



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

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ASMS 2007
UNH Tonic
MP 210: Microwave-assisted O-glycan release (Zhou)
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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

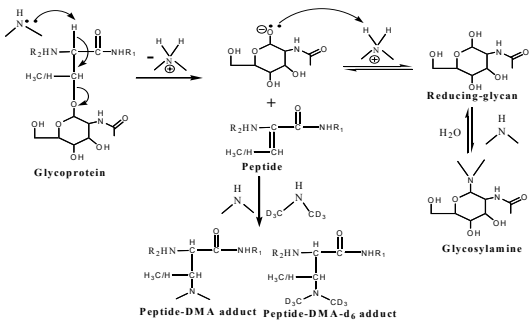
O-glycosylation is an important posttranslational modification of eukaryotic proteins. It has been demonstrated that O-glycosylation plays indispensable roles in many biological processes, such as molecular adhesion, signaling transduction, and recognition. Modulation of these structures has also been 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 E₂-elimination with strong base (NaOH) remains an alternative but requires *in situ* reduction with NaBH₄, converting the released hemiacetal to an alditol¹. Unfortunately, this prevents other functional groups attachment, a requirement for sensitive separation by HPLC or CE or carbohydrate microarray assay. Hydrazinolysis, H₂N₂, requires special equipment to implement safely and also removes native N-acetyl groups². Ammonium-based β -elimination procedures have been introduced to salvage intact the reducing end³. 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 approach⁴.

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/NaBH₄ 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.

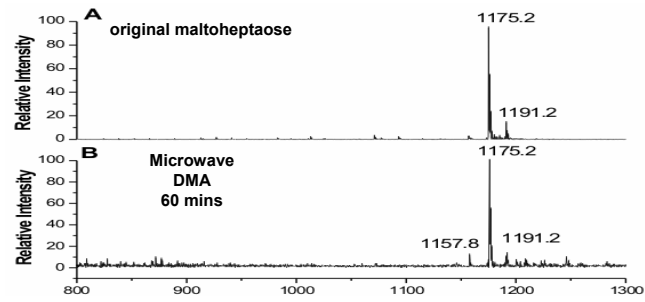
RESULTS AND DISCUSSION

Scheme 1. Proposed mechanism for DMA-based β -elimination and its following modification on glycan and peptides residues. R₁ and R₂ are amino acids representing the peptides.



Control test for peeling products using Glu₇ 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/NaBH₄ (**4A**), and microwave-assisted DMA-based method (**4B**), 25 mins. (♥: non-reduced residue; H: Hexose; N: HexNAc; F: Fucose; A: sialic acid)⁹⁰

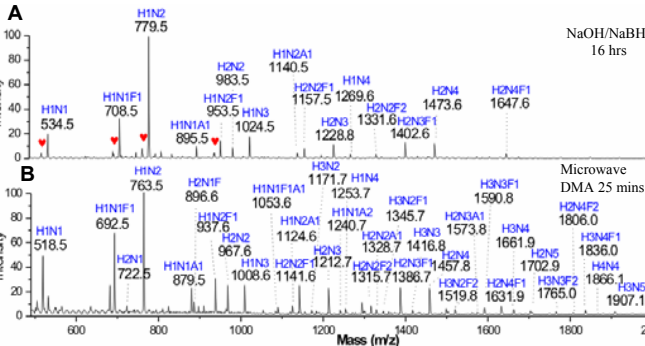


Figure 2. Comparative release of O-glycans from bovine fetuin by three different methods: **A.** NaOH/NaBH₄; **B.** NH₃-based; **C.** DMA-microwave radiation for 25 mins; **D.** DMA-microwave radiation for 50 mins.

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/NaBH₄

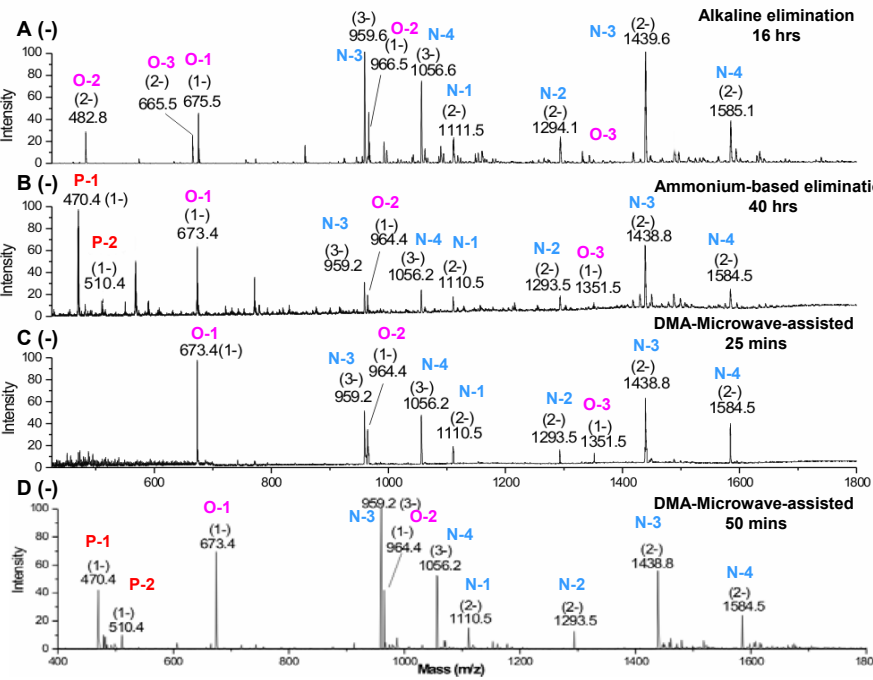
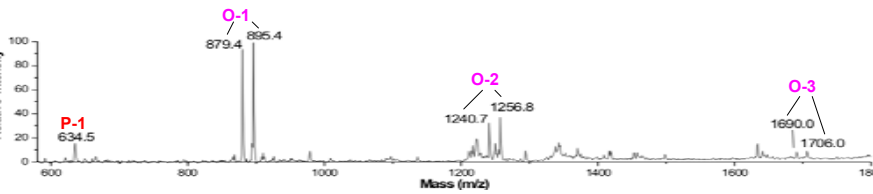


Figure 3. (+) ESI-MS of permethylated glycans from fetuin glycoprotein. 1:1 mixture of non-reduced and reduced glycans from 25 mins of microwave-assisted DMA-based release and classical alkaline release. (16 Da mass difference between non-reduced and reduced glycan)



Future Work

- Internal standard to measure relative quantification precisely
- O-glycosylation site identification by analyses of DMA/DMA-d₆ adduct on peptide portion

References

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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.

Scheme 2: Bovine fetuin O-linked glycans, N-linked glycans and “peeling products” of O-linked glycans

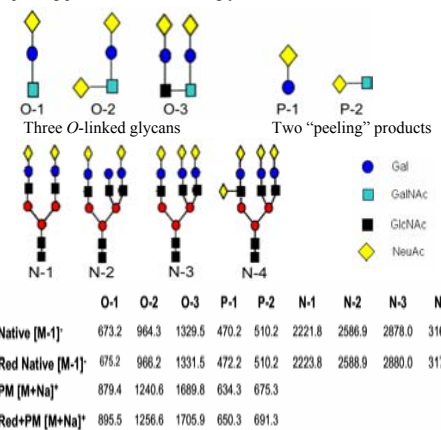


Table 1: Comparison of three O-linked glycan release methods

	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 ₄ OH aq. solution saturated with (NH ₄) ₂ CO ₃ , 60 °C 40 hours	500 μ L DMA aq. solution with (NH ₄) ₂ CO ₃ , 70 °C \approx 30 mins
“peeling”	NO	Significant	Not visible
Reduced/n on-reduce	Reduced	Non-reduced	Non-reduced
Solid salt residue	Excess	NO	NO
Time for a single sample	1-2 days	\approx 4 days	<1 day
O-glycosylation site labeling	NO	N/A	Yes; form DMA adduct on glycosylation site ^{4,5}

Acknowledgements

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