HIGH PRESSURE MEDIATED SAMPLE PREPARATION FOR CAPILLARY ELECTROPHORESIS ANALYSIS OF N-LINKED GLYCANS

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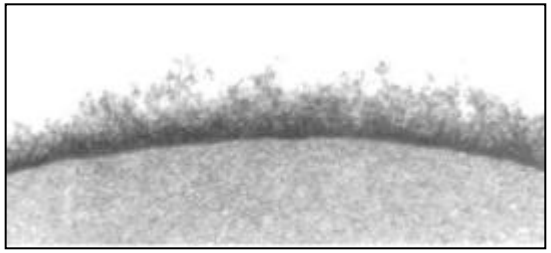
49th EHPRG, Aug 28-Sept 2, 2011 Budapest, Hungary

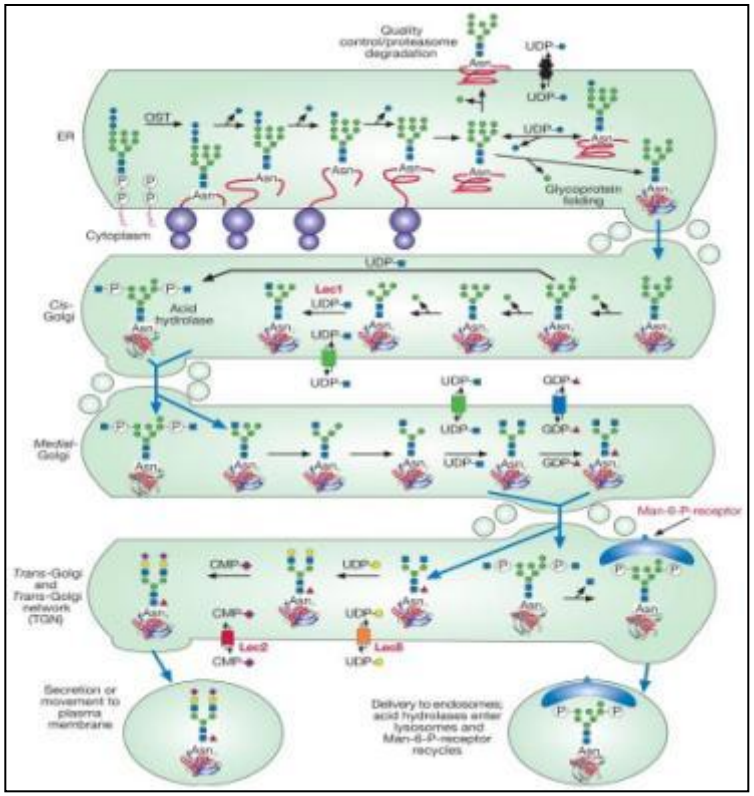
BARNETT INSTITUTE of Chemical and Biological Analysis

Significance of glycosylation

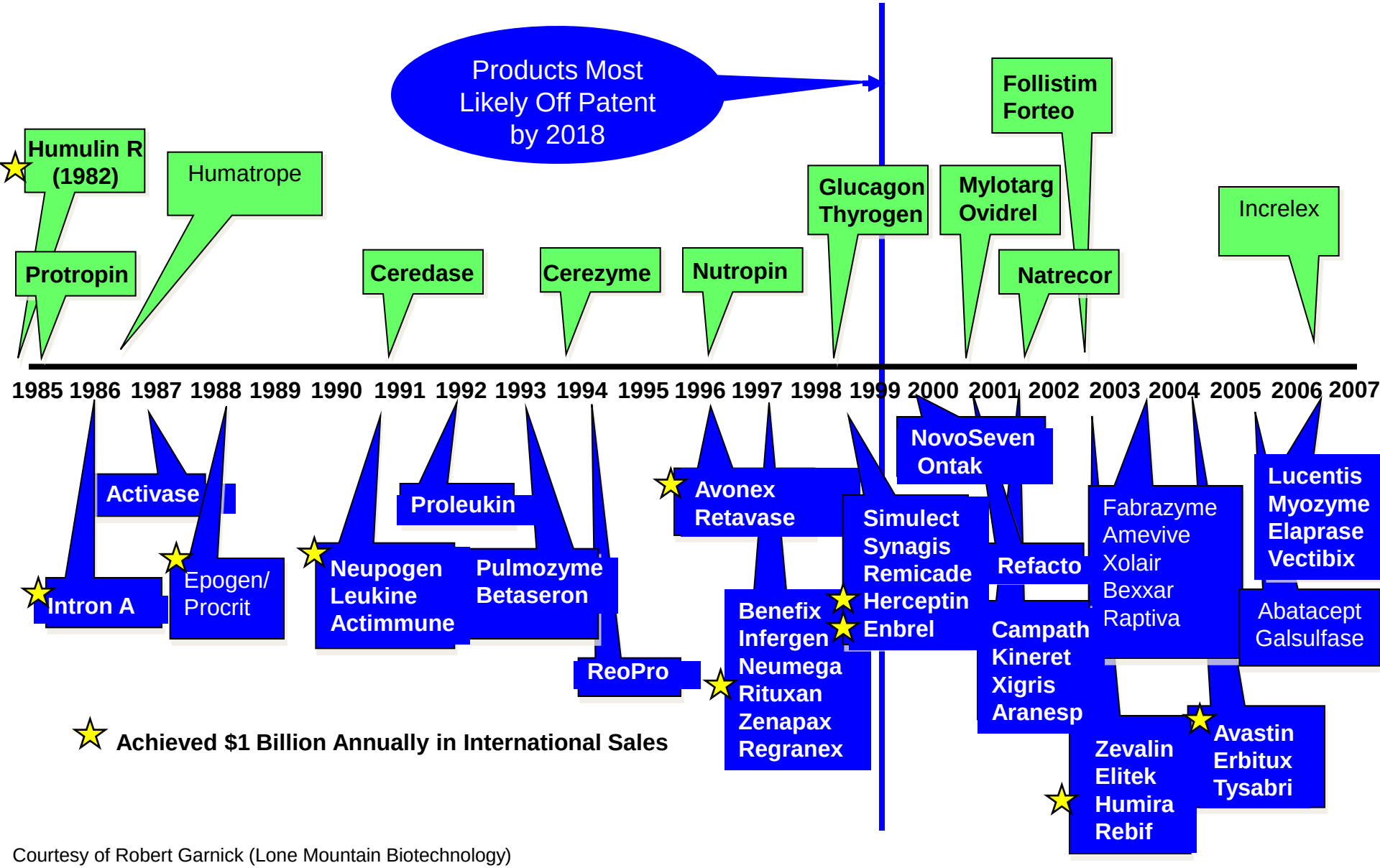
- Glycosylation – prevalent PTM
- Multiple and significant impacts
  - Recognition factors with binding partners
  - Folding
  - Roles in immunogenicity
  - Regulation of bioactivity and final degradation
- Diversity of glycans
  - N- and O-linked
- Analytical challenge



Electron micrograph of the glycocalyx at the surface of an erythrocyte.



Key Biotech Product Approvals (1982 – 2007)



Courtesy of Robert Garnick (Lone Mountain Biotechnology)

Glycan analysis options

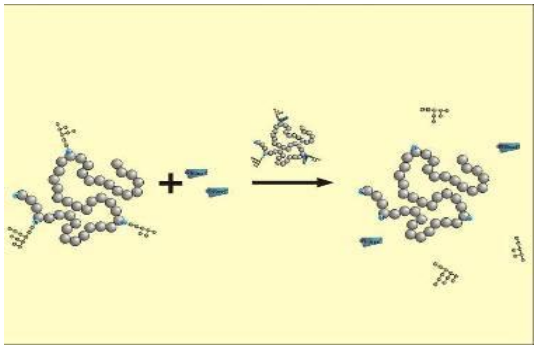
***CHALLENGE**: complex, diversified structures; no chromophore / fluorophore groups; mostly not charged*

Analytical methods in glycan analysis:

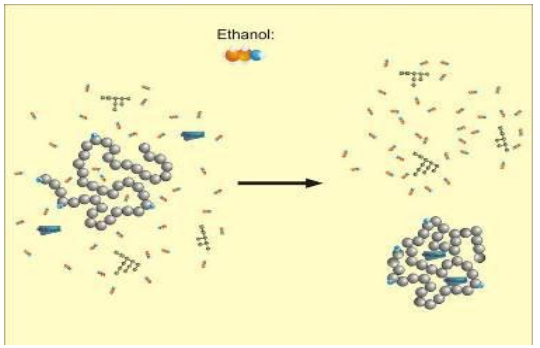
- Gas Chromatography
- HPLC: - HPAE/PAD
  - Normal phase and HILIC
  - UPLC
  - Graphitized carbon / ChipLC
- Structural characterization options: MS and NMR
- PAGE
- Capillary Electrophoresis

Sample preparation for CE based analysis of N-glycans

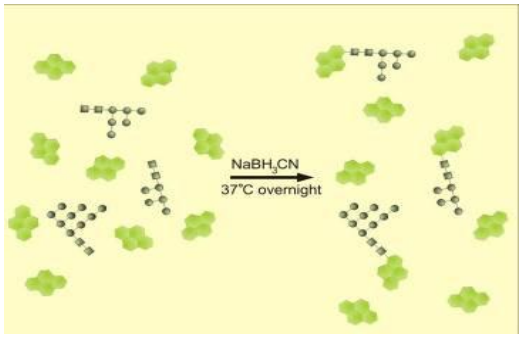
1. Release of N-linked glycan structures by Peptide N-glycosidase F (PNGase F) digestion
   Standard conditions: several hours to overnight; 1:250 – 1:500 enzyme : substrate molar ratio; 37°C



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-induced protein denaturation is different from thermal denaturation

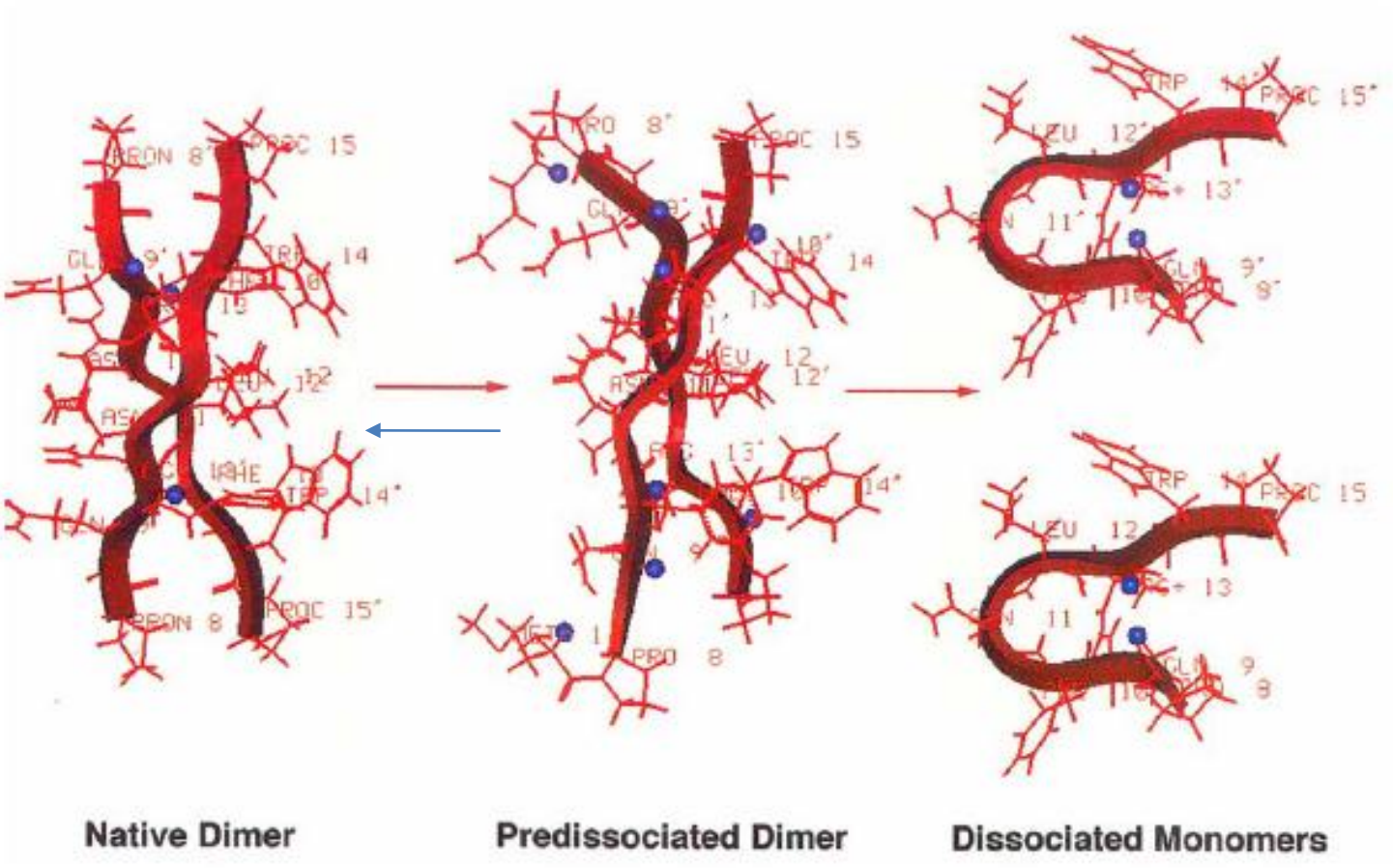


Fig. 2. Proposed β-sheet structure of the Arc repressor in the native state, the predissociated state, and the dissociated molten globule state.[23] (Courtesy of J. Jonas, reproduced with permission from ref. 23. (Copyright 1994, American Chemical Society.)

Differences in compressibility of protein domains

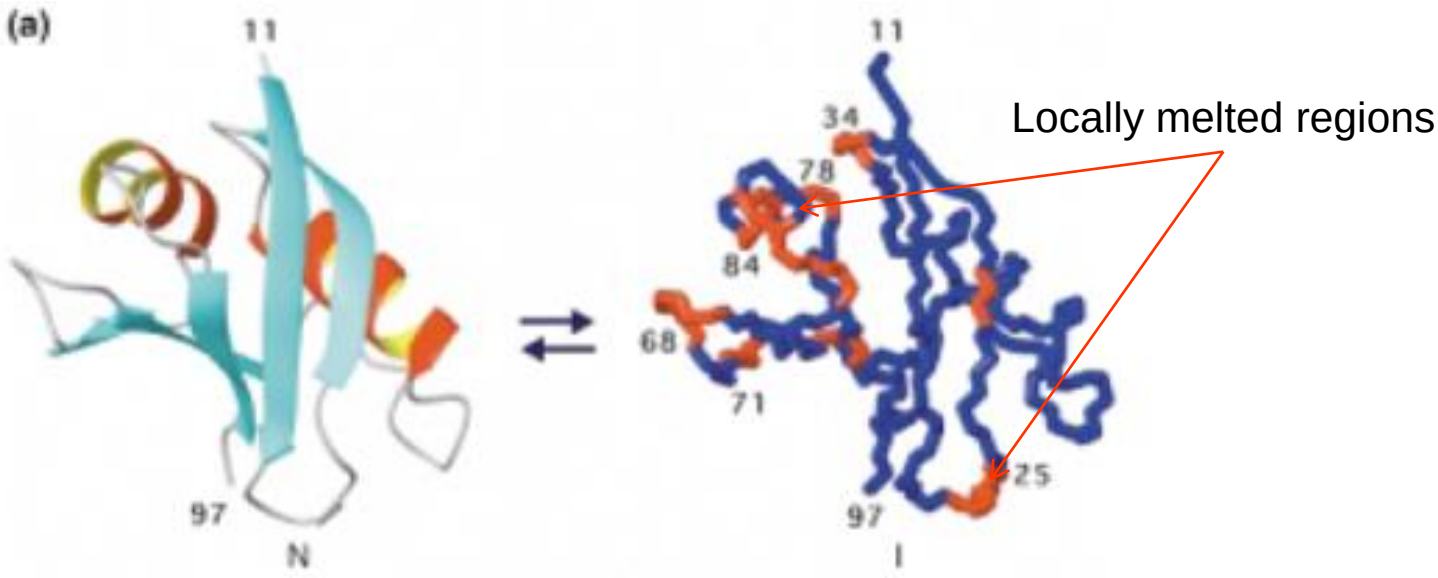


Fig. 3. Structure of pressure-populated folding intermediates.

The native state of the Ras-binding domain of RalGDS (N) is converted by pressure into an intermediate state (I), represented by a structure similar to the native state (blue) but with locally melted regions (red) as determined by nuclear magnetic resonance (NMR)

Inoue, K. *et al. (2000) Nat. Struct. Biol. 7, 547–550*

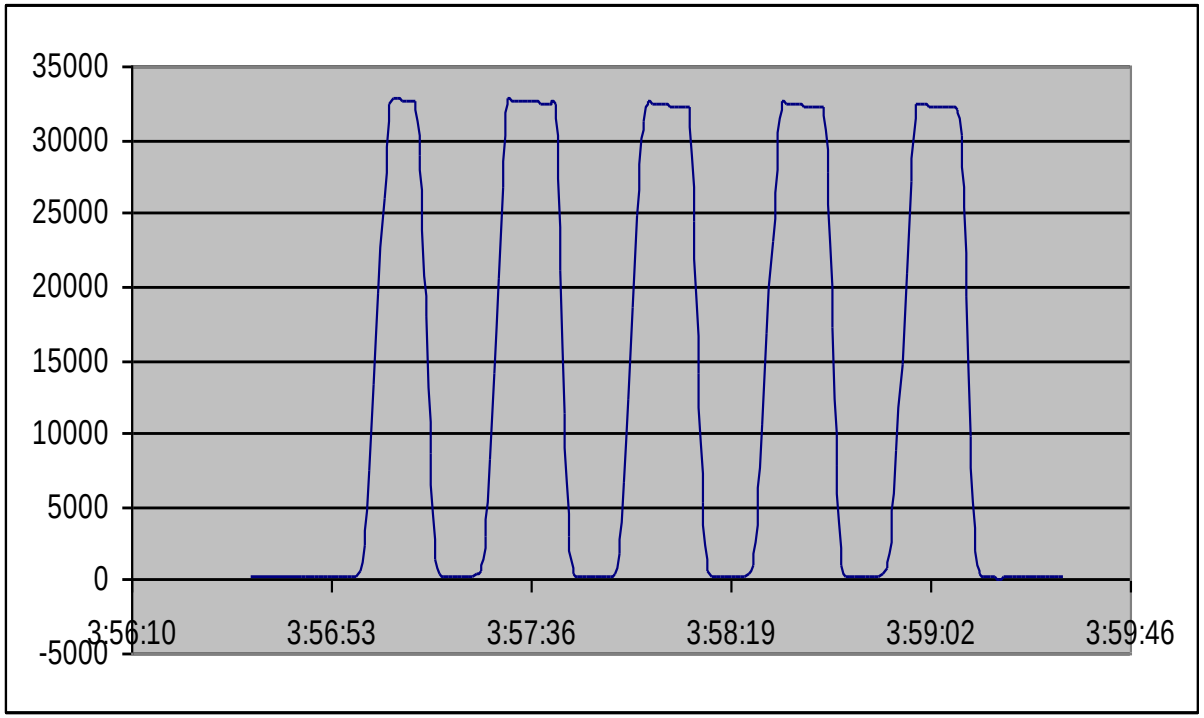
Pressure cycling technology (instrumentation)

Pneumatic system
Single sample capacity
Optional temperature control

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

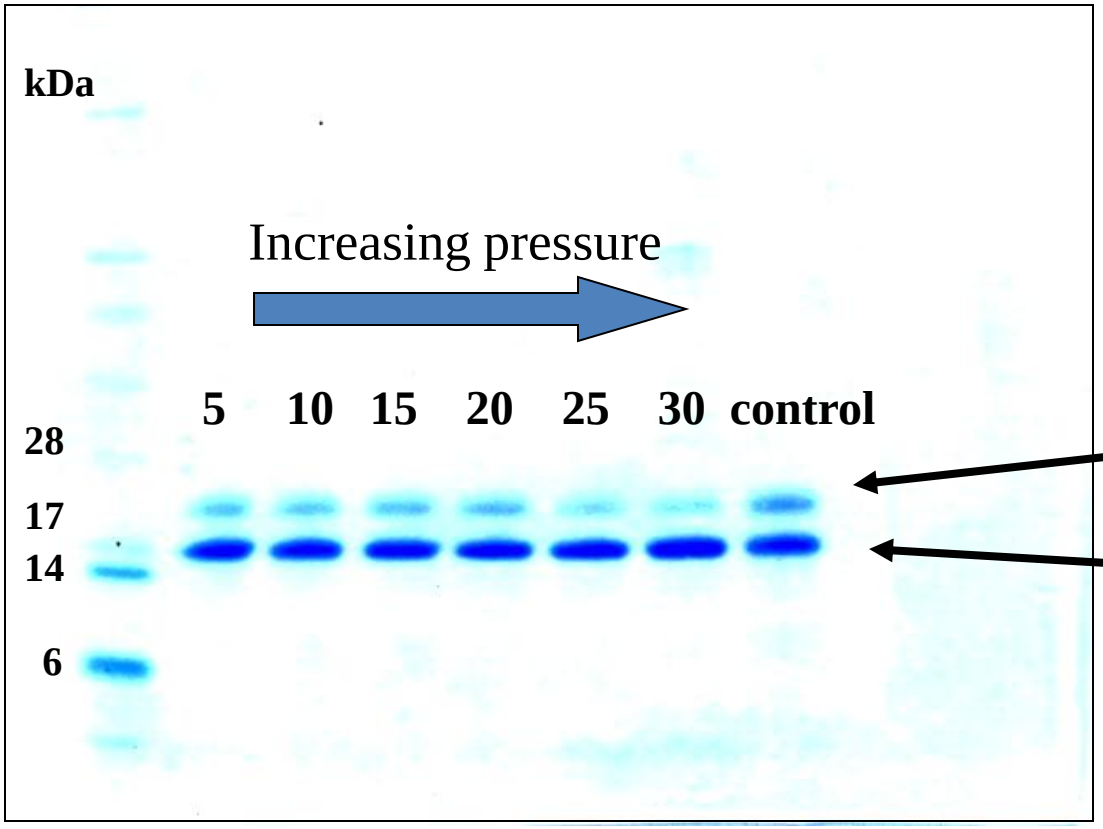
- Inert fluoropolymer material
- Services temperature range of –240 to +205°C
- Non-sticky surface, low binding
- Variety of volumes,
- Flexible workflow
- Up to 48 samples per batch

Pressure Cycling



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

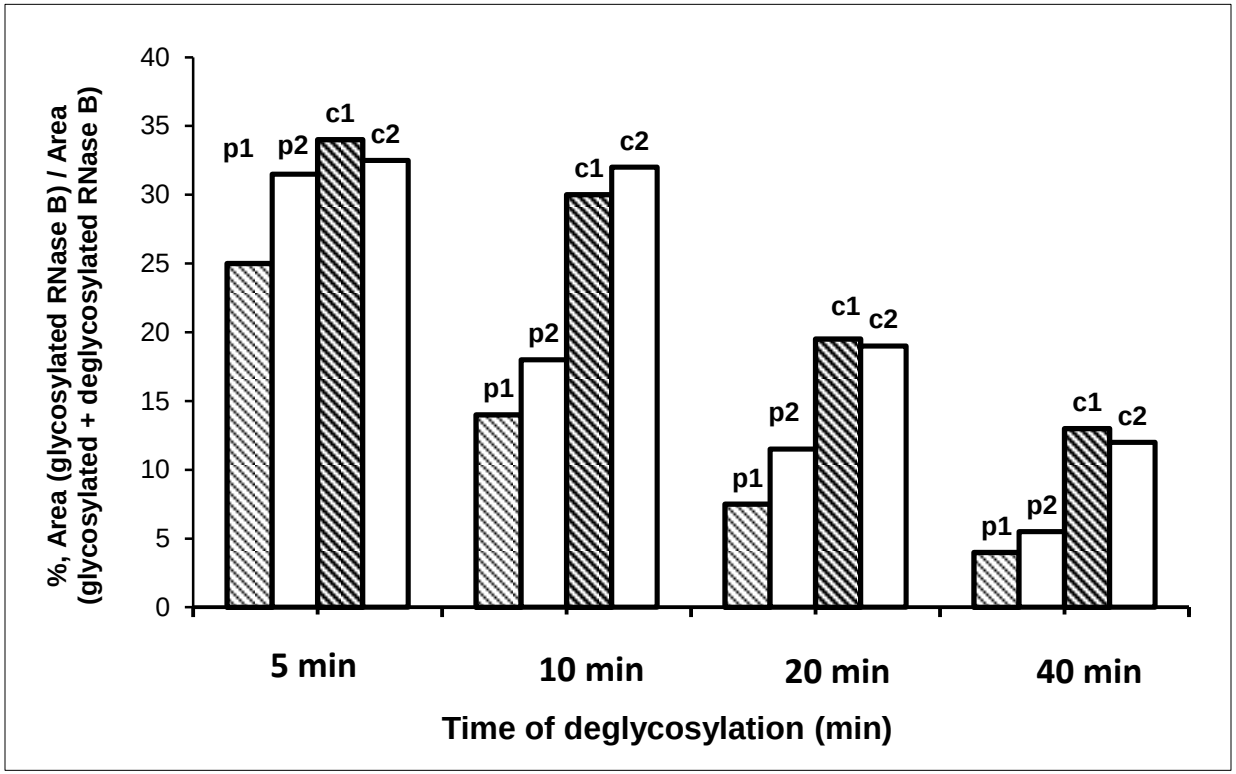
The effect of the maximum pressure level of PCT on PNGase F mediated cleavage of the N-linked sugars from RNase B



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



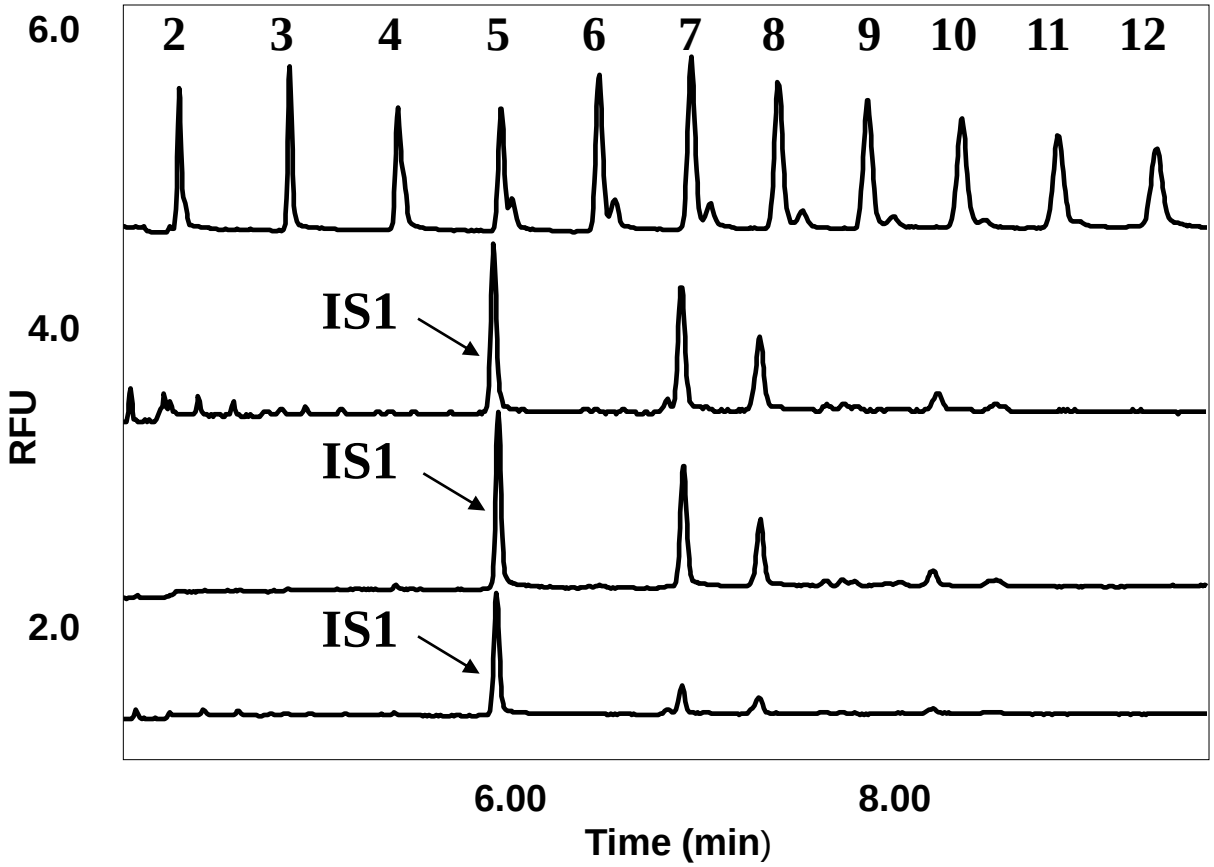
Deglycosylation of RNase B with Triton X-100 under PCT (p1) and atmospheric (c1) conditions and without Triton X-100 in the reaction mixture (p2 and c2)

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^{o}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

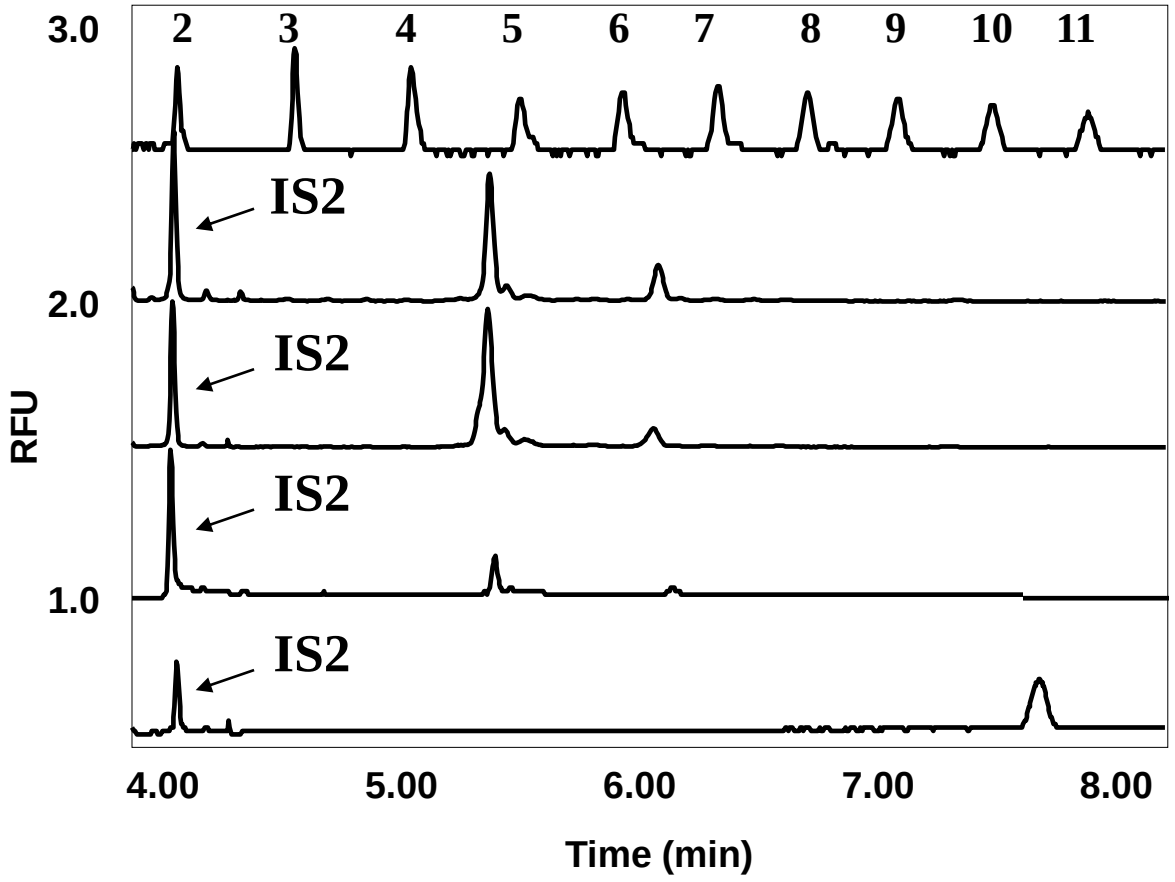
PCT: 30 kPsi, 5 min, 37°C

Atmospheric, 3 hours, 37°C

Atmospheric, 5 min, 37°C

IS1: maltopentaose-APTS

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

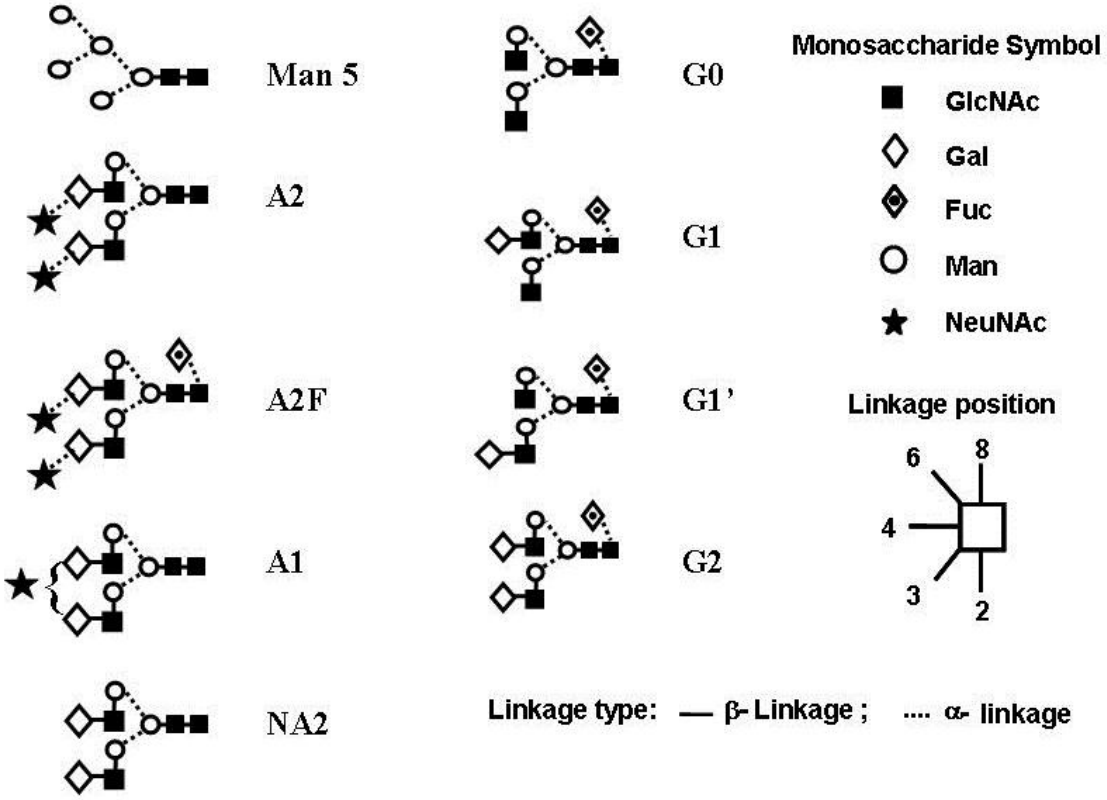
Atmospheric, 3 hours, 37°C

Atmospheric, 20 min, 37°C

Asialotransferrin control

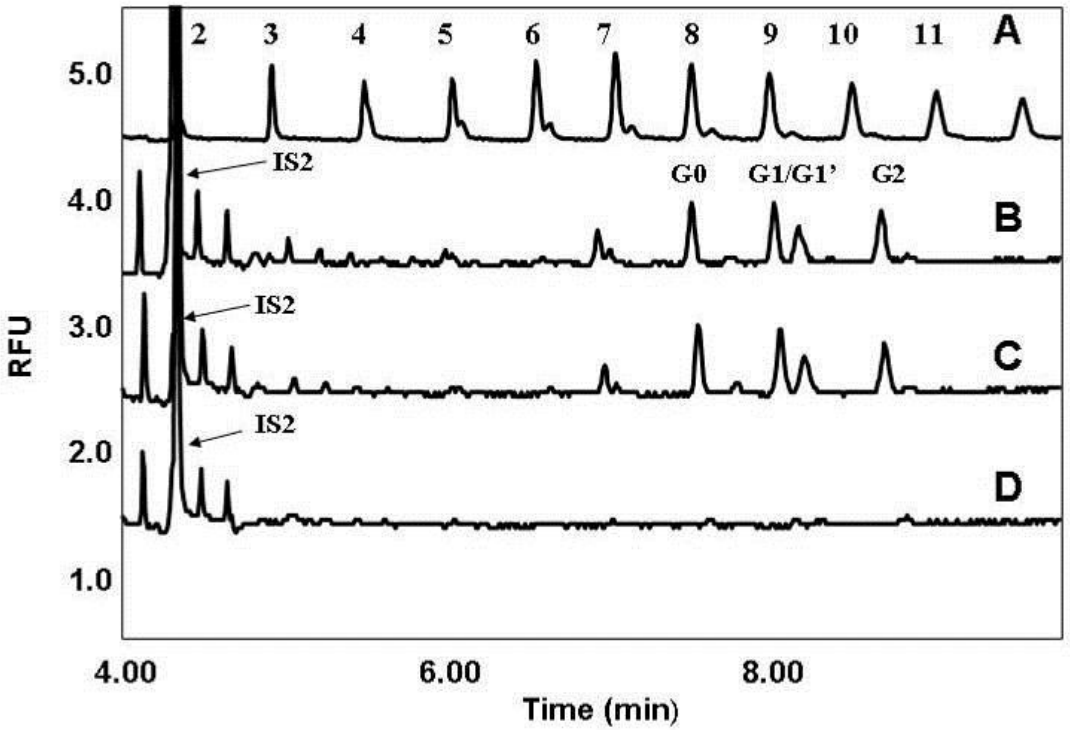
IS2: maltose-APTS

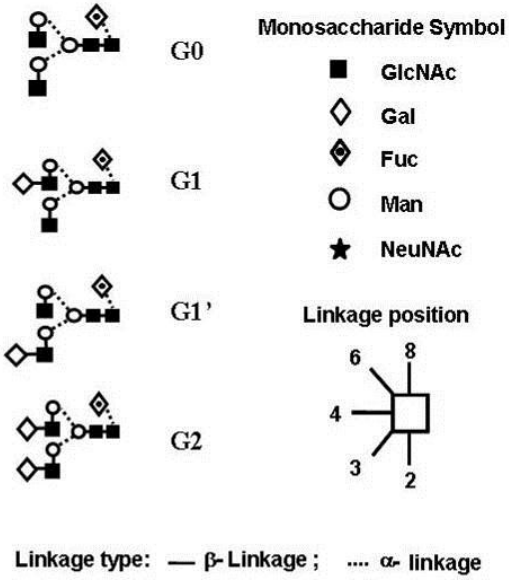
Schematic representation of the glycan structures found in the studies



glycan structures	GU Units	
	measured	calculated
Man 5	6.92	6.82
A2	4.80	4.60
A2F	5.01	4.94
A1	6.48	6.52
NA2	9.93	9.94

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.**

ACKNOWLEDGMENT

Barry L. Karger
Andras Guttman
Tomas Rejtar
Jack Liu

Alexander Lazarev
Edmund Ting