Separation of Sialyated Glycoprotein Isoforms using Strong Anion Exchange (SAX) Chromatography with Controlled pH Gradients

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Introduction

- Sialic acid is a generic term for the N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon backbone.
- Sialic acids are found widely distributed in animal tissues and to a lesser extent in other organisms, ranging from plants and fungi to yeasts and bacteria, mostly in glycoproteins and gangliosides.
- Sialic acid often occupies terminal positions of the oligosaccharides on the glycosylation patterns, yielding an increased negative charge for the glycoprotein. These at the end of sugar chains are connected to the surfaces of cells and soluble proteins.
- The glycosylation patterns which form through sugar-amino acid bonds and the number of carbohydrate units on the protein can lead to isoforms with significantly different biological activity and stability.
- Such charged variants often develop due to difference in protein glycosylation from post translational modifications.
- Difference in glycosylation is exploited in making monoclonal antibody biotherapeutics.

Introduction

- It is critical that protein variants of different glycosylation patterns be isolated for further characterization of their glycan compositions.
- This is often performed using HILIC-MS.
- Ion exchange chromatography (IEX) is a useful analytical tool in the analysis of protein isoforms based on charge heterogeneity.
- This makes strong anion exchange (SAX) an ideal mode for the separation of glycoprotein charged variants as SAX columns utilize a positively charged stationary phase, most commonly functionalized with a quaternary ammonium group.

Introduction

- Often times, the use of salt gradient elution in IEX yields low resolution of glycoprotein isoforms, albeit satisfactory separation.
- The use of controlled pH gradients as an alternative elution method often leads to significantly higher resolution of the protein isoforms with the use of low salt buffer systems[1].
- In SAX separations, the mobile phase pH is gradually reduced, leading to a decrease in the negative charge of the protein isoforms as they approach their individual pI values.
- Due to this mechanism of elution, shallow pH gradients are commonly used to provide high resolution of the individual protein isoforms.

Introduction

- Human transferrin is an iron transport protein with a molecular weight of 80 kDa and is typically present in two forms: Holo (containing iron) and Apo (without iron).
- Analysis of the glycan profile of transferrin has indicated the presence of primarily sialyated biantennary and triantennary N-linked oligosaccharides.
- β_2-glycoprotein is a plasma glycoprotein with a molecular weight of 54 kDa and is essential in the detection of antiphospholipid antibodies for antiphospholipid syndrome.
- Previous work has illustrated that β_2-glycoprotein has extensive glycan heterogeneity, which includes the presence of fucosylation and sialyation[2].
- These heterogenic properties make human transferrin and β_2-glycoprotein complex challenging samples for charge-variant characterization using SAX chromatography.

Materials/Methods

General Chromatographic Conditions:

HPLC System:	Agilent 1200
Column:	TSKgel Q-STAT, 7 µm, 4.6 × 10 cm (R0087-501N)
Flow rate:	1 mL/min
Detection:	UV @ 280 nm (Transferrin)
Temperature:	ambient
Injection vol.:	10 µL (unless noted otherwise)
FLD λ_{ex}:	280 nm, λ_{em}: 330 nm (β_2-Glycoprotein)
Samples:	Transferrin (Holo), β_2-Glycoprotein

pH Gradient (pIsep, CryoBioPhysica):

Mobile phase A:	pIsep buffer (pH 10.01)
Mobile phase B:	pIsep buffer (pH 3.51)
Gradient:	pH 10 - 3.5, 30 min
	pH 5 - 3.5, 30 min

Materials/Methods

pH Gradient (in-house mobile phase):

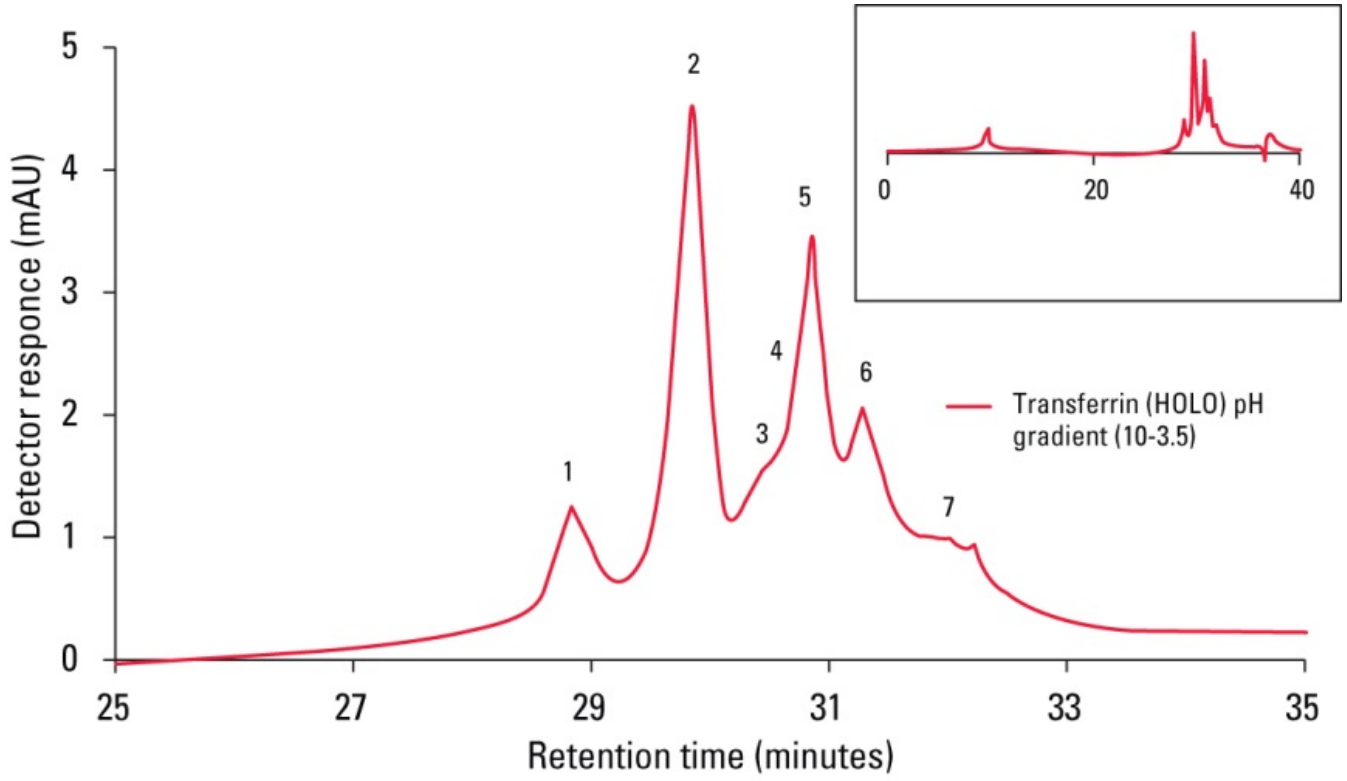
Mobile phase A:	20 mmol/L Triethanolamine + 20 mmol/L Bis-Tris Propane + 20 mmol/L Piperazine + 20 mmol/L N-methylpiperazine, pH 9.7
Mobile phase B:	mobile phase A, pH 3.7, titrated with HCl
Gradient:	pH 9.7-3.7, 30 min pH 9.7-6, 30 min

All pH gradients created using pIsep pH Gradient Maker Plus (CryoBioPhysica)

Salt Gradient:

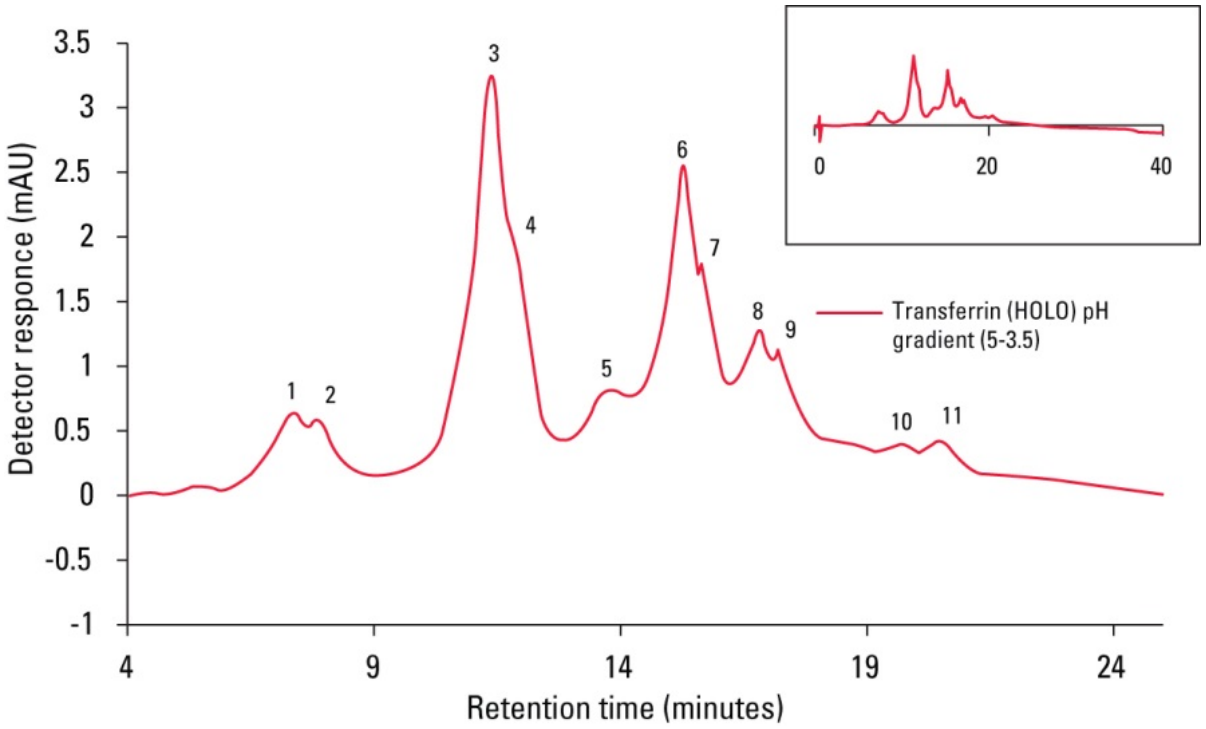
Mobile phase A:	150 mmol/L Tris, pH 9.0
Mobile phase B:	150 mmol/L Tris, pH 9.0 + 0.5 mol/L NaCl
Gradient:	10-30% B, 45 min

Figure 1: Separation of Transferrin (Holo) Isoforms using Wide Range pH Gradient with plsep Kit (pH 10-3.5)



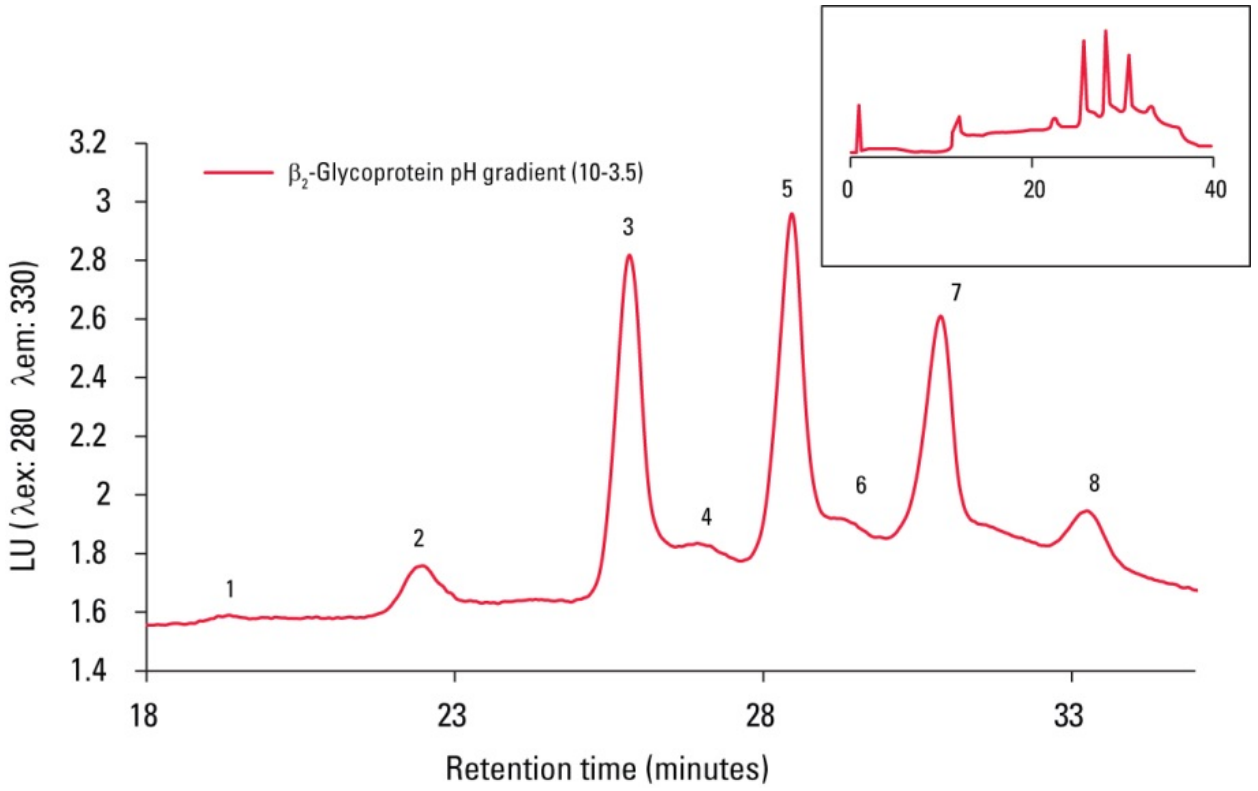
- The wide range pH gradient (pH 10 - 3.5, 30 min) yielded the separation of 7 isoforms of transferrin (Holo).
- Retention factors (k) > 20 indicate significant room for gradient optimization.

Figure 2: Separation of Transferrin (Holo) Isoforms using Narrow Range pH Gradient with pIsep Kit (pH 5-3.5)



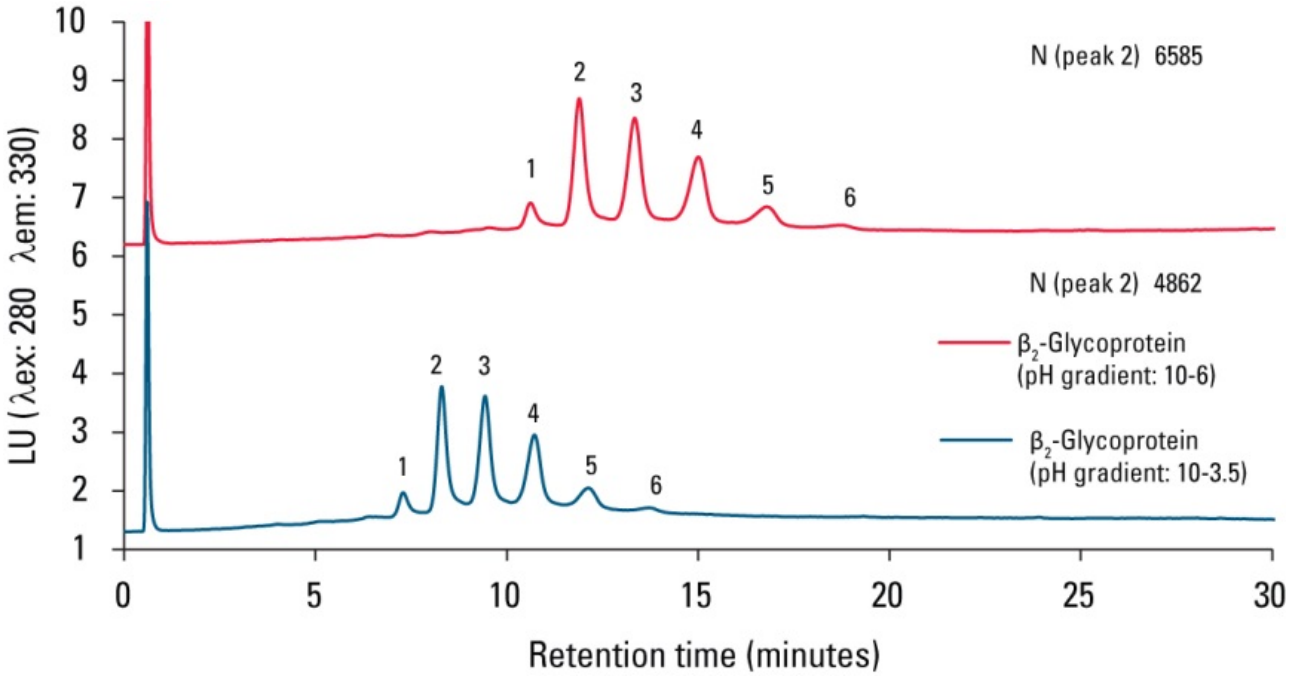
- The narrow range pH gradient (pH 5 - 3.5, 30 min) yielded the separation of 11 isoforms of transferrin (Holo).
- The shallow gradient increased band broadening, yielding a substantial decrease in peak height and sensitivity.
- 25 µL injection volume was used to increase peak height.
- Retention time of all charged variants were reduced by lowering the initial pH.
- pH gradient separation yielded much better separation and resolution of the protein isoforms than under salt gradient conditions, where significant co-elution of protein charged variants occurred (data not shown here).

Figure 3: Separation of β_2-Glycoprotein using Wide Range pH Gradient with plsep Mobile Phase (pH 10-3.5)



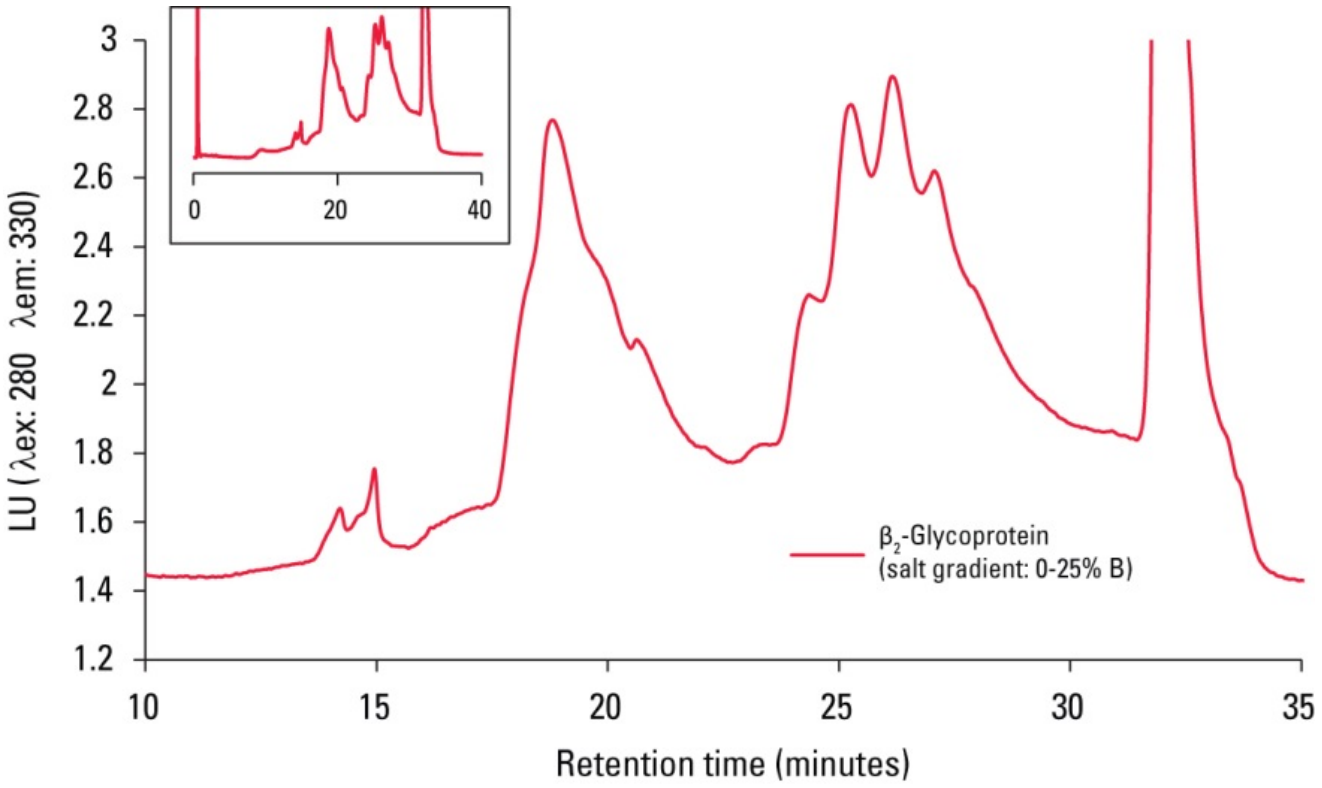
- Due to low UV-VIS signal intensity of 5 µg sample load on column, FLD was used with same load. Injection volume was 50 µL.
- 8 peaks of the glycosylated protein were separated under these conditions.
- Similar to transferrin, high retention factors were observed using the wide range pH gradient conditions.

Figure 4: Separation of β_2-Glycoprotein using pH Gradient with In-House Mobile Phase



- 8 isoforms were separated when β_2-Glycoprotein is separated using the in-house mobile phase with the same pH gradient.
- Under these conditions, the charge protein variants elute in roughly 1/3 of the time observed using the pIsep mobile phase.
- The individual peaks were not further identified by MS or any other analytical technique, so individual peak numbers in these figures don't necessarily represent the same isoform.
- Shallow pH gradient yields an increase in resolution of the isoforms and column efficiency.
- The efficiency of peak #2 as shown in figure 4 is the average of 3 consecutive injections.

Figure 5: Separation of β_2-Glycoprotein using Shallow Salt Gradients (0-25% B)



- Salt gradient of 0-25% B yielded separation profile as shown above.
- Salt gradient separation profile was different from that obtained by pH gradient.
- pH gradient yielded sharper and well resolved peaks compared to salt gradients.

Conclusions

- The use of controlled pH gradients with the TSKgel Q-STAT strong anion exchange column allowed for high resolution separations of charged glycoprotein isoforms.
- For both transferrin and β_2-Glycoprotein, controlled pH gradients using pIsep kit yielded the separation of more protein isoforms relative to the number of isoforms separated by salt gradients.
- The use of our in-house mobile phase yielded very similar separation profiles and faster elution of the charged protein isoforms of β_2-Glycoprotein.
- This study discussed the usefulness of the the separation of glycoproteins using a non-porous strong anion exchange TSKgel Q-STAT column.

References

[1]pH gradient ion exchange chromatography: An analytical tool for design and optimization of protein separations. Journal of chromatography A, 1164 (2007) 181-188.

[2]Characterization of sialylated and fucosylated glycopeptides of β_2-glycoprotein by a combination of HILIC and MALDI MS/MS. J.Sep.Sci. (2010) 33, 891-902.