

Current Trends of O-linked Glycan Release Methodology

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Hui Zhou

Department of Chemistry
The University of New
Hampshire

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N-glycan Release

- N-glycosylation can be release enzymatically by PNGase F for long incubation time
- This enzyme reaction can be accelerated by microwave irradiation; N-glycans can be release efficiently in 30 minutes by microwave reactor¹

Current O-release Techniques

- Hydrazinolysis
- Classical NaOH/NaBH₄
- Borate-ammonia complex
- In-line flow technologies
- AutoGlycoCutter (AGC) equipment
- Ammonia-base release
- Microwave-assisted dimethylamine release

Enzymatic O-glycan Release

- “No universal O-glycanases available”
- A commercial available O-glycosidase only removes the minimum core-1 moiety: Gal(β 1-3)GalNAc; Not useful for general O-glycan release

The Only Option: Chemical Release

Two common chemistry

- Hydrazinolysis (anhydrous hydrazine)
- Alkaline β -elimination
 1. Strong and solid base (NaOH/KOH)
 2. Mild and volatile base (NH₄OH or dimethylamine, or other organic base)

Hydrazinolysis

Feature and Benefits

- Be able to release *O*-glycans (~5 hrs @ 60 °C) and *N*-glycans (~4 hrs @ 90 °C) sequentially¹
- Obtain free reducing end glycans
- No biological constraints; especially good for new and unknown samples²

Disadvantages

- Need an extra step to re-*N*-acetylation; may loss some structural information in some cases
- Totally destroy protein backbone
- “Peeling” unavoidable (especially for *O*-glycans)

1. Patel, T.P. *et al. Meth. Enzymol.* **1994**, 230, 57-66

2. Hanneman, A.J. *et al. Glycobiology* **2006**, 16, 874-890

Alkaline Reductive β -elimination (Classical Carlson Method)

Feature and Benefits

- Usually 0.05 to 0.1M NaOH solution under mild condition (45 – 60 °C for 16 to 8 hrs)
- To prevent “peeling”, 1 to 2M NaBH₄ reducing agent is added
- Simple procedure and widely used

Alkaline β -elimination

(Reductive elimination, continued)

Disadvantages

- High salt residue left after reaction; not practical for small amount proteins
- Glycans are converted to "alditol"; not be able to attach any other functional groups
- Damage of the protein backbones
- Small amount *N*-glycans may be released in some cases
- Long reaction time

Alkaline Reductive β -elimination (Borate-ammonia Approach)

- 5 mg/mL of borate-ammonia complex in 28% Ammonia solution (45°C for 18 - 24 hrs)¹
- Volatile reagents; desalting step may be skipped;
- Possible to obtain high sensitivity
- Release is not efficiently in some cases²

1. Huang, Y.P. *et al. Rapid. Commun. Mass Spec.*, **2002**, 16, 1199-1204

2. Tarelli, E. *Carbohydr. Res.* **2007**, 342, 2322-2325

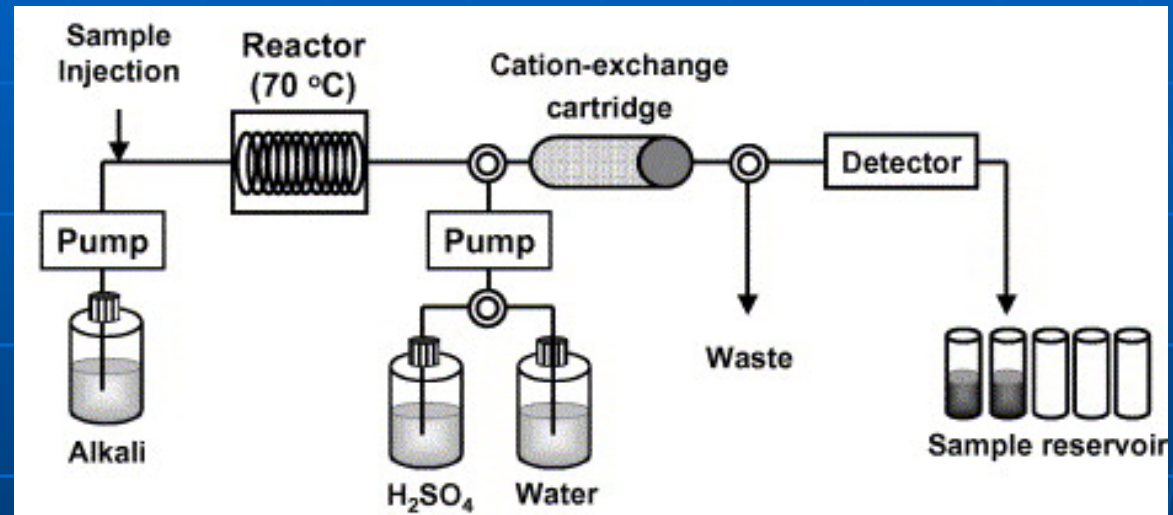
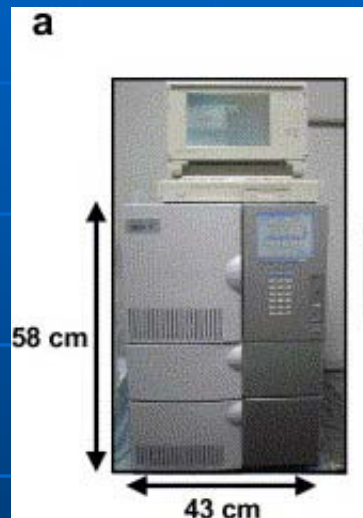
Alkaline Nonreductive β -elimination (In-line flow Approach)

The major goal is to obtain free reducing end glycans!

- PVDF membrane: glycoprotein adsorbed onto membrane; the alkaline flow-through with glycans, quickly neutralized with acetic acid; further workup offline needed (Oh-eda et al., 1996)
- Poros R2 beads: glycoprotein adsorbed onto beads, alkaline flow-through neutralized by cation exchange, with glycan trapping and cleanup on PGC cartridge (Karlsson et al., 2002)

1. Oh-eda, M. *et al. Analytical Biochemistry*, **1996**, 236, 369-371
2. Karlsson, N.G. *et al. Analytical Biochemistry*, **2002**, 305, 173-185

Alkaline Nonreductive β -elimination (New technique: AutoGlycoCutter: AGC)



- 0.5 M LiOH solution; 10m length reaction coil;
- 0.5 ml/min flow rate; 60 °C; UV detection 230 nm
- complete the reaction in 3 minutes; free reducing end glycans
- less peeling; very quick reaction; special instrument
- yield lower than classical NaOH/NaBH₄ method; loss protein

1. Yamada, K. *et al. Analytical Biochemistry*, **2007**, 371, 52-61
2. Matsuno, Y.K. *et al. Analytical Biochemistry*, **2007**, 362, 245-257

Alkaline β -elimination (Non-reductive elimination: Ammonia Base¹)

- 28% ammonia solution sat. with ammonium carbonate, the released glycans are protected as the base-stable glycosylamine-carbonate mixture.
- Maximize the sensitivity by using volatile reagents
- Produce the "Free" reducing glycans
- Time consuming: 40 hours incubation
- No information has been reported on protein recovery

Ideal Goals of O-release Methodology

- Keep intact “Free” reducing end
- Minimize/prevent “Peeling”
- Short reaction time
- No-destruction on the protein backbone during *O*-glycan release reaction

Alkaline β -elimination (Nonreductive elimination: microwave- assisted dimethylamine-base)

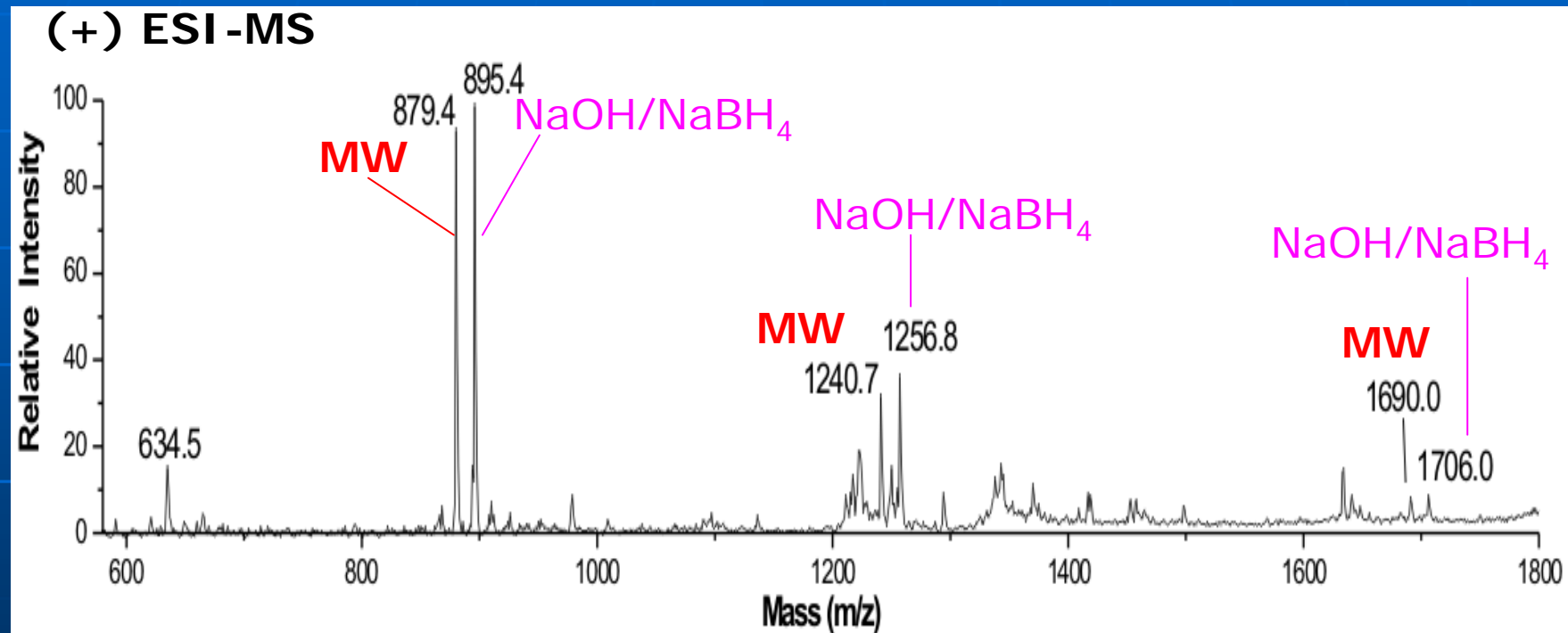


CEM Discover Microwave Reactor

- Dry glycoproteins dissolved in 0.5 mL dimethylamine (40%, aqu. Solution)
- Add small amount ammonium carbonate
- A tiny stir bar
- Ramp 2 mins to 70°C, and hold at 70°C for 25 mins

Release Efficiency

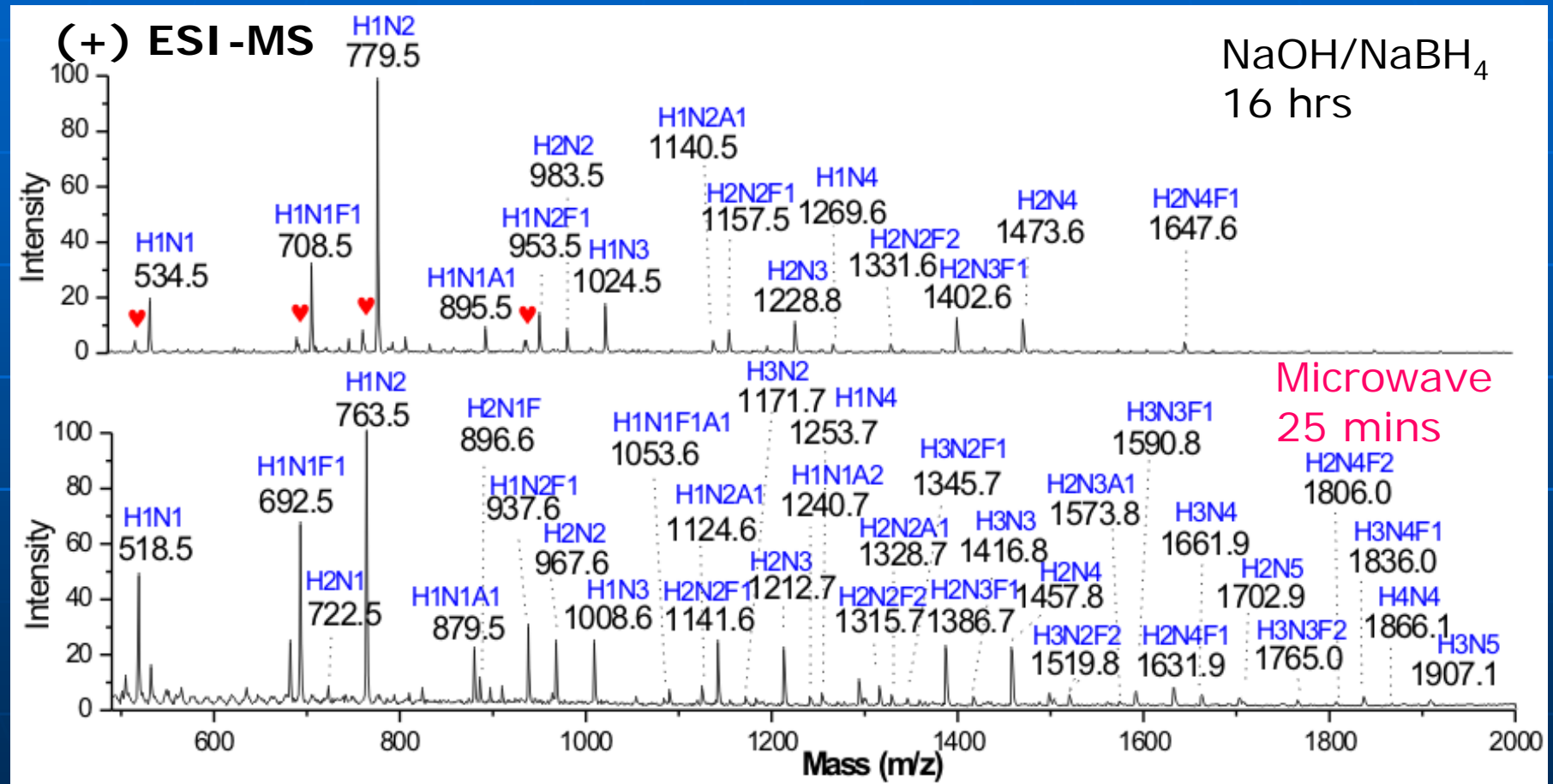
Microwave-assisted comparable to NaOH/NaBH₄ method



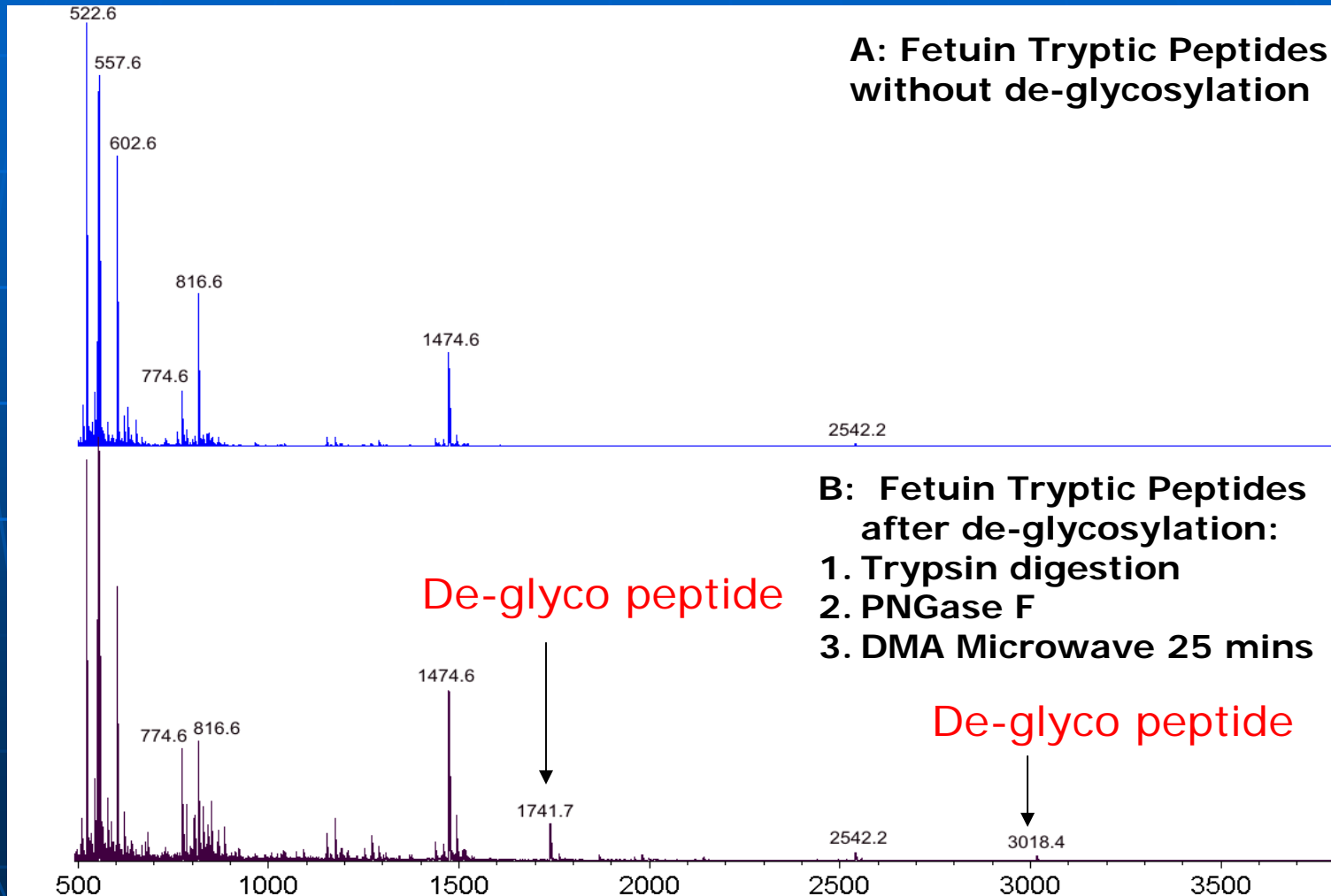
1:1 mixture of glycans from 1 mg fetuin either method
Microwave 25 mins : NaOH/NaBH₄ 16hrs
then permethylation (mass difference 16 Da)

Around 92-94% yield compare to NaOH/NaBH₄ method

Highly O-glycosylated Model: Porcine Stomach Mucin



Fetuin Tryptic Peptides MALDI-MS Comparison



Fetuin Tryptic Peptides MALDI-MS Table

Peptide #	Peptide Sequence	AA	Peptide Mass [Da]	A Native	B De-glyco	Peptide #	Peptide Sequence	AA	Peptide Mass [Da]	A Native	B De-glyco
T1	IPLDPVAGYK	10	1072.3		X	T14	AQFVPLPVSVSVEFAVAATDCIAK	24	2519.3	X	X
T2	EPACDDPDTE QAALAAVDYINK	22	2406.1	X	X	T15	EVVDPTK	7	786.9		
T3	HLPR	4	521.6	X	X	T16	CNLLAEK	7	847.4		
T5	HTLNQIDSVK	10	1154.3	X	X	T17	QYGFCK	6	802.4		
T6	VWPR	4	556.7	X	X	T18	GSVIQK	6	630.7	X	X
T7	RPTGEVYDIEIDT LETTCHVLDPTPL A <u>N</u> CSVR	32	3671.8			T19	ALGGEDVR	8	815.9	X	X
T8	QQTQHAVEGD CDIHVLK	17	1977.9			T20	VTCTLFQTQPVIP QPQPDGAEAEAP <u>S</u> AVPDAAGP <u>TPS</u> AAGPPVASVVVG P <u>S</u> V VAVPLPLHR	61	5960.8		
T9	QDGQFSVLFTK	11	1269.4	X	X	T21	AHYDLR	6	773.8	X	X
T10	CDSSPDSAEDVR	12	1337.5			T22	HTFSGVASVES SSGEAFHVGK	21	2120.3	X	X
T12	LCPDCPLLAPL <u>N</u> DSR	15	1740.8		X	T23	TPIVGQPSIPGG PVR	15	1474.7	X	X
T13	VVHAVEVALAT FNAES <u>N</u> GSYLQL VEISR	28	3017.4	X	X	T24	LCPGR	5	602.3	X	X

A: native peptides without de-glycosylation
B: De-N- and De-O-glycosylation

Microwave-assisted O-release Methodology

- Fast reaction time: 30 minutes
- Minimize “Peeling”;
- Intact “Free” reducing end; good for subsequent labeling
- recover protein/peptides sequence
- Enable the comprehensive strategy to release *N*-glycans (by enzyme), *O*-glycan (by MW-assisted) and recover peptides for peptide mass fingerprint