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Anal Chem. Author manuscript; available in PMC 2011 March 15.

Published in final edited form as:
Anal Chem. 2010 March 15; 82(6): 2421–2425. doi:10.1021/ac902734w.

Rapid De-O-glycosylation Concomitant with Peptide Labeling Using Microwave Radiation and an Alkyl Amine Base

Stephanie Maniatis[1], **Hui Zhou**[1], and **Vernon Reinhold**[1,*]

[1]Department of Chemistry, University of New Hampshire, Durham, NH 03824

Abstract

Procedures are detailed for a quantitative release of O-linked glycans from peptides that now provide a shorter reaction time, a possible identification of O-linked sites, and a quantification of all reaction products. The release was initiated by a mild base, dimethylamine, and accelerated by microwave radiation. Differential analysis using standard glycoproteins has shown improved release efficiency concurrent with facile incorporation of dimethylamine into the former O-linked sites. *In situ* glycan reduction insures protection against peeling, and is synchronous with subsequent studies by high performance MS^n sequencing. The protocols were established with a synthetic O-GlcNAc peptide that would mimic the linkage chemistry and applied to a well characterized glycoprotein bovine fetuin with both N-, and O-linked glycans and a highly-glycosylated swine mucin.

Introduction

Glycosylation is an abundant post-translational modification, which primarily includes N-, and O-linked glycans[1]. Due to their structural complexity, a glycan must be released from the peptide for a high performance sequence analysis (linkage, branching and isomer characterization), a feature clearly not accomplished by MS of glycopeptides. For N-glycans, an endoglycosidase superbly yields unmodified products for further characterization. By comparison, O-linked glycans characterization remains particularly challenging as no analogous endoglycosidase is known, nor is there a recognized consensus sequence for its peptide location. More significant, alternative chemical protocols are either incomplete, lack specificity, or alter one or both products. However, the roles of O-glycosylated proteins exhibit comparable biological function[2,3], are equally diverse, and thereby, challenging to structurally characterize. Thus, improved protocols for their comprehensive evaluation are sorely needed.

The strong base (sodium hydroxide) procedures used for O-glycan release from proteins/peptides cause glycan degradation (peeling) and this was earlier resolved by *in situ* reduction to the alditols, a most successful and long lasting strategy[4]. Peptides/proteins were found to be unstable under these conditions, and in an effort to evaluate both products, ammonia was introduced. This milder base released the glycan by substitution providing the first attempt to identify O-linked peptide sites by MS with the resultant one Da mass shift[5-7]. The substituted NH_2 group was chemically stable under the conditions of MALDI and ES ionization. Further interest in milder conditions was reported using alkyl amines[8] to address a different question;

* To whom correspondence should be addressed: Vernon N. Reinhold, Glycomics Center, Gregg Hall, University of New Hampshire, 35 Colovos Road, Durham, NH 03824, vnr@unh.edu.

To Appear as Supplemental Data. ES-MS^3 spectrum providing sequence of former DMA-peptide, m/z 787.3, (Fig. 3). ES-MS^2 spectrum showed facile loss of DMA residue yielding major fragment, m/z 742.4, the selected precursor providing this spectrum. A series of b-, and y-type ions position the unsaturated threonine in the sequence.

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could reduction be avoided and capture the glycan hemiacetal as an aryl aldiamine for enhanced detection following chromatography?

We have considered these general protocols and selected dimethylamine (DMA) as a mild base and applied microwave radiation to accelerate the substitution. Microwave assisted enzymatic and chemical reactions have been developed for a range of glycomic and proteomic applications with benefits in shorter reaction time and improved efficiency. Such methods include in-gel tryptic digestion9 which resulted in improved yields and shorter reaction times. Microwave radiation has also been shown to release N-glycans using the enzyme PNGase F with completions in 10-60 min, a significant contrast to the usual overnight procedures (>12 hours)10. As might be anticipated, deglycosylation of O-linked glycans could benefit using microwave radiation. This possibility was first evaluated with a synthetic O-GlcNAc peptide (Kindly provided by G. Hart, Johns Hopkins Univ.) which allowed a direct focus on the release kinetics of the glycosylamide linkage under a host of reaction parameters. The goal was to establish a specific and quantitative release in the shortest possible time concomitant with generating stable products (glycans and peptides) suitable for further analysis. The established conditions were subsequently evaluated on a large glycoprotein, bovine fetuin, which showed comparable kinetics, but the product glycans exhibited ion products indicating peeling. Shorter reaction times decreased these undesirable products, but at a cost of release efficiency. Thus, to maintain quantitative and qualitative recoveries, the released glycan hemiacetals were reduced *in situ* to the corresponding alditols, a strategy introduced earlier by D. Carlson[4]. When the microwave-DMA procedure was contrasted to the classical strong base method[4], improved glycan yields were obtained with both bovine fetuin and porcine stomach mucin. The reduced glycans were methylated providing products synchronous for high performance MS^n sequencing.

Experimental

Materials and Reagents

Dimethyl sulfoxide, sodium hydroxide, methyl iodide, ammonium carbonate, 40% aqueous dimethylamine (and DMA-$^2H_6$-isotopically labeled), bovine fetuin, porcine stomach mucin type III, and β-cyclodextrin were available commercially (Sigma-Aldrich, St. Louis, MO). Strong base deglycosylation4 and glycan methylation were carried out essentially as reported5. Two synthesized peptides, one O-GlcNAc glycosylated (OCTD) and the other unglycosylated (CTD) were gifts from G. Hart, (Johns Hopkins). All other solvents (HPLC grade) were purchased from Thermo-Fisher, (Waltham, MA). Graphitized carbon (Carbograph 120/400) was obtained from Alltech Associates, (Deerfield, IL).

Microwave reactions were performed with a CEM Discover LabMate model (CEM Corporation, Matthews, NC). This instrument was equipped with an IR sensor for temperature feedback and control. Temperature was set by the user and the necessary power to accurately maintain that temperature was provide by the microwave power unit. The 10mL Pyrex sample vessel was closed during microwave operation and a small stirring bar insured uniform heating. The peptides (50μg, CTD and OCTD, Scheme 1), were dissolved in 500μL 40% aqueous DMA. Sample glycoproteins (1mg each) were prepared identically but with an internal standard, cyclodextrin (1μg), and a reducing agent, (1M $NaBH_4$). The OCTD peptide showed complete O-GlcNAc elimination in 70min when heated at 70°C, and support for these protocols is detailed below. The reaction is terminated upon the addition of 1ml acetic acid in an ice bath and solvents were removed with a Labconco concentrator, (Kansas City, MO). The borate salts were converted to volatile methyl borate esters by the repeated addition of 1% acetic acid in methanol and evaporation under a stream of nitrogen gas. Dried crude samples were dissolved in water and applied to a hand packed (~0.5cm) porous graphitic carbon (PGC) column equilibrated with water[11]. Samples were washed with water and eluted with 25% acetonitrile

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in water with 0.1% trifluoroacetic acid. The eluants were dried in a vacuum concentrator, methylated5, extracted with methylene chloride, back washed with water and dried. Mass spectral analyses were obtained by dissolving the samples in a 50% aqueous methanol solution containing in 0.1% acetic acid.

Mass Spectrometry

For electrospray, all samples were run on an LTQ instrument (Thermo-Fisher, Waltham, MA), with a spray voltage of 1.4kV in the positive ion mode. This instrument was equipped with a TriVersa Nanomate (Advion, Ithaca, NY), an automated nanoelectrospray ion source. Spectra were collected using Xcalibur 2.0 software (Thermo-Fisher). Signal averaging was accomplished by adjusting the number microscans within each scan, generally ranging between 3 and 20. Collision parameters were left at default values with normalized collision energy set to 35% or to a value leaving a minimal precursor ion peak. Activation Q was set at 0.25, and activation time for 30ms. MALDI-TOF samples were profiled on a Shimadzu Axima-CFR (Manchester, UK) using DHB (10mg/mL in 50% acetonitrile) as the matrix. Samples were analyzed by positive ion extraction using near-axis N2 laser irradiation having a repetition rate of 50Hz and a power setting at 135. The instrument was operated in the reflectron mode with external calibration using UPS2 standard peptides, (Sigma-Aldrich, St. Louis, MO). 1.0μL each of sample and matrix were co-crystallized onto stainless steel target plates under atmospheric conditions. Post extraction was optimized for m/z 1500 ions using a nitrogen laser power of 135. Five hundred laser shots were fired in 10 shot units over the target surface.

Results and Discussion

Optimization of O-Glycan Release Conditions

An ESI-MS spectrum of an equal mixture of the CTD and OCTD peptides showed a distribution of sodiated and protonated ions, (Fig. 1), and MS/MS-CID analysis confirmed their sequence, (Scheme 1). The goal of this effort was to focus on a quantitive de-O-glycosylation of the OCTD peptide in the shortest possible time using the CTD analog as an internal standard. An MS evaluation at 30min and 70°C displayed four product ions, m/z 742.1, 765.3, 787.3, 809.2, for OCTD and two remaining precursor ions at m/z 963.3, 985.2, (Fig. 2, insert). An extension of the reaction time up to 80min diminished the precursor ions, but failed to bring about any qualitative changes. These product ions suggest a combined proton abstraction (Fig. 3, A), and O-GlcNAc elimination coupled with its capture as the alditol by borohydride reduction, (Fig. 3, C). The ion product, m/z 742.2 (Fig. 2) suggests an unsaturated intermediate, (Fig.3, B), which provides the site for DMA addition as well as the opportunity to isotopically label such peptides. Many carboxylic acid analogs undergo electrophilic substitution at an alpha carbon (as in this peptide) and these take place by way of enol tautomer or an enolate anion intermediate, features that require at least one hydrogen on the α-carbon atom. The unique selectivity observed in this study to release only O-linked and not N-linked glycans may have benefited from the greater nucleophilicity of DMA as a consequence of its electron-donating methyl groups.

Summed ion abundance of the precursor and product ions relative to the summed abundance of CTD (internal standard) at differing temperatures provided the reaction curves, (Fig. 4). These data indicate that at 70°C the reaction was complete in 70min. Such conditions provided a release efficiency of better than 95% with about 75% of that being DMA adduct. It would be interesting to evaluate higher concentrations of DMA, but 40% aqueous solutions are saturated so differing solvents or higher pressures could be a consideration. However, other complications, like sample solubility and providing a vessel for microwave heating may induce new impediments. The DMA peptide adduction ion, m/z 787.3 (Fig. 2) was isolated, analyzed by CID which exhibited a facile neutral loss of the DMA with a single product ion (m/z 742.4,

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M - DMA + Na^+). Further analysis of this product ion (MS^3) provided the peptide sequence and specific fragment ions (b_4/y_5) expected for an unsaturated threonine residue at position 4 of this unsaturated peptide.

Isotopic Labeling of Former O-Linked Peptide Sites

De-O-glycosylation coupled with DMA peptide labeling provides a unique mass increment of 27 amu for each former Thr/Ser O-glycosylation site. When equal amounts of deuterated DMA were used (DMA-$^2H_6$/DMA-$^1H_6$) the former OCTD peptide exhibited a 6 Da mass interval that was readily discernable, (Fig. 5). In a complex field of peptides these paired ions of equal abundance would provide a signature, especially when the composition is coupled with a 27 amu Thr/Ser increment. When applied to the OCTD peptide and analyzed by mass spectrometry (MS^2) the peptide ions displayed a facile neutral loss of the DMA molecule providing an unsaturated Thr peptide which was sequenced and identified by MS^3, (supplemental data).

Protein De-O-Glycosylation

Protocols for efficient O-glycan release were established using a synthetic GlcNAc peptide (OCTD) which characterized the stability of the glycosidic linkage, not the applicability or stability of a larger glycan or protein. To be assured such protocols were applicable and equally successful for these samples, the kinetics of release and product stability was first evaluated using bovine fetuin, a well characterized glycoprotein. The results were directly compared with the classical Carlson method[4]. In this study β-cyclodextrin was used as an internal standard and the glycan products were reduced *in* situ and methylated for ESI-MS^n analyses[13]. A time-release study was constructed by following two fetuin glycans, m/z 895.6, 1256.8, (Hex,HexNAc,NeuNAc; Hex,HexNAc,$NeuNAc_2$), and these curves exhibited identical release kinetics as that observed for the glycosylated peptide, OCTD. Even when microwave heating was extended for 90min there was no change in the product plateaus following 70min, strongly suggesting a 70min interval was sufficient. Microwave heating was challenged again with the strong base, heating block method[4] which showed the products to be qualitatively identical, (Fig. 6). However, close quantitative tabulation with an internal standard (β-cyclodextrin) demonstrated a more efficient release, (Table 1). In the same manner a more challenging glycoprotein was selected, commercially available porcine stomach mucin (type III). Again, the two protocols were again contrasted with this mucin which showed the spectra to be qualitatively identical, (Fig. 7) but the quantitative results clearly showed improved yields with the DMA-microwave procedure, (Table 2).

Discussion

A short-time quantitative release of O-linked glycans from peptides and proteins has been detailed. Glycan stability has been insured by *in situ* reduction and these products, following methylation, are directly suited for high performance analysis by MS^n disassembly[13]. A synthetic O-GlcNAc-peptide provided an opportunity to carefully establish conditions for quantitative release in the shortest time. The procedures were directly applied to fetuin and a porcine stomach mucin. These microwave-DMA protocols provided an improved glycan yield when tabulated with an internal standard and compared to the classical strong base procedure[4]. It has been long assumed that the classic conditions[4] using a strong base released O-glycans efficiently. However, this notion is most likely false as these microwave-assisted conditions repeatedly demonstrated more product glycans from both glycoproteins, fetuin and a mucin. The glycan release chemistry was attributed to base-initiated α-proton extraction and DMA addition (Fig. 3). The DMA modification yields a unique peptide mass and when isotopically labeled (DMA-$^2H_6$/DMA-$^1H_6$) provides an identity to former O-linked peptide sites and a six Da interval for easy recognition in a complex peptide field. A study of the peptides from ribonuclease A finds that most peptides are unaffected under the microwave

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release conditions, but some selective modification seems apparent and more detailed studies are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Kevin H. Bullock, (Shiners Burns Institute, Boston, MA) and Zhongfu Wang, (Life Science College, Northwest University, Xi'an, PR. China) made early contributions to this study. Financial support was provided by NIH,NIGMS (GM 54045) and Glycan Connections, Lee, NH.

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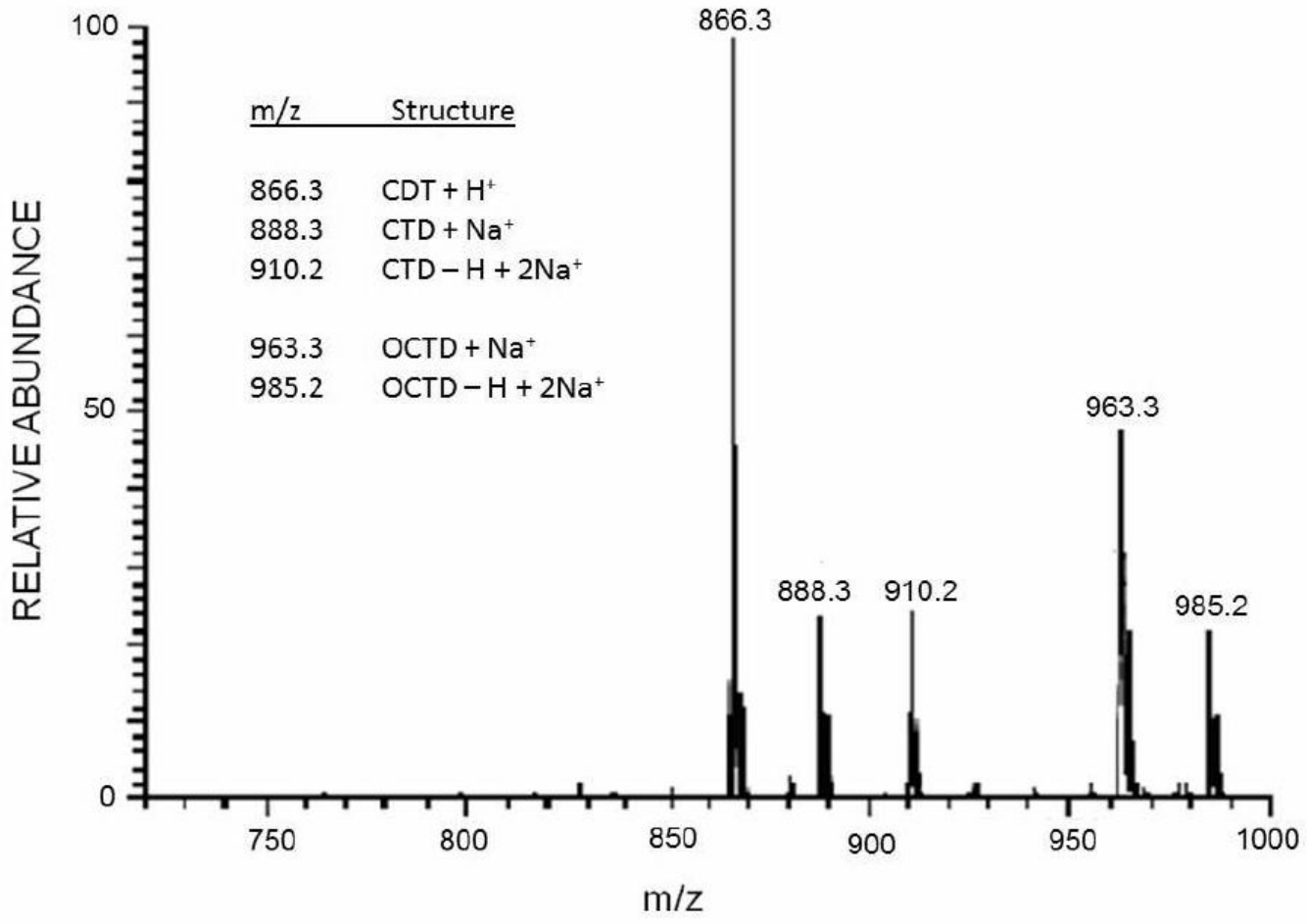

Figure 1.
ESI-MS of two synthetic peptides (Scheme 1) identified as sodiated and protonated ions, (see insert). These multiple ion products from a single structure were equally summed to quantify abundance during temperature and time kinetic studies.

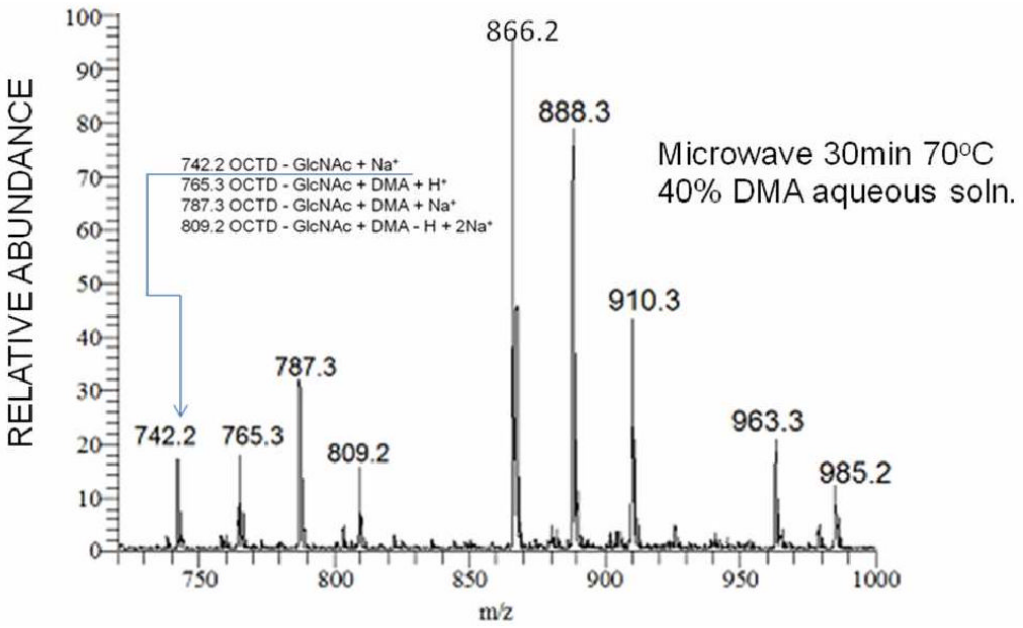

Figure 2.

ES-MS ion profile of OCTD de-O-glycosylation (t = 30min). Protonated and sodiated products identified in figure insert. Internal standard was the CTD peptide, (Scheme 1) with three major ions, m/z 866.2 888.3, 910.3 which remained constant throughout the study. Precursor ions of OCTD, m/z 963.3, 985.2, diminished to zero after one hour.

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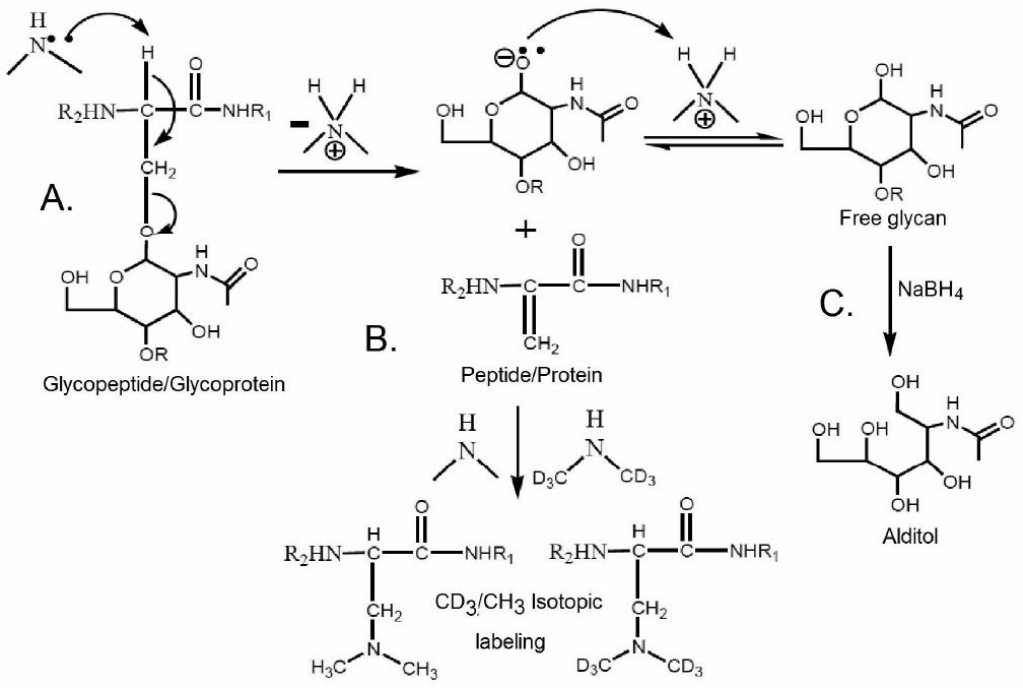

Figure 3.

Probable chemical pathways of microwave catalyzed de-O-glycosylation in aqueous DMA. (A.) Base DMA assisted deprotonation of an alpha-labile proton combine to facilitate glycan elimination; (B.) Substitution of the peptide alkene shown with a 50/50 mix of CD_3/CH_3-DMA to assist peptide ID with 6 Da mass intervals, (see Fig.5); (C.) Released glycan hemiacetal protected from peeling by *in situ* reduction to the alditol with $NaBH_4$.

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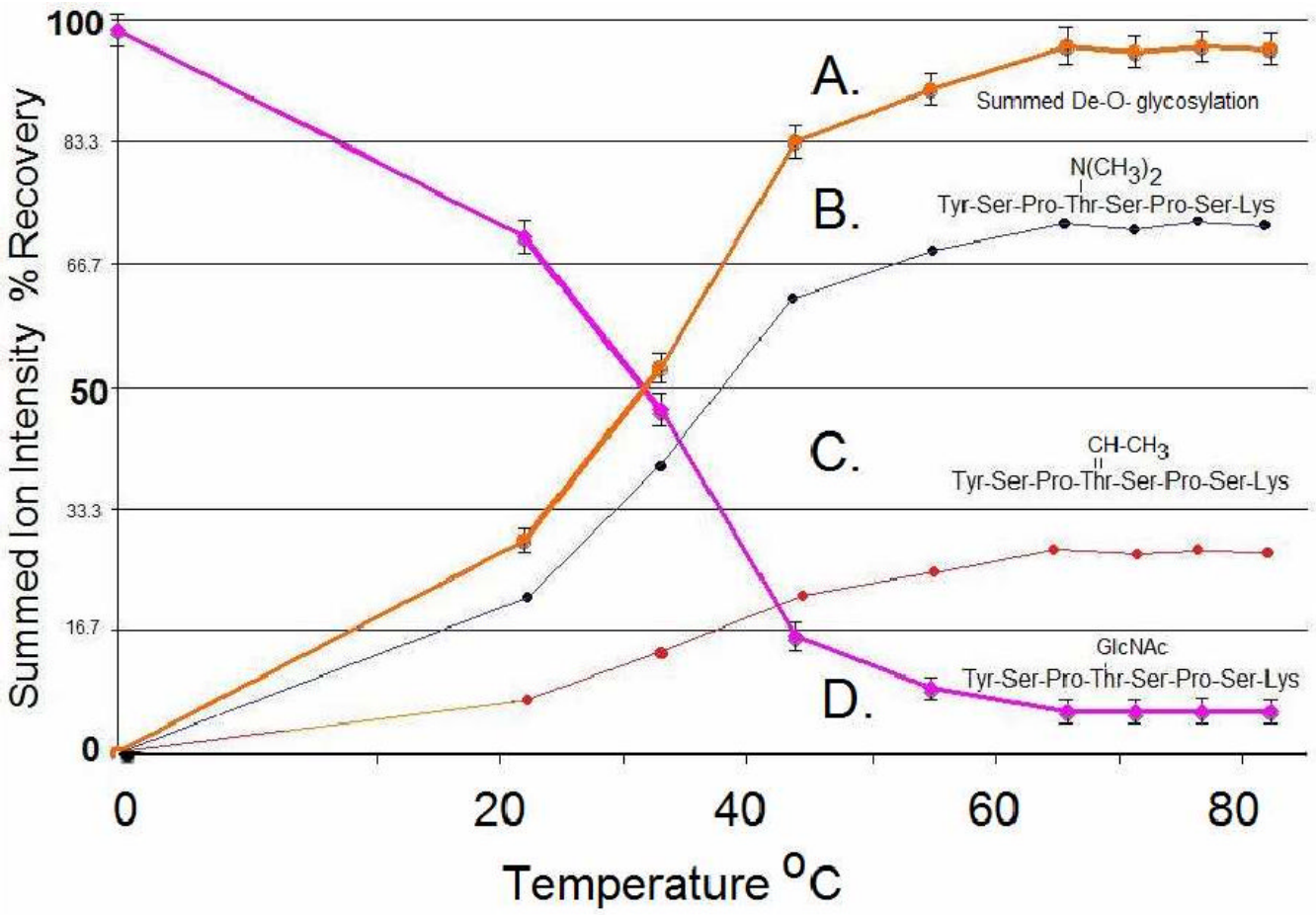

Figure 4.

Temperature dependent precursor-product profiles of de-O-glycosylation over a constant time of 60min. Profiles show de-O-glycosylation approaches completion at temperatures near 70° C. Two products are formed, **B. and C.**, a DMA addition product, ~75%, and an unsaturated peptide, ~25%. Plot **A.**, profiles the summed ion intensities representing de-O-glycosylation, **B.** and **C.** Plot **D.** is the reciprocal of **A.** Plots **A.** and **B.** are averages of 5 independent runs with error bars set for standard deviations.

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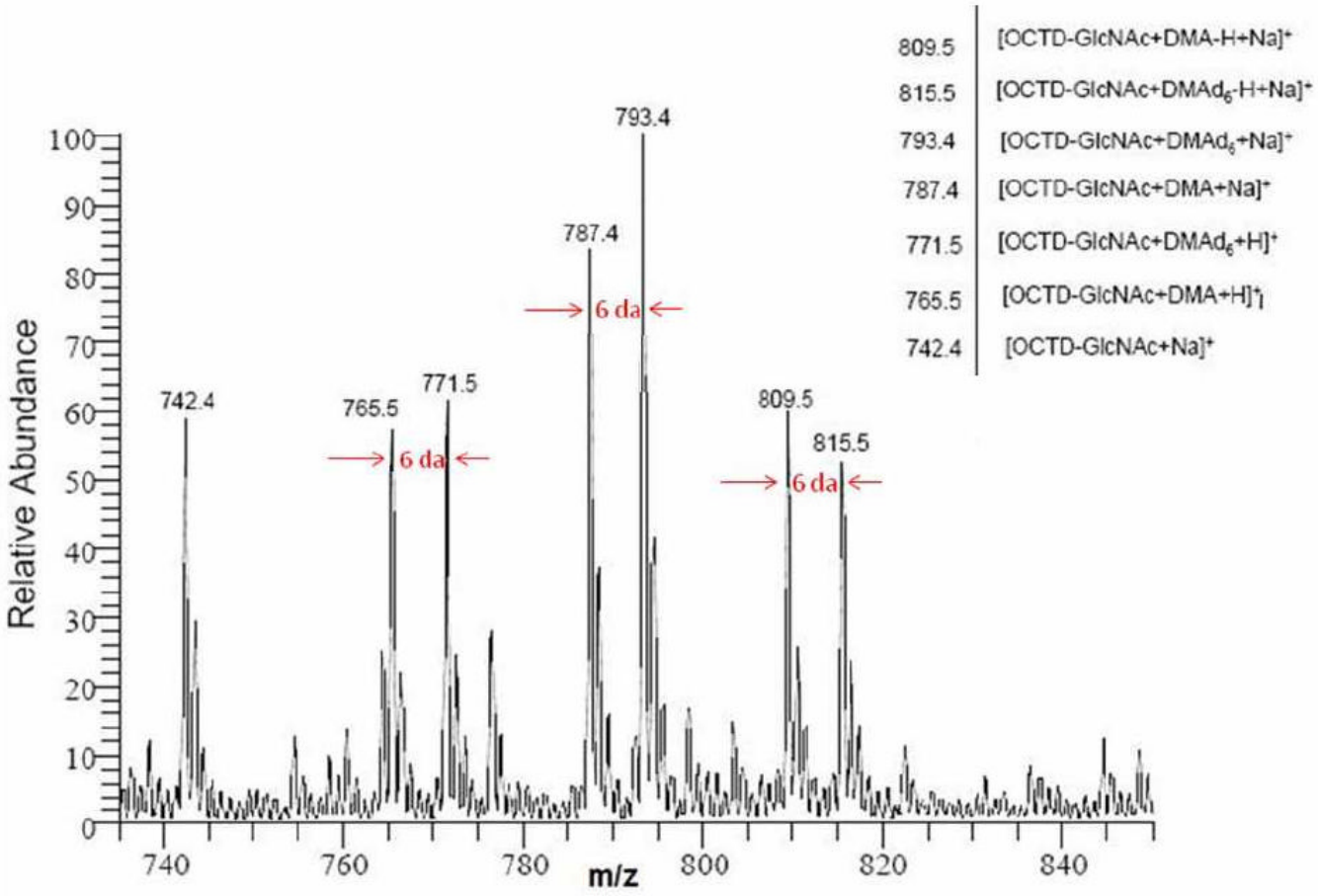

Figure 5.

ES-MS spectrum of DMA isotopic labeling (DMA-$^{2}H_{6}$/DMA-$^{1}H_{6}$) of the OCTD glycopeptide exhibiting 6Da mass intervals. Multiple product ions (insert) representing elimination and DMA addition, this latter ion, (m/z 742.4), a hydrolysis product with no DMA incorporation.

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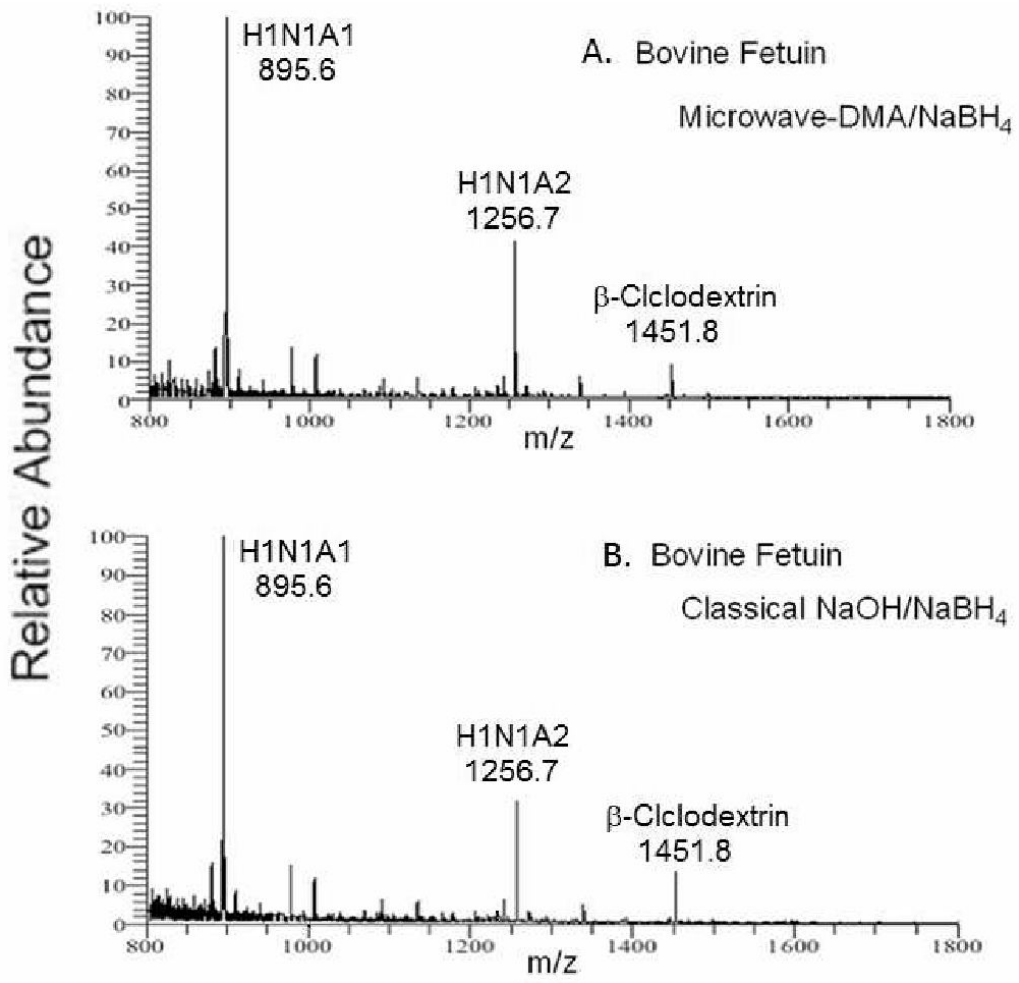

Figure 6.

MALDI-MS Comparison of classical[4] and microwave (this report) de-O-glycosylation protocols on bovine fetuin. (A) Microwave DMA/NaBH4 1hr 70°C and (B) Classical NaOH/$NaBH_4$ 16hrs. 50°C. (H: Hexose; N: HexNAc; A: Neuraminic Acid). Internal standard, m/z 1451.9, β-cyclodextrin. Quantitative results summarized in Table 1.

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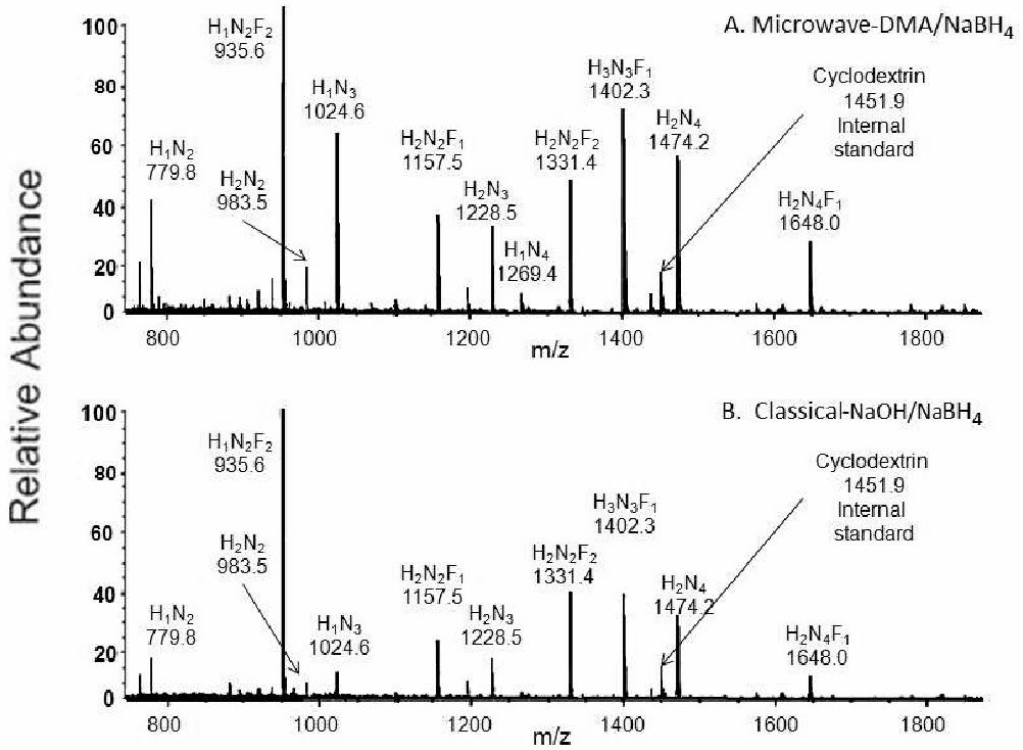
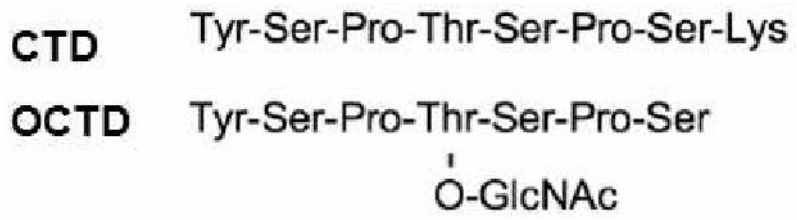


Figure 7.

MALDI-MS Comparison of classical[4] and microwave (this report) de-O-glycosylation protocols on porcine stomach mucin type III. (A) Microwave DMA/$NaBH_4$ 1hr 70°C and (B) Classical NaOH/NaBH4 16hrs. 50°C. (H: Hexose; N: HexNAc; F: Fucose). Internal standard, m/z 1451.9, β-cyclodextrin. Quantitative results summarized in Table 2.



Scheme 1.
Sequence of two synthetic peptides used in this study, (CTD, OCTD). CTD was used as an internal standard to study kinetics of de-O-glycosylation of OCTD.

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Table 1

Comparative efficiencies of classical and microwave assisted O-glycan release from bovine fetuin using β-cyclodextrin as an internal standard. Peak intensities were calculated by summing the ion abundance for the first three isotope peaks for each O-glycan, then contrasted they to the summed ion abundance of the first three isotope peaks of the internal standard in each spectrum for an outcome of relative distribution, [12]. The difference in reaction yield between the two methods is shown in the column 'Reaction yield microwave/classical which is a ratio of the data in the first two columns. A 1.00 would indicate identical results.

O-glycan composition, m/z	Peak intensity Classical method relative to cyclodextrin internal standard	Peak intensity Microwave method relative to cyclodextrin internal standard	Reaction Yield Microwave/Classical
H1N1A1, 895	7.54	12.99	1.71 +/- 0.08
H1N1A2, 1256	2.58	4.99	1.93 +/- 0.09

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Table 2

Comparative efficiencies of classical and microwave assisted O-glycan release from porcine stomach mucin type III using β-cyclodextrin as an internal standard. Calculations were identical to those discussed in Table 1.

O-glycan composition, m/z	Peak intensity Classical method relative to cyclodextrin internal standard	Peak intensity Microwave method relative to cyclodextrin internal standard	Reaction Yield Microwave/Classical
H1N1F1, 708.7	3.05	3.95	1.30 +/- 0.05
H2N2, 779.8	1.05	1.33	1.26 +/-0.03
H1N2F1, 953.6	1.27	1.67	1.31 +/- 0.06
H2N2, 983.5	0.55	0.71	1.29 +/- 0.04
H1N3, 1024.6	1.21	1.55	1.28 +/- 0.05
H2N2F1, 1157.5	0.82	1.01	1.24 +/- 0.05
H2N3, 1228.5	0.82	1.00	1.22 +/- 0.04
H1N4, 1269.4	0.30	0.37	1.22 +/-0.04
H2N2F2, 1331.4	1.04	1.22	1.17 +/- 0.06
H2N3F1, 1402.3	1.84	1.94	1.06 +/- 0.04
H2N4, 1474.2	0.92	1.09	1.18 +/- 0.05
H2N4F1, 1648.0	0.42	0.47	1.13 +/- 0.04