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Intact Glycoprotein Isoforms Separation on Proteomix[®] Strong Anion Exchange (SAX) Chromatography Using Salt and pH Gradients

Application Note PP1010

Protein Separation

Ion exchange chromatography is a versatile separation method for the fractionation of protein mixtures or for profiling variants of biopharmaceutical proteins such as monoclonal antibodies, glycoproteins and Fc-fusion proteins. Both salt gradients and pH gradients provide robust and high resolution separation of proteins. Here we present two protein variants separation (transferrin and β 2-glycoprotein I) using a salt gradient and a pH gradient on Sepax Proteomix[®] strong anion exchange chromatography.

Introduction

Protein glycosylation is one of the most important and complicated post translational modifications. The formation of sugar-amino acid bond and the nature of the carbohydrate units on the protein have significant influences on the protein's biological activity(1). A Number of protein biologics with different glycosylations have been designed for therapeutic use, such as monoclonal antibodies and Fc-fusion proteins. Due to the different glycosylations, variants of these proteins need to be isolated and further characterized for glycan compositions. Often sialic acids occupy the terminal positions of the oligosaccharides on the glycosylation. Sialylation contributes extra negative charges for the glycoproteins. Therefore, strong anion exchange is an ideal chromatography media to separate sialylated glycoproteins. In this application note we demonstrate the SAX separation of two glycoproteins, Transferrin and β 2-glycoprotein I.

Salt gradient is the most common method for ion exchange chromatography. In the case of SAX, the pH of the buffer system is higher than the protein pI. At the specific higher pH, proteins are negatively charged and bind to the positively charged medium or anion exchanger (quaternary amine for Sepax Proteomix[®] SAX). With increasing ionic strength (salt concentration), the proteins are eluted in the order of increasing net charges under the pH of buffer systems. Therefore the protein mixtures with different pIs can be separated. Furthermore, the variants of the same protein can also be separated based on charge differences. In this application, we have applied the method on the separation of transferrin variants before and after the desialylation by neuraminidase.

Although ionic strength based IEX (salt gradient) gives the satisfactory separation of proteins most of the time, it is very protein dependent (pIs). Here we introduce a pH gradient IEX method as an alternative method for glycoprotein variants separation. With SAX, a high pH starting buffer is required to bind all proteins to the column medium. With decreasing pH generated by the LC system, proteins start to lose the negative charges and elute off the column. One of the advantages of a pH gradient is to have low salt in the buffer system and therefore the fractions of different variant peaks. High resolution separation of protein isoforms is the ideal

application for pH gradient-IEX. The buffer compositions for buffer A (high pH) and buffer B (low pH) were reported in the previous pH gradient studies (2).

Proteomix[®] ion-exchange packing support is composed of non-porous, rigid, spherical, highly cross-linked poly (styrene divinylbenzene) (PS/DVB) beads. The PS/DVB resin surface is grafted with a highly hydrophilic, neutral polymer layer with the thickness in the range of nanometer. This hydrophilic coating eliminates non-specific bindings with biological analytes, leading to high efficiency and high recovery separations for bio-molecules. On top of the hydrophilic layer, a layer of ion-exchange functional groups is attached via chemical bonding. A proprietary chemistry was developed to synthesize a densely packed, uniform ion-exchange layer. The IEX phase used in this study is a strong anion exchanger with quaternary ammonium functional groups. The non-porous particle sizes in this study are 5 μ m and 10 μ m.

Glycoprotein Samples

Human serum transferrin is involved in iron transport with two forms Holo (iron containing) and Apo (without iron). It is a glycoprotein with a molecular weight of about 80 kD and a pI between 5 and 6 depending on the iron saturation degree (3). It has two N-glycosylation sites at the C-terminal lobe. Glycan analysis indicated that transferrin contains mainly sialylated biantennary and triantennary N-linked oligosaccharides in the ratio of 85:15 (4). Transferrin has clinical significance. For example, transferrin levels increase during pregnancy and are closely associated with Total Iron Binding Capacity of serum. Hyposialylated transferrin is a biomarker for chronic alcohol consumption due to the influence of alcohols on the modification of terminal sialylation and a loss of an entire oligosaccharide chain (5).

β 2-glycoprotein I is a plasma glycoprotein with a molecular weight of 54 kD and a carbohydrate content of 17%. There were five major protein bands on isoelectric focusing with pIs between 5.1 and 6.1 (6). Glycosylation studies of β 2-glycoprotein I indicate that there is a great degree of glycan heterogeneity, which includes fucosylation and sialylation (7). Previous studies have shown that β 2-glycoprotein I is an

essential protein for the detection of antiphospholipid antibodies in antiphospholipid syndrome (8). β 2-glycoprotein I dependent anticardiolipin antibodies are strong indicators for stroke and myocardial infarction (heart attack) (9).

Experimental

HPLC system

Agilent 1200 HPLC with binary pump

Chemical Reagents

Transferrin Apo and Holo forms from human plasma were purchased from EMD. Neuraminidase from *Vibrio Cholerae* was purchased from Sigma Aldrich. β 2-glycoprotein I was purchased from Haematologic Technologies, inc. PNGase F was purchased from Prozyme. 4-12% Bis-Tris gels and reagents were purchased from Invitrogen.

Columns and LC Method

Size exclusion chromatography analysis of intact protein: ZenixTM SEC-300 (3 μ m, 300 Å), 7.8 x 300 mm, 150 mM phosphate buffer, pH 7.0 as mobile phase, flow rate at 1 mL/min with 280 nm UV detection.

SAX transferrin variants separation (salt gradient): Proteomix[®] SAX, NP10 (10 μ m), 4.6 x 250 mm. Mobile phase A is 150 mM Tris-HCl, pH 9.0, mobile phase B is A + 0.5 M NaCl. Gradients are with varying starting %A.

SAX β 2-glycoprotein I variants separation (pH gradient): Proteomix[®] SAX, NP5 (5 μ m), 4.6 x 250 mm. Mobile phase A is 20mM Piperazine + 20 mM triethanolamine+20mM bis-tris propane+20mM N-methylpiperazine pH 9.7 and mobile phase B is buffer A at pH 3.7 (titrated with HCl). The buffering amines have pKa in regular intervals (piperazine 9.7, triethanolamine 7.7, bis-tris propane 9.0, N-methylpiperazine 4.7). Gradients were indicated on the chromatograms. A descending pH gradient is suitable for SAX chromatography.

Desialylation of transferrin

Holo and Apo transferrin were dissolved in 0.1 M sodium acetate, pH 5 to a 1 mg/mL concentration. 6

mU of neuraminidase were added. The mixture was incubated at 37 °C overnight.

Deglycosylation of β 2-glycoprotein I

100 μ g protein was buffer exchanged into 50 mM Tris, pH 7.6. 5 μ L PNGase F (Prozyme, GKE-5006) was added and the mixture was incubated at 37 °C for 18 hours.

Results

Transferrin Separation

SEC

Figure 1 exhibited an overlay of the size exclusion profile of transferrin Apo and Holo proteins. The chromatogram overlay showed an almost identical elution profile with small amount of protein aggregates at the dimer region. Neuraminidase cleaves the terminal sialic acid at the glycosylation sites. SDS-PAGE gel image compared four different samples (untreated and neuraminidase treated transferrin in both Holo and Apo forms) under non-reducing conditions. Untreated transferrin and desialylated transferrin exhibited no difference with one major band at approximately 70kD. A small amount of dimers were seen for both forms of transferrin. Different glycoforms of transferrin were not resolved on either SEC or SDS-PAGE.

SAX with salt gradient

Transferrin (Holo and Apo) isoforms were separated on Proteomix[®] SAX NP10 (10 μ m, 4.6x250 mm). Panel A in figure 2 presented the separation overlay profiles for transferrin Holo and desialylated forms. Five major peaks for untreated transferrin can be separated. After neuraminidase treatment transferrin Holo was separated into many peaks, which indicated different protein variations. Four major peaks were separated for transferrin Apo. The majority of the desialylated peaks in both Holo and Apo forms were shifted earlier due to the removal of sialic acid groups, which reduced the negative charges of the proteins.

Figure 3 showed the effect of the starting percentage of buffer B with varying starting ionic strengths on the transferrin Holo sample. With increasing ionic strength

(2%B up to 10% B) at a constant gradient (0.5% B/minute), transferrin isoforms were eluted earlier maintaining the resolutions for the five peaks.

The capacity of the SAX column was tested with increasing injection amounts of transferrin Holo at 50 µg, 100 µg and 150 µg. At the 150 µg injection, all five major peaks maintain similar separation resolutions.

β2-glycoprotein I separations

SEC

Both glycosylated and deglycosylated β2-glycoprotein I gave similar SEC elution profiles except that the deglycosylated β2-glycoprotein I was eluted slightly later due to the loss of glycosylation as shown in figure 5 panel A. Panel B presented a SDS-PAGE gel image for both samples. Intact β2-glycoprotein I migrated on the gel, as a typical glycoprotein with different pIs, as one smeared band between 50 kD and 40 kD. After PNGase F digestion, the protein was separated on SDS-PAGE gel as two bands with different molecular weights between 40 kD and 30 kD.

SAX with pH gradient

As seen in figure 6, glycosylated β2-glycoprotein I can be resolved into 7 peaks with baseline separation using a pH gradient from pH 9.7 to pH 3.7 in 25 minutes. With the same gradient, the deglycosylated β2-glycoprotein I exhibited a different separation profile. Major peaks were shifted earlier due to the loss of glycosylation. Further gradient optimization will improve the separation of the deglycosylated protein. Fraction collections of each peak and glycan analysis can further identify the glycosylation structures including the terminal sialic acid variation.

Comparison of salt gradient and pH gradient on the separation Transferrin Holo and β2-glycoprotein I isoforms

Right panel in figure 7 showed transferrin Holo isoform separations on the SAX NP10 column with the same pH gradient used in the β2-glycoprotein I separation. Comparing the two separation profiles of the salt gradient (left panel) and the pH gradient (right panel), it is clear that this specific salt gradient outperformed the pH gradient for the isoform

separations. The protein recoveries were similar when integrating all transferrin peak areas from both methods (data not shown). Further optimization of the pH gradient may improve the separation.

Figure 8 showed the salt gradient SAX separation of β2-glycoprotein I (left panel) in addition to the pH gradient SAX separation. Table 1 summarized the separation efficiencies for both methods. The pH gradient offered a higher resolution and efficiency for β2-glycoprotein I separation. All 5 major peaks had a plate count of more than 20,000 with resolution between 2.2-2.6. Using the salt gradient, all five peaks had much smaller plate counts between 7914 to 9750 and much less resolution between 1.18 and 1.37.

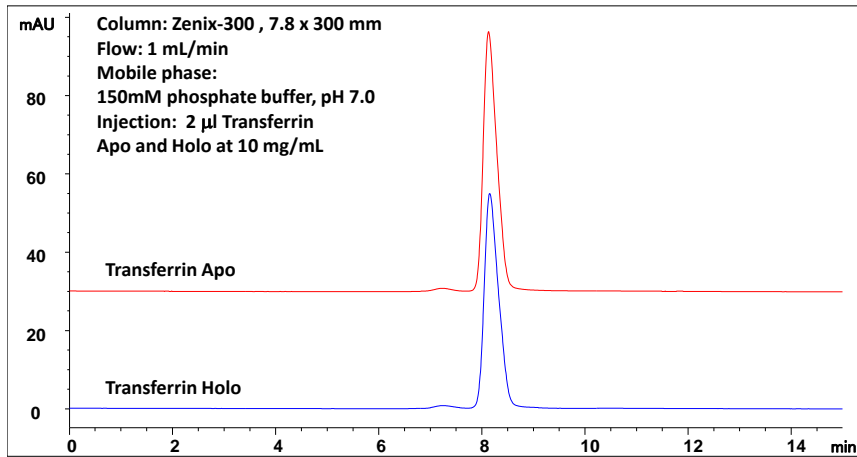
Conclusion

In this study, we presented two successful glycoprotein separations with high resolutions using two different methods: salt gradient and pH gradient on SAX chromatography. Salt gradient and pH gradient are both suitable for glycoprotein isoform separations. The advantage of pH gradient IEX is that the method doesn't require a high salt concentration, which is beneficial to further downstream analysis of variant fractions for glycosylation and sequence variation studies. With salt gradient IEX, pH, initial ionic strength of buffer A and the gradient itself can be modified to achieve optimum protein or protein variant separation. Salt gradients and pH gradients can be complementary to each other in protein or protein variants separation. Both methods are applicable to analytical or preparative scale protein separations and quality control of therapeutic biologics during the manufacturing process.

For more information on Proteomix® products, please visit Sepax website: <http://www.sepax-tech.com/proteomix.php> or contact us at 1-877-SEPAX-US

Figures

A.



B.

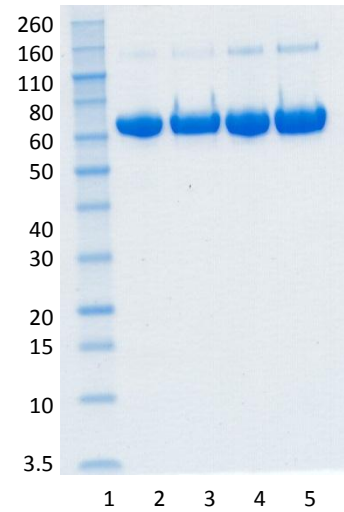
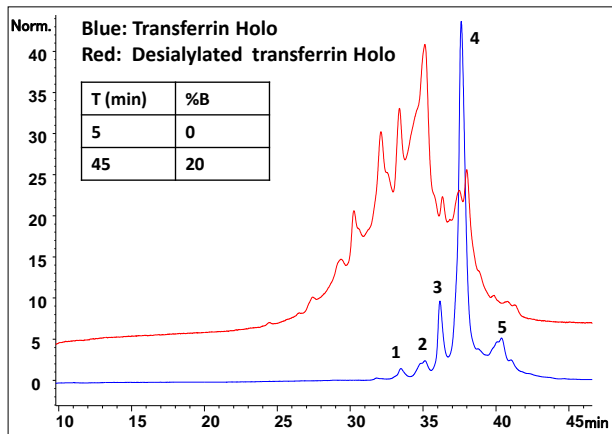


Figure 1. Panel A shows Zenix™ SEC-300 analysis of intact protein Transferrin Apo and Holo. A small amount of aggregates were observed with both forms of transferrin at 10 mg/mL. 20 µg were injected for each sample. Panel B shows the SDS-PAGE gel image of untreated and neuraminidase treated transferrin Holo and Apo proteins. Lane 1: MW marker, lane 2: untreated transferrin Holo, lane 3: desialylated transferrin Holo, lane 4: untreated transferrin Apo, lane 5: desialylated transferrin Apo.

A.



B.

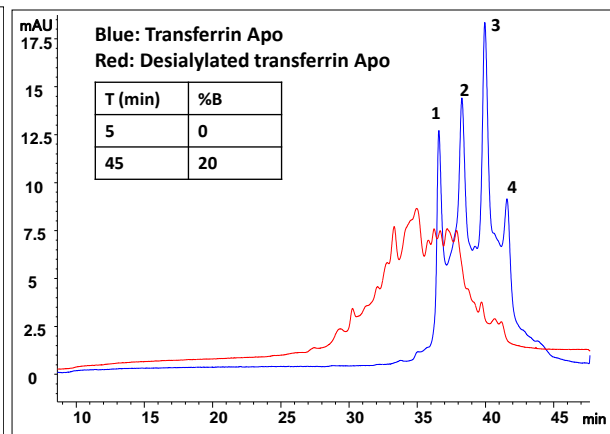


Figure 2. Salt gradient, sialylated and desialylated transferrin Holo separation on Proteomix® SAX NP10, 4.6 x 250 mm. Flow rate was at 1mL/min with UV 280 nm detection. Sample: 50 µg of each untreated and neuraminidase treated transferrin Holo. Mobile phase A: 150 mM Tris-HCl, pH 9.0, Mobile phase B: A + 0.5 M NaCl, gradient: 0-20% B in 40 minutes.

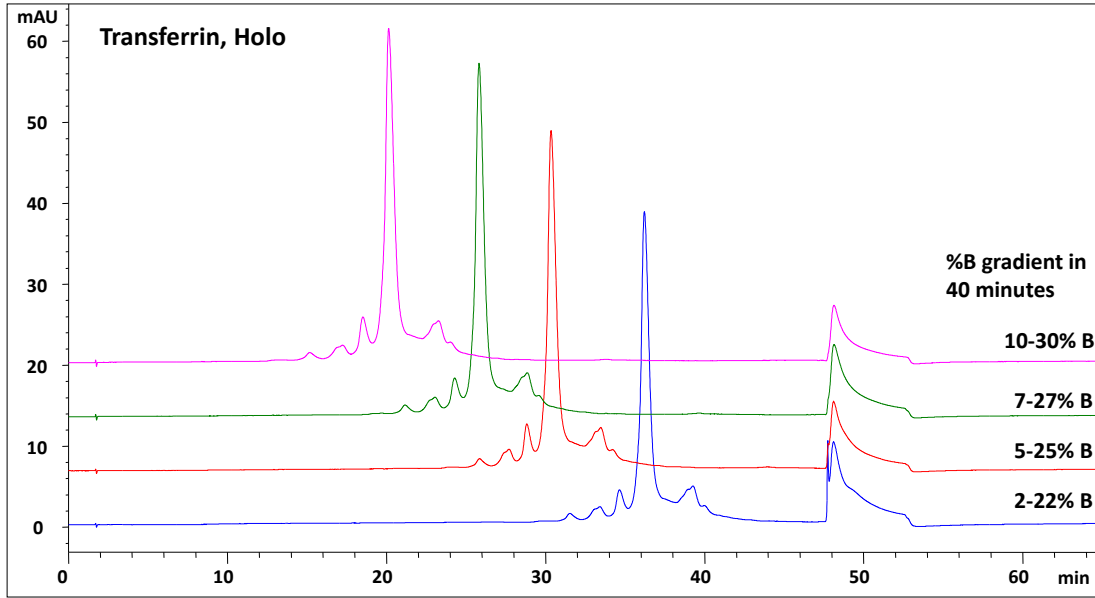


Figure 3. Salt gradient (starting ionic strength) effect on the separation of transferrin, Holo on Proteomix[®] SAX, NP10, 4.6x250 mm. Gradients were indicated on the chromatograms with 0.5%B/min constant for the linear gradients. Mobile phase A: 150 mM Tris-HCl, pH 9.0, Mobile phase B: A + 0.5 M NaCl, flow rate: 1mL/min, UV 280 nm detection.

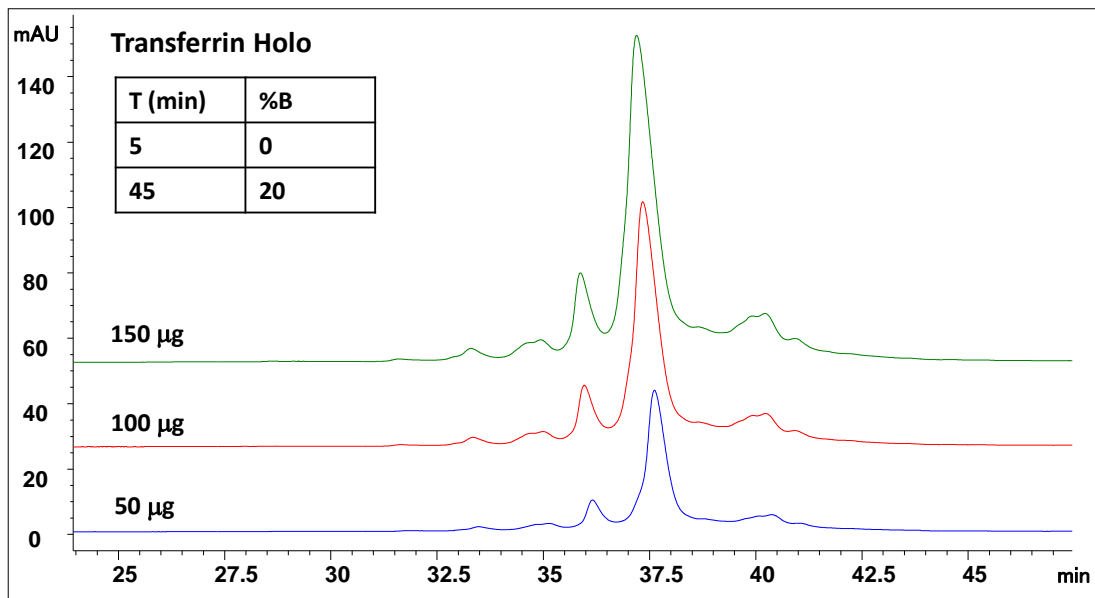
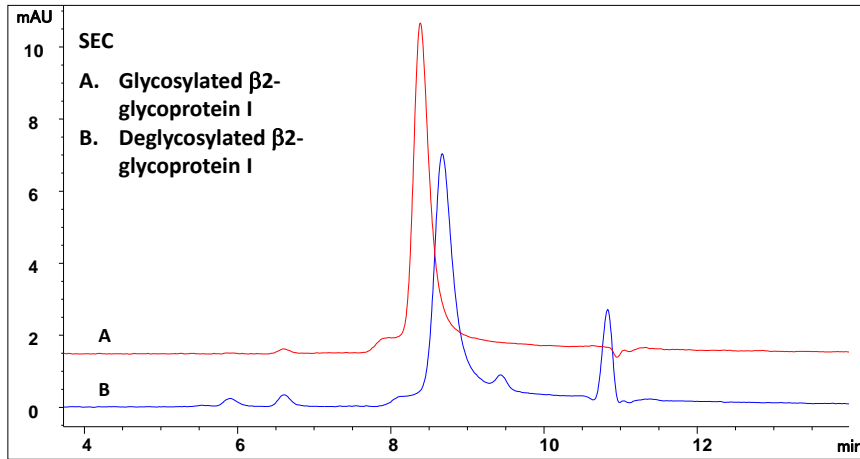


Figure 4. Transferrin Holo loading test on Proteomix SAX[®] NP10, 4.6 x 250 mm. Mobile phase A: 150 mM Tris-HCl, pH 9.0, Mobile phase B: A + 0.5 M NaCl, flow rate: 1mL/min, UV 280 nm detection.

A.



B.

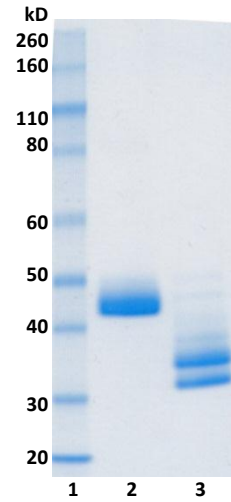


Figure 5. Panel A shows Zenix™ SEC-300 analysis of glycosylated and deglycosylated β 2-glycoprotein I after PNGase F digestion. A small amount of aggregates were observed with both samples. 5 μ g were injected for each sample. Panel B shows the SDS-PAGE gel image of glycosylated and deglycosylated β 2-glycoprotein I (MOPS running buffer). Lane 1: MW marker, lane 2: glycosylated β 2-glycoprotein I (native form), lane 3: β 2-glycoprotein I after deglycosylation.

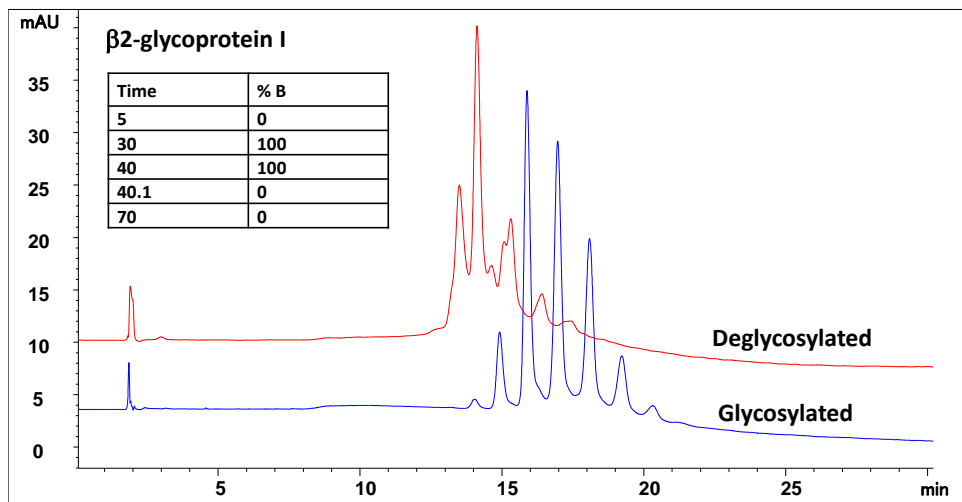


Figure 6. pH gradient, β 2-glycoprotein I separation on Proteomix® SAX NP10, 4.6 x 250 mm. Flow rate was at 1mL/min with UV 280 nm detection. Sample: 50 μ g of glycosylated and deglycosylated β 2-glycoprotein I. Mobile phase A: 20mM Piperazine + 20 mM triethanolamine+20mM bis-tris propane+20mM N-methylpiperazine pH 9.7 (titrate with HCl), B: A at pH 3.7,

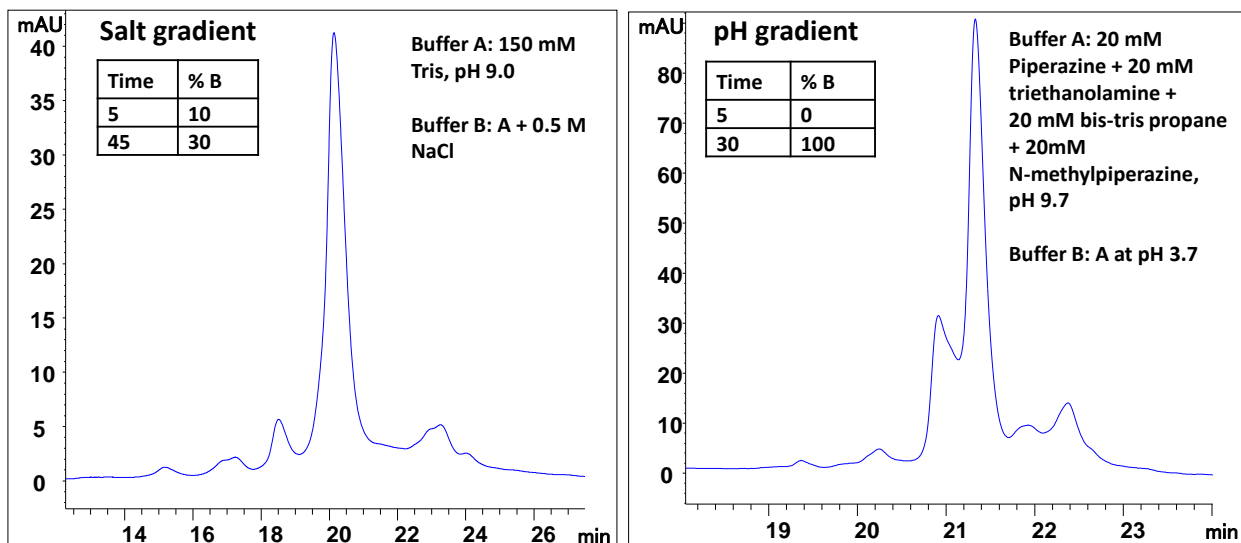


Figure 7. Transferrin Holo separation on Proteomix[®] SAX NP10 4.6 x 250 mm with salt gradient (left panel) and pH gradient (right panel). Buffer system compositions and gradients were indicated on the chromatograms. Flow rates for both methods were 1 mL/min with 280 nm UV detection. 50 μ g of Transferrin Holo were injected for each method.

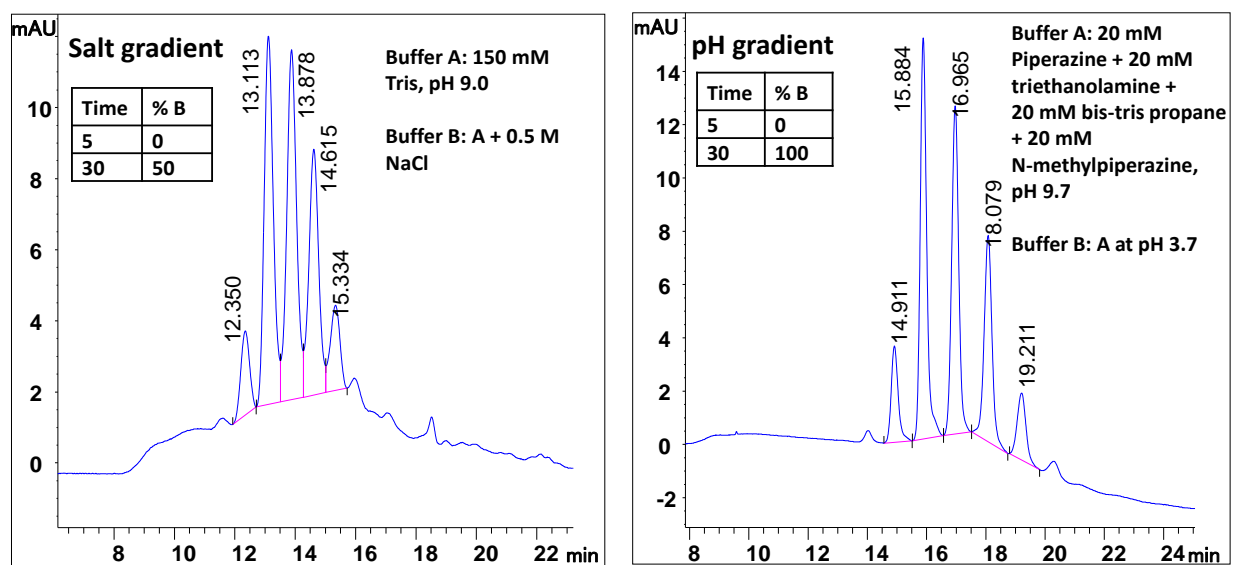


Figure 8. β 2-glycoprotein I separation on Proteomix[®] SAX NP5 4.6 x 250 mm with salt gradient (left panel) and pH gradient (right panel). Buffer system compositions and gradients were indicated on the chromatograms. Flow rates for both methods were 1 mL/min with 280 nm UV detection. 25 μ g of β 2-glycoprotein I buffer exchanged into 50 mM Tris, pH 9.0 were injected for each method.

Table 1. Peak resolution comparison from β 2-glycoprotein I isoform separations with salt gradient and pH gradient on Proteomix[®] SAX NP5 4.6 x 250 mm.

Salt gradient				pH gradient			
RT (min)	Plates	Tailing	Resolution	RT (min)	Plates	Tailing	Resolution
12.35	7914	1.16		14.91	20620	1.41	
13.11	8927	1.17	1.37	15.88	26689	1.38	2.41
13.88	8881	-1.35	1.33	16.97	24419	1.07	2.62
14.62	9750	-1.45	1.25	18.08	23470	0.79	2.45
15.33	9688	1.09	1.18	19.21	20394	0.84	2.24

Reference

1. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* vol. 12 no. 4 pp. 43R-56R, 2002
2. pH-gradient ion-exchange chromatography: An analytical tool for design and optimization of protein separations. *Journal of chromatography A*, 1164 (2007) 181-188
3. Rapid analysis of transferrin-iron complexes by isoelectric focusing and radioactive densitometry on gel. *Pathol, Biol.* 1985 Sep; 33(7), 741-745
4. N-glycosylation site mapping of human serotransferrin by serial lectin affinity chromatography, fast atom bombardment mass spectrometry, and ¹H nuclear magnetic resonance spectroscopy. *Anal. Biochem.*, 206, 53-63
5. The effects of ethanol on the glycosylation of human transferrin. *Glycobiology*, (2003) 12 (3), 191-198
6. Characterization of isoelectric subspecies of asialo- β 2-glycoprotein I. *Biochem. J.* (1989) 260, 531-534
7. Characterization of sialylated and fucosylated glycopeptides of β 2-glycoprotein I by a combination of HILIC and MALDI MS/MS. *J. Sep. Sci.* (2010) 33, 891-902
8. β 2-glycoprotein I, the playmaker of the antiphospholipid syndrome. *Clinical Immunology*, 112 (2004) 161-168
9. β 2-glycoprotein I-dependent anticardiolipin antibodies and risk of ischemic stroke and myocardial infarction: the Honolulu Heart Program. *Stroke* (2001) 32, 1701-1706

Order information

Proteomix[®] SAX

P/N	ID x Length (mm)	Particle Size	Pore Size
403NP10-4625	4.6x250	10 μ m	NP
403NP5-4625	4.6x250	5 μ m	NP
403NP3-4615	4.6x150	3 μ m	NP
403NP2-4615	4.6x150	1.7 μ m	NP

Zenix[™] SEC-300

P/N	ID x Length (mm)	Particle Size	Pore Size
213300-7830	7.8x300	3 μ m	300 Å