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Technical Note 30

Monosaccharide and Oligosaccharide Analysis of Glycoproteins Electrotransferred onto Polyvinylidene Fluoride (PVDF) Membranes

INTRODUCTION

Protein glycosylation is a major co- and post-translational modification that generates a potentially large group of glycoforms from a single polypeptide chain[1]. Over 50% of catalogued protein sequences contain the requisite sequence for N-glycosylation (AsnXxxSer(Thr); Xxx ≠ Pro)[2], and any Ser or Thr can potentially be glycosylated[3]. Since glycosylation can play an important role in the biological and pharmaceutical activity of the glycoprotein, there is increasing interest in identifying and characterizing the oligosaccharide moieties of glycoproteins.

Until recently, this has not been easy. Gel electrophoresis and transfer of proteins to membranes[4] are techniques that are widely used to purify picomole quantities of proteins of interest, minimize preparative losses, and facilitate sample handling for structural analysis. However, analysis of the carbohydrate moiety of immobilized glycoproteins, separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), has been largely limited to the use of lectin probes[5,6,7] or to methods based on the relative specificity of periodate for cleaving carbohydrate[8,9]. Both techniques have their limitations. The former requires that the glycoprotein contain epitopes that bind tightly to available lectins, while the latter can give ambiguous results from non-specific staining.

This Technical Note describes a universal procedure for separating and purifying glycoproteins using SDS-PAGE, followed by electroblotting onto an inert PVDF (polyvinylidene fluoride) membrane. After staining, the purified band of glycoprotein is excised from the membrane and is ready for carbohydrate analysis. This includes acid hydrolysis, which releases monosaccharides for composition analysis, and selective and sequential endoglycosidase digestion, which allows the progressive release of oligosaccharides for mapping and tentative structural assignment. In both cases, saccharides are analyzed by high performance anion exchange chromatography–pulsed amperometric detection (HPAE-PAD). The PVDF is inert and has been shown not to bind carbohydrates[10].

Figure 1 illustrates the experimental approach. This Technical Note is organized into subsections that cover the following procedures:

I. Separation and purification of the glycoprotein sample by SDS-PAGE.
II. Electrotransfer of the glycoprotein from the gel to the PVDF membrane.
III. Acid hydrolysis and monosaccharide analysis of the purified glycoprotein on the membrane.
IV. Glycosidase digestion and oligosaccharide mapping of the glycoprotein on the membrane.

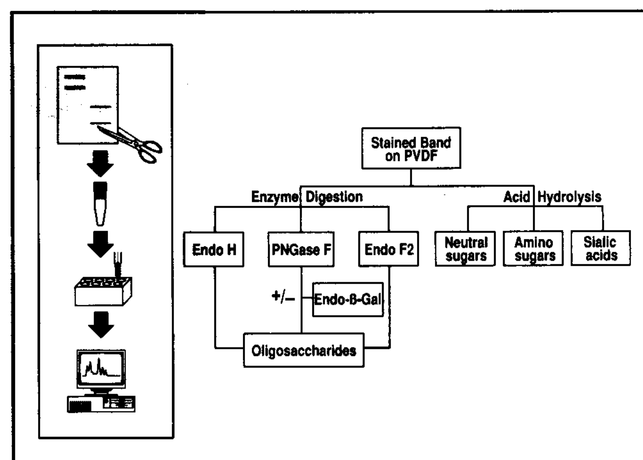

Figure 1 General experimental strategy. Once the glycoproteins have been electrotransferred to the membrane, the bands are stained and excised. They are then either hydrolyzed in acid for total monosaccharide composition, or digested with an array of enzymes for oligosaccharide mapping.

The analysis of oligosaccharides by HPAE-PAD is described in detail in several publications[10,11,12] and in Dionex Technical Note 20, "Analysis of Carbohydrates by Anion Exchange Chromatography with Pulsed Amperometric Detection". Please ask your local Dionex representative for a copy of Dionex Technical Note 20.

***CAUTION:* Maintaining purity is critical during every step of the procedures outlined in this technical note.**

- **Use care to avoid contact with any source of contaminating carbohydrate, protein, or glycoprotein.**
- **All water used for the preparation of buffers, eluents, and electrophoresis solutions should be glass-distilled. In dispensing the water, avoid the use of non-glass tubing that may support microbial growth and yield artifactual sugars.**
- **Wear powderless gloves when handling samples, gels, and membranes to avoid contamination by proteins and carbohydrates.**
- **Use only the highest quality ultrapure electrophoresis-grade reagents.**

I. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

The procedure described here is for a standard discontinuous buffer system first described by Laemmli[13] and designed to be used with 12 x 10-cm mini-gels between 0.5 and 1.5 mm thick. For more detailed information on SDS-PAGE, refer to one of the many excellent articles on the subject[14].

Equipment

Electrophoresis unit suitable for running mini-gels 12 x 10 cm, 0.5 to 1.5-mm thick

Power Supply

Reagents*

Acrylamide

Bis-acrylamide

Sodium dodecyl sulfate (SDS)

Glycine

Tris (Tris[hydroxymethyl]amino methane)

Ammonium persulfate

TEMED (N,N,N',N'-Tetramethylethylene diamine)

Glycerol

Hydrochloric acid

2-Mercaptoethanol

Butanol

Bromophenol blue

Protein molecular weight standards

*Use only the highest quality ultrapure electrophoresis-grade reagents.

Stock Solutions

Separation in SDS-PAGE is determined by the molecular weights of the protein or glycoprotein species and the pore size of the gel. The pore size of polyacrylamide gels decreases as the concentration of acrylamide plus bis-acrylamide (expressed as %T) increases. Pore size is also affected by the extent to which the gel is cross-linked. Increasing the proportion (expressed as %C) of the cross-linker (bis-acrylamide) relative to the total monomer (acrylamide *plus* bis-acrylamide) up to 5% by weight further decreases the pore size.

Choose an appropriate acrylamide concentration for separating proteins in the following molecular weight ranges in the presence of 1% SDS as follows:

MW 12,000 – 45,000:	5%T
MW 16,000 – 70,000:	10%T
MW 60,000 – 200,000:	15%T

Make up the following stock solutions and filter through a Whatman No. 1 filter or equivalent.

Solution 1: Monomer Solution—30%T, 2.7%C

*Acrylamide, 58.4 g

*Bis-acrylamide, 1.6 g

Add water to a total volume of 200 mL. Store at 4°C in the dark. The percent cross-linking (%C) is determined by the ratio (w/w) of bis-acrylamide to acrylamide plus bis-acrylamide. For gels with a different %C, adjust the ratio of the two components appropriately.

**Warning*: Acrylamide and bis-acrylamide are neurotoxins and must be handled with caution.

Solution 2: Resolving Gel Buffer—1.5 M Tris-Cl, pH 8.8
Tris, 36.3 g; dissolve in water, and adjust pH to 8.8 with hydrochloric acid. Add water to bring solution to a total volume of 200 mL. Store at 4°C.

Solution 3: Stacking Gel Buffer—0.5 M Tris-HCl, pH 6.8
Tris, 3.0 g; dissolve in water, and adjust pH to 6.8 with hydrochloric acid. Add water to bring solution to a total volume of 50 mL. Store at 4°C.

Solution 4: 10% SDS
SDS, 50 g; dissolve in water to a final volume of 500 mL.

Solution 5: Initiator—10% ammonium persulfate
Ammonium persulfate, 0.5 g; add water to a final volume of 5.0 mL. Make up just before use.

Solution 6: TEMED
Use as supplied. Store undiluted at 4°C in a dark bottle.

Solution 7: 2X Treatment Buffer—0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol
Tris, 2.5 mL (Solution 3)
SDS, 4.0 mL (Solution 4)
Glycerol, 2.0 mL
2-mercaptoethanol, 1.0 mL
Add water to a total volume of 10.0 mL. Divide into aliquots and store frozen.

Solution 8: Running Buffer—0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% SDS
Tris-Base, 12 g
Glycine, 57.6 g
SDS, 40 mL (Solution 4)
Add water to a total volume of 4 L. Store at 4°C.

Prepare the resolving and stacking gel solutions using the recipes provided in Tables 1 and 2. The resolving gel recipe is for making a 10%T, 2.7%C gel. Quantities of acrylamide should be adjusted appropriately to make gels of different pore size (%T) and the ratio of bis-acrylamide to acrylamide should be adjusted to make gels with a different percent cross-linking (%C).

Pouring the Gels

Make the resolving gel first. Combine all the ingredients except the TEMED in a vacuum flask and degas for 1 minute using an aspirator. Add the TEMED, gently swirl, and pour the resulting mixture into the clean and assembled gel plate system. Layer a saturated solution of butanol in water over the gel to give a clean interface and allow the gel to polymerize (about 1 hour).

Prepare the stacking gel solution in the same manner as the resolving gel. Rinse the surface of the resolving gel with some of the stacking gel mixture. Add TEMED to the stacking gel mixture, pour the stacking gel, and immediately insert the comb to create sample wells. Once the gel has polymerized, transfer the mini-gel assembly to the electrophoresis unit and fill the electrode chambers with running buffer (Solution 8), making sure that the gel is

Table 1 Recipe for Resolving Gel

Reagent	Stock Sol.	Vol. Req.	Final Conc.
Acrylamide/ Bisacrylamide	Solution 1	3.33 mL	10% T, 2.7%C
Tris-HCL, pH 8.8	Solution 2	2.50 mL	0.375 M
SDS	Solution 4	0.10 mL	0.1%
Ammonium persulfate	Solution 5	50 µL	0.05% w/v
TEMED	Solution 6	5 µL	0.05% v/v
H_2O		4.0 mL	
Total Volume		**10 mL**	

Table 2 Recipe for Stacking Gel

Reagent	Stock Sol.	Vol. Req.	Final Conc.
Acrylamide/ Bisacrylamide	Solution 1	0.67 mL	4% T, 2.7%C
Tris-HCL, pH 6.8	Solution 3	1.25 mL	0.125 M
SDS	Solution 4	50 mL	0.1%
Ammonium persulfate	Solution 5	25 µL	0.05% w/v
TEMED	Solution 6	2 µL	0.04% v/v
H_2O		3.0 mL	
Total Volume		**5 mL**	

completely covered. Gently remove the comb when the samples are ready to be loaded. If the gels are not to be used immediately, they can be stored for several weeks at 4°C if they are wrapped in plastic wrap.

Sample Preparation

Add an equal volume of the 2X Treatment Buffer (Solution 7) to the glycoprotein solution and heat at 100°C for 10 minutes. Store frozen until needed. Before loading onto the gel, add bromophenol blue as a tracking dye to a final concentration of 0.002%. A maximum of approximately 35 µg of glycoprotein should be added to each well. Load suitable molecular weight markers onto the two outside lanes of the slab gel.

Electrophoresis

Run a 10 x 12-cm gel at 15 mA constant current until the tracking dye has traversed the stacking gel, then raise the current to 20 mA and run until the tracking dye reaches the end of the resolving gel (about 2 hours). It may be necessary to experiment with the electrophoresis conditions to optimize them for the equipment in use.

II. ELECTROBLOTTING AND STAINING

Equipment

Electrotransfer equipment

Cooling unit

Power supply

Reagents*

Methanol

Acetic acid

Tris

Glycine

Coomassie blue

*Use only the highest quality ultrapure electrophoresis-grade reagents.

Blotting Membrane

PVDF (Immobilon PSQ 15 x 15 cm, 0.1-µm pore size) from Millipore Corporation. Some glycoproteins may pass through the membrane during electroblotting if PVDF of larger pore size is used.

Solutions

Transfer buffer:

20% methanol in 25 mM Tris, 190 mM glycine buffer, pH 8.8.

Staining solution:

0.1% Coomassie brilliant blue R-250, in water : methanol : acetic acid (5:4:1).

Destaining solutions:

1) 50% methanol/10% acetic acid in water.
2) 5% methanol/7% acetic acid in water.

Electrotransfer

When electrophoresis is complete, switch off the power and disassemble the equipment. Remove the clamps holding the gel sandwich together and carefully lift off one of the plates, leaving the gel lying on the other. Gently loosen the gel from this plate by squirting distilled water from a wash bottle under the gel, then carefully lift it off and place in a tray containing transfer buffer. Use gloves throughout this procedure to avoid transfer of protein from the skin to the gel.

Cut out a sheet of the PVDF transfer membrane to the same dimensions as the gel and wet with methanol. Transfer to a tray of water.

A typical transfer assembly is shown in Figure 2. The sandwich consists of the gel and transfer membrane enclosed between layers of blotting paper and sponge. Soak a piece of blotting paper (Whatman No. 1 or equivalent) in transfer buffer and place on one of the layers of sponge. Take the gel out of the tray of transfer buffer and place it on top of the blotting paper. Carefully place the PVDF membrane on top of the gel, taking care not to trap bubbles beneath the membrane. (If the pore size of the PVDF is greater than 0.1 µm, two layers of membrane should be used, as some glycoprotein may pass through the first membrane.) Complete the sandwich by placing another sheet of soaked blotting paper on top of the PVDF membrane, followed by the second layer of sponge.

Place the completed transfer assembly in the electroblotting tank oriented so that the transfer membrane is between the gel and the positive electrode. Fill the tank with transfer buffer and cool the device by circulating refrigerated water through the equipment. Turn on the power and adjust to run for a total of 300 to 400 volt hrs. Transfer times will vary depending on thickness and porosity of the acrylamide gel and on the size and hydrophobicity of the transferred proteins. Conditions should be optimized empirically.

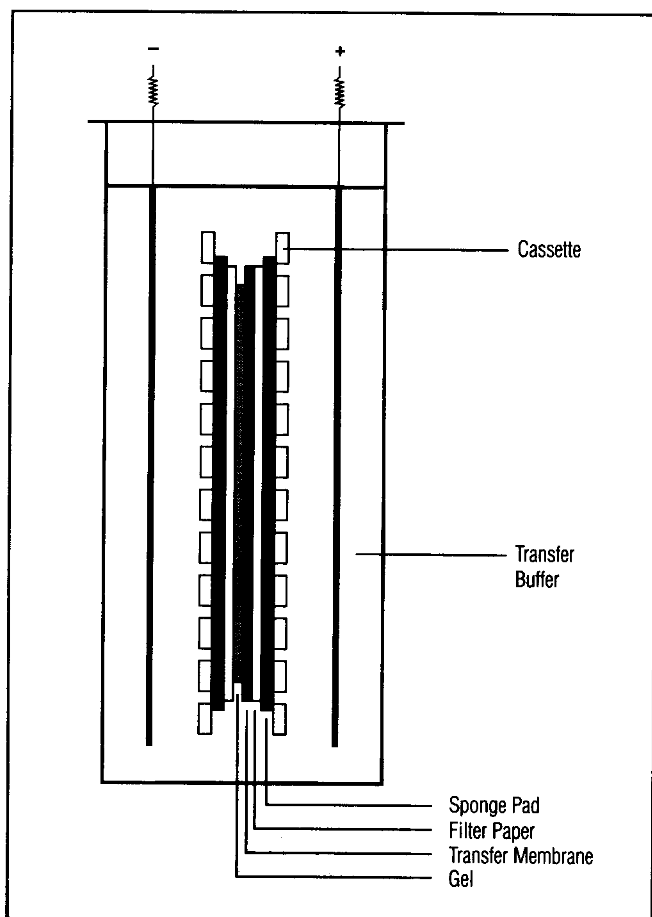

Figure 2 *A schematic diagram of a typical electrotransfer apparatus.*

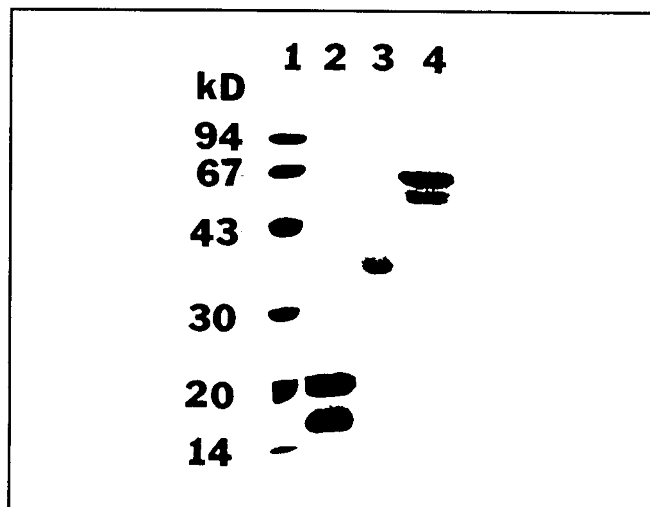

Figure 3 *Typical stained electroblot. Lane 1: molecular weight markers, sizes indicated in column to the left; Lane 2: ribonuclease B; Lane 3: recombinant erythropoeitin (rEPO); Lane 4: bovine fetuin.*

Staining

After electrotransfer is complete, disassemble the electrotransfer device. Transfer the PVDF membrane to a tray and stain in the 0.1% Coomassie blue solution for 10 min. To remove the nonspecifically adsorbed stain, destain the membrane in multiple washes of 50% methanol/ 10% acetic acid, followed by multiple washes of 5% methanol/7% acetic acid, and finally rinse in distilled water. Dry on a filter paper for about 30 min and store at 4°C until needed. A typical blot is shown in Figure 3. The stained protein bands are clearly visible.

To determine if the electrotransfer is complete, stain the gel in 0.1% Coomassie blue for several hours. Destain using 50% methanol/10% acetic acid until the background is clear. There should be no visible bands in the gel if the transfer is complete.

III. MONOSACCHARIDE COMPOSITION ANALYSIS

Introduction

For composition analysis, the carbohydrate moiety of the glycoprotein is first acid hydrolyzed to monosaccharides. The hydrolysate is then analyzed using HPAE-PAD. In this section, the hydrolysis and sample preparation steps for the analysis of neutral and amino monosaccharides is described. The chromatography is described in detail in Dionex Technical Note 20, "Analysis of Carbohydrates by Anion Exchange Chromatography with Pulsed Amperometric Detection", and by Weitzhandler et al.[10] and Hardy et al.[11] These references provide an in-depth discussion of hydrolysis conditions appropriate for glycoconjugates and oligosaccharides. We recommend hydrolysis at 100°C for 4 hours in 2 N TFA or 6 N HCl for the quantitative release of neutral or amino monosaccharides, respectively. Release of Neu5Ac or NeuGc requires milder hydrolysis conditions, for example, 0.1 N HCl at 80°C for 1 hour.

Reagents

Methanol

Concentrated (13 N) trifluoroacetic acid (TFA) in sealed 1-mL ampules

-or-

6 N Hydrochloric acid (sequencing-grade)

MonoStandards (available from Dionex)

Equipment

1.5-mL polypropylene microcentrifuge tubes with caps and O-rings

Vacuum centrifuge/concentrator, refrigerated trap and vacuum pump

Sample Preparation

Using forceps and a razor blade, excise the stained glycoprotein bands of interest from the dried, blotted PVDF and transfer the membrane bands to capped polypropylene microcentrifuge tubes. To make a membrane blank, cut out a band from an unstained area of the membrane. Wet the PVDF bands with 5 to 10 µL of methanol and submerge in 400 µL of fresh 2 N TFA (or 400 µL of fresh 6 N HCl). Incubate the tubes at 100°C for 4 hrs, occasionally monitoring them to ensure that the PVDF bands remain completely submerged in acid. Remove the membrane band(s) and evaporate the supernatant to dryness using equipment such as the Speed-Vac.

Reconstitute the samples in a suitable volume of deionized water. They are now ready for HPAE-PAD analysis. HPAE-PAD chromatography of acid hydrolyzed blotted recombinant erythropoietin is shown in Figure 4.

IV. OLIGOSACCHARIDE MAPPING

Introduction

Glycoprotein oligosaccharide mapping involves the release of oligosaccharides from glycoproteins by the use of specific enzymes. This is followed by analysis of the liberated oligosaccharides by HPAE-PAD. Tentative structure assignments can sometimes be made by comparing oligosaccharide retention times with those of known standards. By digesting with an array of enzymes of differing specificity, different classes of oligosaccharides can be sequentially released and analyzed from the same immobilized glycoprotein band. A detailed description of enzyme digestion of PVDF-bound glycoproteins and subsequent HPAE-PAD analysis can be found in Weitzhandler et al.[10] Further discussion of HPAE-PAD analysis is given in Technical Note 20 (see above) and in Townsend et al.[12]

This section covers the digestion of blotted glycoproteins with peptide-N^4-(N-acetyl-D-glucosaminyl)asparagine amidase (PNGase F). We have also obtained equally successful digestion and release of oligosaccharides with two endo-N-acetylglucosaminidases (Endo H and Endo F_2) and endo-β-galactosidase.[10]

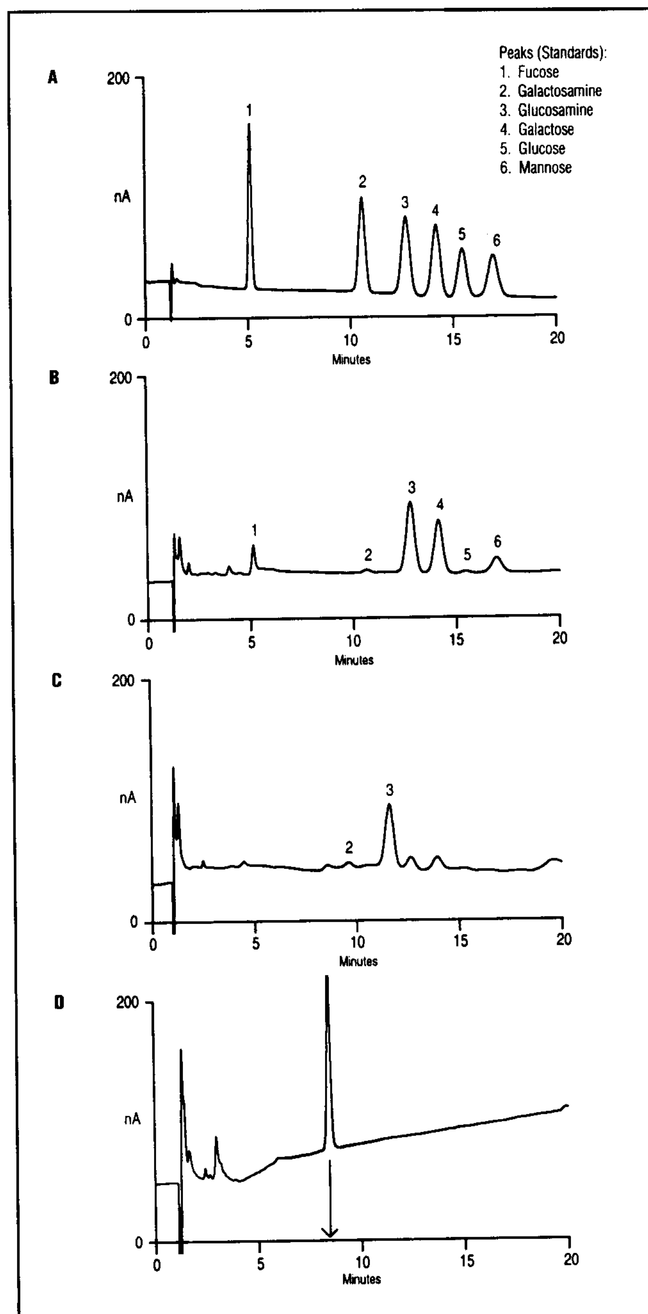

Figure 4 HPAE-PAD profiling of monosaccharides released from PVDF blotted recombinant erythropoietin by acid hydrolysis and separated on a CarboPac PA1 column. A, B, and C were eluted isocratically using 16 mM sodium hydroxide. D was eluted using a 50 to 180 mM sodium acetate gradient in 100 mM sodium hydroxide.

A: Separation of fucose, galactosamine, glucosamine, galactose, glucose, and mannose standards.

B: 4-hour 2 N TFA hydrolyzate (100°C) of PVDF blotted erythropoietin (8 µg rEPO loaded per lane).

C: 4-hour 6 N HCl hydrolyzate (100°C) of PVDF blotted erythropoietin (8 µg rEPO loaded per lane).

D: Sialic acid analysis of PVDF blotted erythropoietin. The stained band was treated with 0.1 N HCl for 1 hour (80°C). The elution position of standard Neu5Ac is shown by the arrow.

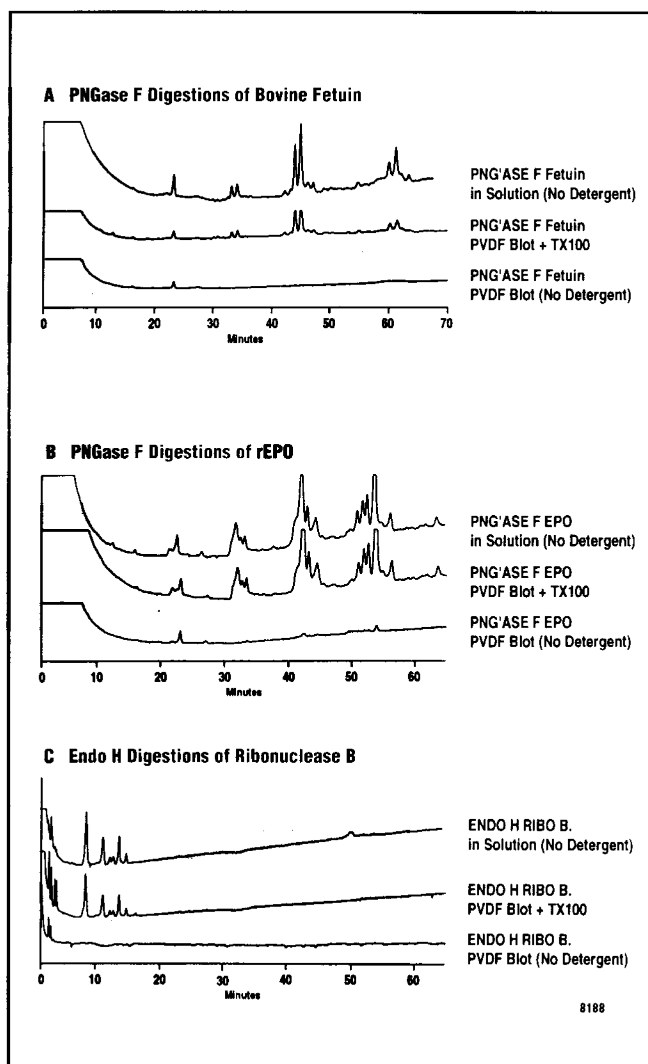

Figure 5 *HPAE-PAD profiling of oligosaccharides released by PNGase F or Endo H digestion and separated on a CarboPac PA-100 column. A gradient of 20–200 mM sodium acetate in the presence of 100 mM sodium hydroxide was developed over 1 hour. Chromatograms within panels A, B, and C are as follows—*
Top trace: Digestion in a free solution (no detergent present).
Middle trace: Digest of blotted glycoprotein, Triton X-100 present.
Bottom trace: Digest of blotted glycoprotein Triton X-100 absent.

Reagents

Methanol

Sodium dihydrogen phosphate

Reduced Triton X-100

PNGase F (available from Boehringer-Mannheim Corporation)

OligoStandards (available from Dionex)

Equipment

1.5-mL Polypropylene microcentrifuge tubes with caps and O-rings

Solutions

Digestion Buffer: 10 mM sodium phosphate, pH 7.6

Dissolve 1.38 g of sodium dihydrogen phosphate in approximately 800 mL water and mix until dissolved. Adjust the pH of the buffer solution to 7.6 by addition of 50% sodium hydroxide solution. Adjust the final volume of the buffer to 1 L.

PNGase F Digestion and Sample Preparation

To release N-linked oligosaccharides, excise the stained glycoprotein bands of interest from the dried, PVDF blot and transfer to capped polypropylene microcentrifuge tubes. Wet the membrane bands with 5 to 10 µL of methanol and submerge in 200 µL of 10 mM sodium phosphate, pH 7.6, 0.1% reduced Triton X-100. Add 1 to 1.5 µL PNGase F (1 to 5 units) and digest at 37°C for 48 hrs. The released oligosaccharides are now ready for HPAE-PAD analysis.

During PNGase F digestion a glycosyl amine intermediate is formed. At elevated pH, it is possible that this intermediate is still present after the digest is complete. It is readily converted to the reducing oligosaccharide by acidification of the sample. Add 4 µL of a 1:100 dilution of glacial acetic acid to the 200 µL digest and incubate at room temperature for 2 hours. (Under the conditions given here for PNGase F digestion, we have not found this step to be necessary.)

Loading approximately 20 µg of glycoprotein per gel lane is frequently sufficient to generate a good oligosaccharide map. If the PNGase F is supplied stabilized in glycerol, the prominent void peak seen upon HPAE-PAD chromatography is largely due to the glycerol in the preparation. It generally does not interfere with the oligosaccharide peaks. Figure 5 shows the PNGase F digestion of two glycoproteins. The figure also shows oligosaccharides released by Endo H digestion of blotted ribonuclease B. The profiles obtained from the blotted glycoproteins are very similar to those obtained from the identical glycoproteins digested in free solution. Note that little oligosaccharide is released from the PVDF-blotted glycoproteins in the absence of Triton X-100 detergent.

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LPN 034883 5M 2/99

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