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
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; 37ºC*

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

3. Labeling of the released sugar structures by reductive amination using 1-aminopyrene-3,6,8-trisulfonic acid (APTS)
   *Standard conditions: 1 : ≥100 glycan : APTS molar ratio; 55ºC /2 hours (37ºC / 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
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.

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

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.
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

<table>
<thead>
<tr>
<th>PNGase F : Substrate ratio</th>
<th>1:10000</th>
<th>1:5000</th>
<th>1:2500</th>
<th>1:1666</th>
<th>1:1250</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCT (% intact RNase B)</td>
<td>83</td>
<td>71</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Atmospheric (% intact RNase B)</td>
<td>84</td>
<td>69</td>
<td>20</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>RFU</th>
<th>IS1</th>
<th>APTS labeled maltooligo-saccharide ladder</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3</td>
<td>IS1</td>
<td>PCT: 30 kPsi, 5 min, 37°C</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>IS1</td>
<td>Atmospheric, 3 hours, 37°C</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>IS1</td>
<td>Atmospheric, 5 min, 37°C</td>
</tr>
</tbody>
</table>

IS1: maltopentaose-APTS
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 maltooligosaccharide ladder

PCT: 30 kPsi, 20 min, 37°C

Atmospheric, 3 hours, 37°C

Atmospheric, 20 min, 37°C

Asialotransferrin control

IS2: maltose-APTS
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. 37°C
C) Atmospheric pressure, 3 hours, 37°C
D) Atmospheric pressure, 5 min, 37°C
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°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.
**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.
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

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

Barry L. Karger
Andras Guttman
Tomas Rejtar
Jack Liu