibrated with 0.015 M NaOH for 15 min before injecting the next sample (program 1). A post column solution of 0.3 M NaOH was passed through the detector cell. Water was degassed with helium prior to adding 50% NaOH to the required NaOH concentration.

For the separation of sialic acids and reduced sialylated oligosaccharides, a gradient from 0.2 M NaOH to 0.25 M sodium acetate in 0.2 M NaOH over 30 min was used; the column was reequilibrated with 0.2 M NaOH for 15 min before injecting the next sample (program 2). A post column solution was not used in this procedure.

*Monosaccharide analysis*

For neutral sugar and hexosamine analysis, mucins were hydrolysed in 2 N trifluoroacetic acid for 3 h at 100°C as described by Hardy *et al.* [19]. A known amount of 2-deoxygalactose was added as an internal standard before separating the sugars (program 1). Hexosamines were also determined after hydrolysis in 4 N HCl for 6 hours. Sialic acids were separated as described above (program 2) after hydrolysis in 0.1 N HCl at 80°C for 1 h. A known amount of 2-deoxygalactose was added after the hydrolysis step. *N*-Acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) standards were hydrolysed and separated in a similar manner. Since sialic acid is partially destroyed in the hydrolysis step, an appropriate correction factor was used in the analysis of mucin samples.

*Alkaline elimination procedure and chromatography of resulting reduced oligosaccharides*

Mucin (100–500 μg) was treated with 0.05 M NaOH and 1 M $NaBH_4$ (400 μl) at room temperature for 2 days [20]. The sample was cooled in ice and neutralized with acetic acid (4 M). The sample was then deionized by passage over a Dowex 50 ($H^+$) column (1.0 ml) and subsequent evaporation with methanol (3 × 1 ml). The sample was finally dissolved in water (100 μl) and 10 or 20 μl aliquots were analysed using detector settings of 300 or 1000 nA, respectively. Since there can be some variation in the elution times between different chromatographic runs, the identity of peaks was confirmed by mixing with an authentic sample and rechromatographing the mixture.

*Sialidase treatment*

Reduced oligosaccharide sample (30 μl) was treated with *Vibrio cholerae* sialidase (1 unit, 15 μl; Calbiochem, CA, USA) and 5 μl NaCl (0.15 M) + sodium phosphate (0.02 M) + $CaCl_2$ (0.1 mg $ml^{-1}$) + $MgCl_2$ (0.1 mg $ml^{-1}$), pH 7.0 buffer at 37°C for 16 h. The hydrolysed sample was analysed directly by injecting 20 μl into the chromatograph.

*Analysis of sugar composition of individual peaks*

For the further analysis of individual peaks, a larger sample (100–500 nmol sugar) of the alkaline borohydride-treated samples was fractionated (with the detector set at 10 000 nA). Fractions (0.2 ml) were collected. Aliquots of individual fractions (20 μl) were reanalysed to confirm the elution position of the component (using a detector setting of 300 nA). Appropriate fractions were pooled, deionized by passing through a column of Dowex 50 $H^+$ (1.0 ml) and then freeze dried. Suitable aliquots were then analysed for their sugar content as described above.

## Results

*Chromatography of standard oligosaccharides*

Table 1 lists the elution times of 15 monosaccharides and reduced oligosaccharides representing structures commonly found in mucins. Neutral reduced oligosaccharides (samples **3–8**) elute very rapidly (2–3 min) and are very poorly separated from each other. Somewhat improved separations can be obtained by eluting isocratically with 1.5 mM NaOH, but even under these conditions the elution times are much faster than those of the corresponding reducing oligosaccharides [21]. In contrast, sialic acid containing reduced oligosaccharides are well retained on the column and usually separate from each other. A notable feature of these analyses is the much longer elution times given by NeuGc containing compounds than by the corresponding NeuAc containing species (compare **9** with **10**, and **14** with **15**). Even the monosaccharides NeuAc and NeuGc separate from each other by 12 min under these chromatographic conditions.

*Separation of reduced oligosaccharides derived from mucins: ovine submaxillary mucin (OSM)*

Ovine submaxillary mucin treated with NaOH + $NaBH_4$ yielded a major peak eluting at a position corresponding to NeuAcα2-6GalNAc-ol (**9**) and a smaller peak eluting at 2.45 min, which is probably *N*-acetylgalactosaminitol (Fig. 1(a)). Some samples also contained small amounts of free NeuAc (**1**). Following sialidase treatment, peak **9** disappeared, confirming that it contained sialic acid, and a new peak (**1**) corresponding to NeuAc appeared. The peak at 2.45 min corresponding to GalNAc-ol, increased in size (Fig. 1(b)).

*Bovine submaxillary mucin (BSM)*

Alkaline borohydride treatment of BSM gave a more complicated pattern of products (Fig. 2(a)). The major peaks corresponded to structures previously identified in BSM [20, 22, 31]. Thus **9** and **10** correspond to the sialylated disaccharides NeuAcα2-6GalNAc-ol and NeuGcα2-6GalNAc-ol, respectively. Peak **12** eluted at a position corresponding to GlcNAc[NeuAcα2-6]β1-3GalNAc-ol. An early eluting peak is probably a mixture of GalNAc-ol and Galβ1-3GalNAc-ol. Treatment of the sample with sialidase eliminated

**Table 1.** Structures and elution times of standard sugars and reduced oligosaccharides.

| *Designation* | *Oligosaccharide/sugar* | *Elution time* (min) |
|---|---|---|
| 1 | NeuAc | 11.0 |
| 2 | NeuGc | 23.0 |
| 3 | GalNAc-ol | 2.5 |
| 4 | Galβ1-3GalNAc-ol | 2.5 |
| 5 | GalNAcα1-3GalNAc-ol | 2.7 |
| 6 | Fucα1-2Galβ1-3GalNAc-ol | 3.0 |
| 7[a] | Galβ1-4GlcNAcβ1-3GalNAc-ol | 2.7 |
| 8[b] | Fucα1-2Galβ1-3GlcNAcβ1-3GalNAc-ol | 2.8 |
| 9 | NeuAcα2-6GalNAc-ol | 8.0 |
| 10 | NeuGcα2-6GalNAc-ol | 17.0 |
| 11 | NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAc-ol | 16.0 |
| 12 | GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol | 9.0 |
| 13 | Galβ1-4GlcNAcβ1-3(NeuAcα2-6)GalNAc-ol | 9.5 |
| 14 | Fucα1-2Galβ1-4GlcNAcβ1-3(NeuGcα2-6)GalNAc-ol | 8.5 |
| 15 | Fucα1-2Galβ1-4GlcNAcβ1-3GalNAc-ol | 15.5 |

[a] Derived from neuraminidase treatment of **13**.
[b] Derived from neuraminidase treatment of **14**.

peaks **9**, **10** and **12**, confirming that they were sialylated, and liberated NeuAc (**1**) and NeuGc (**2**) (Fig. 2(b)).

*Porcine submaxillary mucin (PSM)*

Alkaline borohydride treatment of two PSM $A^-$ samples gave three major acidic peaks (**10**, **16** and **17**) and two fast eluting neutral peaks. The latter probably corresponding to Galβ1-3GalNAc-ol and Fucα1-2Galβ1-3GalNAc-ol, respectively (Fig. 3(a)). PSM $A^+$ gave a similar profile except that one additional acidic peak (**18**) was observed and peak **17** was much smaller than in the PSM $A^-$ profile (Fig. 3(b)). Neuraminidase treatment confirmed that peaks **10**, **16**, **17** and **18** were acidic and contained NeuGc (data not shown). Since characterized oligosaccharides derived from PSM were not available, the sugar composition of each of the peaks was determined. As shown in Table 2, component **16** contained galactose, component **17** contained fucose and galactose, whereas component **18** contained fucose, *N*-acetylgalactosamine and galactose (in addition to *N*-acetylgalactosaminitol and NeuGc which were present in all the samples). From these data it was concluded that the components derived from $A^-$ and $A^+$ PSM correspond to the four major compounds previously isolated from these mucins by other investigators [22–25] (Fig. 4).

The presence of peak **18** in the $A^+$ but not the $A^-$ PSM sample is consistent with this interpretation. Minor peaks (eluting between 10 and 13 min) were not identified but may be NeuAc-containing reduced oligosaccharides.

## Discussion

This study shows that HPAE chromatography under high pH conditions is an effective method for the separation of reduced oligosaccharides derived from mucins by alkaline borohydride treatment. As with N-linked oligosaccharides, closely related oligosaccharides are well resolved [12–16]. The procedure is most effective with sialylated oligosaccharides. As has been reported previously [21], neutral reduced oligosaccharides in the di- to tetrasaccharide range are poorly retained on the ion exchange column. This is apparently due to the high acidity of the anomeric hydrogen atom in reducing sugars, resulting in column binding, which

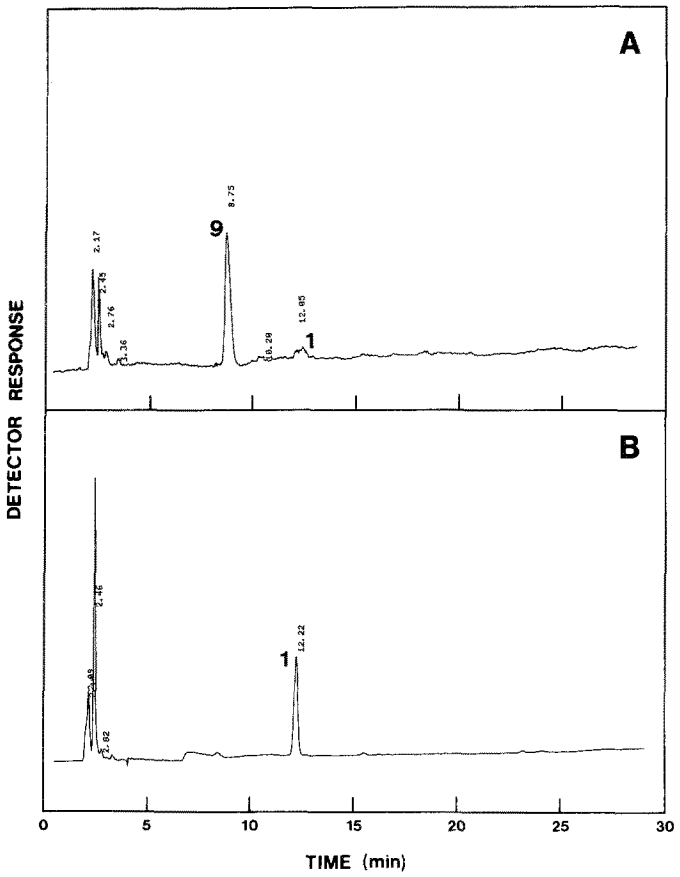


**Figure 1.** Chromatograms showing HPAE separations of reduced oligosaccharides from ovine submaxillary mucin before (a) and after (b) sialidase treatment using Program 2. Peaks are numbered according to Table 1.

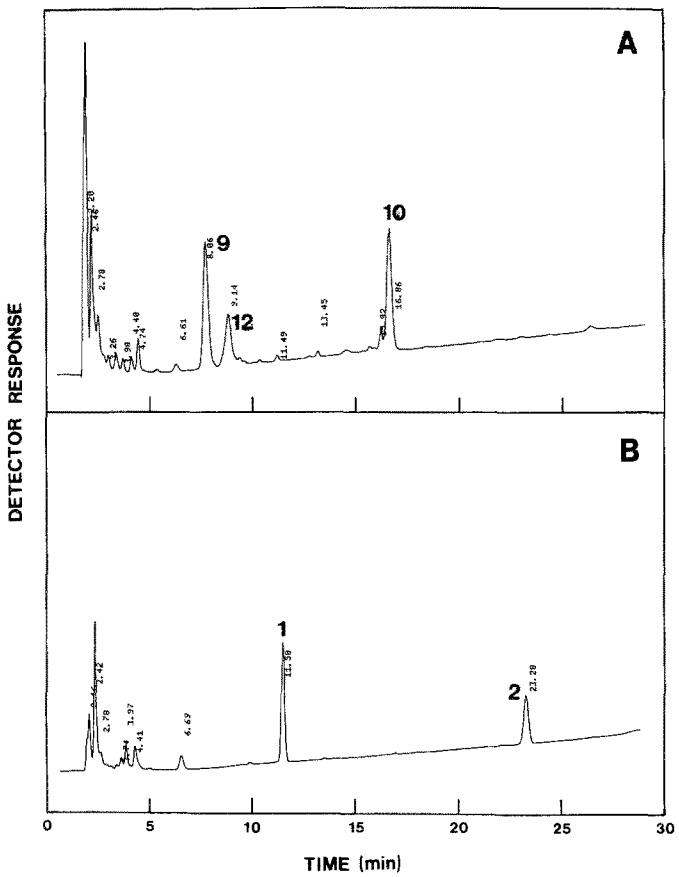


**Figure 2.** Chromatograms showing HPAE separation of reduced oligosaccharides from bovine submaxillary mucin before (a) and after (b) sialidase treatment using Program 2. Peaks are numbered according to Table 1.

is lost in the corresponding alcohols. In contrast, acidic reduced oligosaccharides are strongly bound to the column. Even NeuAcα2-6GalNAc-ol has a retention time of 8 min under the relatively rigorous elution conditions used (Table 1). For this reason, the method, as it now stands, is most useful for acidic (sialylated) oligosaccharides derived from certain classes of mucins, and may be less useful for mucins containing mainly neutral oligosaccharides (e.g., ovarian cyst mucins).

A particularly striking finding of this study were the greatly enhanced retention times of NeuGc-containing oligosaccharides over the corresponding NeuAc-containing compounds (compare **9** with **10**, and **14** with **15**). A similar dramatic separation is observed for the free sialic acids (cf. [26, 27]). This effect is probably due to the introduction of additional acidic hydrogen atom (the OH of the *N*-glycolyl group) into these structures. This result shows the clear advantage of this method over chromatographic methods relying on charge differences in neutral buffers [28].

Analysis of the three model mucins using this method gave results closely corresponding to published data for the major oligosaccharide structures present in these mucins. Thus, OSM gave one major sialylated peak corresponding to the disaccharide NeuAcα2-6GalNAc-ol previously reported to the major acidic oligosaccharide in this mucin [29, 30]. BSM yielded three major acidic peaks with elution times corresponding to the structures reported by Tsuji and Osawa [20] and by Savage *et al.* [17, 31]: NeuAcα2-6GalNAc-ol, NeuGcα2-6GalNAc-ol and GlcNAcβ1-3(NeuGcα2-6)GalNAc-ol. PSM from A blood group hogs gave four major acidic peaks whereas $A^-$ mucin gave three major acidic peaks (Fig. 3(a,b)). The extra peak in the $A^+$ sample was attributed to the A-active oligosaccharide (**18**). The other peaks were assigned to structures **10**, **16**, and **17** (H-active oligosaccharide). These structures correspond to those reported for PSM by Carlson [23], and by Van Halbeek *et al.* [24]. The PSM samples also contained major neutral peaks that were not identified.

The high sensitivity of the pulsed amperometric detector enables small samples of mucins to be analysed effectively without resorting to radiolabelled products. Thus a complete sugar composition can be obtained on 1 nmol. A profile of released reduced oligosaccharide requires about 10–50 nmol (including confirmation of acidic oligosaccharides by

**Table 2.** Sugar composition of reduced oligosaccharides isolated from porcine submaxillary mucin.

| *Mucin and peak number* | *Fuc* | *Gal* | *GlcNAc* | *GalNAc* | *GalNAc-ol* | *NeuGc* |
|---|---|---|---|---|---|---|
| PSM A$^-$ | | | | | | |
| **10** | —[a] | — | — | — | 1.0 | +[b] |
| **16** | — | 0.82[c] | — | — | 1.0 | + |
| **17** | 1.08 | 1.25 | — | — | 1.0 | + |
| PSM A$^+$ | | | | | | |
| **10** | — | — | — | — | 1.0 | + |
| **16** | — | 1.21 | — | — | 1.0 | + |
| **17** | 1.04 | 0.97 | — | — | 1.0 | + |
| **18** | 1.40 | 1.02 | — | 1.20 | 1.0 | + |

[a] —, not detected
[b] Present but not quantified
[c] Values relative to GalNAc-ol.

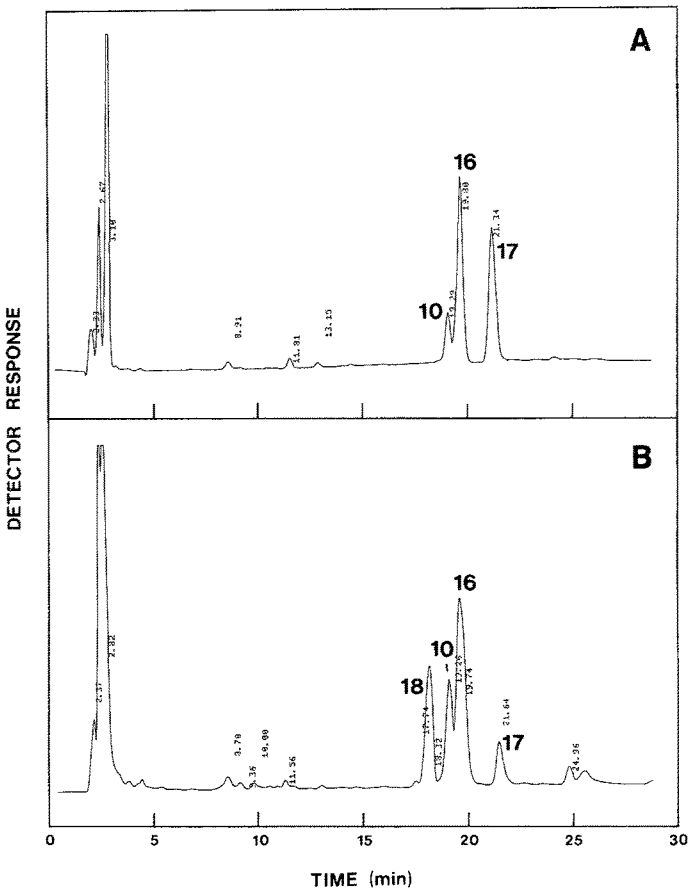


**Figure 3.** Chromatograms showing HPAE separation of reduced oligosaccharides from A-negative (a) or A-positive (b) porcine submaxillary mucin using Program 2. Peaks are numbered according to Fig. 4 and Table 1.

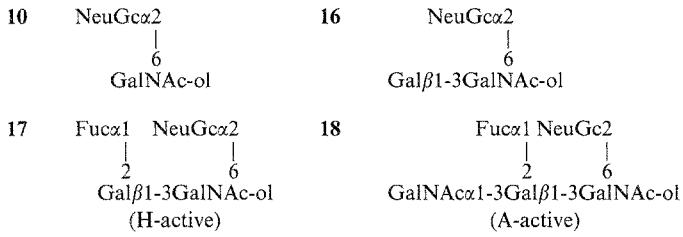


**Figure 4.** Major compounds isolated previously from porcine submaxillary mucin.

sialidase treatment and rechromatography). With a larger amount of sample (100–500 nmol), the separated components can be isolated and analysed for sugar composition. Isolated components could also be subjected to other procedures (e.g., glycosidase treatment, NMR spectroscopy, mass spectrometry or methylation analysis) for a complete structural analysis. HPAE chromatography under these conditions promises to be an important additional tool for the analysis of mucins.

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