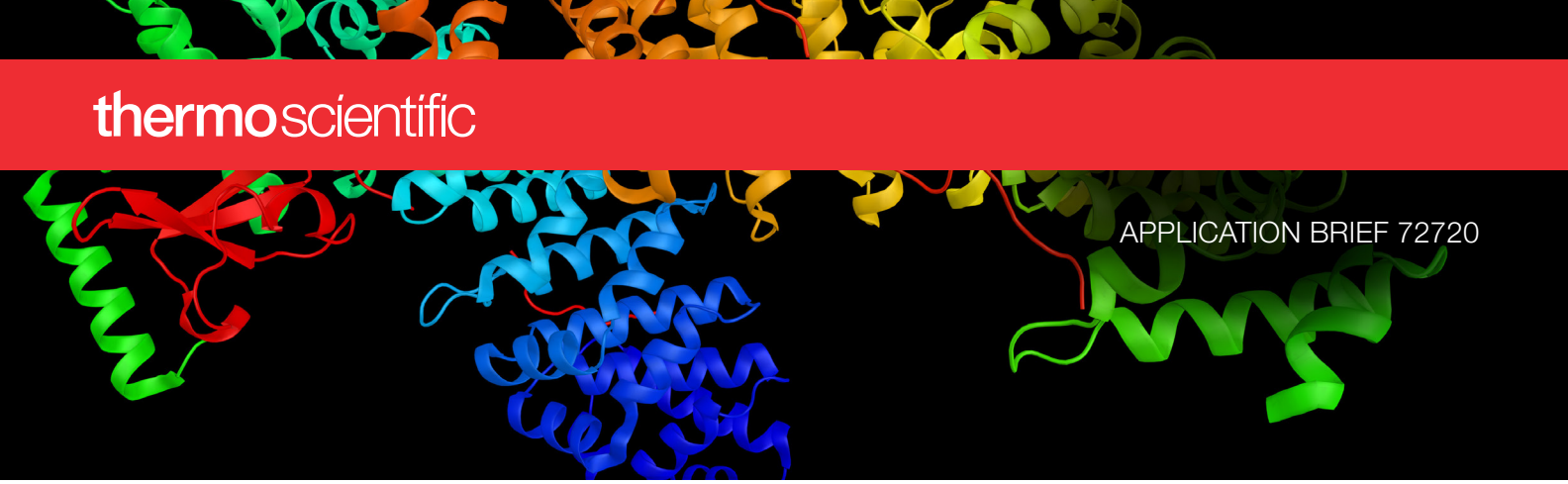

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APPLICATION BRIEF 72720

A glycopeptide standard for improving glycoprotein hydrolysis reaction yield for accurate monosaccharide content determination

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Keywords

CarboPac PA20-4µm-Fast column, HPAE-PAD, protein glycosylation, RFIC system, biopharmaceutical

Goal

To demonstrate the application of a glycopeptide standard for improving accuracy of glycoprotein monosaccharide quantification using HPAE-PAD on a Thermo Scientific™ Dionex™ CarboPac™ PA20-4µm-Fast column.

Introduction

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well-established method for glycoprotein carbohydrate analysis[1-3]. One of the key applications of HPAE-PAD is determination of the monosaccharide composition in a glycoprotein[4]. This assay is a key quality control assay for glycoprotein-based therapeutics. Accurate determination of glycoprotein monosaccharides requires complete and reproducible hydrolysis. Variation in hydrolysis conditions affects monosaccharide yield and leads to inaccurate monosaccharide determination.

In this study, we demonstrate the use of a commercially available glycopeptide standard as a control to determine glycoprotein hydrolysis reaction efficiency. Because the monosaccharide composition of glycopeptide is known, hydrolysis efficiency under a set of conditions can be easily calculated. Here the glycopeptide and two glycoproteins fetuin and alpha1 acid glycoprotein (AGP) were subjected to two different hydrolysis conditions using 1) HCl, which is ideal for quantifying amino sugars like galactosamine and glucosamine and 2) TFA, which is ideal for quantifying neutral sugars like mannose, fucose, and galactose. The monosaccharide yield for the glycopeptide was compared with that of two glycoproteins under identical conditions. This comparison revealed efficient hydrolysis using the conditions tested here. Minimally the glycopeptide standard serves as a positive control for glycoprotein monosaccharide determination.

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Conditions

Columns: Dionex CarboPac PA20-Fast-4µm, 2 x 100 mm column (P/N 302749), Dionex CarboPac PA20-Fast-4µm 2x 30 mm guard column (P/N 302750)

Column Temperature: 30 °C Compartment Temperature: 30 °C

Flow Rate: 0.22 mL/min

Eluent: KOH

Eluent Source: Dionex EGC 500 KOH Eluent Generator Cartridge (P/N 075778)

Working Electrode: Gold disposable on PTFE (P/N 066480) Sampler Tray Temperature: 4 °C

Injection Volume: 5 µL (partial_loop_LS)

Typical Background: 37 nC

Typical Backpressure: 3000 psi

Elution Conditions: 10 mM KOH for 8 min, 100 mM KOH from 8.01 to 14 min, 10 mM KOH from 14.01 to 20 min

Equipment

- A Thermo Scientific™ Dionex™ ICS-5000⁺ Reagent-Free Ion Chromatography (RFIC™) system was used in this work. The Dionex ICS-5000⁺ system is a modular ion chromatograph that includes:
  - SP single pump module (P/N 061707) or DP Dual Pump (P/N 061712) with degas option
  - DC detector compartment (P/N 061767) with single-temperature zone
  - Electrochemical detector (P/N 061719) and cell (P/N 061757)
  - pH-Ag/AgCl reference electrode (P/N 061879)
  - Dionex ICS-5000⁺ EG Eluent Generator module (P/N 079973)
  - Dionex EGC 500 KOH Eluent Generator Cartridge (P/N 075778) with Dionex CR-ATC 500 Continuously Regenerated Anion Trap Column (P/N 075550)
  - Carbohydrate disposable Au working Electrode, pack of 6 (six 2.0 mil gaskets included) (P/N 066480)
  - VP vacuum pump kit (P/N 066463)
- Thermo Scientific™ Dionex™ AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- Sterile assembled microcentrifuge tubes with screw cap, 1.5 mL (Sarstedt P/N 72.692.005)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ 0.2 µm filter units, 1000 mL, nylon membrane, 90 mm diameter (Thermo Scientific P/N 164-0020)

Table 1. Carbohydrate 4-Potential Waveform for the ED.

Time (s)	Potential (V)	Gain	Ramp Region	Integration
0	0.1	Off	On	Off
0.2	0.1	On	On	Off
0.4	0.1	Off	On	Off
0.41	-2	Off	On	Off
0.42	-2	Off	On	Off
0.43	0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.5	-0.1	Off	On	Off

Reference electrode used in Ag mode (Ag/AgCl reference).

Reagents and Standards

Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better

Human serum IgG (Sigma P/N I4506)

Bovine Serum Fetuin (Sigma P/N F2379)

Alpha 1 acid glycoprotein from human plasma (Sigma P/N G9885)

Thermo Scientific Pierce Trifluoroacetic Acid (TFA), sequencing grade (P/N 28904)

Thermo Scientific Pierce Hydrochloric Acid (P/N 24308)

Thermo Scientific™ Dionex™ MonoStandard (P/N 043162)

Ludger BioQuant™ GPEP A2G2S2 glycopeptide standard (QA-Bio, P/N BQ-GPEP-A2G2S2)

Carbohydrate standards

Dissolve the contents of one Dionex MonoStandard 100 nmol vial in 1.0 mL of DI water and mix to prepare a stock standard solution containing 0.1 mM (100 pmol/µL) of each monosaccharide. Immediately freeze unused stock standard at <-10 °C. Avoid repeated freeze/thaw cycles. Deterioration can occur within 24–48 h at room temperature.

Eluents

The KOH eluents were prepared by the eluent generator

equipped and an EGC-KOH cartridge. Though not tested, this method can also be run with manually prepared sodium hydroxide eluents. Sodium hydroxide eluents should be prepared accorded to the instructions in Technical Note 71[5]. Manually prepared eluents may require the column to be washed for a longer period of time and with 200 mM NaOH due to the presence of carbonate in the eluent.

Methods

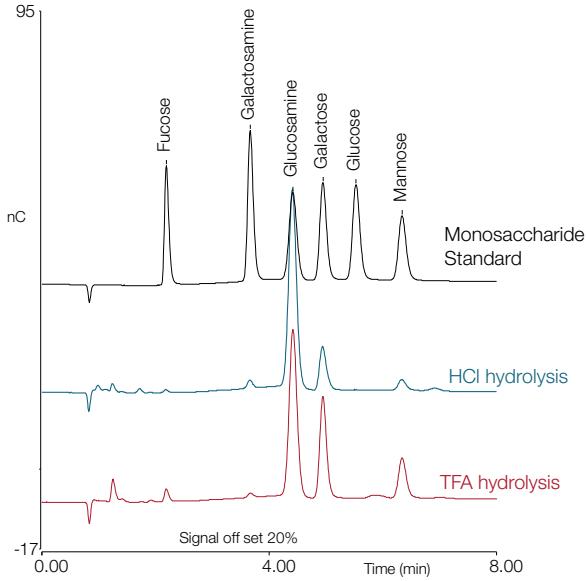
Acid hydrolysis reactions for the glycopeptide as well the glycoproteins used here were set up as follows:

1. TFA Hydrolysis:
   TFA hydrolysis reactions were prepared by combining 20 µL of 3 mg/mL fetuin and AGP or 0.1 mg/mL for glycopeptide, 150 µL DI water, and 30 µL of neat TFA in a 1.5 mL microcentrifuge tube.
2. HCl Hydrolysis:
   HCl hydrolysis reactions were set up by combining 400 µL of 6 M HCl with 20 µL of 3 mg/mL fetuin and AGP or 0.1 mg/mL for glycopeptide solution in a 1.5 mL microcentrifuge tube.
3. All the solutions were heated for 4 h at 100 °C and then dried overnight at room temperature in a Thermo Scientific™ Savant™ SpeedVac™ concentrator equipped with an acid trap.
4. Each vial was reconstituted with 300 µL of DI water.
5. All the samples were vortexed for 30 s and centrifuged for 5 min. The fetuin and AGP samples were diluted 10-fold. A 5 µL aliquot of the supernatant (0.1 µg protein per injection) into the ion chromatography system.

Results

Acid hydrolysis reactions using the glycopeptide, AGP, and fetuin were set up as described in the methods section. After hydrolysis and drying the reactions were dissolved in DI water and injected directly on to the column. Representative chromatograms for the glycopeptide HCl and TFA hydrolysis reactions are shown in Figure 1. The chromatograms show all expected monosaccharide peaks based on the glycopeptide structure. The monosaccharide yields, (glucosamine and galactosamine from HCl hydrolysis, mannose and galactose yields from TFA hydrolysis) were calculated. Table 1 contains monosaccharide composition data for the glycopeptide and both proteins as moles of monosaccharide per mole protein. For calculations, the molecular weight and quantity information provided by the glycopeptide supplier were used. For both glycoproteins, the molecular weights provided by the supplier and

Figure 1. TFA and HCl hydrolysis reactions of the glycopeptide standard used in this study.



protein concentration calculated at A_{280} were used.

The trends for monosaccharide yield from glycoproteins are consistent with that of the glycopeptide. The amino sugar yields from HCl hydrolysis reactions are close to the theoretical maximum indicating complete release of amino sugars. We have consistently gotten higher yield for AGP[6] which could be related to the protein preparation. The yields from TFA hydrolysis are lower for the glycopeptide as well as both the glycoproteins. This points to general difficulty of getting good yields for TFA hydrolysis under these conditions. Fan, et al. published a strategy for optimizing acid hydrolysis of glycoproteins for monosaccharide analysis[7]. Overall, the results imply that the monosaccharide yield data from

Sample	HCl Hydrolysis- Yield-mole/mole of protein			
	Galactosamine		Glucosamine	
	Theoretical	Experimental	Theoretical	Experimental
Fetuin	3	3.07	15	14.65
AGP			22–28	34.60
Bioquant peptide			4	4.06

Sample	TFA Hydrolysis- Yield-mole/mole of protein			
	Galactose		Mannose	
	Theoretical	Experimental	Theoretical	Experimental
Fetuin	12	7.53	9	4.08
AGP	14-25	17.80	11–14	10.74
Bioquant peptide	2	1.40	3	1.57

glycopeptide hydrolysis can be used to improve yields from the glycoproteins.

Conclusion

An approach for optimizing monosaccharide yields from acid hydrolysis of glycoproteins is described here. Monitoring release of monosaccharides from a standard glycopeptide with known composition should allow for improving accuracy in determining glycoprotein monosaccharide composition.

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