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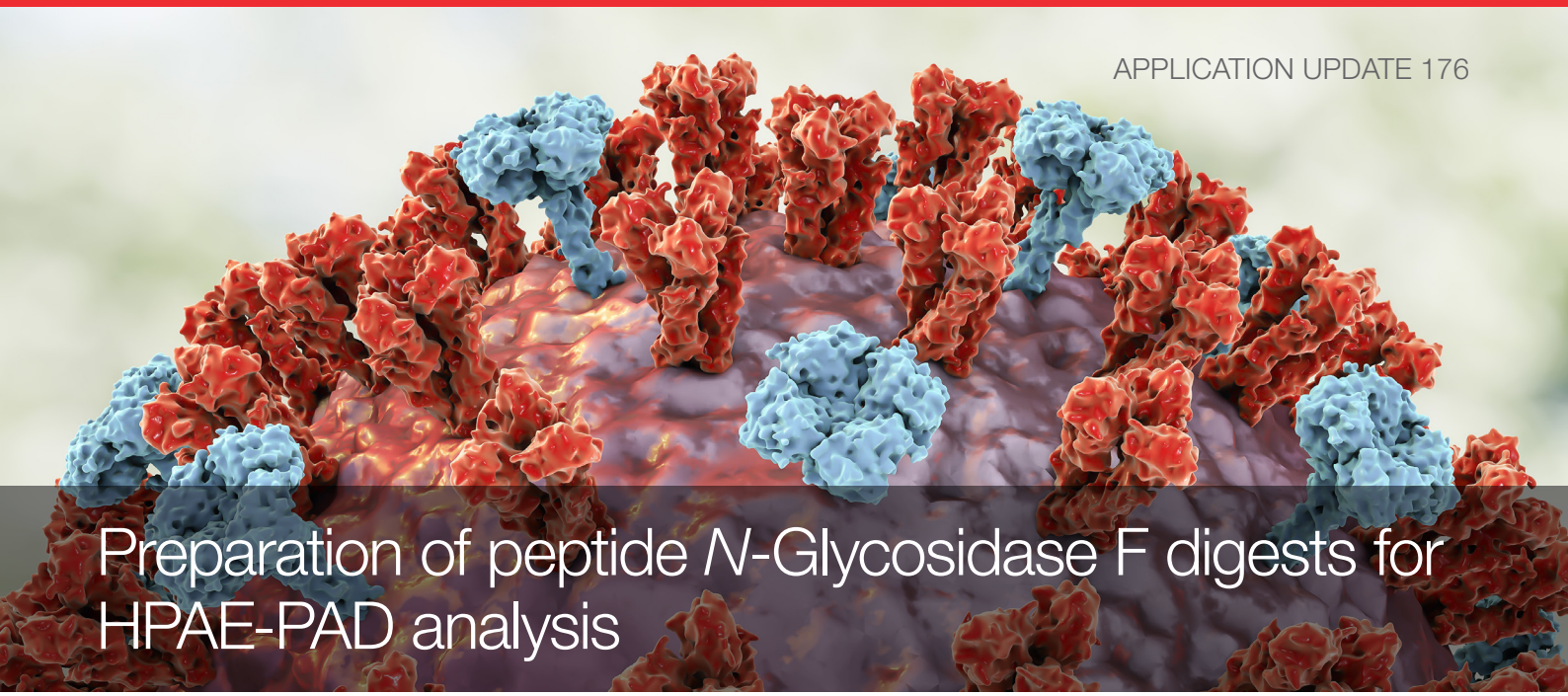
APPLICATION UPDATE 176



Preparation of peptide *N*-Glycosidase F digests for HPAE-PAD analysis

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Keywords

PNGase F, glycan, oligosaccharide, *N*-linked oligosaccharide, CarboPac PA200 column

Goal

To develop a protocol to prepare PNGase F digests of glycoproteins for HPAE-PAD

Introduction

Glycosylation is one of the most important post-translational modifications in eukaryotic cell proteins. Glycoproteins are involved in a wide range of biological functions ranging from cell adhesion and antibody recognition to cell signaling. These functions are often dependant on the glycoprotein's attached oligosaccharides, particularly those linked through an asparagine side chain (Asn- or *N*-linked).[1] The biological activity and function of carbohydrates linked through the side chains of serine or threonine (*O*-linked) are less understood. The biotechnology industry has already produced a number of glycoprotein therapeutics and more are being investigated for their therapeutic potential. This requires that companies understand the oligosaccharide content of the glycoprotein and monitor it to ensure that it is consistent from one lot of therapeutic to another. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) often is one of the tools used to study and monitor glycoprotein oligosaccharides.[2]

To study and monitor a glycoprotein's oligosaccharide content by HPAE-PAD, the oligosaccharide chains first must be released from the glycoprotein. Both chemical and enzymatic methods may be used to remove oligosaccharides from glycoproteins. Chemical methods can be harsh and non-specific,

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whereas enzymatic methods are gentle, with no protein degradation. Peptide *N*-Glycosidase F (PNGase F) is the most effective enzymatic method for removing *N*-linked oligosaccharides from glycoproteins. PNGase F deaminates the asparagine residue to aspartic acid, leaving the oligosaccharides intact for further analysis. Although some glycoproteins can be simply treated with PNGase F in a phosphate buffer, the glycosylation sites of some proteins are obstructed by the secondary and/or tertiary structure of the protein, thereby interfering with oligosaccharide release by PNGase F. For these proteins, denaturation of the glycoprotein by heating with SDS and β-mercaptoethanol (BME) greatly enhances the rate of deglycosylation by PNGase F. It is essential to pretreat glycan-containing samples released under these conditions prior to analysis by HPAE-PAD. The SDS can gradually consume the anion-exchange column's capacity and the other digestion components (e.g., NP40 and BME) can interfere with oligosaccharide analysis. Traditionally, size-exclusion columns with low- to medium-molecular weight cut-off resins are used for sample pretreatment. However, most size-exclusion methods require large amounts of sample, are time consuming, and also run the risk of losing charged sugars.[3]

The work shown here describes glycoprotein deglycosylation with PNGase F, followed by an efficient sample pretreatment using desalting and detergent removal spin columns to remove high concentrations of salt and detergents from small-scale PNGase F digestions. The HPAE-PAD technique was used to separate and detect the oligosaccharides. Oligosaccharides were separated using the Thermo Scientific™ Dionex™ CarboPac™ PA200 column. The Dionex CarboPac PA200 column is a nonporous, high-efficiency, pellicular anion-exchange column that provides high-resolution separations required for oligosaccharide mapping and analysis.[4, 5]

Equipment

Thermo Scientific™ Dionex™ ICS-3000 system* including:

SP Single Pump module (P/N 079820) or DP Dual Pump (P/N 079825) with degas option

DC Detector Compartment (P/N AAA-061767), single-temperature zone

Electrochemical Detector (P/N 079831) and cell (P/N 061757)

pH-Ag/AgCl Reference electrode (P/N 061879)

Carbohydrate Disposable Au Working Electrode, pack of 6 (two 2.0 mil gaskets included) (P/N 060139)

Thermo Scientific™ Dionex™ AS Autosampler (P/N 061289) with cooling tray option (recommended)

Centrifuge (Eppendorf 5400 series)

* A Thermo Scientific™ Dionex™ ICS-5000 or ICS-5000+ IC system can be used for equivalent or superior results

Consumables

Dionex CarboPac PA200 Analytical column, 3 × 250 mm (P/N 062896)

Dionex CarboPac PA200 Guard column, 3 × 30 mm (P/N 062895)

Spin desalting columns, 7K MWCO, 0.5 mL (P/N 89882)

Detergent removal spin columns, 0.5 mL (P/N 87777)

Disposable filtration units, 0.20 µm Nylon membrane (Nalgene® P/N 164-0020)

Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarsted P/N 72.692.005)

1.5 mL polypropylene autosampler vials, with caps and split septa (P/N 061696)

Reagents and standards

Sodium acetate, anhydrous (Fluka P/N 71183)

Sodium hydroxide, 50% (Fischer P/N SS254-500)

PNGase F, 15000 units, 500000 U/mL, a unit is defined as the amount of enzyme required to remove > 95% of carbohydrate from 10 µg denatured RNase B in 1 h at 37 °C in a total reaction volume of 10 µL (New England BioLabs P/N P0705S), enzyme is supplied with 10× glycoprotein denaturing buffer (5% SDS, 10% β-mercaptoethanol), 10× G7 buffer (0.5 M sodium phosphate, pH 7.5 at 25 °C), 10% NP40

Reagent-grade water Type I, 18 MΩ-cm resistance or better, filtered through a 0.2 µm filter immediately before use

Thermo Scientific™ Dionex™ Oligo Standard, Sialylated *N*-Linked Alditols, 25 nmol (P/N 043064)

Human transferrin, ≥ 98% pure by agarose gel electrophoresis (Sigma P/N T3309)

Conditions

Columns: Dionex CarboPac PA200, 3 × 250 mm (P/N 062896) and guard, 3 × 50 mm (P/N 062895)

Flow rate: 0.5 mL/min

Inj. volume: 20 µL sialylated fetuin
PNGase F digest 50 µL human transferrin PNGase F digest

Temperature: 30 °C

Tray temp: 4 °C

Detection: PAD

Temperature: 30 °C

Waveform: Carbohydrate (standard quad)

Time	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

*Settings required when using the Dionex ICS-3000 and ICS-5000 systems but not used for older Dionex systems.

Eluents: A: 100 mM NaOH
B: 1 M Sodium acetate, 100 mM NaOH

Gradient conditions

Time (min)	A%	B%
0.0–2.0	98.0	2.0
2.0–90.0	85.0	15.0
90.1–115.0	98.0	2.0

Preparation of solutions and reagents

100 mM NaOH

Transfer 5.2 mL of 50% sodium hydroxide into a plastic 1 L volumetric flask containing approximately 800 mL degassed and filtered DI water using a plastic serological pipette. Mix by inverting the volumetric flask and bring to volume with degassed and filtered DI water.

100 mM NaOH, 1 M sodium acetate

Dissolve 82.04 g high-purity anhydrous sodium acetate into approximately 800 mL DI water. Vacuum filter the solution through a 0.22 µm filter to remove any particulates. Transfer the filtered solution into a 1 L plastic volumetric flask and add 5.2 mL of 50% sodium hydroxide using a plastic serological pipette. Bring to volume with degassed and filtered DI water. Consult Thermo Scientific TN 71 for additional details on hydroxide and hydroxide/acetate eluent preparation for HPAE-PAD.

Preparing the 10× diluted PNGase F

The vial contains 30 µL PNGase F, which was diluted 1:10 by adding 270 µL DI water.

Sample preparation

PNGase F digestion

Control without denaturation

Add 13 µL of 10× G7 buffer to 867 µL of DI water. Follow this by the addition of 80 µL of diluted PNGase F. Incubate the total reaction volume of 960 µL for 20 h at 37 °C.

Digestion of protein without denaturation

Add 240 µL of 4 mg/mL transferrin to 627 µL of DI water. Follow this by the addition of 13 µL 10× G7 buffer and 80 µL diluted PNGase. Incubate the total reaction volume of 960 µL for 20 h at 37 °C.

Control with denaturation

1. Add 10 µL of 10× glycoprotein denaturing buffer to 710 µL of DI water. Incubate the mixture at 100 °C for 10 min.
2. Allow the 720 µL of mixture in step 1 to cool. Follow this by the addition of 13 µL of 10× G7 buffer, 10 µL NP40 and 80 µL of diluted PNGase F. Add DI water (137 µL) to the mix to bring the final reaction volume to 960 µL. Incubate the mixture at 37 °C for 20 h.

Digestion of protein with denaturation

1. Add 240 µL of 4 mg/mL transferrin to 470 µL of DI water. Follow this by the addition of 10 µL of 10× glycoprotein denaturing buffer. Incubate the total reaction volume of 720 µL at 100 °C for 10 min.
2. Add 13 µL of 10× G7 buffer to the 720 µL of mixture in step 1. Follow this by the addition of 10 µL of NP40 and 80 µL diluted PNGase F. Add 137 µL of DI water to the mix to bring the final reaction volume to 960 µL. Incubate the mixture at 37 °C for 20 h.

Desalting PNGase F digests with protein denaturation

1. Prepare a 7K MWCO, 0.5 mL, desalting spin column by removing the column's bottom closure and loosening the cap.
2. Place a 1.5 to 2.0 mL collection tube at the bottom of the column and centrifuge at 1500 × g for 1 min to remove the storage solution.
3. Add 500 µL DI water to the resin, place another collection tube at the bottom of the desalting column, and centrifuge at 1500 × g for 1 min to wash the resin.
4. Place a new tube at the bottom of the desalting column, remove the cap, and add 100 µL of sample to the center of the compacted resin bed.
5. Allow the sample to incubate on the resin bed for 10 min.
6. Centrifuge at 1500 × g for 2 min to collect the desalted sample.

Detergent removal from PNGase F digests with protein denaturation

1. Repeat steps 1 through 5 from the Desalting PNGase F Digests with Protein Denaturation section, substituting the detergent removal spin column for the 7K MWCO desalting spin column.
2. Centrifuge at 1500 × g for 2 min to collect the detergent-removed sample.

Results and discussion

Figure 1 shows the separation of the *N*-linked oligosaccharides released from bovine fetuin. The Dionex CarboPac PA200 column used for the separation is a nonporous, high-efficiency, polymeric anion-exchange column that provides high-resolution separations of oligosaccharides. The pellicular resin structure of this column allows for good mass transfer. The detail observed in the minor peaks between 30 and 40 min is indicative of the higher efficiency of the column. The Dionex CarboPac columns separate sialylated glycans based on the extent of sialylation. The higher the degree of sialylation, the longer the glycoforms are retained on the column. Observe the families of di-, tri-, and tetrasialylated oligosaccharides from bovine fetuin in Figure 1.

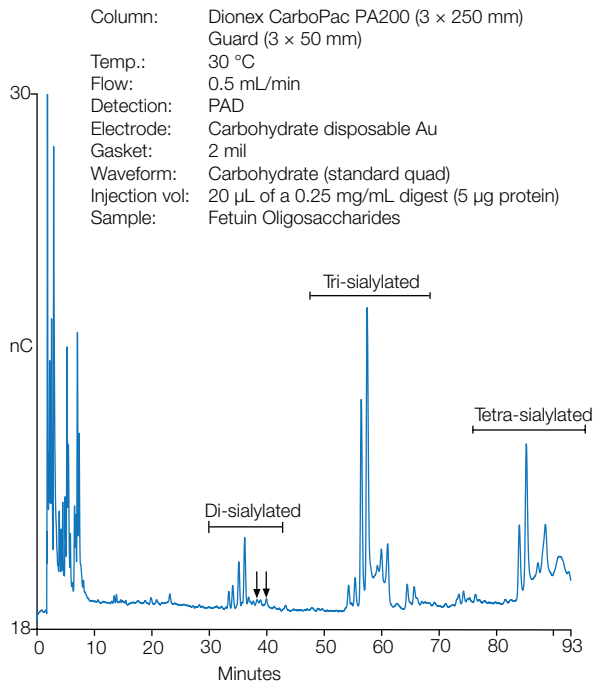

Figure 1. Fetuin oligosaccharide map showing the positions of the sialylated oligosaccharides.

Transferrin is an iron-binding glycoprotein with a molecular mass of 79.6 kDa in a single polypeptide chain. Carbohydrate makes up 4.4 kDa of the molecular mass and is attached to two asparagines in the polypeptide chain. Transferrin glycosylation varies from species to species. Transferrin glycoforms present in human serum are comprised of species having terminally sialylated bi-, tri-, and, tetraantennary oligosaccharides. The most dominant glycoforms are the biantennary oligosaccharides located at both asparagine positions. Denaturation is required for efficient glycan release by PNGase F.[6, 7]

The Dionex CarboPac PA200 column is a strong anion exchanger; therefore, the presence of high salt concentrations and anionic detergents must be avoided. Human transferrin was treated with PNGase F with and without denaturation. The denatured samples were cleaned using desalting and detergent removal columns to remove salt and anionic detergents from the sample. The desalting columns contain a proprietary high-performance desalting resin that rapidly removes 95% of the salts and other small molecules (< 1000 MW) with high protein recovery. Following desalting, the detergent was removed from the samples using detergent removal columns.

These columns contain an affinity resin that effectively and specifically binds a wide variety of detergents and surfactants commonly used during protein extractions and biological sample preparations. Denaturation of PNGase F can occur by SDS, so NP40 is used to protect against denaturation. Elution of NP40 occurs early and this may cause challenges during analysis of the digest by HPAE-PAD, due to interferences with early eluting and neutral oligosaccharides. The amount of NP40 used for the PNGase F digest was evaluated empirically (data not shown) because the presence of lower amounts of NP40 would require less removal during sample pretreatment. The amount of NP40 used in the PNGase F digestions is less than specified by the enzyme manufacturer. The described pretreatment procedure successfully removed NP40 prior to sample analysis.

Figure 2A shows the separation of the *N*-linked oligosaccharides released from human transferrin when treated with PNGase F without any prior sample treatment or denaturation. The chromatogram is not what is expected for transferrin, suggesting that this protein requires denaturation for efficient release of glycans. The peaks near 10 min may be monosialylated oligosaccharides, but there is no evidence of the disialylated oligosaccharides that would be expected for human transferrin and expected to elute around 30 min (based on the bovine fetuin *N*-linked oligosaccharide separation).

Figure 2B shows separation of *N*-linked oligosaccharides released from human transferrin when treated with the glycosidase PNGase F with prior sample denaturation followed by sample pretreatment. Human transferrin is known to have a high concentration of disialylated oligosaccharides; however, the sialylation of human transferrin varies with physiological state. Conditions like alcoholism and cancer affect the sialylation state. Commercially available human transferrin can vary in the degree of sialylation, due to either variations in the pooled serum from which the transferrin is isolated, or loss of sialylation during purification.[6] Peak area analysis of the different sialylated oligosaccharide peaks shows that the sample contains 55% monosialylated oligosaccharides and 45% disialylated oligosaccharides.

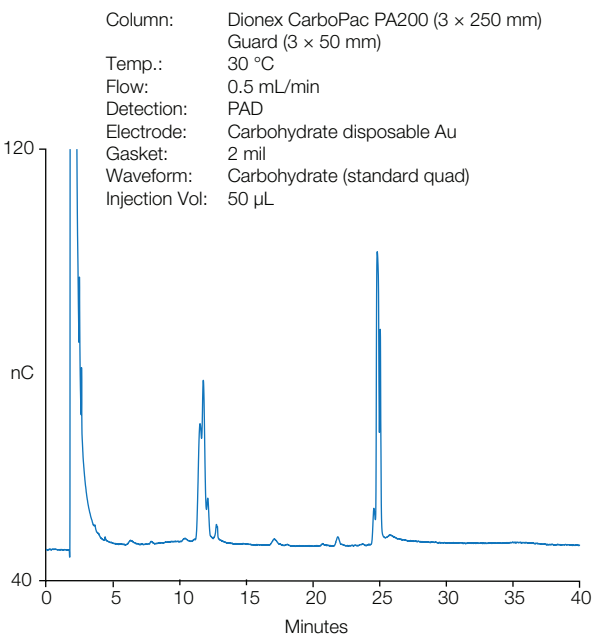

Figure 2A. Separation of non-denatured transferrin treated with PNGase F.

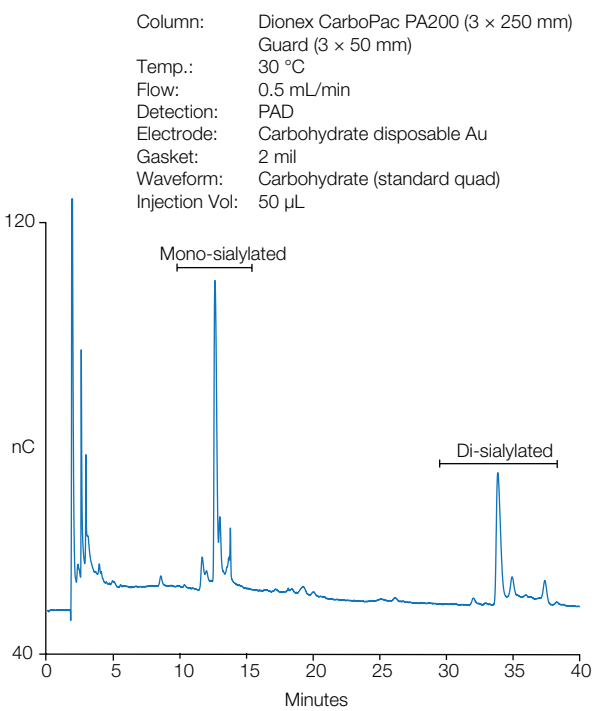

Figure 2B. Separation of oligosaccharides from denatured transferrin treated with PNGase F.

Conclusion

This work describes a simple sample pretreatment method for small-scale PNGase F digests that use protein denaturation. After pretreatment, the samples are amenable to HPAE-PAD oligosaccharide analysis with the Dionex CarboPac PA200 column, making it possible to study and monitor the oligosaccharide content of a glycoprotein.

Precautions

The PNGase F must be glycerol-free to minimize interferences with HPAE-PAD applications.

Handle human transferrin as a substance capable of transmitting infectious diseases. Wear protective clothing to prevent contact with skin and eyes. Contact a licensed professional waste-disposal service to dispose of this material. Disposal must be made in accordance with existing disposal practices employed for infectious waste at your institution. Observe all federal, state, and local environmental regulations.

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Suppliers

Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO, 63103, U.S.A. Tel: 800-521-8956. www.sigmaaldrich.com

Thermo Fisher Scientific (Pierce Biotechnology, Nalgene, Mallinckrodt Baker, J.T. Baker, and Savant Instruments), 308 Ridgefield Court, Asheville, NC, 28806-2210, U.S.A. Tel: 866- 984-3766. www.thermo.com

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