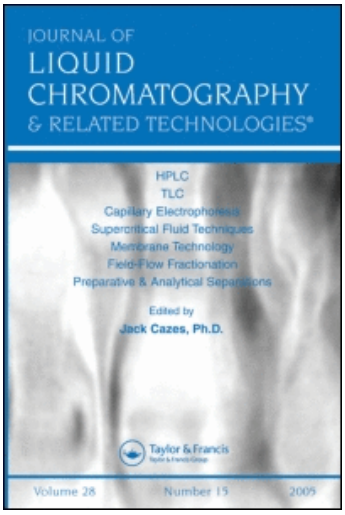

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of Reducing Oligosaccharides Derived from Glycoproteins on Stable Polymeric HPLC Packings

George Vella[a]; Charles Phoebe Jr[a]; Brad Bendiak[a]
[a] Millipore, Waters Chemical Products Division 34 Maple Street Miliford, Massachusetts 01757 and The Biomembrane Institute, Washington

To cite this Article Vella, George , Phoebe Jr, Charles and Bendiak, Brad(1989) 'Separation of Reducing Oligosaccharides Derived from Glycoproteins on Stable Polymeric HPLC Packings', Journal of Liquid Chromatography & Related Technologies, 12: 8, 1333 — 1346
To link to this Article: DOI: 10.1080/01483918908049510
URL: http://dx.doi.org/10.1080/01483918908049510

JOURNAL OF LIQUID CHROMATOGRAPHY, 12(8), 1333-1346 (1989)

SEPARATION OF REDUCING OLIGOSACCHARIDES DERIVED FROM GLYCOPROTEINS ON STABLE POLYMERIC HPLC PACKINGS

GEORGE VELLA, CHARLES PHOEBE, JR., AND BRAD BENDIAK
Millipore, Waters Chemical Products Division
34 Maple Street
Milford, Massachusetts 01757
and
The Biomembrane Institute
201 Elliot Avenue West
Seattle, Washington 98119

ABSTRACT

A multidimensional high performance liquid chromatographic approach has been designed to purify and isolate oligosaccharides derived from glycoproteins prior to structure elucidation. After release by chemical or enzymatic means, crude oligosaccharide mixtures can be fractionated into acidic and neutral glycans by using a stable polymeric anion exchange column, Glyco-Pak DEAE. Acidic fractions can be further separated on the same column by changing mobile phase selectivity. Neutral oligosaccharides are resolved using partition mode chromatography first on a silica-based Carbohydrate Analysis column followed by a hydroxylated polymeric Glyco-Pak N column. Crude mixtures containing only neutral glycans can be resolved directly on Glyco-Pak N. These stable polymeric columns do not shed packing materials which may interfere with post-column structural analyses such as NMR. This multicolumn strategy is demonstrated by using acidic and neutral oligosaccharides derived from hydrazinolysis of fetuin and ovomucoid respectively and by using high mannose compounds released by Endohexosaminidase H treatment as examples which support this approach.

INTRODUCTION

Carbohydrates constitute a class of compounds that are ubiquitous in all living organisms. Sugars which are covalently bound to proteins to form glycoproteins are believed to mediate important biological functions such as control of uptake by cells, regulate lifetime of proteins in circulation, speciate human blood types and stabilize protein conformation[1], [2]. In order to facilitate many of these functions, complex carbohydrates derived from glycoproteins vary in structure. Structure elucidation of these glycan moieties, including the assignment of linkages and anomeric configurations, has been a challenging problem for many years.

Oligosaccharides derived from glycoproteins are classified into two types: N-linked and O-linked glycans. N-linked oligosaccharides contain N-acetylglucosamine (GlcNAc)β-linked to the amide nitrogen of asparagine (ASN). N-acetylgalactosamine, galactose or xylose bound to the hydroxyl group of serine, threonine, hydroxylysine or hydroxyproline constitute the several types of O-linked oligosaccharides that are known to occur. Since proteins may contain asparagine, serine and/or threonine, N-linked and O-linked peptide linkages are not mutually exclusive and both can occur on the same glycoprotein. The N-linked oligosaccharides can be further classified as high mannose, hybrid and multianntenary complex. The latter containing sialic acid at the non-reducing terminus. Hybrid structures at times may contain sialic acid or alternatively may be phosphorylated. Glycoproteins treated chemically with anhydrous hydrazine[3] or enzymatically[4] with a specific amidase, releases the oligosaccharide by hydrolysing the peptide carbohydrate bond. Oligosaccharides are either neutral or acidic and the nature of the acidic character is attributed to sialic acid content, phosphorylation or sulfation rendering negatively charged species. In addition, microhetrogeneity within the glycan moieties results in a diversity of structural isomers and carbohydrates which vary in size. Each of these compounds must be purified in sufficient quantity so that the structure may be elucidated unequivocally.

Techniques such as high voltage paper electrophoresis and paper chromatography, traditionally used to purify complex carbohydrates, do not have the resolving power to adequately separate closely related compounds. These methods

are also tedious and time consuming. High performance liquid chromatography is now emerging as the method of choice to purify these oligosaccharides. High resolution packing materials provide the most efficient separations in shorter times. In addition, separations can be scaled-up to preparatively isolate and purify larger amounts of complex oligosaccharides.

Silica-based columns have been widely used and packing materials which contain an amino functionality are the most common in carbohydrate analysis[5]. A major disadvantage in all amino-bonded silica surface chemistries is that contaminants from the packing material slowly leach out of the column thus causing variable retention times and decreased column life time. The contaminants, which are probably solubilized silica due to locally high pH of the amino functionality, interfere with structural elucidation methodologies, such as NMR. More recently stable polymeric based supports have demonstrated to be free from contaminants. This paper discusses the strategies employed to isolate and purify N-linked oligosaccharides by high resolution HPLC after chemical or enzymic release of the glycans from glycoproteins. Stable polymeric supports can be used to preparatively isolate larger quantities of carbohydrates and in the case of neutral reducing oligosaccharides, can be taken directly to NMR after D_2O exchange[6].

MATERIALS

Glycoprotein Derived Oligosaccharides

Ovomucoid and fetuin (Sigma) were used to obtain mixtures of oligosaccharides after treating the glycoproteins with anhydrous hydrazine (Aldrich). Endohexosaminidase H (Endo H) released high mannose compounds were generously supplied by Dr. S. Hirani, Genzyme Corporation, Boston, Massachusetts 02111.

Chromatographic Instrumentation and Columns

Separation of acidic and neutral oligosaccharides was carried out on either two M510 pumps controlled by an M680 gradient controller or an M600

multisolvent delivery system. Both systems were equipped with an M490 programmable multiwavelength UV/VIS detector monitoring between 197-205nm. Either an M712 WISP autosampler or U6K manual injector was used to apply samples to the systems.

Column chemistries included Glyco-Pak DEAE, a polymeric anion exchange column (7.5 x 75mm), Glyco-Pak N, a hydroxylated resin based column (7.8 x 300mm) and Waters Carbohydrate Analysis (3.9 x 300mm) amino bonded silica column.

METHODS

Hydrazinolysis of Glycoproteins

The procedure of hydrazinolysis, re-N-acetylation and mild acid hydrolysis was followed as described by Bendiak, et al[3], [6]. Briefly, glycopeptides derived from Pronase digestion of ovomucoid or fetuin (30g) were dried under vacuum for 3 days at 5 mTorr. The dry residue was dissolved in 500ml of anhydrous hydrazine and the mixture, maintained under dry argon, was heated to 100°C for 36 hours. Upon cooling, the reaction mixture was rotorary evaporated using a dry ice trap. The residue was dissolved in 500ml of 0.5M $NaHCO_3$ and the pH adjusted to 7.2 with acetic acid and stored frozen. Aliquots were applied to a Sephadex G-25 (5 x 100cm) equilibrated in water and eluted fractions (20ml) were collected. Hexose containing fractions were assayed by a micro-scale phenol-sulfuric acid test[7] and fractions 37-50 and 55-75 represented two pooled fractions. Amino containing fractions eluted between fractions 80 to 140 as monitored by ninhydrin[8]. Aliquots of the second pooled fraction (30ml) were re-N-acetylated by first evaporating to dryness in the presence of methanol (1:1 V/V) and dissolving the residue in 80ml of saturated $NaHCO_3$. Acetic anhydride (2 x 5ml aliquots) was added within 10 minutes and after 1 hour, solid $NaHCO_3$ was added to adjust the pH to 6. The mixture was diluted to 140ml and applied to a Sephadex G-25 column (5 x 100cm). Hexose containing fractions were pooled and treated with 0.1N HCl for 1 hour at 40°C to hydrolize the β-
-acetohydrazide.

CHROMATOGRAPHIC CONDITIONS

Method 1 - Glyco-Pak DEAE was utilized by employing a linear NaCl gradient elution. The column was equilibrated with water (Solvent A) and a gradient to 50mM NaCl was run over 30 minutes at a flow rate of 0.6 ml/min. Samples were dissolved in water prior to injection. This method was used to fractionate crude oligosaccharide mixtures containing both acidic and neutral glycans after release by hydrazinolysis.

Method 2 - Isocratic elution utilizing the Glyco-Pak DEAE was accomplished with 12.5 mM NaH_2PO_4 buffer at pH 5.4 at a flow rate of 0.8 ml/min over 60 minutes. This method was used to further resolve acidic fractions collected from Method 1.

Method 3 - The Waters Carbohydrate Analysis column was used with an acetonitrile/water (65:35 (V/V)) mobile phase at 1 ml/min. This column was employed to further fractionate neutral oligosaccharides collected from Method 1. Samples were dissolved in 50:50 (V/V) acetonitrile/water prior to injection.

Method 4 - Glyco-Pak N column was used to separate neutral oligosaccharides prior to subsequent structural analysis by NMR. The mobile phase is comprised of acetonitrile/water ranging from 65:35 (V/V) to 77:25 (V/V) depending on the size of the oligosaccharides. At a flow rate of 1 ml/min, run times vary from 35 minutes to 240 minutes. Samples were dissolved in 70:30 (V/V) acetonitrile/water prior to injection.

RESULTS

Fetuin Derived Oligosaccharides:

The crude mixture of complex carbohydrates obtained by the hydrazinolysis of fetuin was separated on the Glyco-Pak DEAE column employing Method 1. Figure 1 shows the chromatographic profile obtained by monitoring at 205nm. Four major fractions were observed. The peak centered at 41 minutes was collected and stored frozen. Rechromatography of this fraction using Method 2 resulted in increased

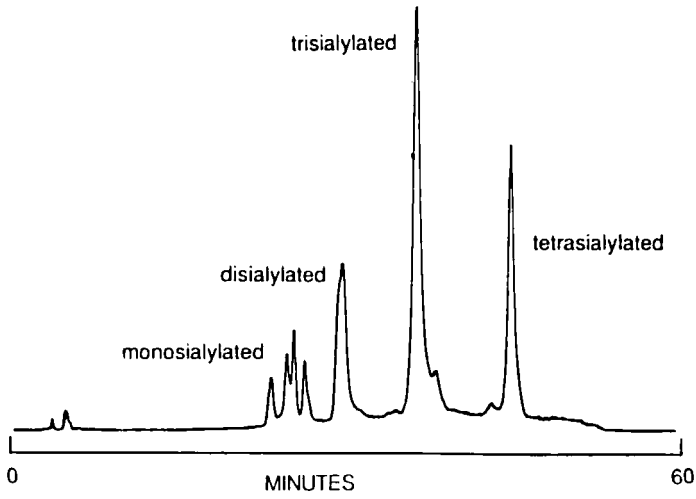

Figure 1: High resolution HPLC separation of acidic oligosaccharides derived from fetuin using Method 1. The main peak at 41 min. was collected and further analysed by Method 2. Detection was at 205nm with baseline subtraction.

resolution of what appeared as a single peak in Method 1. At least four peaks were discernable (Figure 2).

Ovomucoid Derived Oligosaccharides:

Oligosaccharides derived from ovomucoid were separated on Glyco-Pak DEAE using Method 1. The elution profile indicated a major peak eluting at or near the column void volume (Figure 3). Two other smaller peaks were observed at 24 and 27 minutes. The unretained peak was collected and stored frozen for further analysis. An aliquot (200ug) of this fraction was separated using Method 3 employing the Waters Carbohydrate Analysis column. Figure 4 shows the resolution of several major and minor peaks. The peak at 17 minutes was collected and analyzed by Method 4. The Glyco-Pak N column eluted with 75% acetonitrile further resolved the fraction from Method 3 into at least five components (Figure 5).

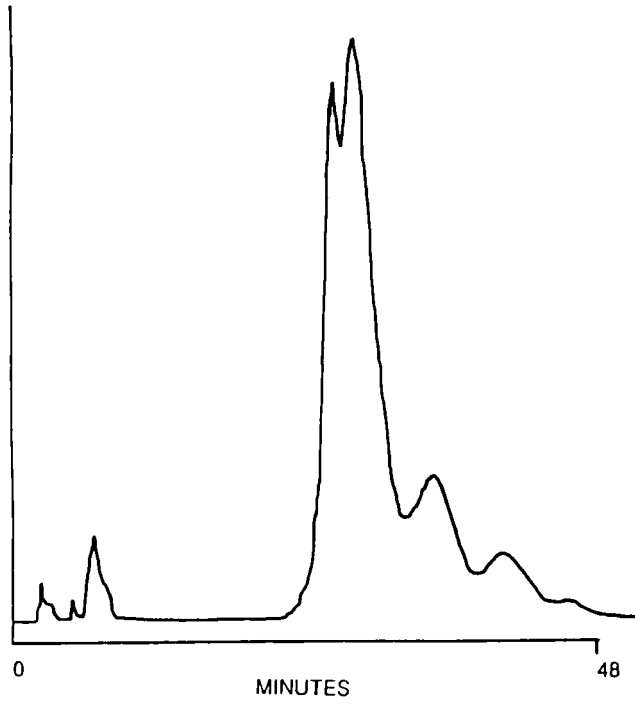

Figure 2: HPLC elution profile of fraction collected at 41 minutes (Method 1) using Glyco-Pak DEAE isocratically in 12.5mM NaH_2PO_4 buffer pH 5.4 (Method 2).

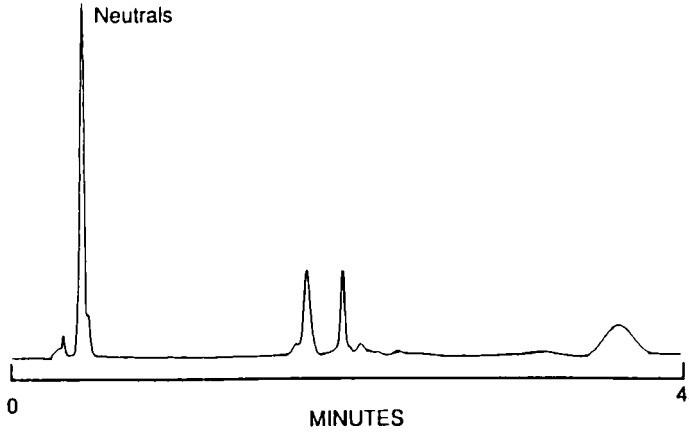

Figure 3: Elution profile of HPLC separation of ovomucoid oligosaccharides (Method 1). Peak at void volume was collected for further analysis on Water Carbohydrate Analysis column.

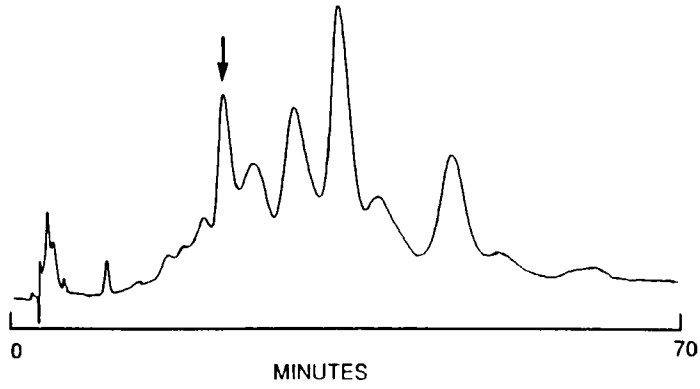

Figure 4: Separation of neutral ovomucoid oligosaccharides obtained from void volume of Glyco-Pak DEAE column. Waters Carbohydrate Analysis column eluted isocratically with 65:35 (V/V) acetonitrile water at 1ml/min. (Method 3). Fraction indicated by arrow was collected for analysis on Glyco-Pak-N (Method 4).

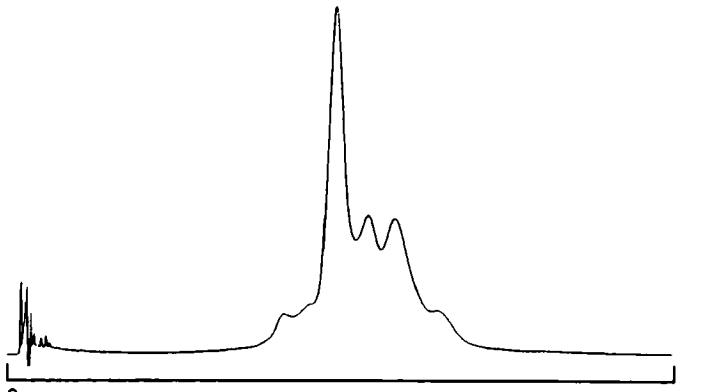

Figure 5: Elution profile of neutral ovomucoid oligosaccharides separated on Glyco-Pak N (Method 4). Sample was collected from prior separation on Water Carbohydrate Analysis column.

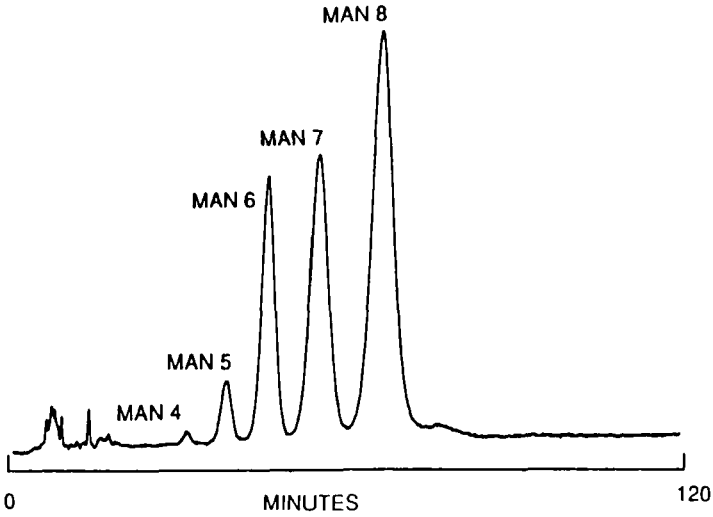

Figure 6: HPLC elution profile of Endo H released high mannose compounds using Glyco-Park N (Method 4).

High Mannose Oligosaccharides:

Endohexosaminidase H released oligosaccharides were resolved by using Method 4. Five peaks were baseline resolved within 90 minutes (Figure 6). The mobile phase was 70% acetonitrile and 100ug of sample was applied.

DISCUSSION

Elucidating the oligosaccharide structure of a glycoprotein is a challenging task and it is becoming more prevalent as new glycoproteins are discovered or as recombinant products are quality controlled to assure that the correct glycosylation from a given host cell. Since such a large number of structural isomers can exist, chromatographic retention times alone cannot be used as a means of identifying an oligosaccharide. Subsequent analyses by NMR[9], MS[10] or permethylation[11] is usually employed to unequivocally elucidate structures of complex carbohydrates. It is for this reason that HPLC plays an important role in the purification of oligosaccharides.

By employing high efficiency columns, oligosaccharides of similar structures can be resolved, collected and subjected to post column analytical techniques. Stable polymeric columns alleviate the difficulty of fractions being contaminated by packing material which may interfere with proper structural elucidation. Polymer based packing materials are now becoming more common with a variety of surface chemistries. By taking advantage of the different selectivities which these column chemistries provide, separation of oligosaccharides based on their molecular and physical properties can be achieved. Figure 7 demonstrates the strategy of oligosaccharide purification using a multidimensional approach.

The polymeric anion exchange column, Glyco-Pak DEAE separates oligosaccharides based on charge. As shown by the resolution of the fetuin oligosaccharides (Method 1, Figure 1) separation of neutral, mono, di-, tri- and tetrasialylated glycans can be achieved by employing a linear gradient. The fraction collected was further separated on the same column by changing mobile phase selectivity (Method 2, Figure 2). This fraction has been shown to contain only trisialylated compounds and structure elucidation by NMR will be published in the near future.[12], [13]

The ovomucoid oligosaccharides were also initially fractionated on the Glyco-Pak DEAE (Method 1, Figure 3). The unretained peak constitutes the neutral glycans and can be further fractionated by employing the Waters Carbohydrate Analysis column in the partition mode (Method 3, Figure 4). Although this is a silica based amino bonded packing material, fractions isolated from this column are further resolved by the Glyco-Pak N column (Method 4, Figure 5) containing a hydroxylated polymeric packing material. This column separates any contaminants from the silica based column and permits the isolation of compounds which can be analyzed directly by NMR after D_2O exchange[6]. Glyco-Pak N also works in a partition mode but by employing a hydroxylated packing material the selectivity is different from that of the Waters Carbohydrate Analysis column. This can be seen by the different acetonitrile concentration used and the run times required for the separation. Glyco-Pak N is particularly sensitive to very small changes in acetonitrile concentration and hence can be used to "fine-tune" a particular separation.

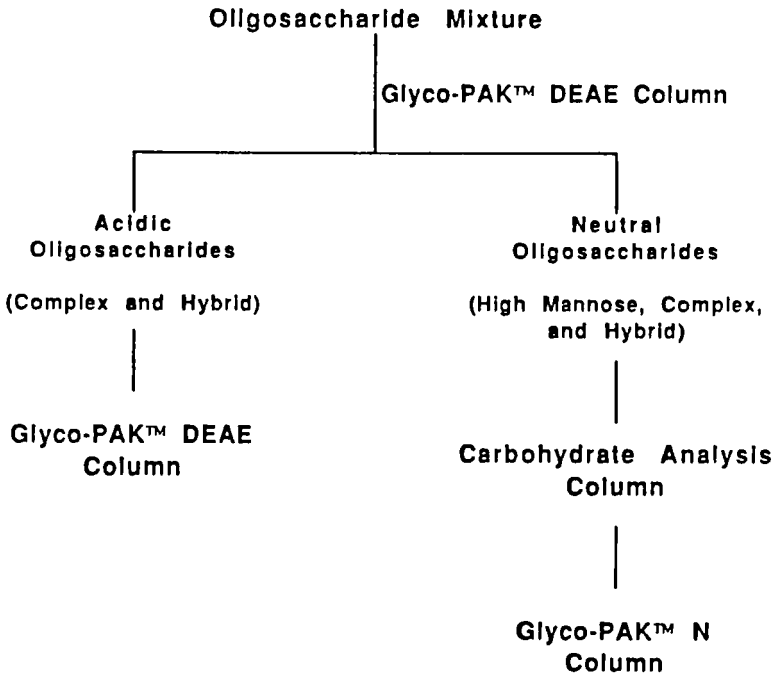

Figure 7: Flow chart outlines the multidimensional approach to purify glycoprotein derived oligosaccharides released by hydrazinolysis or by specific amidase.

Should the crude oligosaccharide mixture contain only neutral oligosaccharides, such as the Endo H released high mannose compounds, employing Method 4 directly would be appropriate. Figure 6 demonstrates the baseline resolution of the high mannose compounds. The resolution of compounds with 4, 5, 6, 7 and 8 mannose residues can easily be achieved within 90 minutes.

The multidimensional approach for the purification and isolation of glycoprotein derived oligosaccharides takes advantage of non-destructive detection by monitoring absorbance at the low UV range. Detection limits of 1 nmole are easily obtained for oligosaccharides containing the N-acetyl moiety. A minimum of 20-30 nmoles are typically required for 500MHz NMR analysis. Therefore adequate sensitivity is

obtained by monitoring the low UV range thereby permitting structural elucidation on intact carbohydrates.

In addition, a multicolumn strategy for the purification and isolation of complex carbohydrates using stable polymeric supports of different selectivities will enable researchers to fractionate closely related oligosaccharides free from column contaminants and will permit a more convenient methodology so that structure/function relationship of glycoprotein derived oligosaccharides may be better understood.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Jeanne Orr for her technical assistance and Ms. Jerry Nelson for her expert secretarial skills.

REFERENCES

1. Montreuil, J., "Primary Struture of Glycoprotein Glycans", Adv. Carbohydr. Chem. and Biochem., 37, 157, (1980).

2. Warren, L., Cossu, G., "The Bound Carbohydrates of Glycoproteins in Normal and Pathological States", Annals. N.Y. Academ. Sci. 401, 85, (1982).

3. Bendiak, B., Cumming, D.A., "Hydrazinolysis-N-Reacetylation of Glycopeptides and Glycoproteins. Model studies using 2-Acetamido-1-N-(L-aspart-4-oyl)-2-Deoxy-β-D-Glucopyranosylamine", Carbohydr. Res., 144, 1, (1985).

4. Hirani, S., Bernasconi, R.J. and Rasmussen, J.R., "Use of N-Glycanase to Release Asparagine-Linked Oligosaccharides for Structural Analysis", Anal. Biochem., 162, 485, (1987).

5. Honda, S., "High Performance Liquid Chromatography of Mono-and Oligosaccharides", Anal. Biochem., 140, 1, (1984).

6. Bendiak, B., Orr, J., Brockhausen, I., Vella, G., Phoebe, C., "Separation of Neutral Reducing Oligosaccharides Derived from Glycoproteins by HPLC on a Hydroxylated Polymeric Support", Anal. Biochem., 174, (1988) (In Press).

7. Kobata, A., "Methods in Enzymology", Edited by Ginsbury, V., 28, (1972), Academic Press, New York, p. 262.

8. Spies, J.R., "Methods in Enzymology", Edited by Colowick, S. and Kaplan, N.O., 3 (1957), Academic Press, New York, p. 467.

9. Carver, J.P. and Grey, A.A., "Determination of Glycopeptide Primary Structure by 360 MHz PMR", Biochemistry, 20, 6607, (1981).

10. Dell, A., Morris, H.R., Egge, H., Von Nicolai, H., Strecker, G., "Fast Atom Bombardment Mass Sepctrometry for Carbohydrate Structure", Carbohydr. Res., 115, 41, (1983).

11. Rauvala, H., Finne, J., Krusius, T., Karkkainen, J., "Methylation Techniques in the Structural Analysis of Glycoproteins and Glycolipids", Adv. Carbohydr. Chem. Biochem., 38, 389, (1981).

12. Bendiak, B., Harris-Brandts, M., Michnick, S.W., Carver, J.P., and Cumming, D.A., "Separation of the Complex Asparagine-linked Oligosaccharides of the Glycoprotein Fetuin and Elucidation of Three Triantennary Structures Having Sialic Acids Linked Only to Galactose Residues", Manuscript in preparation.

13. Cumming, D.A., Harris-Brandts, M., Michnick, S.W., Helleiqvist, C.A., Carver, J.P. and Bendiak, B., "Structures of Asparagine-linked Oligosaccharides of the Glycoprotein Fetuin having Sialic Acid Linked to N-acetylglucosamine", Manuscript in Preparation.