RAPID SAMPLE PREPARATION METHODS FOR THE ANALYSIS OF N-LINKED GLYCANS

Zoltan Szabo, András Guttman, Tomas Rejtar and Barry L. Karger

Barnett Institute, Boston, MA, USA

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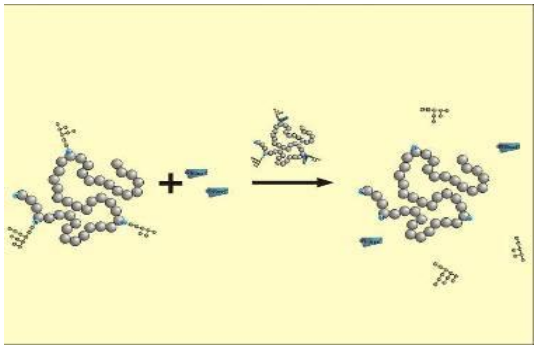
Glycan Analysis Options

Challenge: complex, diversified structures; no chromophore / fluorophore groups, no charge

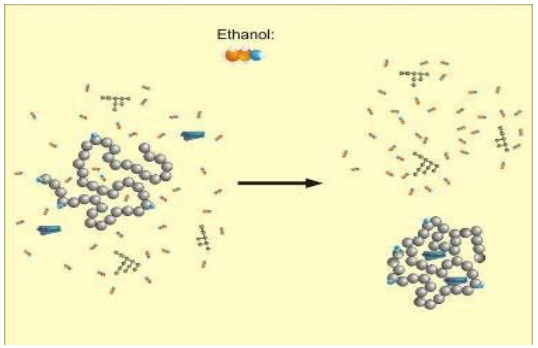
- GC requires derivatization resulting in stereoisomers and complex separation patterns
- HPAE/PAD: limited separation efficiency, non-specific detector response;
- Graphitized carbon chromatography: recovery issues
- NP-HPLC / HILIC: solubility problems in high organic solvent
- MS: limited information on anomeric configuration and other isoforms
- NMR: high quantity and purity requirements
- PAGE: slow, labor intensive and not quantitative
- CE: fast, efficient, automated, sensitive, easy multiplexing; however, requires charged UV / fluorophore tags to assure electromigration and detectability.

Sample Preparation for CE Based Analysis of N-glycans

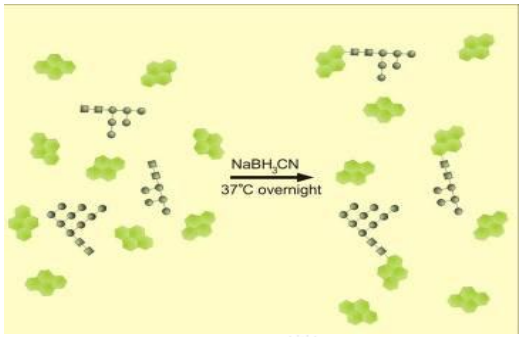
1. Release of N-linked glycan structures by Peptide N-glycosidase F (PNGase F) digestion
   Standard conditions: several hours to overnight; 1:250 – 1:500 enzyme : substrate molar ratio; 37°C



2. Removal of the deglycosylated proteins
   Standard method: ice-cold ethanol precipitation



3. Labeling of the released sugar structures by reductive amination using l-aminopyrene-3,6,8-trisulfonic acid (APTS)
   Standard conditions: 1 : ≥100 glycan : APTS molar ratio; 55°C /2 hours (37°C / overnight for sialylated structures), acetic acid catalyst



Methods to Accelerate Enzyme Catalyzed N-deglycosylation of Glycoproteins

- Microwave assisted deglycosylation of N-linked glycans
- Immobilized PNGase F enzyme reactors in capillary columns
- Integrated microfluidic chip for rapid deglycosylation
- Pressure cycling technology (PCT)

PCT- enhanced enzyme reactions

- Kinetic advantage: pressure promotes water dissociation
- Many hydrolytic reactions are accelerated
- Substrate binding – pressure reversibly denatures substrate protein, revealing hindered cleavage sites
- PCT accelerates and improves reduction/alkylation
- Enzymes: Trypsin, Chymotrypsin, Pepsin, Lys-C, Glu-C, Asp-N, Proteinase K, PNGase F tested to date – all positive
- Both in-solution and in-gel digestion protocols benefit from PCT

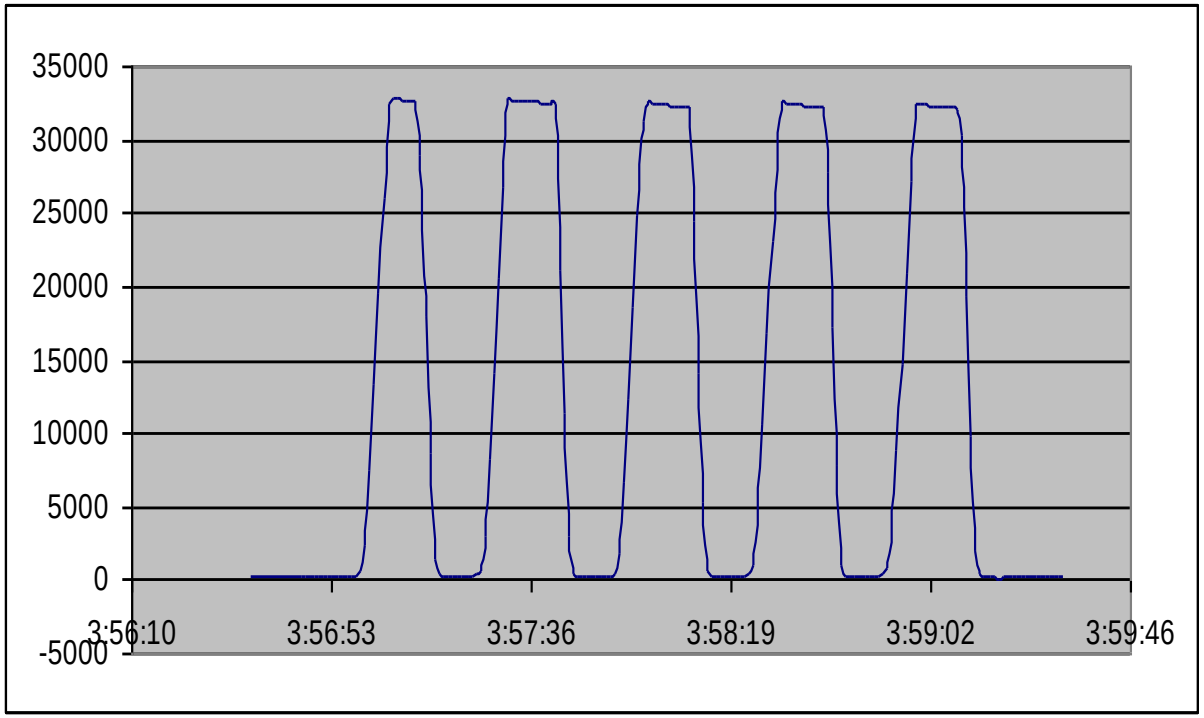
Pressure cycling technology (instrumentation)

Pneumatic system
Single sample capacity
Optional temperature control

Cartridge system permits pressure cycling and incubation at temperatures above boiling point

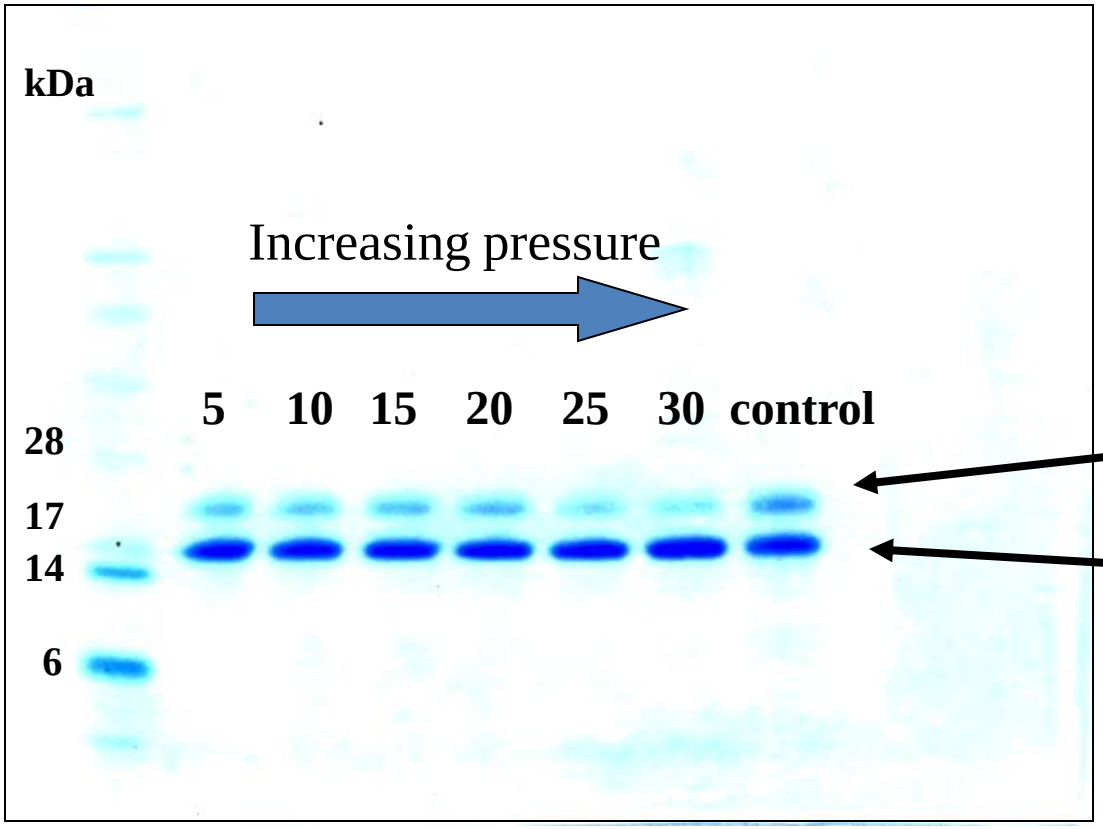
Inert fluoropolymer material
Service t° range –240 to 205°C
Non-stick surface, low binding
Variety of volumes, flexible workflow
Up to 48 samples per batch

Pressure Cycling



Cycles of hydrostatic pressure between ambient and ultra high levels allow to control biomolecular interactions

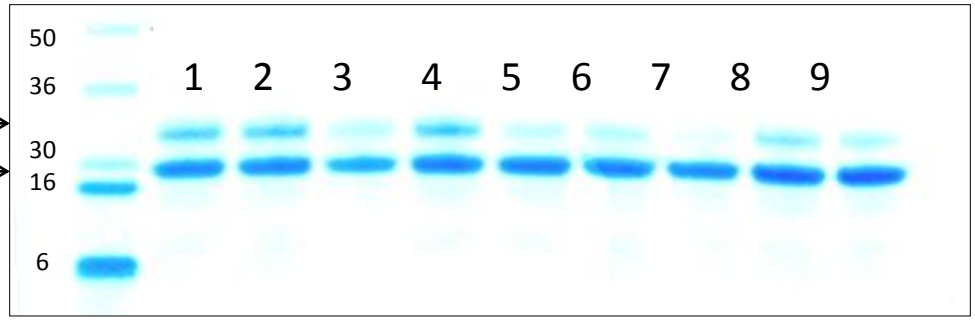
The Effect of the Maximum Pressure Level of PCT on PNGase F Mediated Cleavage of the N-linked Sugars from RNase B



1:2500 enzyme:substrate molar ratio, 5 min, 37°C.
Pressure cycles: 50 s pressure/10 s atmospheric.

Szabo, Z., Guttman, A., Karger, B.L., *Analytical Chemistry* 82 (2010) 2588-2593.

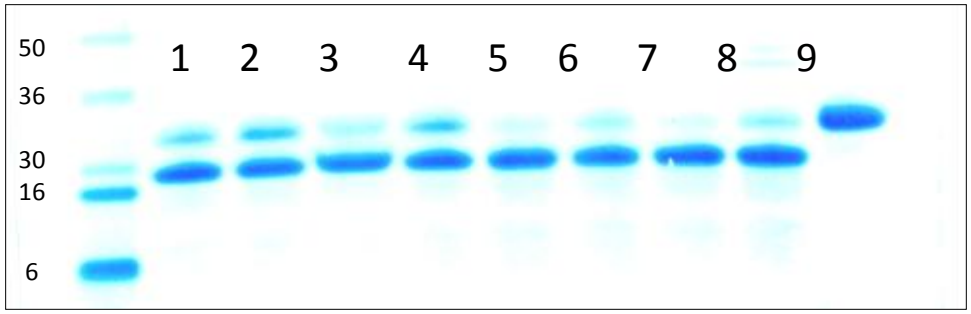
Influence of the presence of non-ionic detergent (Triton X-100) on enzyme activity



Glycosylated (RNase B) →

Deglycosylated (RNase A) →

No Triton X-100



Glycosylated (RNase B) →

Deglycosylated (RNase A) →

1: 5 min pressure; 2: 5 min atmospheric; 3: 10 min pressure; 4: 10 min atmospheric; 5: 20 min pressure; 6: 20 min atmospheric; 7: 40 min pressure; 8: 40 min atmospheric; 9: Control 3 h, atmospheric.

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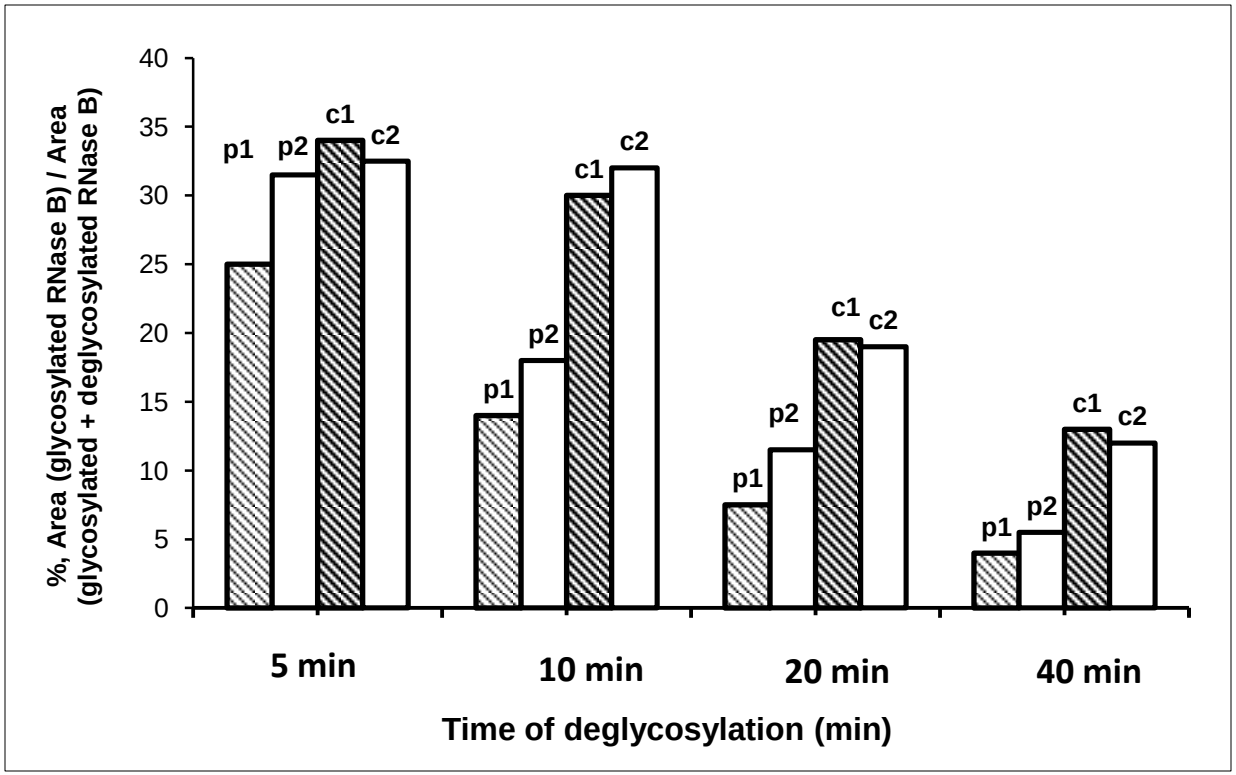
Influence of the Presence of Non-ionic Detergent (Triton X-100) on Enzyme Activity



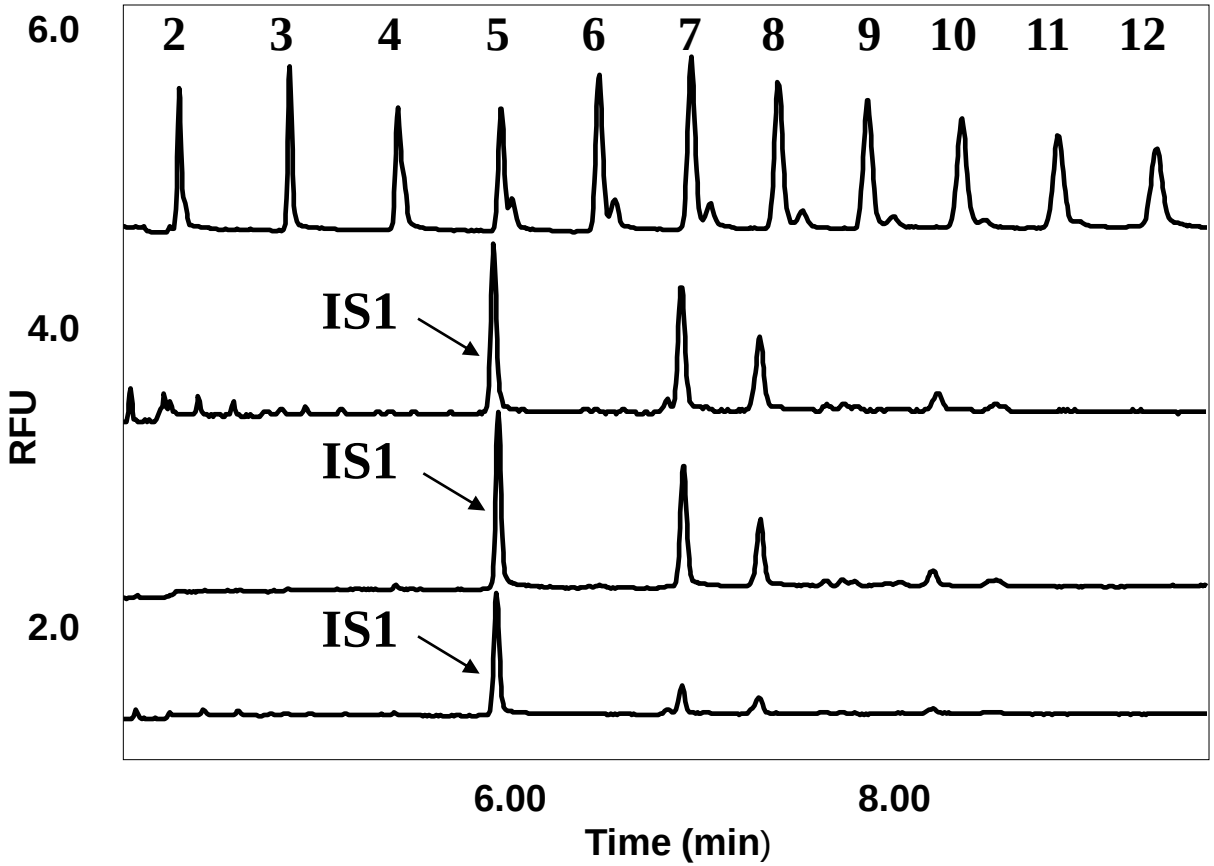
Deglycosylation of RNase B with Triton X-100 under PCT (p1) and atmospheric (c1) conditions and without Triton X-100 in the reaction mixture (p2 and c2)

Effect of the PNGase F Concentration on N-deglycosylation Efficiency under PCT vs. Atmospheric Reaction Conditions

PNGase F : Substrate ratio	1:10000	1:5000	1:2500	1:1666	1:1250
PCT (% intact RNase B)	83	71	**7**	7	7
Atmospheric (% intact RNase B)	84	69	**20**	7	8

Pressure cycle level: 20 kPsi,
Reaction time: 20 min
Temperature: 37°C.

Comparative CE Analysis of APTS Labeled Released Glycans from RNase B by PCT and Atmospheric N-deglycosylation Using 1:2500 Enzyme:Substrate Molar Ratio



APTS labeled maltooligo-saccharide ladder

PCT: 30 kPsi, 5 min, 37°C

Atmospheric, 3 hours, 37°C

Atmospheric, 5 min, 37°C

IS1: maltopentaose-APTS

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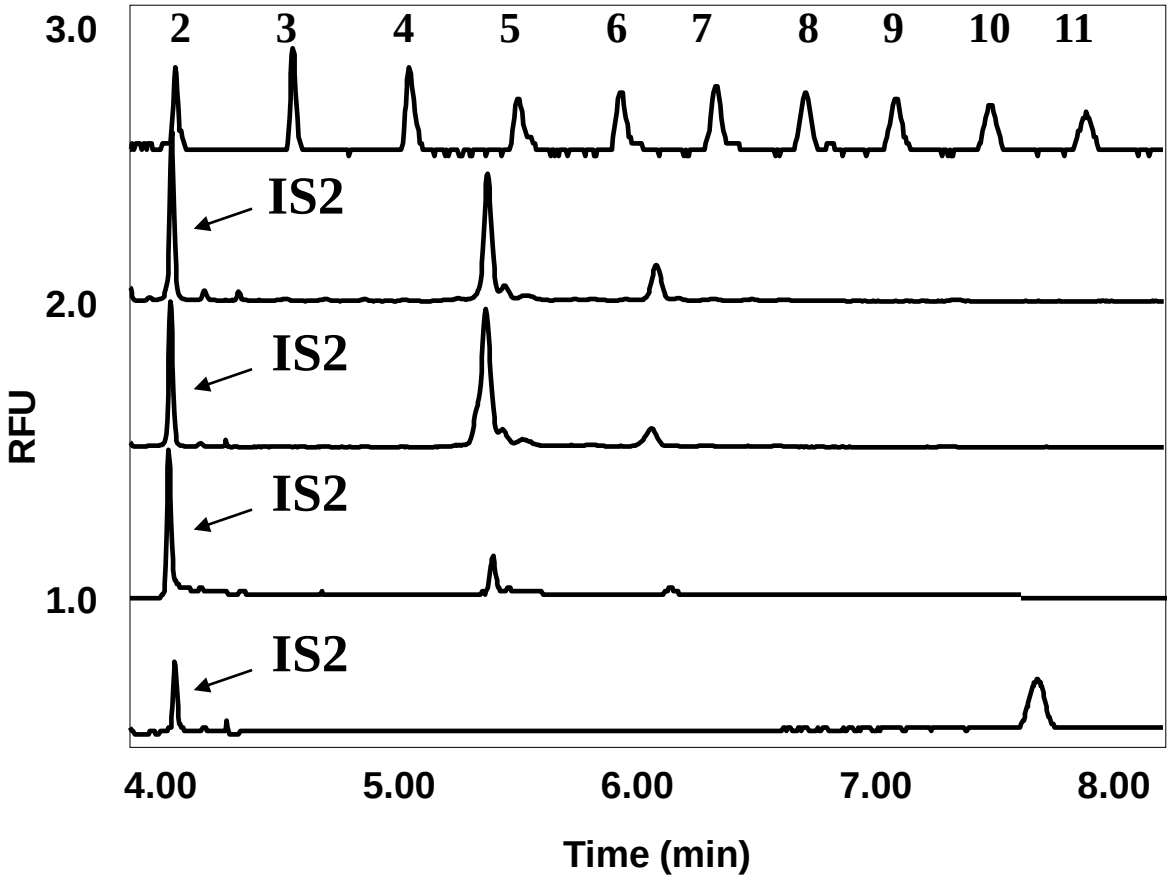
Comparative CE Analysis of APTS Labeled Released Glycans from Human Transferrin by PCT and Atmospheric N-deglycosylation Using 1:2500 Enzyme:Substrate Molar Ratio



APTS labeled maltooligo-saccharide ladder

PCT: 30 kPsi, 20 min, 37°C

Atmospheric, 3 hours, 37°C

Atmospheric, 20 min, 37°C

Asialotransferrin control

IS2: maltose-APTS

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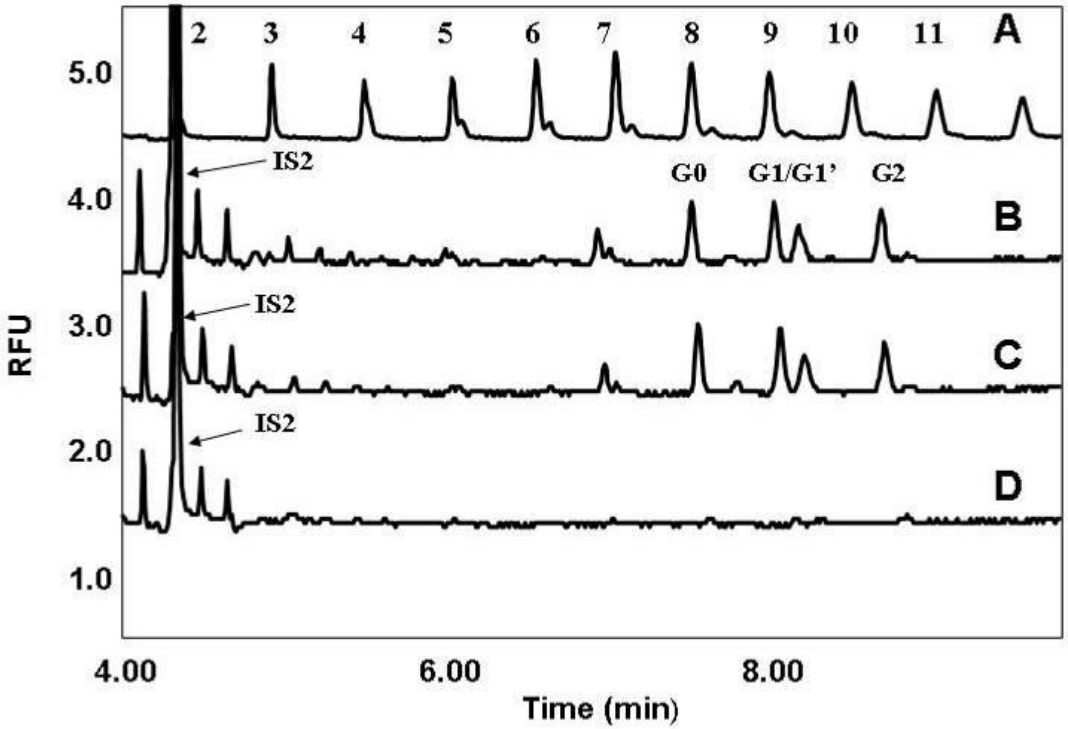
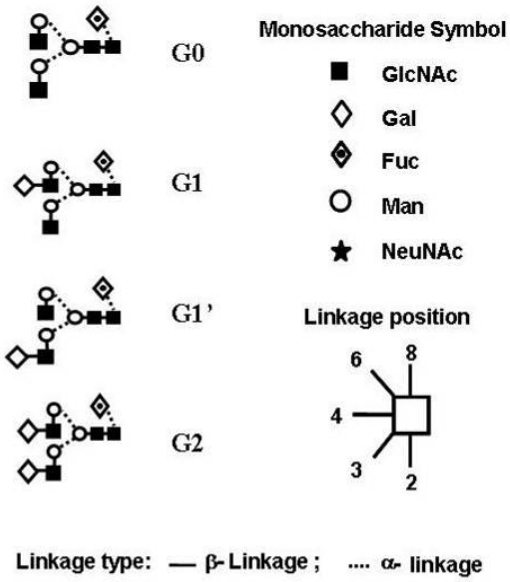
Comparative CE Analysis of APTS Labeled Released Glycans from Polyclonal Human IgG Using PCT and Atmospheric N-deglycosylation with 1:2500 Enzyme:Substrate Molar Ratio.



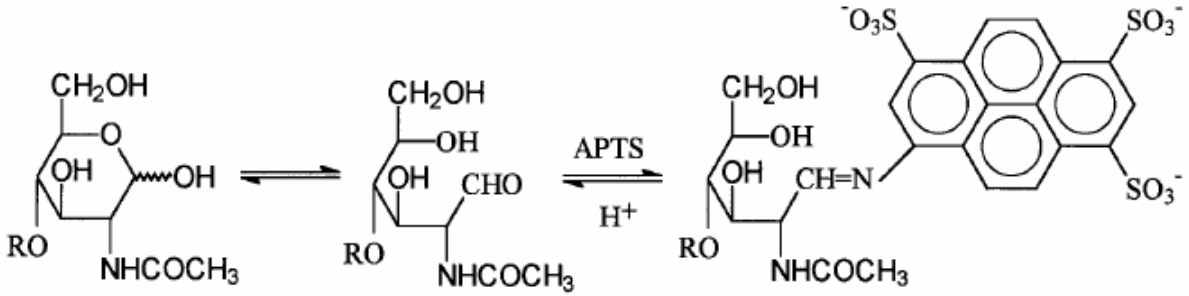


A) APTS labeled maltooligosaccharide ladder
B) PCT: 30 kPsi, 5 min. 37ºC
C) Atmospheric pressure, 3 hours, 37ºC
D) Atmospheric pressure, 5 min, 37ºC
IS2: maltose - APTS.

Advantages of Pressure Cycling Technology (PCT) Assisted Enzymatic N-deglycosylation

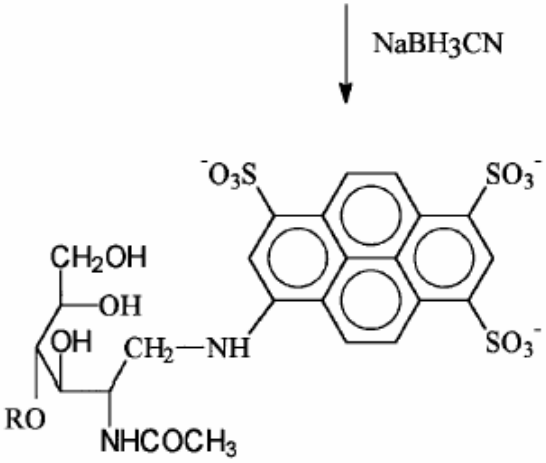
- **The high pressure facilitates conformation changes of the target glycoprotein, increasing the accessibility of the endoglycosidase to the cleavage sites.**
- **1:2500 enzyme : substrate molar ratio at 30 kPsi and 37^{o}C quantitatively released the asparagine linked glycans in minutes.**
- **Pressure cycling apparently did not lead to any loss of sialic acid residues.**
- **The microliter scale reaction volume alleviated possible precipitation related issues.**
- **PCT offers simultaneous processing of 12 samples.**

Sugar Labeling by APTS



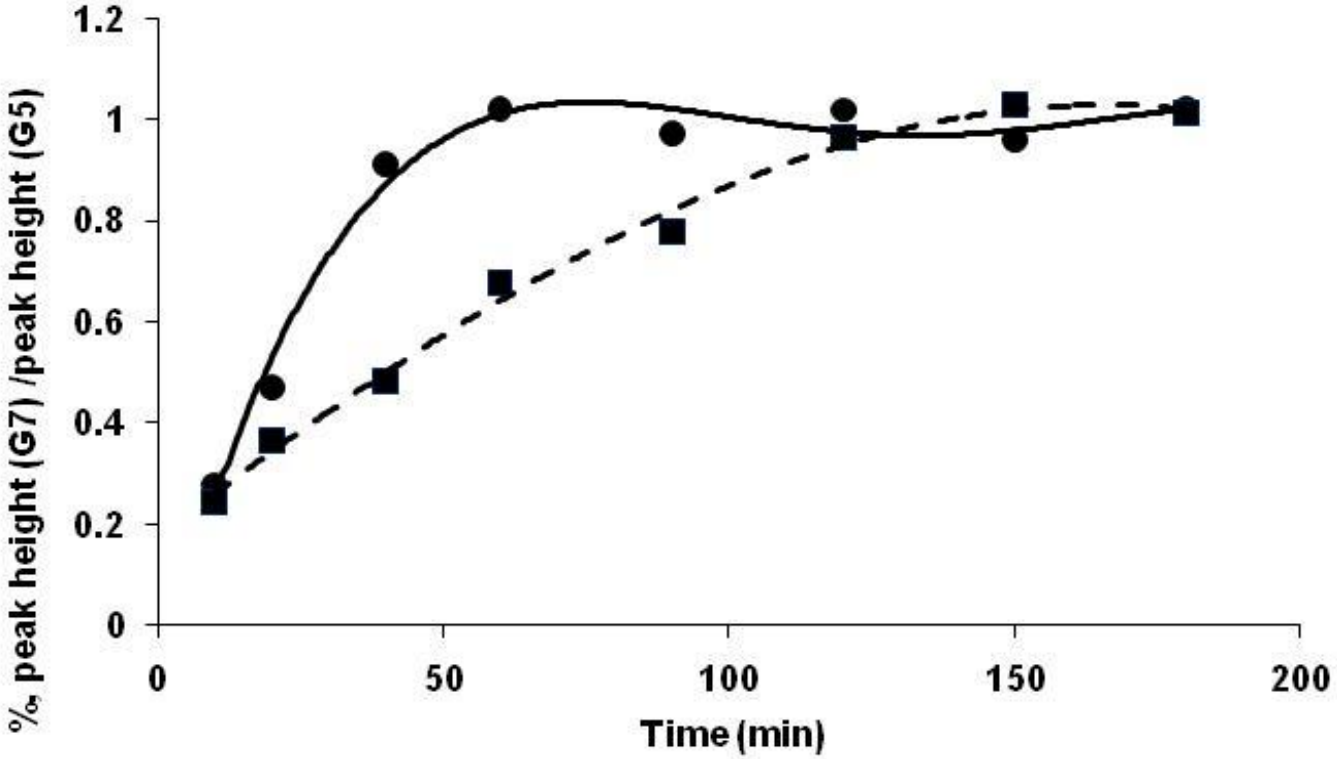
Purpose:

Introduction of label and charge



- **Reductive amination**
- **Sugar reducing ends only**
- **ex 488 / em 520 nm LIF/LedIF, excellent sensitivity**
- **Simple, one step reaction**
- **Great efficiency (over 90%) under optimized conditions (reagent concentration, time, temperature, pH, solvent)**
- **Non-selective: uniform labeling for most structures**
- **Easy quantification: one fluorophore per sugar molecule**

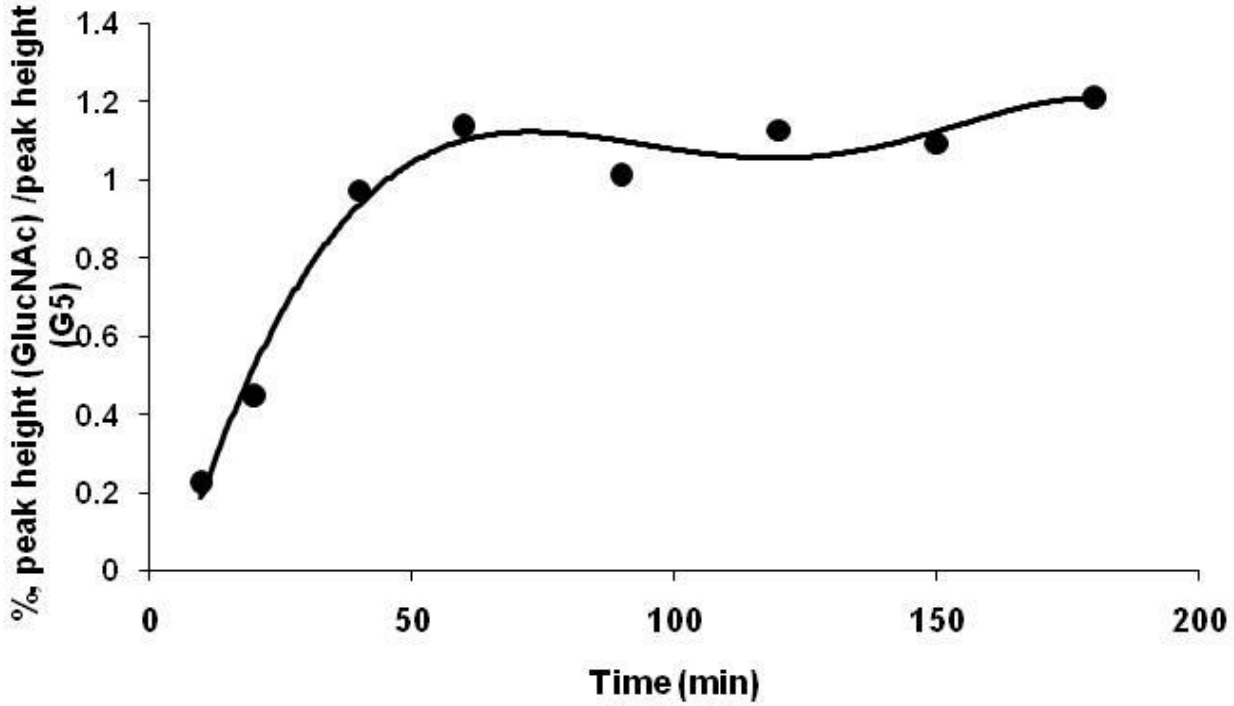
Time Dependence of APTS Derivatization of 5 nmol Maltoheptaose



Solid line: 10x excess of APTS, 0.6 M citric acid catalyst
Dotted line: 100x excess of APTS, 7.5% acetic acid catalyst
Reaction temperature: 55°C

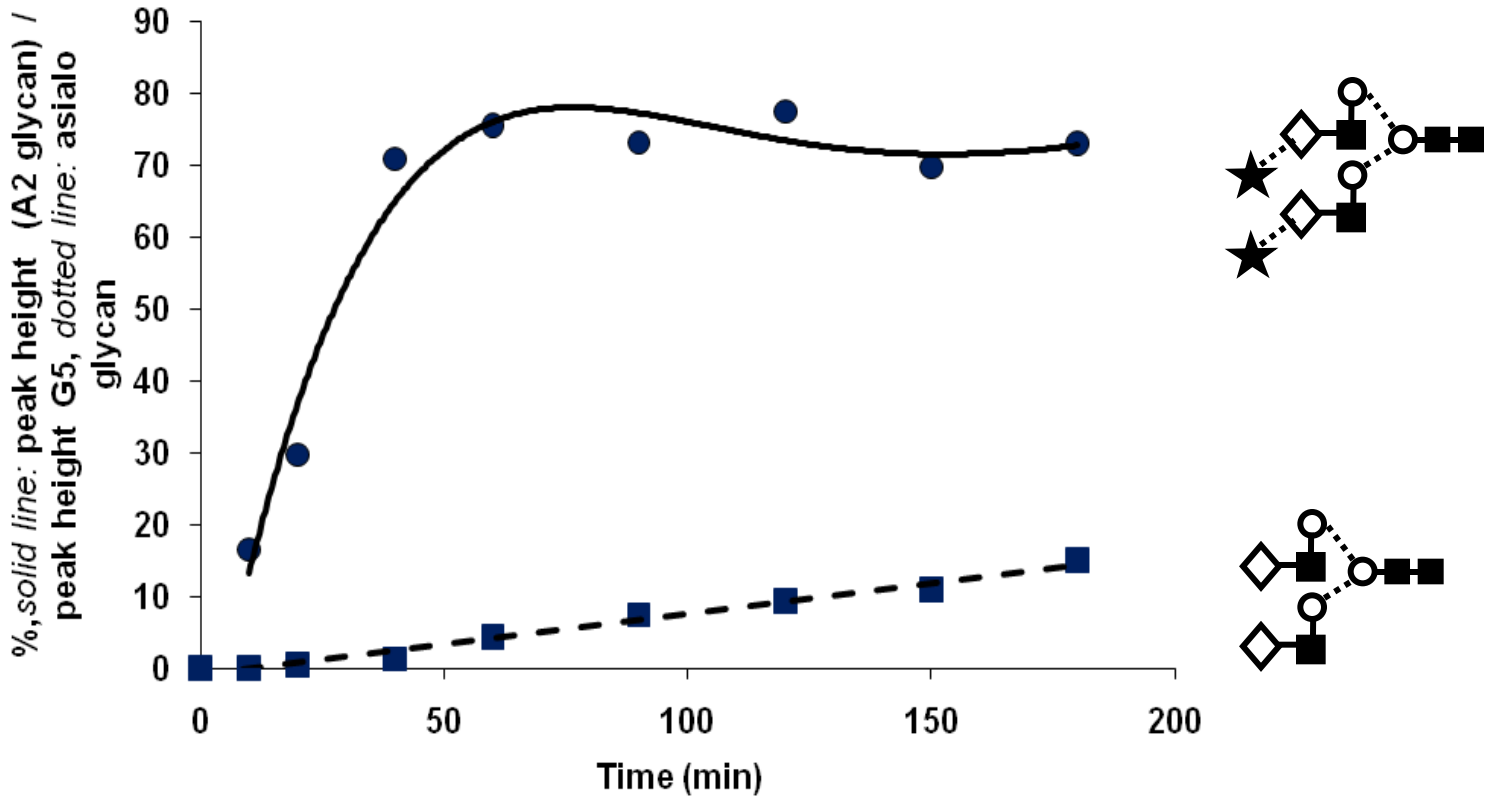
Szabo, Z., Guttman, A., Rejtar, T., Karger, B.L., Electrophoresis 31 (2010) Published online

GlcNAc Labeling Efficiency by APTS as a Function of Reaction Time



Reaction temperature: 55°C, 10x excess of APTS, 0.6 M citric acid catalyst

APTS Labeling Efficiency of Disialo-galactosylated Biantennary Oligosaccharide



Solid line: 10x excess of APTS, 0.6 M citric acid catalyst

Dotted line: sialic acid loss (measured by the amount of asialo-galactosylated biantennary structure

Reaction temperature: 55°C

Symbols: ■ GlcNac; ○ Man; ◊ Gal; ∗ Neu5Ac, – β linkage; … α linkage.

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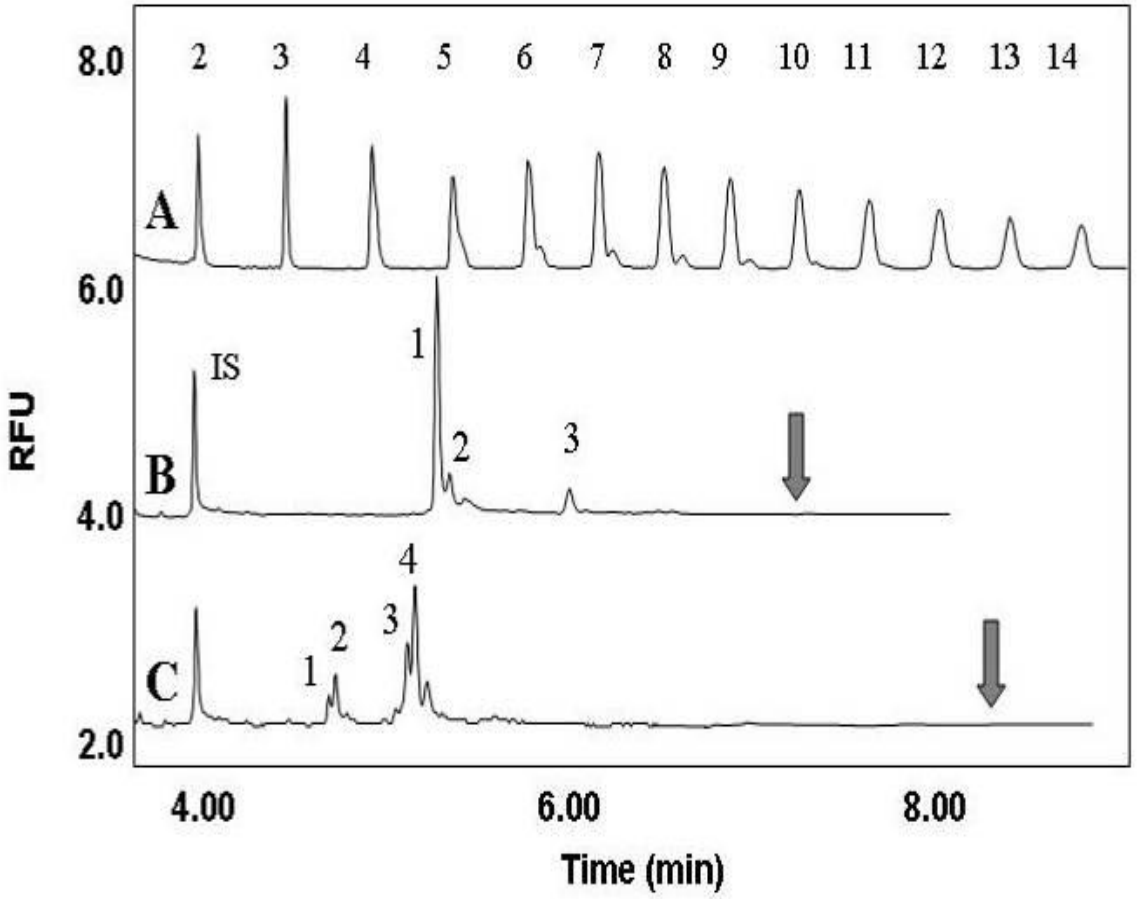
Labeling of Transferrin and Fetuin N-glycans for 50 min at 55°C in the Presence of 0.6 M Citric Acid Using 10:1 APTS to Glycan Molar Ratio



APTS labeled maltooligo-saccharide ladder

APTS labeled transferrin glycans

APTS labeled fetuin glycans

IS: maltose-APTS

The arrows depict the calculated migration positions for desialylated transferrin (trace B, GU=9.94) and fetuin (trace C, GU=11.6) glycans.

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Improved Fluorophore Derivatization Protocol for N-linked Glycans

- Standard CE sample preparation protocol generally requires derivatization times of 2h 55°C or overnight at 37°C, using >100 fold excess of fluorophore reagent.
- Replacing the conventionally used acetic acid catalyst with citric acid, as low as 1 : 10 glycan to fluorophore molar ratio maintained the >95% derivatization yield at 55°C with only 50 minutes reaction time.
- Terminal sialic acid loss was negligible at 55°C during the derivatization process.
- The reduced relative level of APTS simplified the removal of excess reagent.

ACKNOWLEDGMENT

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