Microwave-Assisted Nonreductive Release of *O*-linked Glycans: A Method with Numerous Analytical Advantages

Hui Zhou, David J. Ashline, and Vernon N. Reinhold

the Glycomics Center, Department of Chemistry, UNH, Durham, NH 03824

ASMS 2007
UNH Topics

MP 210: Microwave-assisted O-glycan release (Zhou)

MP 211: Glycosylation biomarkers of malignancy (Prien)
TOE (3:50): Ion traps and glycoproteomics (Reinhold)
WOB (8:35): Identification of unique N-glycan structures by MS^n (Ashline)
WP 288: F-tag on fungal glycosphingolipids (Arigi)

OVERVIEW

PURPOSE:

- To develop a nonreductive *O*-linked glycan release method

METHODS:

- Dimethylamine (DMA)-based β-elimination in microwave-reactor

RESULTS:

- *O*-linked glycan from bovine fetuin could be release efficiently in 25 minutes without "peeling" products

INTRODUCTION

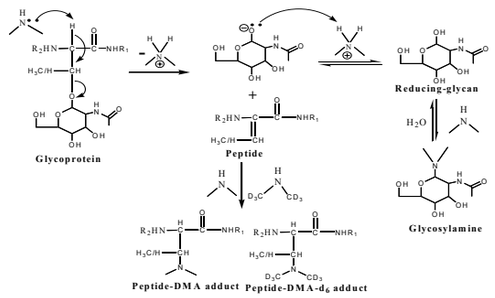
O-glycosylation is an important posttranslational modification of eukaryotic proteins. It has been demonstrated that *O*-glycosylation plays indispensable roles in many biological processes, such as molecular adhesion, signaling transduction, and recognition. Modulation of these structures has also been reported in cancer cells and may be a potential biomarker for early diagnosis. The characterization of *O*-linked glycans has remained more challenging due to the lack of generic releasing enzyme. Chemical E_2-elimination with strong base (NaOH) remains an alternative but requires *in situ* reduction with $NaBH_4$, converting the released hemiacetal to an alditol[1]. Unfortunately, this prevents other functional groups attachment, a requirement for sensitive separation by HPLC or CE or carbohydrate microarray assay. Hydrazinolysis, H_2N_2, requires special equipment to implement safely and also removes native *N*-acetyl groups[2]. Ammonium-based β-elimination procedures have been introduced to salvage intact the reducing end[3]. Unfortunately, all of these procedures also release *N*-linked glycans and exhibit significant "peeling" products. Herein we introduce a more specific strategy using molecular excitation with electromagnetic (microwave) radiation which completes the release in a few minutes. Importantly, this shorter time avoids the slower "peeling" processes which could be detected at lower mass in our earlier DMA-based β-elimination approach[4].

MATERIALS AND METHODS

- Bovine fetuin and porcine stomach mucin (Sigma) were used as model glycoproteins.
- Microwave-assisted reactions were carried out using a Discover Labmate microwave (CEM Inc., Matthews, NC). Dry glycoproteins were dissolved in 0.5 mL of 40% aqueous dimethylamine solution with small amount of ammonium carbonate in a 10-mL Pyrex glass sample holder. A small stirring bar equalized heating during the reaction. The reaction temperature was set at 70 °C and 2 mins was the maximum ramp time to reach 70 °C. Once the reaction was complete, (usually under 30 min), the reagents were removed by repeated evaporation under a stream of nitrogen gas. When no visible residue was observed, the reaction mixture was dissolved in 1mL of water, and pass through a hand-made PGC cartridge (alltech, Deerfield, IL). After washing with 3 mL of water, the glycans were eluted with 3 mL of 25% ACN in 0.1% TFA solution. Classical release with $NaOH/NaBH_4$ as published.[1]
- ESI-MS was performed on an LTQ (ThermoFinnigan, San Jose, CA) equipped with a Triversa Nanomate (Advion, Ithaca, NY).
- MALDI-MS were performed on an Kratos AXIMA-CFR instrument (Shimadzu) with DHB as matrix.

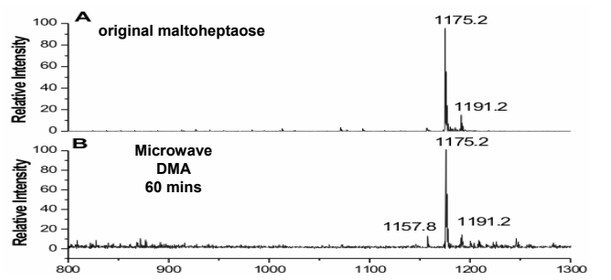
RESULTS AND DISCUSSION

Scheme 1. Proposed mechanism for DMA-based β-elimination and its following modification on glycan and peptides residues. R_1 and R_2 are amino acids representing the peptides.



Control test for peeling products using Glu_7 in microwave reactor under reaction conditions

Figure 1A. MS spectrum of the original maltoheptaose; **1B**. MS spectrum of maltoheptaose subjected to DMA/ammonium carbonate reaction in microwave-reactor for 60 minutes



For highly *O*-glycosylated mucin, microwave-assisted method provide high sensitivity

Figure 4. Comparative (+) ESI-MS spectra of methylated *O*-glycans released from 1 mg porcine stomach mucin using $NaOH/NaBH_4$ (**4A**), and microwave-assisted DMA-based method (**4B**), 25 mins. (♥: non-reduced residue; H: Hexose; N: HexNAc; F: Fucose; A: sialic acid)90

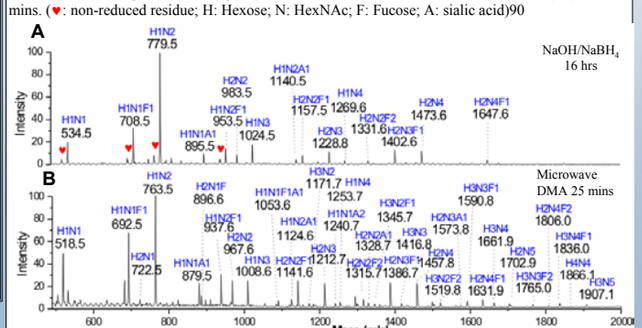


Figure 2. Comparative release of *O*-glycans from bovine fetuin by three different methods: **A.** $NaOH/NaBH_4$; **B.** NH_3-based; **C.** DMA-microwave radiation for 25 mins; **D.** DMA-microwave radiation for 50 mins.
Results: For this glycoprotein, condition **C.** for 25 mins provided the highest abundance of *O*-linked products without visible "peeling" products, and is comparable to Carlson's classical $NaOH/NaBH_4$

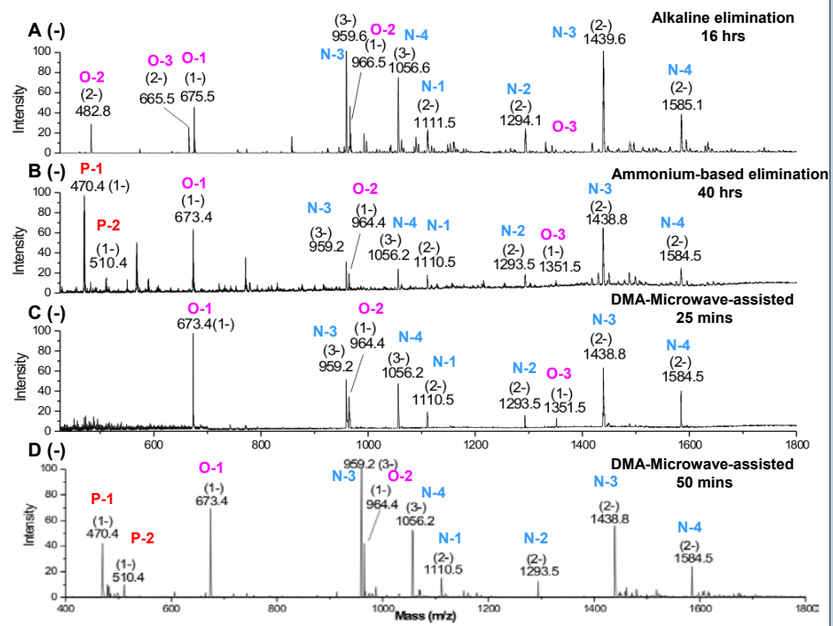
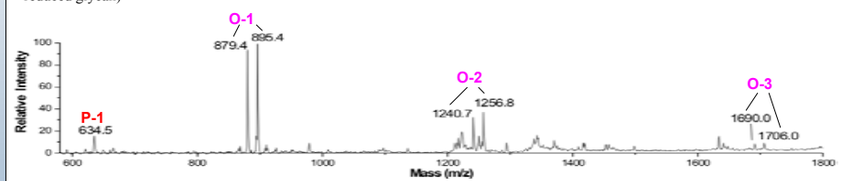


Figure 3. (+) ESI-MS of permethylated glycans from fetuin glycoprotein. 1:1 mixture of non-reduced and reduced glycans from 25 mins of microwave-assisted DMA-based release and classical alkaline release. (16 Da mass difference between non-reduced and reduced glycan)



Scheme 2: Bovine fetuin *O*-linked glycans, *N*-linked glycans and "peeling products" of *O*-linked glycans

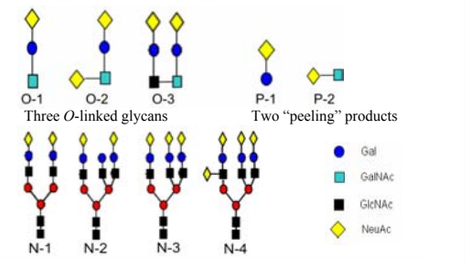


Table 1: Comparison of three *O*-linked glycan release methods

	O-1	O-2	O-3	P-1	P-2	N-1	N-2	N-3	N-4
Native $[M-1]^-$	673.2	964.3	1329.5	470.2	510.2	2221.8	2586.9	2878.0	3169.1
Red Native $[M-1]^-$	675.2	966.2	1331.5	472.2	510.2	2223.8	2588.9	2880.0	3171.1
PM $[M+Na]^+$	879.4	1240.6	1689.8	634.3	675.3				
Red+PM $[M+Na]^+$	895.5	1256.6	1705.9	650.3	691.3				

	Alkaline-based release	Ammonium-based release	Microwave-assisted DMA-based release
Typical release condition	50 - 100 mM NaOH / 0.8 - 1M $NaBH_4$ 45-55 °C 16 - 24 hours	1mL NH_4OH aqu. solution saturated with $(NH_4)_2CO_3$, 60 °C 40 hours	500 µL DMA aqu. solution with $(NH_4)_2CO_3$, 70 °C ≈30 mins
"peeling"	NO	Significant	Not visible
Reduced/non-reduce	Reduced	Non-reduced	Non-reduced
Solid salt residue	Excess	NO	NO
Time for a single sample	1-2 days	≈ 4 days	<1 day
O-glycosylation site labeling	NO	N/A	Yes; form DMA adduct on glycosylation site[4,5]

Future Work

- Internal standard to measure relative quantification precisely
- *O*-glycosylation site identification by analyses of DMA/DMA-d_6 adduct on peptide portion

References

1. Carlson, D.M. etc. J. Biol. Chem. 1966, 241(12), 2984.
2. Patel, T.P. etc. Methods in Enzym. 1994, 230, 57.
3. Huang, Y.P. etc. Anal. Chem. 2001, 73(24), 6063.
4. Wang, Z. etc. ASMS Poster, 2004, #ThP 031
5. Wang, Z. Reinhold, V.N., unpublished data.

Acknowledgements

Funding provided by NIGMS R01 GM54045 (VNR) is gratefully acknowledged.