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Improved fractionation of sialylated glycopeptides by pellicular anion-exchange chromatography

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Abstract

The glycoprotein bovine fetuin was treated with trypsin and the Asn-81 tryptic glycopeptide was purified (90% pure by Edman sequencing) by reversed-phase chromatography (RP-HPLC). The Asn-81 glycopeptide, which eluted as a single peak by RP-HPLC, was separable into five peaks on the NucleoPac PA100 column, a pellicular anion-exchange column. Each of the five Asn-81 glycopeptide peaks was shown to contain N-linked oligosaccharides by treatment of each peak with peptide N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase F (PNGase F) and subsequent oligosaccharide analysis by high-pH anion-exchange chromatography with pulsed amperometric detection. High-pH anion-exchange chromatography–pulsed amperometric detection oligosaccharide analysis revealed that each peak contained a different population of sialylated N-linked oligosaccharides. Hence each peak contained a different group of glycopeptide glycoforms. It was observed that the longer the retention time of the Asn-81 glycopeptide peak on the anion-exchange column, the greater the oligosaccharide sialylation. Two glycopeptide peaks which differed in their distribution of disialylated oligosaccharides demonstrated that the glycopeptide separation was a result of something more than gross differences in sialic acid content. The two other N-linked tryptic glycopeptides of fetuin were also separated into multiple peaks on the NucleoPac PA100 column and these separations were shown to be due to differences in oligosaccharide sialylation. The separations of the three fetuin N-linked glycopeptides demonstrate that pellicular anion-exchange chromatography offers improved separation speed and resolution for the separation of sialylated glycopeptides.

1. Introduction

Glycoproteins are microheterogenous with respect to their attached oligosaccharide structures. This heterogeneity arises because glycoproteins may differ in number of sites of oligosaccharide attachment and in the degree of occupancy of each site. Additionally, each site may possess its own set of oligosaccharides. Glycoproteins having an identical polypeptide but differing in glycosylation site occupancy, oligosaccharide structure, or location of the oligosaccharide structures, are called glycoforms. Similarly, glycopeptides having an identical peptide but differing in oligosaccharide content, are glycopeptide glycoforms. IgG has 30 glycoforms which vary reproducibly with physiological state and were shown to be a marker of rheumatoid arthritis [1]. The population of glycoforms present when glycoproteins are produced recombinantly is dependent upon cell type and cell culture conditions (for review, see ref. 2), and a

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glycoprotein's oligosaccharide structure can affect the protein's specific activity, clearance rate and immunogenicity [3].

The separation of glycoprotein and glycopeptide glycoforms is a challenging analytical problem. Recently, capillary electrophoresis has shown promise in separating glycoprotein glycoforms, but capillary electrophoresis techniques are limited because fractions can not be collected for further analysis [4–7]. Concanavalin A–crossed affinoimmunoelectrophoresis has been used to separate human transferrin into three groups of glycoforms based on the oligosaccharide branching at the protein's two N-linked (*i.e.*, asparagine-linked) glycosylation sites [8]. Rice *et al.* [9] showed that RP-HPLC at pH 7 could separate each of the three tryptic N-linked glycopeptides of bovine fetuin into groups of glycoforms based on the number of sialic acid residues on each glycopeptide.

Pellicular anion-exchange resins have been successfully used to separate oligonucleotides [10], protein isoforms due to deamidation [11] and oligosaccharide linkage isomers [12]. This paper shows that the Asn-81 glycopeptide of bovine fetuin (amino acid residues 54–84), which elutes as a single peak by RP-HPLC, can be separated into five peaks on a pellicular anion-exchange column. These peaks are glycopeptides which possess different populations of sialylated oligosaccharides. Hence each peak has a different population of sialylated glycopeptide glycoforms. Additionally, this manuscript shows that the two other N-linked tryptic glycopeptides of fetuin can be separated into multiple peaks on the pellicular anion-exchange column.

2. Experimental

2.1. Materials

Bovine fetuin was obtained from Gibco (Grand Island, NY, USA; lot No. 14P7696). Acetonitrile and 50% NaOH were obtained from Fisher Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid (TFA) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Polypropylene tubes (1.5 ml) for fraction collection were purchased from Sarstedt (Catalogue No. 72-692; Princeton, NJ, USA). Glycerol-free peptide N^4-(N-acetyl-β-D-glucosaminyl)asparagine amidase F (PNGase F) and *Arthrobacter ureafaciens* neuraminidase were purchased from Boehringer Mannheim (Indianapolis, IN, USA). The water for all eluents and sample preparations was deionized water which was glass-distilled by a Corning Mega-Pure system. The distilled water was collected directly into a glass container.

2.2. Purification of the fetuin glycopeptides

Fetuin was reduced, alkylated, and treated with trypsin as previously described [13]. About 600 μg of a fetuin tryptic digest were separated on a DuPont Zorbax C_{18} RP-300 column (15 × 0.93 cm, Dionex) and fractions were collected [FOXY II fraction collector (ISCO)] into 100 × 13 mm polypropylene tubes (Fisher, Catalogue No.14-956-7A). This and all other separations were on a GlycoStation (Dionex) equipped with a variable-wavelength detector (VDM-2). The first five minutes of the separation were isocratic at 5% eluent B [0.1% TFA in acetonitrile–water (90:10)] and 95% eluent A (0.1% aqueous TFA). This was followed by a linear gradient of 5 to 52% of B in the final 55 min. The flow-rate was 4.0 ml/min. Fractions were collected with the slope sensitivity set to "normal". The maximum fraction size was set to 4.0 ml. Fractions were immediately dried in a SpeedVac (Savant, Model SVC100). Two preparations of each glycopeptide, which were previously identified [13], were combined, dried and redissolved in water (600 μl). To assess the purity of each glycopeptide, an aliquot (2 μl) was rechromatographed on an analytical (15 × 0.46 cm) reversed-phase column (Zorbax C_{18} RP-300) with the same gradient used for the purification. The flow-rate was 1.0 ml/min.

The experimental strategy for analyzing the purified fetuin Asn-81 glycopeptide is shown in Fig. 1.

2.3. Peptide sequence analysis

The amino terminal sequence was determined for the fetuin Asn-81 glycopeptide to assess its

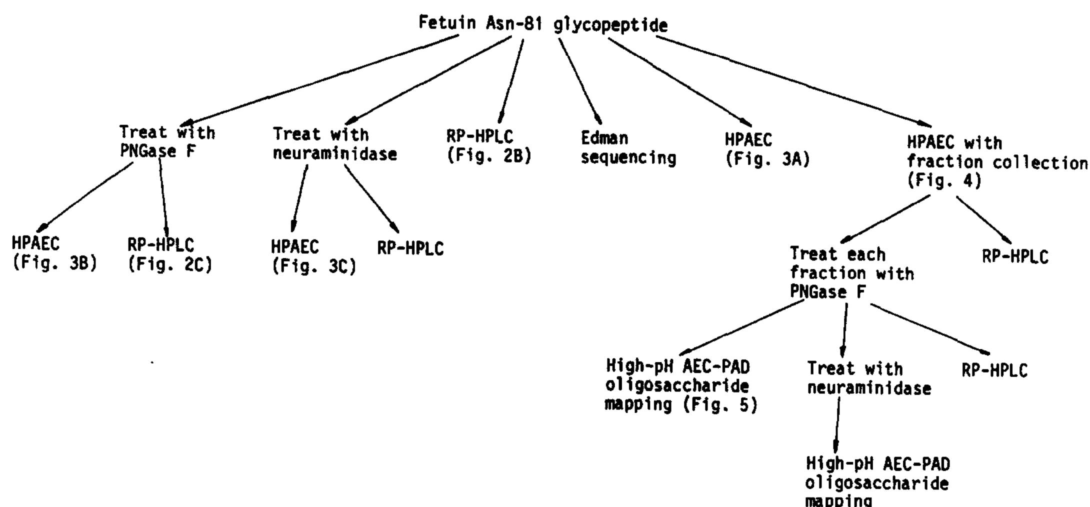

Fig. 1. Experimental scheme for the analysis of the fetuin Asn-81 glycopeptide after RP-HPLC purification. The right side of the diagram outlines the analysis of individual fractions from the anion-exchange separation (AE-HPLC) of the glycopeptide.

purity. A Porton Instruments (Tarzana, CA, USA) PI2090E integrated microsequencing system was used for the analysis. About 400 pmol (10 μl) of the glycopeptide was sequenced after deposition on a glass fiber "peptide support" (Porton). There was greater than 83% repetitive yield.

2.4. Anion-exchange separation of the fetuin glycopeptides

Each fetuin glycopeptide preparation and the neuraminidase and PNGase F digestions of these preparations were separated on a NucleoPac PA100 column (25 × 0.4 cm, Dionex). Tris–HCl (15 mM) pH 8.0 was installed as eluent A and 0.5 M NaCl, 15 mM Tris–HCl pH 8.0 was installed as eluent B. Both eluents were filtered through a 0.22-μm filter. The glycopeptide was separated with a gradient of 25 to 250 mM NaCl (5 to 50% B) in 30 min. The column was then returned to starting conditions over 2 min and held at starting conditions for 15 min prior to the next injection. The flow-rate was 1.0 ml/min and the absorbance was monitored at 215 nm. Fractions were prepared from a separation of 140 μl of the Asn-81 glycopeptide with a fraction collector slope sensitivity setting of "high" and the maximum fraction size set to 1 ml. The fractions were dried in the SpeedVac, redissolved in water (125 μl), and dialyzed against water using a microdialyzer (Pierce, System 100) with M_r 1000 cut-off dialysis tubing (Spectra, Los Angeles, CA, USA). After microdialysis, the fractions were dried.

2.5. Enzyme digestions

An aliquot (20 μl) of each fetuin glycopeptide was dried, and then dissolved in 100 μl of 50 mM sodium phosphate buffer pH 7.6 containing 10 mM EDTA and 0.5 units of PNGase F. EDTA was omitted from the buffer in the analyses of the Asn-138 and Asn-158 glycopeptides to make the chromatograms easier to interpret. These PNGase F digests were mixed and allowed to incubate for 18 h in a 37°C water bath. Fractions from the anion-exchange separation of the fetuin Asn-81 glycopeptide were redissolved in water (50 μl) and each added to a 1.5-ml polypropylene tube containing 50 μl of 50 mM sodium phosphate + 10 mM EDTA pH 7.6 and 0.5 units of PNGase F. To create a larger sample volume to work with, all PNGase F digests were diluted with 100 μl of water. An aliquot (40 μl) of each PNGase F digestion was analyzed by RP-HPLC (the same method described in section 2.2) to determine if the digestion was complete [14].

An aliquot (20 μl) of each fetuin glycopeptide

and an aliquot (50 μl) of each PNGase F digest of fractions from the anion-exchange separation of the fetuin Asn-81 glycopeptide were treated with 10 μl of a 1 mU/μl solution of *Arthrobacter ureafaciens* neuraminidase (in 0.1 *M* sodium acetate pH 5.0) and then brought to a total volume of 200 μl with 0.1 *M* sodium acetate pH 5.0. All samples were mixed and incubated at 37°C for 24 h.

2.6. Oligosaccharide analysis

PNGase F and neuraminidase digests were analyzed for oligosaccharides with a CarboPac PA100 column (25 × 0.4 cm, Dionex) and its guard column (5 × 0.4 cm). NaOH (100 m*M*) and 100 m*M* NaOH + 1.0 *M* sodium acetate were installed as eluents C and D, respectively. The NaOH eluents were prepared from appropriate dilutions of 50% NaOH. The sodium acetate eluent was filtered through a 0.22-μm filter. Oligosaccharides were separated with 100 m*M* NaOH and 20 m*M* sodium acetate (2% D) for 5 min followed by 100 m*M* NaOH and a gradient of 20 to 200 m*M* sodium acetate (2 to 20% D) over the next 65 min. The eluent was returned to starting conditions over the next 2 min and kept there for 15 min prior to the next injection. The flow-rate was 1.0 ml/min and the detector sensitivity was 100 nA.

3. Results

Fig. 2A shows the RP-HPLC separation of 7.5 μg of a bovine fetuin tryptic digest. The peak with a retention time of 41.9 min was previously identified as one of the three fetuin N-linked glycopeptides [13]. This glycopeptide has 31 amino acid residues (residues 54–84) and the carbohydrate is attached to Asn-81. Rechromatography of the purified fetuin Asn-81 glycopeptide (2 μl) is shown in Fig. 2B. Fig. 2C shows the RP-HPLC separation of the Asn-81 glycopeptide PNGase F digestion. PNGase F treatment removes the glycopeptide's oligosaccharide. The deglycosylated peptide elutes 2.1 min later than the native peptide, which suggests

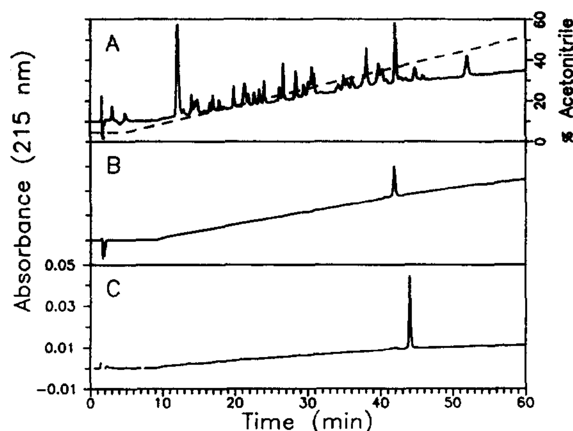

Fig. 2. RP-HPLC separations of bovine fetuin tryptic peptides (A), the purified fetuin Asn-81 glycopeptide (B), and the purified fetuin Asn-81 glycopeptide digested with PNGase F (C). The gradient is displayed with the dashed line in panel A. The separation conditions are described in section 2.2.

that the PNGase F digestion is complete [14]. RP-HPLC of glycopeptide PNGase F digestions is used in this manuscript to assess the extent of deglycosylation.

Fig. 3A shows the anion-exchange separation of the fetuin Asn-81 glycopeptide preparation. This preparation, which is one peak when separated by RP-HPLC (Fig. 2B), is separated into

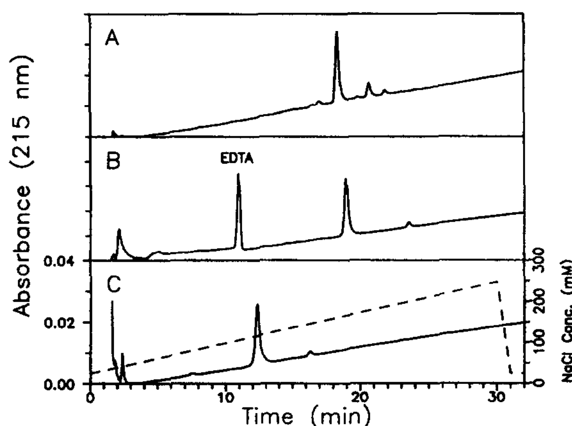

Fig. 3. Anion-exchange separations of the fetuin Asn-81 glycopeptide (A), the PNGase F digestion of the fetuin Asn-81 glycopeptide (B), and the neuraminidase digestion of the fetuin Asn-81 glycopeptide (C). The gradient is displayed with the dashed line in panel C. Each chromatogram represents 4 μl of the glycopeptide. The separation conditions are described in section 2.4.

seven peaks. Fig. 3B shows the anion-exchange separation of a PNGase F digest of the fetuin Asn-81 glycopeptide. The PNGase F treatment reduces the number of peaks. The peaks in the first 5 min of the chromatogram are also found in the blank (no substrate) digest (chromatogram not shown). The peak with a retention time of 10.5 min is EDTA, a component of the digestion buffer. The peak at 19 min is believed to be the deglycosylated fetuin Asn-81 glycopeptide. When this peak was collected and then separated by RP-HPLC, it coeluted with the deglycosylated fetuin Asn-81 glycopeptide (chromatogram not shown). The identity of the peak with a retention time of 23.5 min is unknown. Fig. 3C shows the anion-exchange separation of a neuraminidase digestion of the fetuin Asn-81 glycopeptide. This treatment also reduces the number of peaks. The peaks in the first 5 min of the chromatogram are also found in the blank digest (chromatogram not shown). The peak with a retention time of 12.5 min is believed to be the desialylated fetuin Asn-81 glycopeptide. When this peak was collected and then separated by RP-HPLC, it coeluted with the desialylated fetuin Asn-81 glycopeptide (chromatogram not shown). The identity of the peak at 16.5 min is unknown. When the Asn-81 glycopeptide preparation is incubated in either the PNGase F or the neuraminidase digest buffer and separated by anion-exchange chromatography, there is no change in the retention times of any peaks or the evolution of any new peaks (chromatograms not shown).

Fig. 4 shows the anion-exchange separation of 140 μl of the fetuin Asn-81 glycopeptide preparation. Seven peaks, numbered 1–7, were collected for further analysis. Each of the seven peaks was analyzed by RP-HPLC and only peaks 1 and 7 did not coelute with the Asn-81 glycopeptide peak. Using initial yields from Edman sequencing, the yield and purity of the fetuin Asn-81 glycopeptide was estimated to be 24 nmol and 90.8%, respectively. Edman sequencing of about 400 pmol (10 μl) of the glycopeptide preparation revealed that there were three peptide impurities. The amino-terminal sequences of the three contaminating peptides could be found in the amino acid sequence

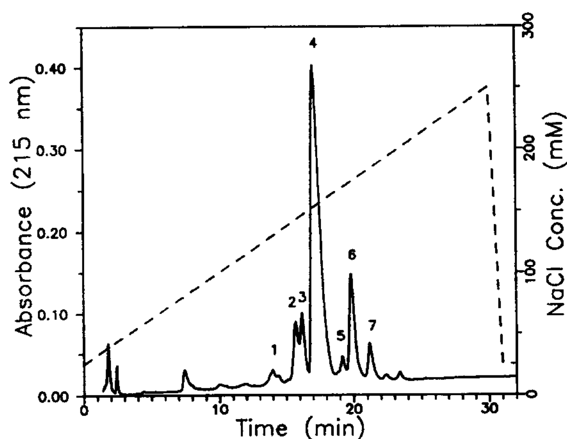

Fig. 4. Preparation of fractions from the anion-exchange separation of the fetuin Asn-81 glycopeptide. Peak numbers correspond to fractions digested with PNGase F. The dashed line shows the NaCl gradient. This is a separation of 140 μl of the glycopeptide. The separation conditions are described in section 2.4.

of bovine fetuin [15] and match the amino-terminal sequences of the following three fetuin tryptic peptides, residues 228–288, residues 103–113 and residues 295–315, which will be designated peptides A, B and C, respectively. Peptides A, B and C, which had been previously purified by RP-HPLC and sequenced [16], were analyzed on the anion-exchange column. Peptide A, which contains fetuin's three O-linked (*i.e.*, Ser/Thr-linked) glycosylation sites, coeluted with peak 1 and had the same doublet shape. Peptide A also had a small peak that coeluted with peak 3. Peptide B coeluted with peak 5 and peptide C coeluted with peak 1.

Fig. 5 shows the respective oligosaccharide profiles when the RP-HPLC purified fetuin Asn-81 glycopeptide (panel A) and individual fractions of the anion-exchange separation of the fetuin Asn-81 glycopeptide (panels B–E) are treated with PNGase F. The oligosaccharide profile in panel A shows a direct injection of 40 μl of the fetuin Asn-81 glycopeptide PNGase F digest. The peaks labeled 1 and 2 in Fig. 5A are trisialylated triantennary oligosaccharides and peaks 3 and 4 are tetrasialylated triantennary oligosaccharides. Townsend *et al.* [12] identified these structures in the high-pH anion-exchange

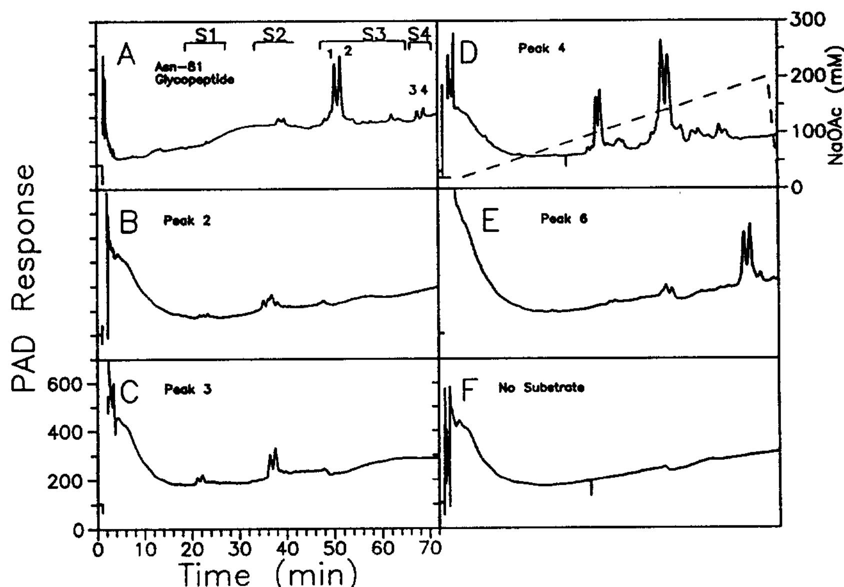

Fig. 5. High-pH AEC–PAD oligosaccharide maps of the PNGase F digestions of the fetuin Asn-81 glycopeptide and its anion-exchange fractions. (A) PNGase F digestion of the fetuin Asn-81 glycopeptide, (B–E) PNGase F digestions of fractions 2, 3, 4 and 6, respectively, (F) enzyme blank. The gradient is displayed with the dashed line in panel D. The injection volume for panel A was 20 μl and the injection volumes for panels B–F were 50 μl. The approximate retention times of mono-, di-, tri- and tetrasialylated oligosaccharides are indicated by S1, S2, S3 and S4, respectively. The separation conditions are described in section 2.6. The peak numbers are discussed in the text.

chromatography (AEC)–pulsed amperometric detection (PAD) chromatogram of fetuin N-linked oligosaccharides. The peaks at 20–24 min and 35–40 min are at the retention times where mono- and disialylated oligosaccharide standards elute, respectively. Panels B and C show the oligosaccharide profiles of peaks 2 and 3 (collected fractions, Fig. 4). Both peaks possess mainly disialylated oligosaccharides with some monosialylated oligosaccharides. The distribution of mono- and disialylated oligosaccharides differs between peaks 2 and 3. The oligosaccharide profile of peak 4, the main peak in the anion-exchange separation of the fetuin Asn-81 glycopeptide, is shown in panel D. Panel D shows that peak 4 contains di- and trisialylated oligosaccharides. Many of the peaks between 50 and 62 min have been shown to be other trisialylated triantennary oligosaccharides [12]. Peak 4 does not have any monosialylated oligosaccharides or either of the two known tetrasialylated oligosaccharides of fetuin. Panel E shows the oligosaccharide profile of peak 6. This peak contains the two tetrasialylated oligosaccharides of fetuin as well as a small amount of two trisialylated oligosaccharides. Panel F shows the blank (no substrate) digestion. No oligosaccharides were observed in the PNGase F digests of peaks 1 and 7 and a small amount of trisialylated oligosaccharides was observed in the PNGase F digest of peak 5 (chromatograms not shown). A RP-HPLC analysis of each PNGase F digest confirmed that all digestions were complete. Each PNGase F digest was treated with neuraminidase and analyzed by high-pH AEC–PAD oligosaccharide mapping (chromatograms not shown). This analysis confirmed the identification of sialylated oligosaccharides. The oligosaccharide analysis confirms that five of the seven peaks (peaks 2–6) contain the fetuin Asn-81 glycopeptide and the other two peaks (peaks 1 and 7) are impurities.

To determine if the pellicular anion-exchange column could be used to fractionate other

sialylated glycopeptides, the two other glycopeptides of fetuin (Asn-138 and Asn-158) were separated on the NucleoPac PA100 column. These glycopeptides were purified by RP-HPLC [13] and rechromatography suggested that both glycopeptides contained impurities (data not shown). It was previously shown that the Asn-138 glycopeptide elutes in two fractions by the RP-HPLC [13]. The earlier-eluting fraction was used for this study due to its greater purity (contains one peptide impurity [13]). Panels A and D of Fig. 6 show the high-performance (HP) AEC separations of the Asn-138 and Asn-158 glycopeptides, respectively. These glycopeptides are fractionated into multiple peaks by HPAEC. Treatment of the glycopeptides with PNGase F reduces the number of HPAEC peaks (panels B and E). Injection of equal volumes of the enzyme blank (no substrate, panels C and F) shows that many of the peaks in panels B and E are derived from the enzyme preparation and the digestion buffer. The peak with a retention time of 10.5 min coelutes with EDTA, a component of the enzyme preparation. Treatment of the Asn-138 and Asn-158 glycopeptides with neuraminidase abolishes all binding to the column except for one small peak with a retention time

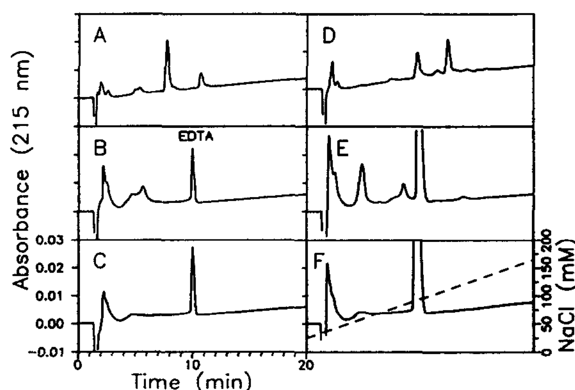

Fig. 6. Anion-exchange separations of the fetuin Asn-138 glycopeptide (A), the PNGase F digest of the Asn-138 glycopeptide (B), the PNGase F blank (C and F), the fetuin Asn-158 glycopeptide (D), and the PNGase F digest of the Asn-158 glycopeptide (E). The dashed line in panel F shows the NaCl gradient. The injection volumes for panels A–F are 4, 40, 40, 10, 100 and 100 μl, respectively. The separation conditions are described in section 2.4.

of 7 min in the Asn-158 glycopeptide sample (chromatograms not shown). The HPAEC analysis of the Asn-138 and Asn-158 glycopeptides shows that the NucleoPac PA100 column can fractionate other sialylated glycopeptides and the separation is due to oligosaccharide sialylation.

4. Discussion

The N-linked carbohydrate of bovine fetuin, a glycoprotein isolated from fetal calf serum, is microheterogeneous. Most of this microheterogeneity resides in the amount of N-acetylneuraminic acid, its location, and its linkage to galactose [17]. Additionally, fetuin's three N-linked glycosylation sites differ in their sialylated oligosaccharide content [13]. In this paper, the fetuin Asn-81 tryptic glycopeptide was purified to a single peak by RP-HPLC and then fractionated by pellicular AEC (NucleoPac PA100 column). The pellicular anion-exchange separation yielded seven peaks, five of which were identified as the Asn-81 glycopeptide. Using high-pH AEC–PAD in combination with enzyme treatments, the Asn-81 glycopeptide peaks were shown to differ in their sialylated oligosaccharide content. In the order of their elution, the first two peaks (peaks 2 and 3) have mainly mono- and disialylated oligosaccharides, the third and fourth peaks (peaks 4 and 5) have mainly trisialylated oligosaccharides, and the fifth peak (peak 6) has mainly tetrasialylated oligosaccharides. Thus, as the sialylation of the Asn-81 glycopeptide increases, its retention time on the NucleoPac PA100 column increases. Additionally, this manuscript shows that the NucleoPac PA100 column can fractionate two other sialylated glycopeptides of fetuin due to their attached oligosaccharide.

The relationship between the Asn-81 glycopeptide's sialylation and its retention on the NucleoPac PA100 column suggests that oligosaccharide sialylation is controlling the separation of this glycopeptide on the NucleoPac PA100 column. For peptides larger than 20 amino acids, secondary and tertiary structure develops as chain length increases [18]. This

three-dimensional structure makes it difficult to predict the peptide's RP-HPLC retention time from the peptide's amino acid composition [19]. It has been hypothesized that when large peptides (greater than 20 amino acids) and proteins are separated on reversed-phase or ion-exchange resins, only part of the molecule interacts with the resin [18]. This part of the molecule has been defined as the chromatographic contact area. The fetuin tryptic N-linked glycopeptides are 31 (Asn-81), 15 (Asn-138) and 28 (Asn-158) amino acids in length. Both neuraminidase and PNGase F treatments support the conclusion that the oligosaccharide of each glycopeptide is at least a portion of the glycopeptide's chromatographic contact area on the NuceloPac PA100 column. Neuraminidase treatment collapsed the five Asn-81 glycopeptide peaks into one peak with a shorter retention time. Treatment of the Asn-81 glycopeptide with PNGase F collapses the five glycopeptide peaks into one peak with a longer retention time than the largest of the five peaks. Unlike neuraminidase which only removes sialic acids, leaving the remainder of the oligosaccharide intact, PNGase F removes the whole N-linked oligosaccharide. The results of the PNGase F digestion suggest that the removal of the Asn-81 glycopeptide's carbohydrate exposes at least some of the glycopeptide's six acidic amino acids. This exposure increases the peptide's retention on the NucleoPac PA100 column.

Using RP-HPLC at pH 7, Rice *et al.* [9] have shown that individual fetuin glycopeptides can be separated into groups of glycoforms based on their degree of sialylation. Unlike the separation of the Asn-81 glycopeptide reported here, these glycoforms elute in the order of greatest to least number of sialic acid residues with a single peak for each charge group. When tryptic digests of glycoproteins are separated by RP-HPLC at pH 2, it has been observed that glycopeptide peaks are broad [20]. Examination of glycopeptide peaks with on-line mass spectrometry has shown that the heaviest glycoforms elute at the front of the glycopeptide peak [21,22]. The results of Rice *et al.* [9] at neutral pH are consistent with a heaviest to lightest glycoform elution order.

In conclusion, pellicular AEC can be used to fractionate sialylated glycopeptides. This fractionation is not only based on the number of sialic acids, but on the structure of the attached sialylated oligosaccharides. This is exhibited by peaks 2 and 3 in Fig. 4 which have different populations of disialylated oligosaccharides. The fractionated sialylated glycopeptides can be purified for further study. As noted by Rice *et al.* [9], glycopeptide glycoforms can be used as substrates for glycopeptidase studies and to study the influence of peptide sequence on oligosaccharide conformation.

5. Acknowledgements

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6. References

[1] P.M. Rudd, R.J. Leatherbarrow, T.W. Rademacher and R.A. Dwek, *Molec. Immunol.*, 28 (1991) 1369.
[2] D.A. Cumming, *Glycobiology*, 1 (1991) 115.
[3] T.W. Rademacher, R.B. Parekh and R.A. Dwek, *Ann. Rev. Biochem.*, 57 (1988) 785.
[4] S.-L.Wu, G. Teshima, J. Cacia and W.S. Hancock, *J. Chromatogr.*, 516 (1990) 115.
[5] Y.W. Yim, *J. Chromatogr.*, 559 (1991) 401.
[6] A.D. Tran, S. Park, P.J. Lisi, O.T. Huynh, R.R. Ryall and P.A. Lane, *J. Chromatogr.*, 542 (1990) 459.
[7] E. Watson and F. Yao, *J. Chromatogr.*, 630 (1993) 442.
[8] T.J. Hahn and C.F. Goochee, *J. Biol. Chem.*, 267 (1992) 23982.
[9] K.G. Rice, N.B.N. Rao and Y.C. Lee, *Anal. Biochem.*, 184 (1990) 249.
[10] S.M. Gryaznov and R.L. Letsinger, *Nucl. Acids Res.*, 20 (1992) 1879.
[11] A. Tuong, M. Maftouh, C. Ponthus, O. Whitechurch, C. Roitsch and C. Picard, *Biochemistry*, 31 (1992) 8291.
[12] R.R. Townsend, M.R. Hardy, D.A. Cumming, J.P. Carver and B. Bendiack, *Anal. Biochem.*, 182 (1989) 1.
[13] J.S. Rohrer, G.A. Cooper and R.R. Townsend, *Anal. Biochem.*, 212 (1993) 7.
[14] J.S. Rohrer and H.B. White III, *Biochem. J.*, 285 (1992) 275.
[15] K.M. Dziegielewska, W.M. Brown, S.-J. Casey, D.L. Christie, R.C. Foreman, R.M. Hill and N.R. Saunders, *J. Biol. Chem.*, 265 (1990) 4354.

[16] J.S. Rohrer and G.A. Cooper, unpublished results.
[17] E.D. Green, G. Adelt, J.U. Baenzinger, S. Wilson and H. van Halbeek, *J. Biol. Chem.*, 263 (1988) 18253.
[18] F.E. Regnier, *LC·GC*, 5 (1987) 230.
[19] J.L. Meek, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 1632.
[20] R.J. Harris, S.M. Chamow, T.J. Gregory and M.W. Spellman, *Eur. J. Biochem.*, 188 (1990) 292.
[21] V. Ling, A.W. Guzzetta, E. Canova-Davis, J.T. Stults, W.S. Hancock, T.R. Covey and B.I. Shushan, *Anal. Chem.*, 63 (1991) 2909.
[22] M.E. Hemling, G.D. Roberts, W. Johnson, S.A. Carr and T.R. Covey, *Biomed. Environ. Mass Spectrom.*, 19 (1990) 677.