Intact Protein and Glycoprotein Separations by HILIC

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Agenda

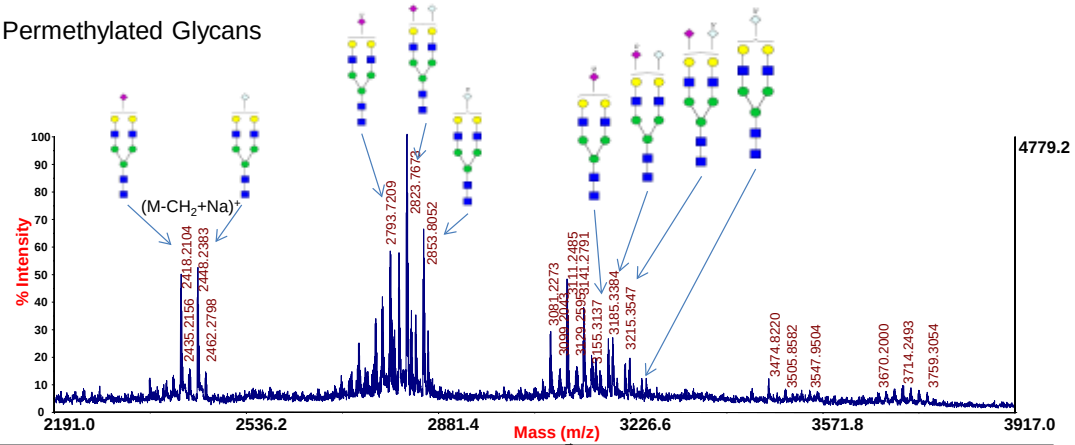
- HILIC separations have utility in glycoscience applications
- Polar interactions for glycans, glycopeptides and glycoproteins lead to useful separations
- Materials and methods of use for such separations are improving, but use remains complex

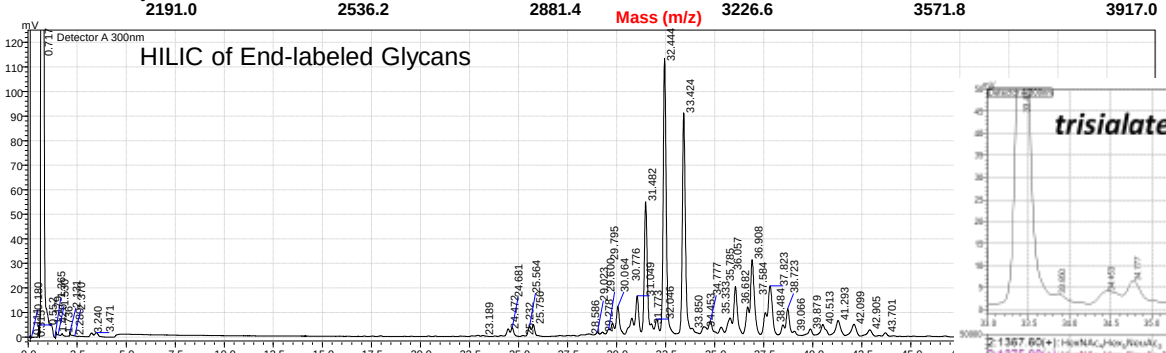
HILIC for Glycoscience: Oligosaccharides

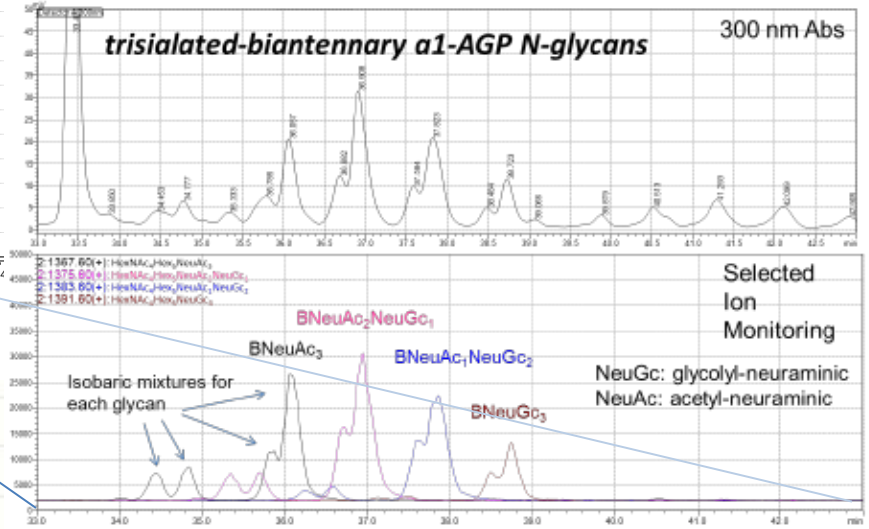
Penta-HILIC Separations of Abundant bov. a1-AGP N-glycans

2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 mL/min.

MALDI of Permethylated Glycans







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Peptide Separations by HILIC

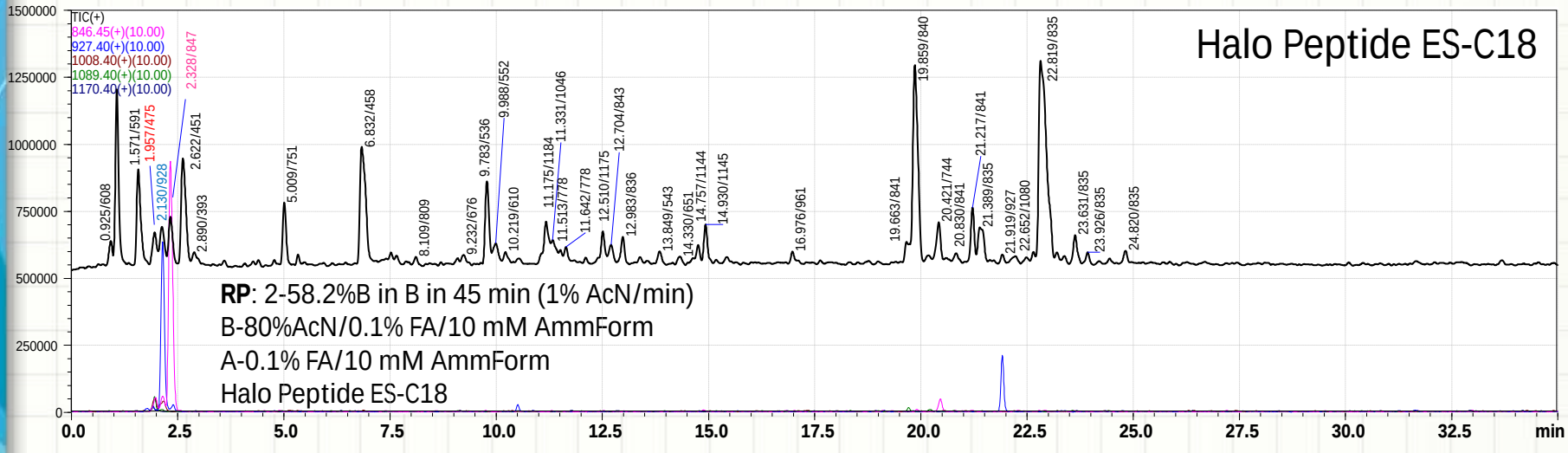
Generalities:

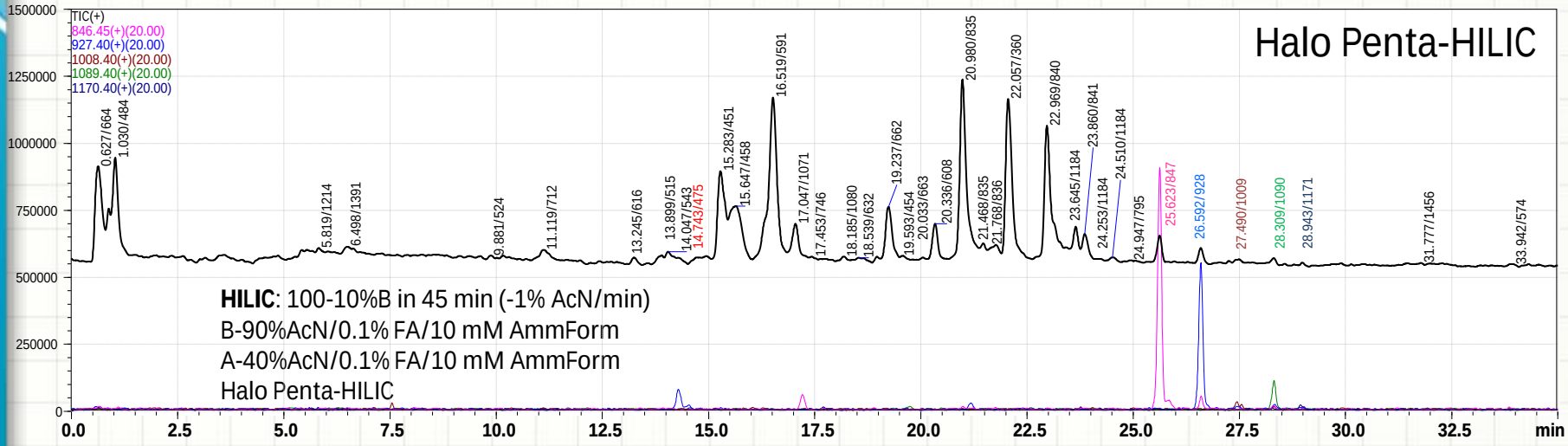
- acidic conditions (TFA, FA, HOAc, Phosphoric, etc.)
- can exhibit orthogonality in retention, relative to RP
  - M.Gilar, et al., Anal. Chem. 82 (2010) 265, and more
  - Boyes, et al., ISPPP 2011
- do not have to be at a disadvantage for efficiency
- can be very MS friendly
- can show some solubility problems
- additional “polar modes” of operation are possible, eg., ERLIC, HILIC/SALT
- exhibit useful recognition of polar PTMs, compared to RP
- can allow separations that are very difficult using RP

Penta-HILIC Strongly Retains N-linked Glycopeptides

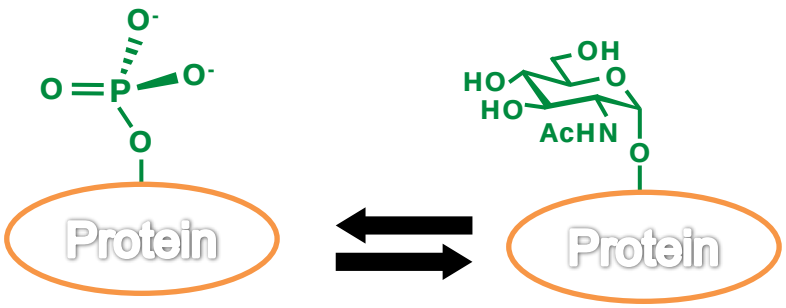
2.1 mm ID x 100 mm, 0.35 mL/min, 40 C, MS: SQ TIC (+ 300-2000 m/z) @ 0.35/s

20 µg Bovine Ribonuclease B tryptic digest (CAM)



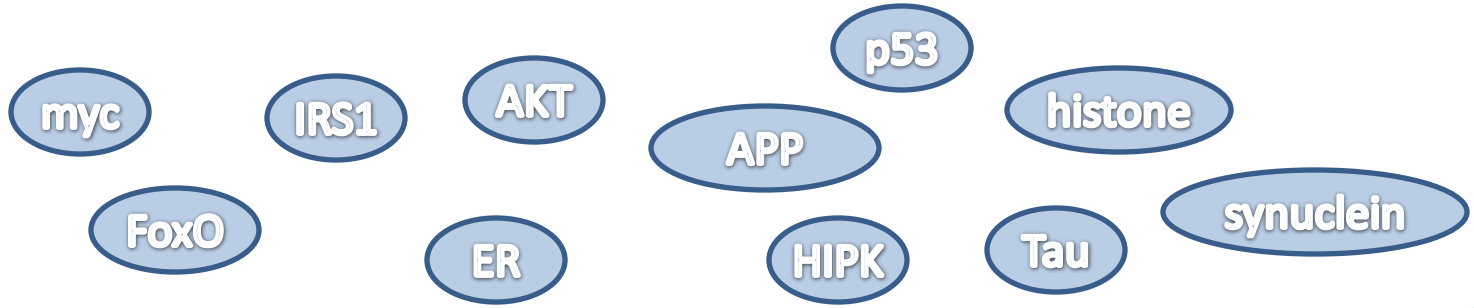


N-acetylglucosamine are What?



O-GlcNAc modifies Ser and Thr. O-GlcNAc is a modifier of biological activity, in some cases, via competition for phosphorylation. Multiple sites on a particular protein can be modified by –P or –GlcNAc, close by, or far apart.

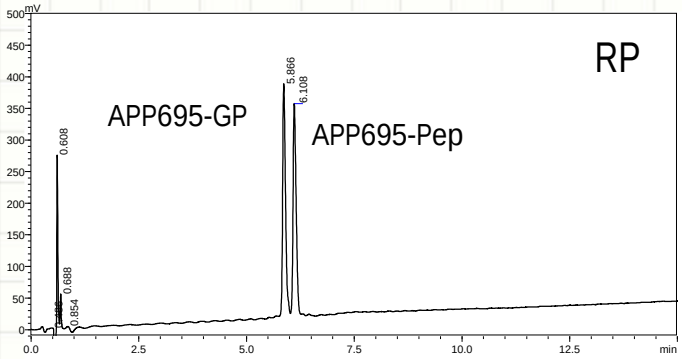
Hundreds of proteins that are implicated in the progression of diseases such as cancer, diabetes and neurodegeneration are modified by O-GlcNAc.

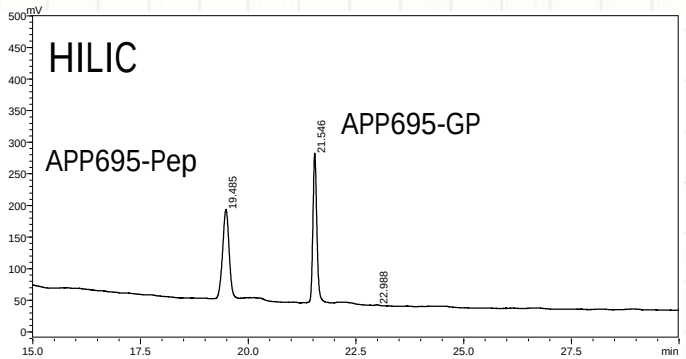


LC/MS of O-GlcNAcylated Peptides

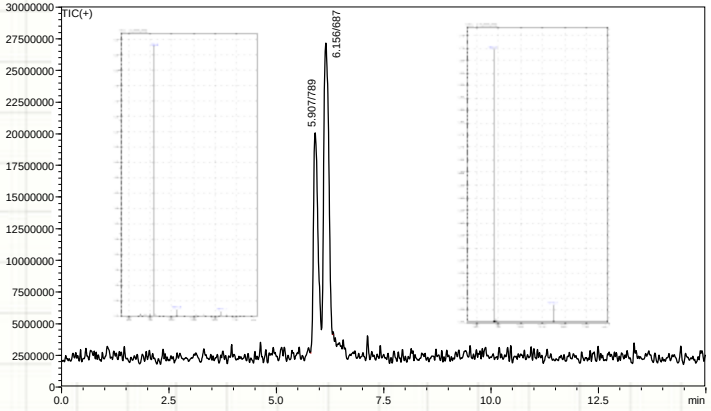
2.1 x 100 mm Halo Penta-HILIC, 0.4 mL/min at 60°C.
Gradient conditions: A – 0.1% formic acid/10 mM ammonium formate; B – 90% Acetonitrile in A.
500 pmol, MS operated +4.5 kV, with 0.45 s scan from 500 to 2000 m/z (TIC)
RP Gradient - 4% to 34% AcN/30 min (1%/min); HILIC Gradient - 90% to 60% AcN/30 min (-1%/min).

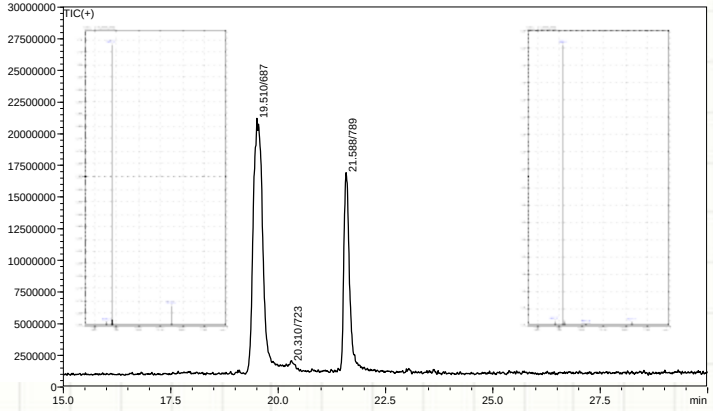
A_{210}





MS





APP695-14GPep	**VPTT(OGlcNAc)AASTPDAVDK**
APP695-14Pep	**VPTTAASTPDAVDK**

Protein Separations by HILIC

- Limited use relative to RP
  - Tetaz, et al., J. Chromatogr. 1218 (2011) 5892.
  - Carrol, et al., P.N.A.S. U.S.A. 103 (2006) 16170.
  - Focused on membrane proteins, histone and lipoprotein PTM modifications
- Expected to show very different selectivity than Reversed Phase separations of proteins
- Challenge of using high organic mobile phases for proteins
- Not clear what features are needed to improve intact protein separations – mobile phase, additives, stationary phases, or combinations of all

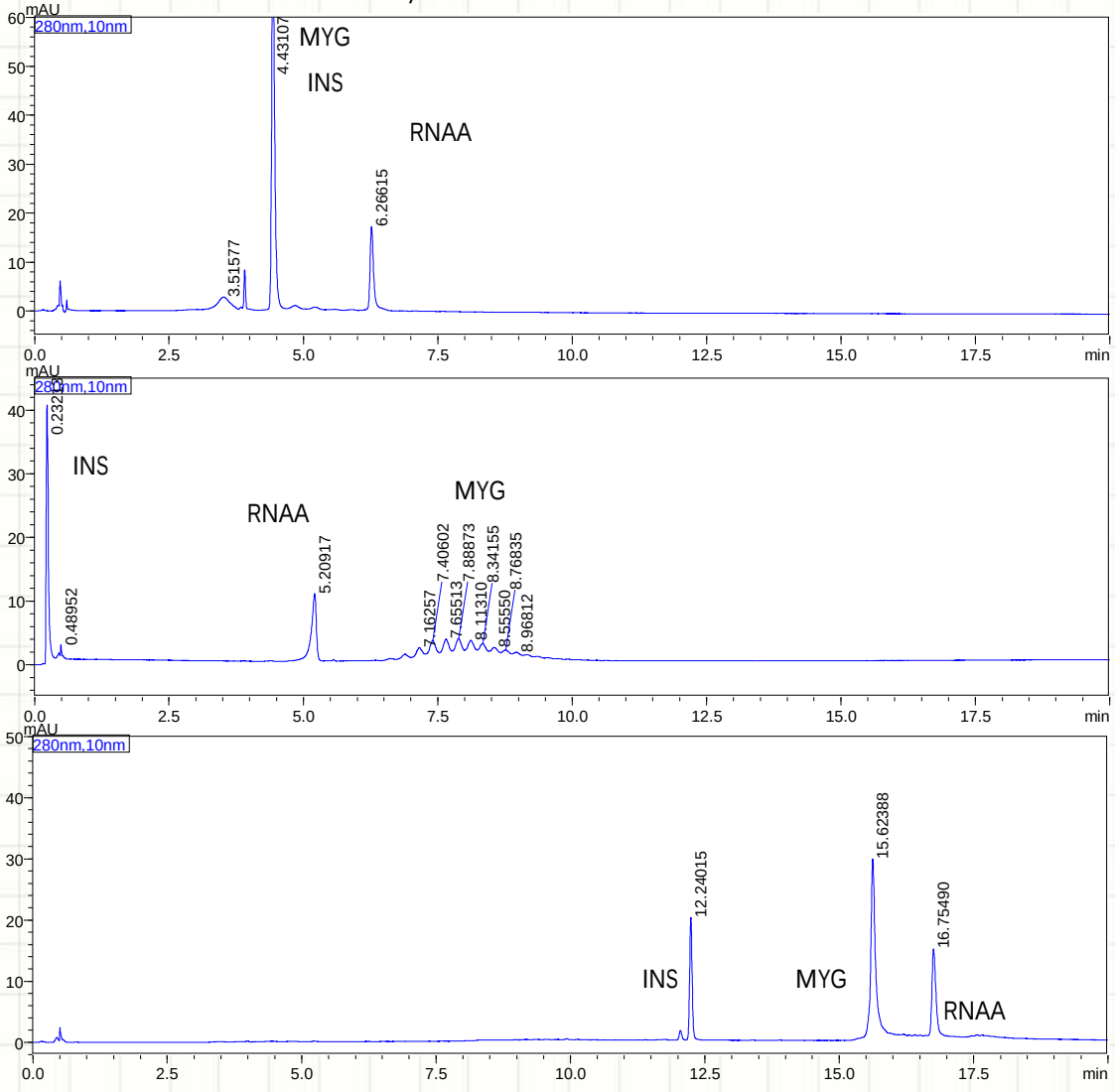
This could be a longer term project!

Operational Considerations

- Mobile phases components:
  - TFA, Formic Acid, FA/AF (0.05%-1%)
  - Acetonitrile, *i*-propanol, *n*-propanol
  - HFIP, TFE, others
- Stationary phase characteristics:
  - 2.7 µm Halo Penta-HILIC
    - hydroxylated neutral HILIC bonded phase
    - Fused-Core particle with 0.25 um SPP layer
  - 90 Å and 160 Å pore size
- Aqueous soluble proteins and glycoproteins

Effect of Acid Modifier

2.1 x 100 mm Halo Penta-HILIC 90Å, 0.5 mL/min at 50°C.
Gradient conditions: A – 0.1% acid; B – 90% Acetonitrile in A. Gradient - 90% to 50% AcN/20 min (-2%/min).



0.1% TFA

0.1% FA

0.1% FA/10 mM AF

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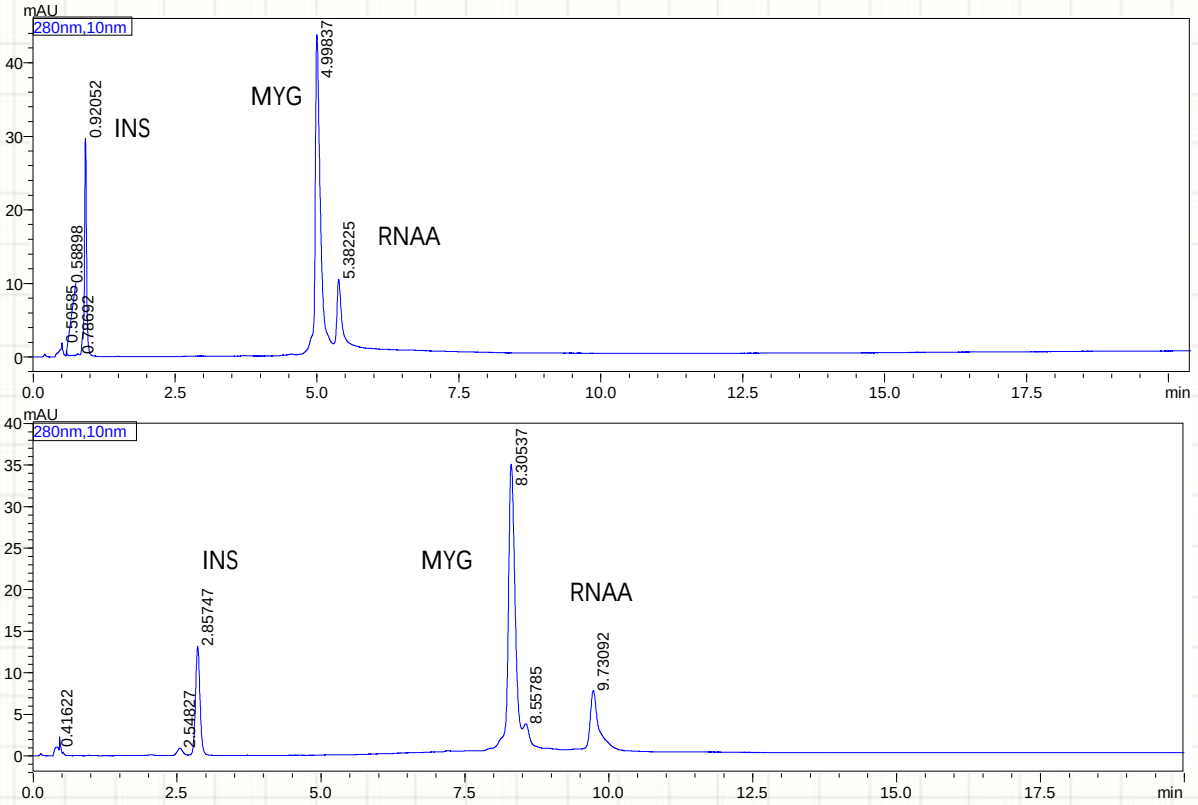
Current Consensus MP & Pore Size

- FA/AF buffer (0.2%/20 mM) with 0.5% HFIP (does not HURT!)
- Mixture of Acetonitrile/*i*-Propanol (40%/35%) to 10% iPA
- Elevated column temperature: 40-60°C
- Pore Size not a dominant factor for smaller proteins: use with these conditions *sometimes* works
- Adjusting sample is crucial – addition of FA to 1-2% resolves many solubility problems

2.1 x 100 mm Halo Penta-HILIC, 0.5 mL/min at 50°C; Gradient conditions: B – 0.2% FA/20 mM AF/40% AcN/35% IPA/ 0.5% HFIP; A – 0.2% FA/20 mM AF/10% IPA Gradient - 90% to 55% B in 20 min.



160 Å

90 Å

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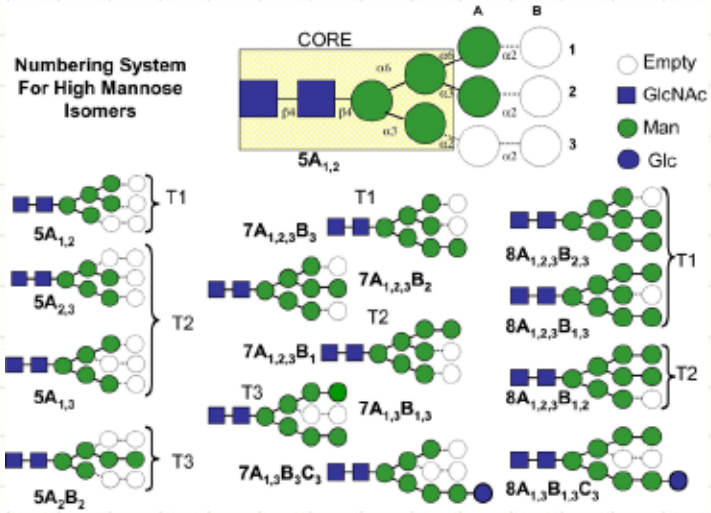
Bovine Ribonuclease as a Glycoprotein

- Composition:
  - RNase A - 124 aa residues, 4 disulfide bridges, 13.7 kDa
  - RNase B – Asn^{34} is the site of glycosylation, with 5 high mannose structures M5-M9 , leading to enzyme heterogeneity, 14.9-15.5 kDa
  - In addition to compositional heterogeneity, isomers of the high mannose oligosaccharides M5, M7, M8 are indicated

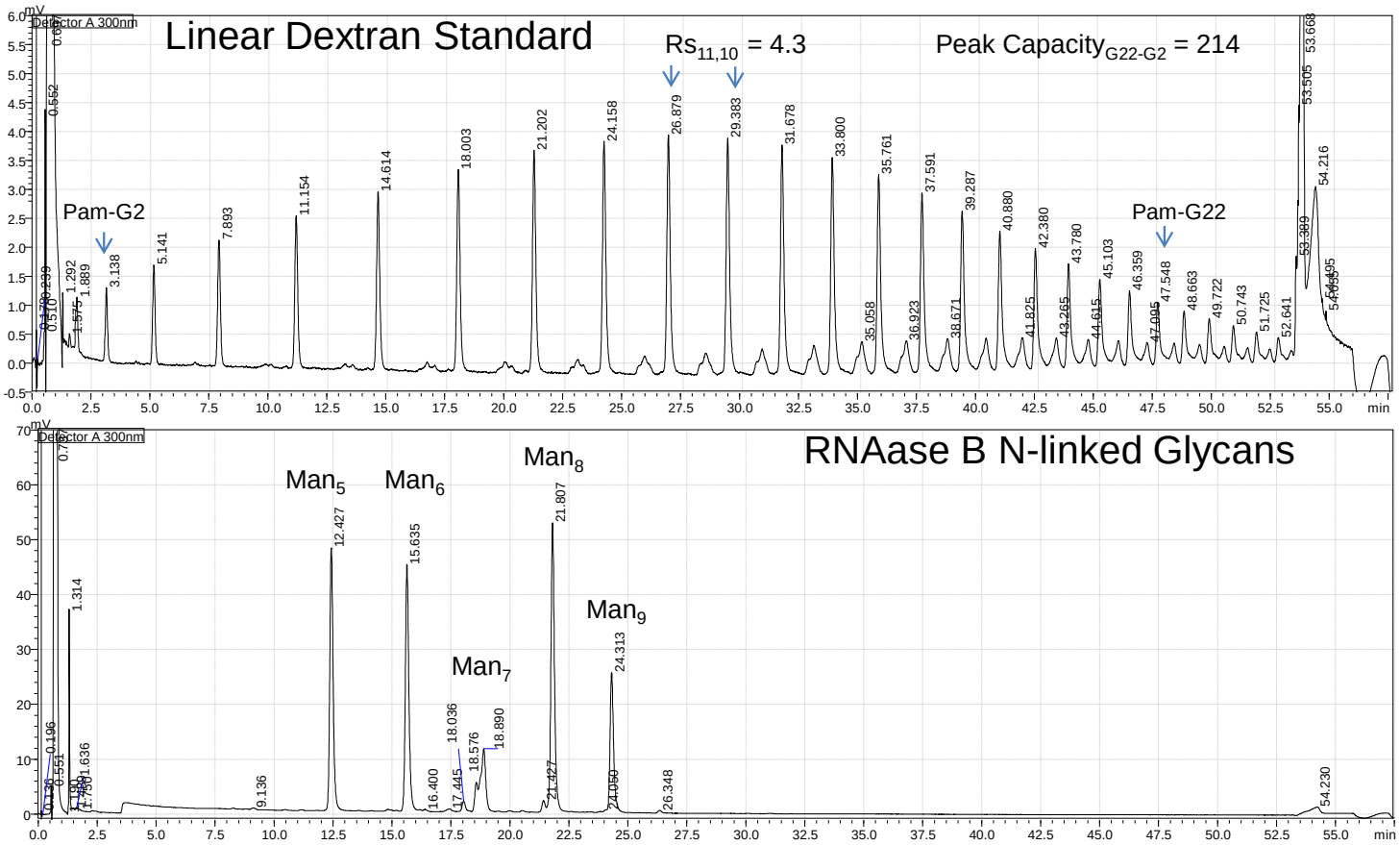


Prien, et al., J. A.S.M.S. 20 (2009) 539

Separations of RNase B Glycans

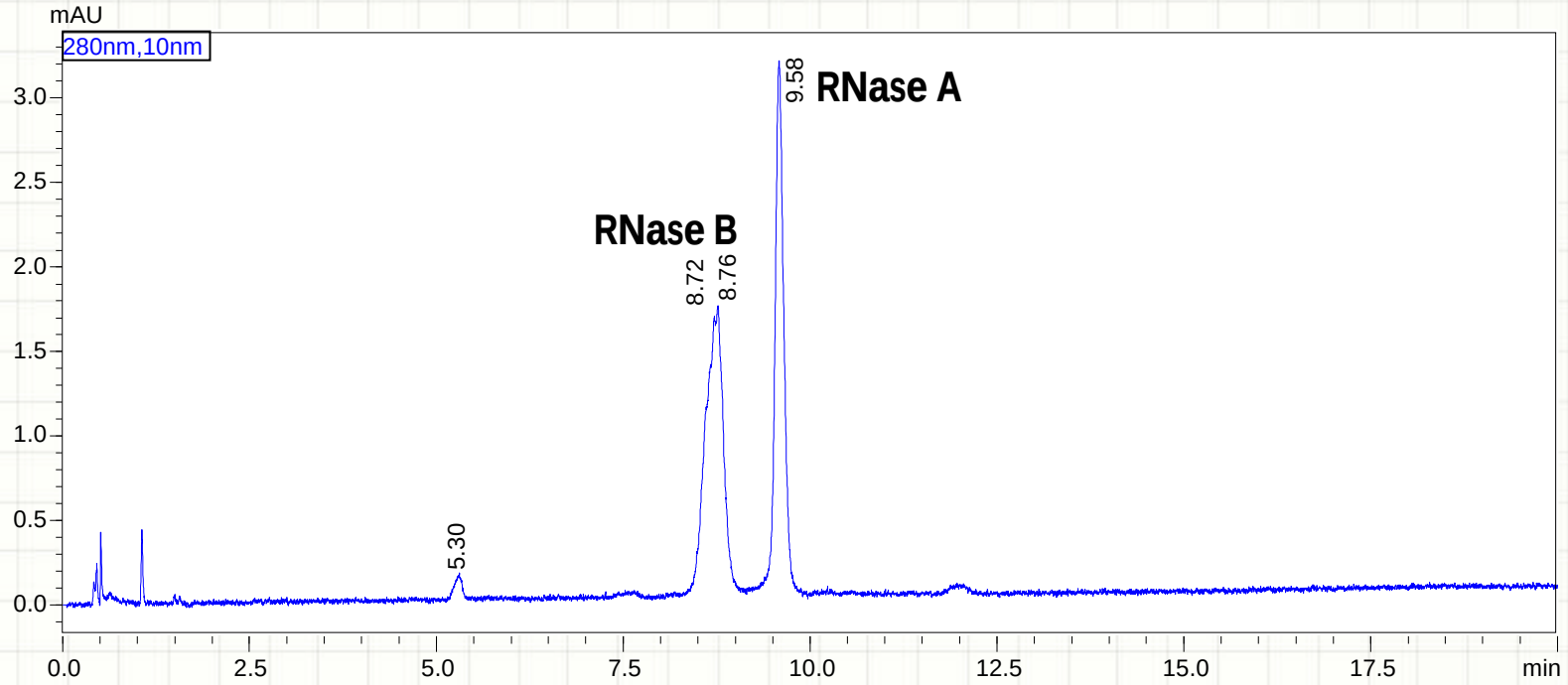
Penta-HILIC Separations of Labeled Oligosaccharides and Glycans

2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 mL/min.
Detection: 300 nm Abs; ESI-MS (MS-2020, (+) 4.2 kV, 400-2000 with SIM)



Separation of RNases by RP

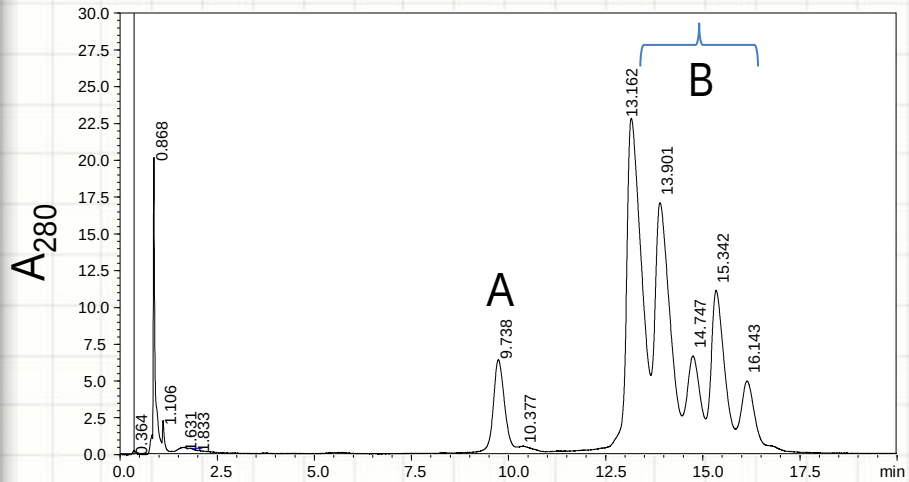
2.1 x 100 mm Halo Peptide ES-C18, 0.5 mL/min at 50°C; Gradient conditions: A – 0.1% TFA/water, B – 0.1% TFA in 80% AcN; Gradient - 30% to 40% B in 20 min (0.4% AcN/min), 0.5 µg each.

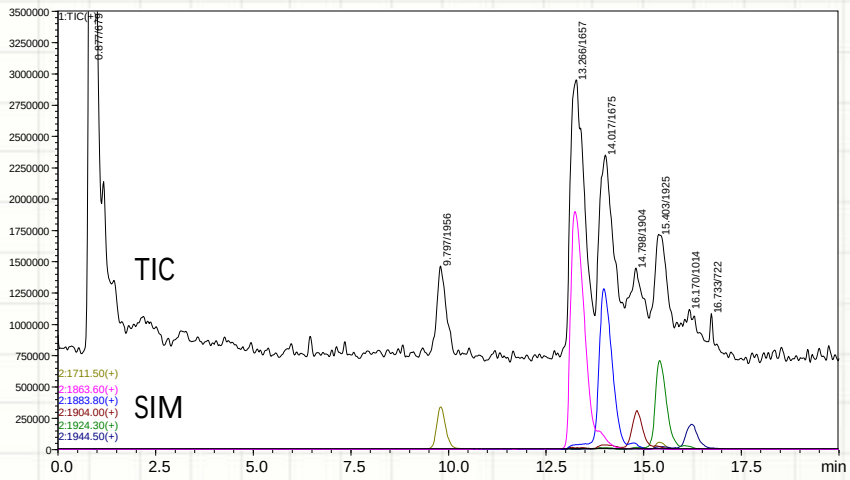


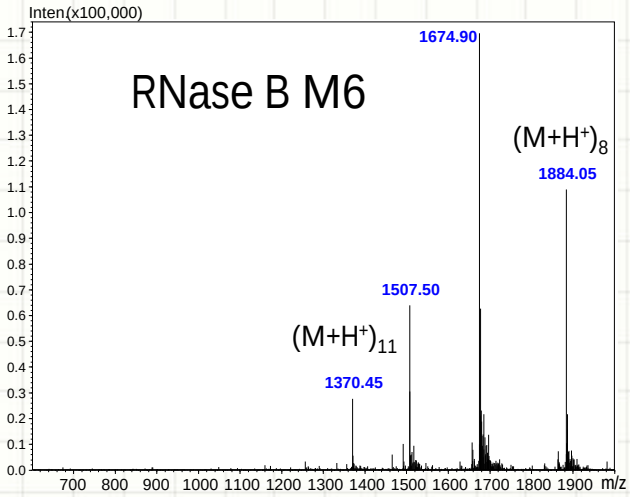
Some resolution of A/B, little resolution of B glycoforms

HILIC Separation of RNase B Glycoforms

2.1 x 100 mm Halo Penta-HILIC, 0.35 mL/min at 50°C; Gradient conditions: B – 0.2% FA/20 mM AF/40% AcN/35% IPA/ 0.5% HFIP; A – 0.2% FA/20 mM AF/10% IPA Gradient - 85% to 75% B in 20 min.





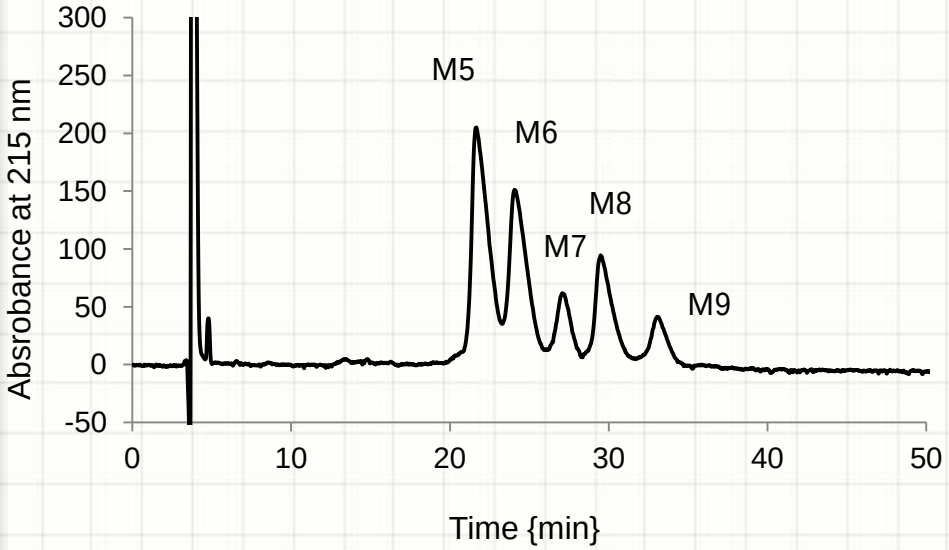


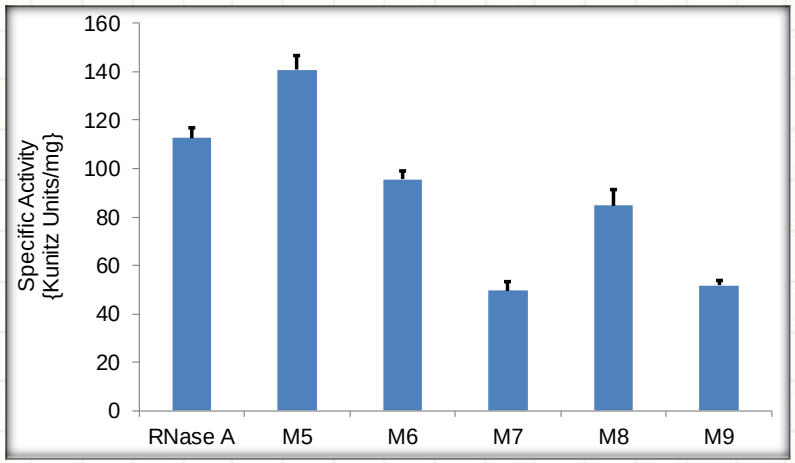
ID	Mass (Da) Theory	Mass (Da) Observed
RNA A	13,681	13,686
RNA B M5	14,898	14,903
RNA B M6	15,060	15,068
RNA B M7	15,222	15,227
RNA B M8	15,384	15,390
RNA B M9	15,564	15,550

Isolated RNase B Glycoforms

Separation Conditions: 4.6 x 250 mm Penta HILIC 2.7 µm 90Å, 60 C, 0.4 mL/min, 215 nm, 78-75 % B over 60 min, B – 40% AcN/35% IPA/0.2%FA 20 mM AF/0.5% HFIP

Assay Conditions: 50 mM NaOAc, pH 5.0, 25 C, 0.5 mg/mL RNA, ΔA_{300}





More on Glycoproteins

- Protein structure contributes to retention
- Mass and nature of glycans will contribute
- Few glycoproteins are as simple as RNase
- LC/MS resolution requirements increase a lot with size
- MS Sensitivity is a problem as size and glycosylation rise

TABLE 3

Characteristics of Glycoproteins Chosen to Test the Organic Solvent Precipitation/Extraction Methodology

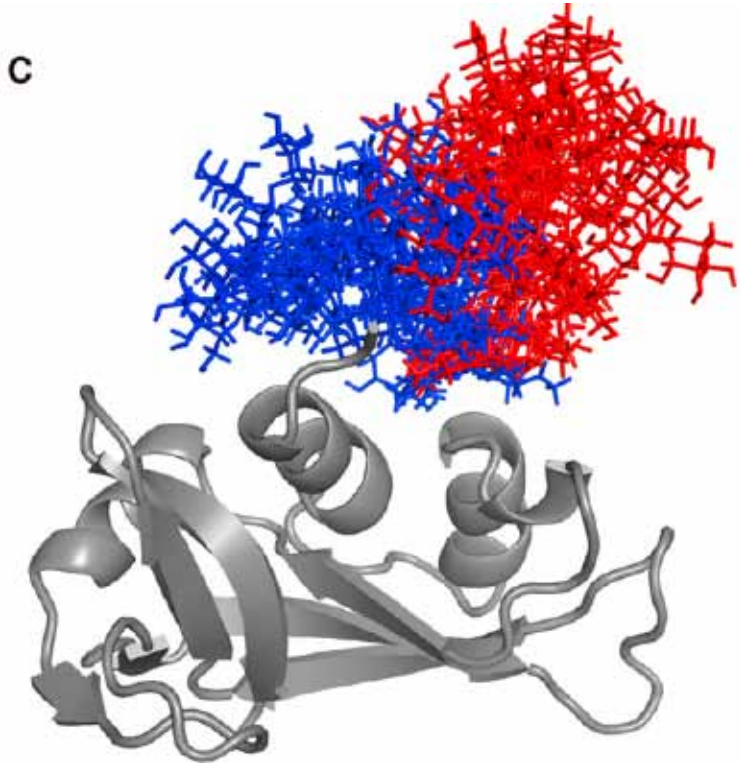
Glycoprotein	Source	Monomer (kDa)	Native form	CH_2O[a] Content (g/100 g)	Oligosaccharides/monomer: N-linked: High mannose	Oligosaccharides/monomer: N-linked: Complex	Oligosaccharides/monomer: O-linked	Amount treated (mg)
RNase B	Bovine	13	Monomer	11	1	0	0	1, 20
Ovalbumin	Hen	43	Monomer	3	1	0	0	5 to 60
Invertase	Wt yeast	60	Octamer	50	9–10	0	0	20
	Sec18 yeast			28	9–10	0	0	10
Thyroglobulin	Bovine	330	Dimer	9	5–6	13–14	0	20, 40
Fetuin	Bovine	48	Monomer	23	0	3	3	40
BSSL	Human/*P. pastoris*	76.3	Dimer	16	0.7	0	27	65, 80, 100

[a] Carbohydrate.

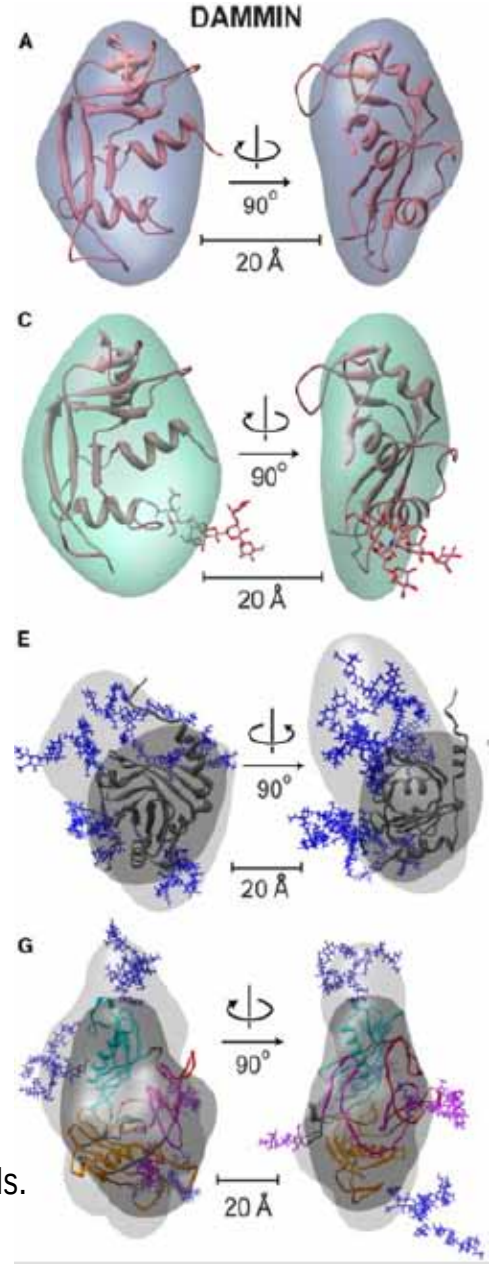
Verostek, et al., Anal. Biochem. 278 (2000) 111.

Protein and Glycoprotein Surfaces

Guttman, et al., Structure 21 (2013) 321.



RNaseA (A and B),
RNaseB (C and D),
a1AGP (E and F),
and Fetuin (G and H)



Glycan positions are shown for the ten best (red) and poorest (blue) fitting models.

Conclusions

- HILIC separations methods have impact in glycobiology at the glycan, glycopeptide and glycoprotein structure level.
- Initial results with intact protein and glycoprotein LC/MS separations show promise, with complexity (SP, MP, use condition development)
- It took effort for RPC to mature, and HILIC will be the same
- Selectivity for glycan separations, in the context of peptide and protein structures, appears excellent
- RNase B glycoforms can be well resolved, under conditions that retain activity

Thank you for your attention!

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