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Rapid removal of N-linked oligosaccharides using microwave assisted enzyme catalyzed deglycosylation

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Abstract

The removal of N-linked oligosaccharides from glycoproteins is commonly performed during the preparation of samples for mass spectrometry. A reduction in the protein's structural heterogeneity is sometimes essential to obtain a mass for the intact protein. Alternatively, removal of the sugar may be desired to facilitate oligosaccharide analysis. A typical approach to deglycosylation employs overnight digestion with the enzyme peptide N-glycosidase F (PNGase F). We report a method for the accelerated removal of N-linked oligosaccharides using PNGase F assisted by microwave irradiation. Complete deglycosylation was achieved in less than 30 min for most proteins without compromising the integrity of protein samples. This method was tested on a variety of glycoproteins, including antibodies, at the microgram level.

Keywords: Proteomic; Microwave; Deglycosylation; PNGase F; Oligosaccharide

1. Introduction

The vast micro-heterogeneity of the eukaryotic proteome is due to several genetic and proteomic events including, but not limited to: genomic splice variation, intracellular processing and the dynamic process of post-translational modification (PTM). Among the PTMs, glycosylation stands as one of the most common, yet complex, modifications. The glycosylation and deglycosylation process *in vivo* plays an important role in key proteomic functions such as protein folding [1], protein and cellular trafficking [2], protein stabilization [3], protease protection [4] and quaternary structure [5] to name a few. Glycosylation can also have profound effects on receptor binding and inflammation. Indeed, the onset or recovery from many diseases, for example HIV-2 [6], Creutzfeldt–Jakob disease [7], rheumatoid arthritis [8] and tuberculosis [9] has been linked to the presence, diversity or lack of glycosylation sites.

Glycosylation sites are classified as either N-linked (via the amide nitrogen of asparagine) or O-linked (via the hydroxyl groups of serine, threonine and occasionally hydroxylysine or hydroxyproline) [10]. Due to the diverse nature of carbohydrate structures, characterization of glycoproteins has proven to be challenging. Many avenues have been followed in the pursuit of glycosylation site mapping, including glycol-enrichment using lectin affinity resins [11], beta elimination followed by Michael addition for O-linked sugar residues [12] and utilization of chemoenzymatic properties by engineering the galactostransferase enzyme to selectively label O-GlcNAc proteins with a ketone-biotin tag followed by affinity selection [13]. Although many of the techniques listed above are specific to the mapping of the more cumbersome O-linked glycosylation sites, mapping of N-linked moieties can often also prove challenging. The most common technique for their characterization is comparative chromatographic mapping/profiling of the enzymatic cleavage products of a protein before and after deglycosylation, sometimes in conjunction with mass spectrometric analysis.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; MAAH, microwave-assisted acid hydrolysis; MAb, monoclonal antibody; MQ, Milli-Q water; PNGase F, peptide N-glycosidase F; PTM, post translational modification; PVDF, polyvinylidine fluoride; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TFMSA, trifluoromethane sulfonic acid; TOF, time of flight

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For the chromatographic mapping protocol mentioned above, and for other analytical scenarios, complete deglycosylation of both proteins and peptides is often desired. For example, deglycosylation may reduce smearing during protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Deglycosylation also proves particularly useful when looking at intact molecular weights of proteins that may be skewed due to heterogeneity from an abundance of PTMs. One example of this can be seen in the case of naturally expressed and recombinant antibodies produced in eukaryotic cell lines. The majority of these immunoglobulins posses one or more N-linked glycosylated heavy chains. In the case of therapeutic monoclonal antibodies (MAb), deglycosylation is routinely performed to characterize modifications such as the presence of C-terminal lysines, or in the case of conjugated monoclonal antibodies [14], to monitor the number of small molecules coupled to the immunoglobulin. For these and a plethora of other scenarios it is often advantageous to deglycosylate the glycoproteins.

Over the past decade, several published techniques have described improvements over traditional overnight incubation of glycoproteins with their respective deglycosylating enzymes/chemicals. These have included: optimization of conditions using anhydrous trifluoromethane sulfonic acid (TFMSA) to cleave all sugar residues from the glycoprotein [15], PVDF-immobilization of a glycosylated protein of interest followed by incubation with a deglycosylating enzyme [16], on-chip deglycosylation using hydrophobic and hydrophilic chip technology [17] incubation of glycoproteins with PNGase F in the presence of enzyme-friendly surfactants [18], and engineering of hybrid de-glycosylation enzymes for facile immobilization on cellulose [19]. In addition to in-solution or on-membrane immobilized deglycosylation protocols, in-gel methods have proven successful for O-glycosylated proteins of up to 150 kDa [20].

Despite these advances, the shortest time documented for complete deglycosylation of standard glycoproteins such as Fetuin and RNase B is 2 h [15–19] and some glycoproteins require incubation overnight or longer [21]. Although the need for a rapid deglycosylation protocol is not paramount to the typical researcher, a faster and more complete deglycosylation strategy could be advantageous in industrial settings and in high-throughput laboratories.

In recent years, microwave radiation technology has been introduced into the proteomics arena, allowing faster reaction times in comparison to traditional conductive/convective (i.e., water bath, oven or thermocycler) methods. This has been demonstrated for (but not limited to): amino acid protein hydrolysis [22], tryptic digestion [23,24] and microwave-assisted acid hydrolysis (MAAH) [25–27]. Utility of MAAH was recently demonstrated for characterizing oligosaccharide moieties of glycopeptides by Lee et al. [28] using TFA, whereby peptide maps could be produced from cleavage of acid labile sites, without interference to the integrity of the PTMs.

We have investigated accelerated deglycosylation of N-linked proteins using microwave-assisted deglycosylation. Digestions using PNGase F that typically take up to 24 h to complete using conventional protocols may be performed in 10–60 min with partial deglycosylation occurring in less than 60 s. The investigation and optimization of the necessary parameters for microwave-assisted N-linked deglycosylation are hereby described.

2. Experimental

2.1. Materials

Herceptin® (trastuzumab) and Avastin® (Bevacizumab) were produced in-house at Genentech Inc. RNase B, PNGase F, ethylenediaminetetraacetic acid (EDTA), HCl, NaCl and formic acid were purchased from Sigma (St Louis, MO). All solvents including methanol and acetonitrile were from Burdick and Jackson (Muskegon, MI). Dithiothreitol (DTT) was purchased from Promega (Madison, WI). Tris buffer was from Biorad (Hercules, CA) and trifluouroacetic acid (TFA) from Applied Biosystems (Foster City, CA). Milli-Q (MQ) water system was from Millipore (Billerica, MA).

2.2. DOTA conjugation

The DOTA-NHS-ester was dissolved in dimethylacetamide (DMA, Fluka Chemika, Switzerland) and prepared to concentrations of 60–100 mg/mL. Typical procedures involved buffer exchanging the MAb into PBS with 2 mM EDTA at pH 7.2. Reactions were performed at a ratio of 1 molecule MAb to 4 DOTA molecules (1:4) and carried out at 25 °C while gently stirring on a Thermomixer plate (Eppendorf, Westbury, NY).

2.3. N-linked deglycosylation

Herceptin, Avastin, RNase B and a DOTA-conjugated antibody (10 μg each) were diluted in 0.1 M Tris containing 50 mM DTT to a final volume of 20 μL. Reduction occurred at room temperature for 30 min. PNGase F was added to the sample at an enzyme:substrate ratio of 1:20 (0.5 μg) and was exposed to microwave irradiation using a Discover microwave (CEM Corporation, Newark, CA) at varying temperature and time conditions. Control samples were incubated in traditional water baths at the corresponding temperature. Samples were taken at intervals of 2–60 min at temperatures ranging from 37 to 60 °C using the microwave power settings of 1–20 W. In all cases reactions were stopped immediately with 5% TFA (2 μL). Samples were refrigerated until online LC–MS analysis.

2.4. On-line LC/MS analysis

Intact mass measurements were performed using a TSQ Quantum Triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA). Samples were diluted 1:2 in 0.1% TFA (Solvent A) and 10 μL (approximately 25 pmol) was loaded by auto-sampler onto a PLRP-S 300 Å reverse-phase micro-bore column (50 mm × 2.1 mm, Polymer Laboratories, Shropshire, UK) heated to 75 °C. Compounds were separated with a 12 min gradient from 0 to 60% B (solvent A: 0.05% TFA in water and

solvent B: 0.05% TFA in acetonitrile) and eluant was directly ionized using the electrospray source. Mass spectrometric conditions were as follows: spray voltage was set at 4000 V, sheath gas pressure to 55 psi, ion sweep gas pressure to 3 psi, capillary temperature to 340 °C and the tube lens to 204 V. Data was collected in the mass range 300–3000 Da. Data were collected using the Xcalibur data system and deconvolution was performed using ProMass software.

3. Results and discussion

3.1. Deglycosylation of antibodies

Fig. 1a shows the mass spectrum of intact Avastin prior to reduction with DTT. The starting material (Fig. 1a) contains two carbohydrate additions of 1445 Da each representing a G0 (Asn 297-GlcNac-GlcNac-Mannose-[Mannose-GlcNac]$_2$) (in which GlcNac is *N*-acetylglucosamine) residue on each heavy chain (MW 1445 Da each) as well as a smaller percentage of the galactose capped species (G1). (For proprietary purposes the exact molecule weights are not shown for the antibody examples, instead the peaks are labeled as a mass delta from the naked parent ion molecular weight.) For example $[M+H+2890]^+$ represents the mass for Avastin plus two G0 oligosaccharides, one per heavy chain. The peak observed at $[M+H+3214]^+$ is consistently observed and is thought to be due to an additional mannose residue in the oligosaccharide. After 5 min (Fig. 1b) of microwave irradiation at 37 °C new masses corresponding to a loss of 1445 and 2890 Da, respectively, are observed; these correspond to Avastin with the partial and complete loss of the two heavy chain oligosaccharides, respectively. After 10 min in the microwave (Fig. 1c), deglycosylation is complete. In comparison, complete deglycosylation of the control sample (water bath) was seen after incubation for 1 h (Fig. 2). The peak at $[M+H+203]^+$ represents a small amount of GlcNac remaining on the antibody heavy chain after deglycosylation.

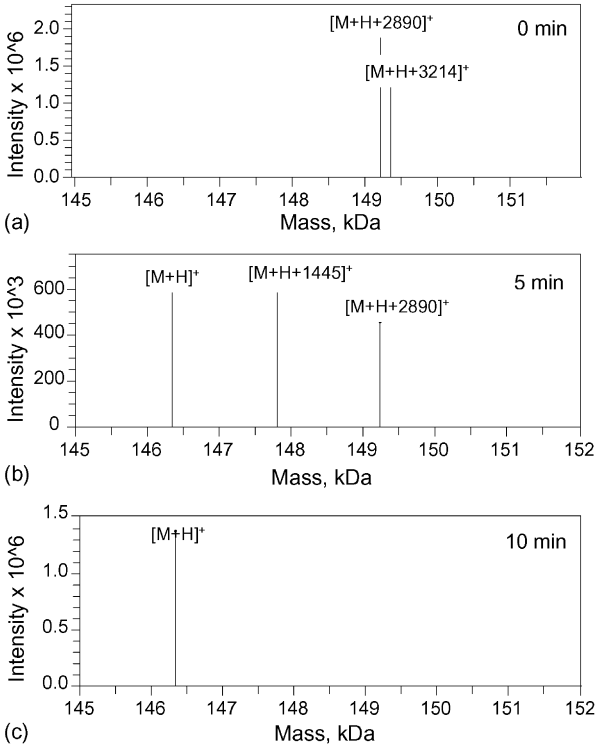

Fig. 1. (a) Deconvoluted mass spectrum of non-reduced, glycosylated Avastin, (b) non-reduced Avastin after 5 min incubation in the microwave at 37 °C with PNGase F at an enzyme:substrate ratio of 1:20, and (c) the same sample after 10 min microwave-catalyzed incubation.

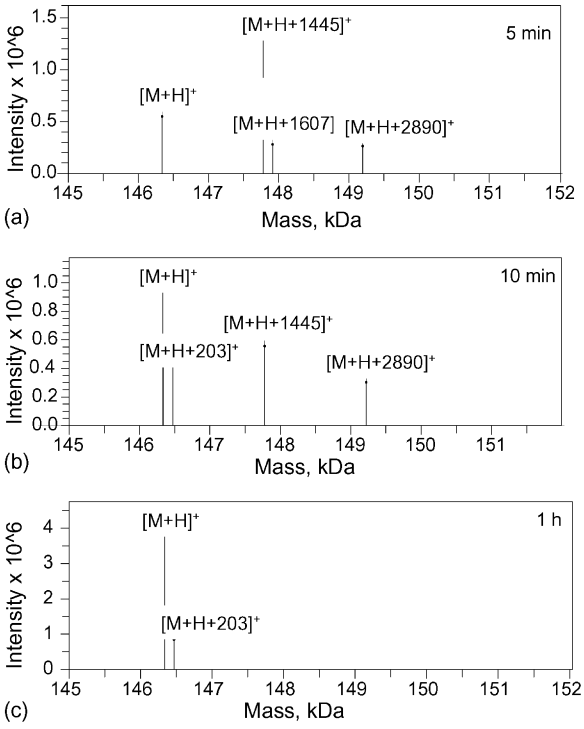

Fig. 2. (a) Deconvoluted mass spectrum of non-reduced Avastin after 5 min incubation in a water bath at 37 °C with PNGase F at an enzyme:substrate ratio of 1:20, (b) after 10 min incubation, and (c) after 1 h incubation.

A similar time profile was observed with reduced Avastin. Deglycosylation in the microwave occurred at a considerably faster rate than in the water bath. Fig. 3a shows the glycosylated heavy chain of Avastin (51,160 Da). The heavy chain is approximately 40% deglycosylated after 5 min in the microwave (Fig. 3b) and is completely deglycosylated after 10 min in the microwave (Fig. 3c). Fig. 4 shows the same time points for deglycosylation under identical conditions in a water bath. As can be seen, at 10 min, the molecule still contains an oligosaccharide residue and 1 h of incubation is necessary to achieve complete deglycosylation under these conditions. Irradiation at 60 °C was also performed (data not shown) and although rapid deglycosylation was observed (less than 2 min), significant loss of protein is observed.

The microwave-assisted deglycosylation procedure was also tested on other in-house antibodies, e.g., Herceptin, and similar results were observed, i.e., complete deglycosylation after 10 min of incubation with PNGase F. Comparing microwave-assisted deglycosylation to the overnight standard deglycosylation protocol for PNGase F, this is a remarkable decrease in incubation time.

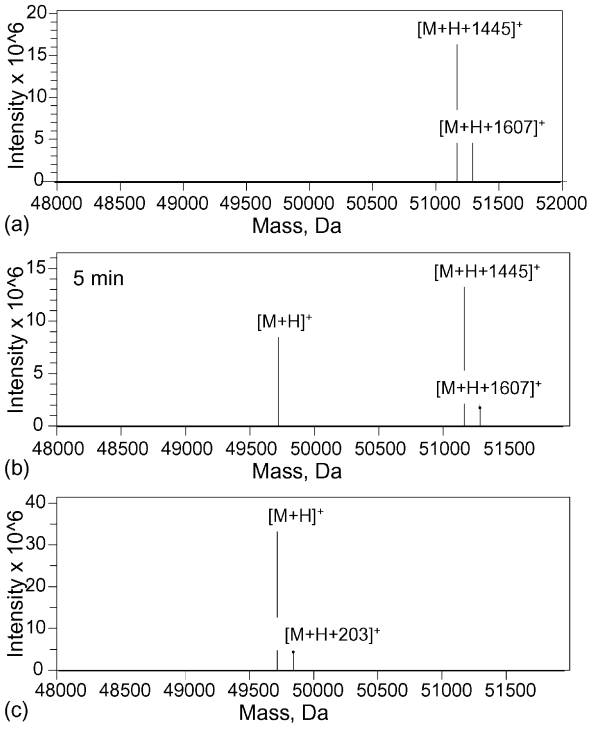

Fig. 3. (a) Deconvoluted mass spectrum of reduced Avastin heavy chain prior to deglycosylation, (b) after 5 min microwave-assisted deglycosylation at 37 °C, and (c) after 10 min microwave assisted incubation at 37 °C.

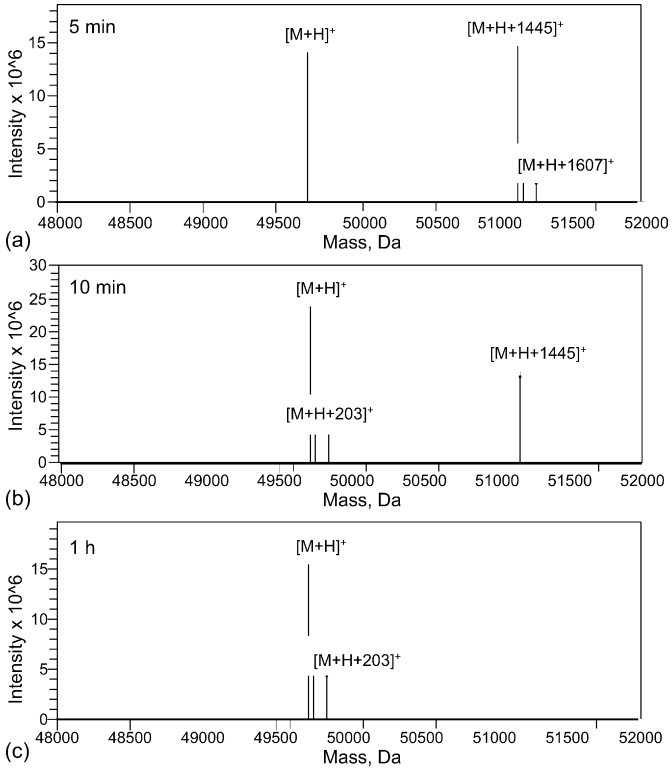

Fig. 4. (a) Deconvoluted mass spectrum of reduced Avastin heavy chain after 5 min incubation at 37 °C in the water bath, (b) 10 min incubation, and (c) 1 h incubation.

3.2. Deglycosylation to allow determination of antibody conjugate sites

Antibodies may be conjugated with bi-functional chelating agents (BCA), which in turn bind radioactive metal ions, thus enabling radio-imaging to track the distribution of the therapeutic antibodies in animal models [29]. These BCAs attach a chelating moiety to a biological targeting vector, in this case an antibody, to incorporate a radioisotope for bio-distribution determination [29]. DOTA (1, 4, 7, 10 tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) is a well characterized reagent that is favored for its ability to produce physiologically stable complexes with trivalent radio-metals. Mono *N*-hydroxysuccinimide derivatives of DOTA (DOTA-NHS-ester) are often employed for attaching DOTA to proteins because of the simple chemistry and consistency in conjugation yields. Due to the heterogeneity of the number of DOTA molecules on an antibody, deconvolution of the mass spectra from these antibodies is somewhat more cumbersome than regular "naked" antibodies and is often indecipherable (Fig. 5a). Combination of DOTA and glycosylation heterogeneity, results in extremely complicated spectra, and makes determination of loading of antibodies and other glycosylated proteins difficult.

To further test the efficiency of the microwave-assisted deglycosylation protocol, conditions necessary for deglycosylation of DOTA conjugated antibodies were explored. Once initial results ascertained that these molecules were unaltered (in terms of mass and signal intensity) during microwave irradiation, time points were taken using both microwave-assisted deglycosylation and the conventional PNGase F conditions.

For microwave-assisted N-linked deglycosylation, 1 h was found to be sufficient to remove all the oligosaccharides, which allowed for mass spectrometric interpretation of the molecular weights for the conjugated antibody so that DOTA quantitation was possible (Fig. 5b). For conventional convection heating, it is necessary to incubate the antibody overnight in order to get sufficiently complete deglycosylation for the accurate deconvolution of the data and inference of the number of DOTA conjugates (Fig. 5c). The DOTA molecules add a mass of 386 Da to the antibody mass and as can be seen in Fig. 5b and c, the heavy chain exists as the native form and also with one and two DOTA molecules conjugated. The light chain is not shown in this data as in this particular case, the light chain was neither glycosylated or conjugated to DOTA. This technique has been tested on several DOTA-conjugated antibodies and all have shown a similar pattern, i.e., that the DOTA molecule is stable to microwave irradiation, and that all reduced conjugated antibodies tested so far can be completely deglycosylated within 1 h.

3.3. Deglycosylation of other proteins

To demonstrate the wide applicability of the microwave-assisted N-linked deglycosylation, experiments were performed on RNase B, a well-characterized glycoprotein. RNase B is known to have a high mannose N-linked oligosaccharide attached *via* Asn-60. Fig. 6a represents a typical deconvoluted mass spectrum of reduced RNase B prior to any exposure to

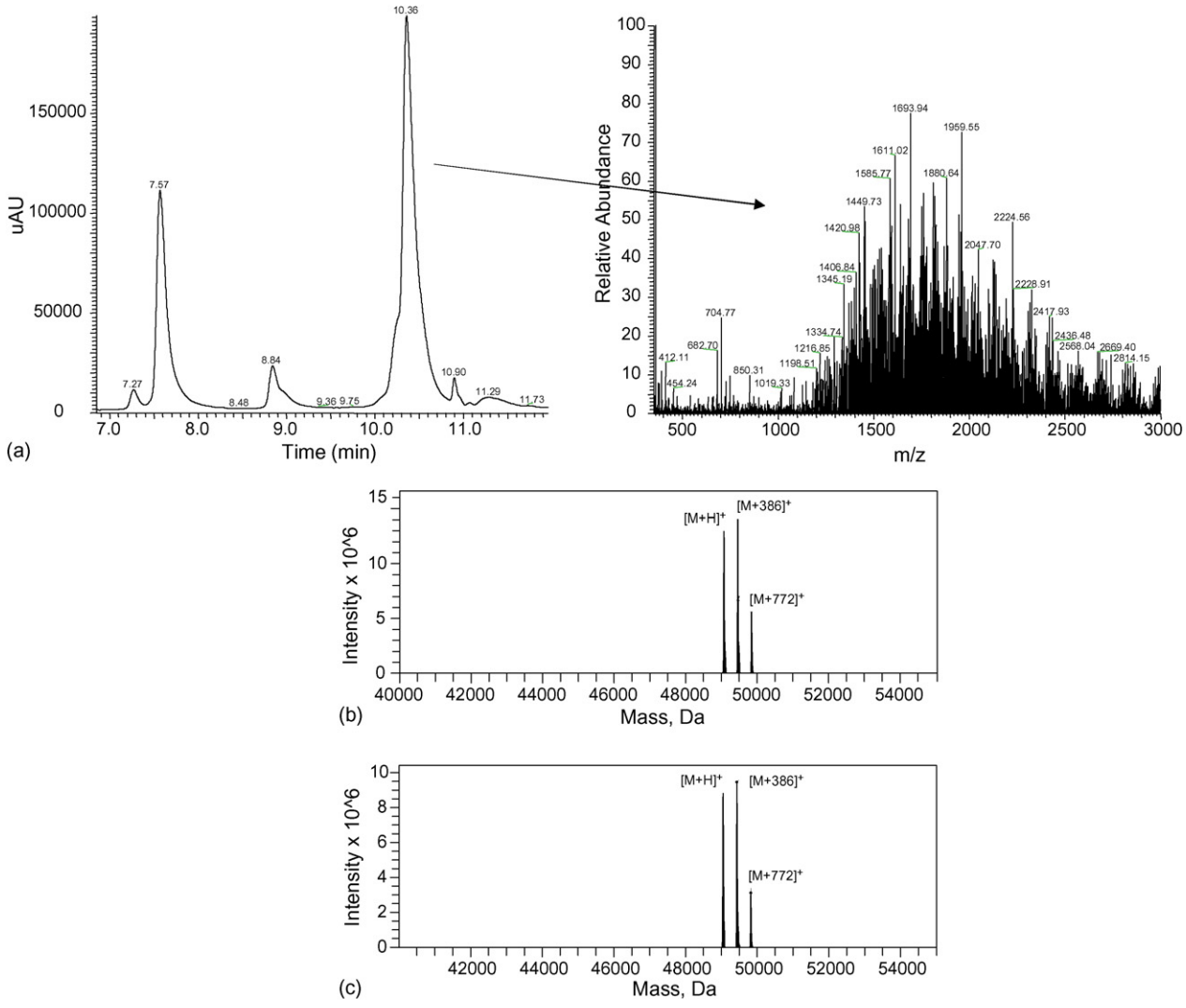

Fig. 5. (a) Raw mass spectrum of the reduced glycosylated anti-CD4 heavy chain conjugated to DOTA along with the TIC trace. *Note*: the spectrum could not be deconvoluted due to the complex heterogeneity of the glycosylation and DOTA conjugation (b) mass spectrum of the DOTA conjugated anti-CD4 antibody after 1 h incubation at 37 °C in the microwave with PNGase F. Conjugation adds a mass of 386 Da per DOTA molecule to the biomolecule (c) the same antibody after incubation with PNGase F in the water bath at 37 °C overnight.

PNGase F. Fig. 6b and c demonstrate the same protein after deglycosylation in the microwave for 10 min and 1 h, respectively. Fig. 7a and b shows the same data generated by deglycosylation in the water bath for 1 h and overnight. Complete deglycosylation of RNase B after 1 h in the microwave is observed. Comparing this to the same time point in the water bath, it can be demonstrated that deglycosylation has occurred at a much faster rate when performed in the presence of microwave energy. Indeed, even after overnight incubation in the water bath at 37 °C, complete deglycosylation is not yet observed.

The acid-labile surfactant Rapigest^{TM} has previously been shown to accelerate the deglycosylation of proteins under conventional conditions [18]. To see if the reaction could be accelerated even further in the microwave, the addition of enzyme friendly surfactants such as Rapigest^{TM} and the addition of organic solvent (10–20% ACN) was investigated on a range of glycoproteins including Avastin and RNase B. Under these denaturing conditions, enzymatic reactions may be accelerated due to increased accessibility of the active site of the enzyme to its substrate. Addition of organic solvent did not show any marked increase in deglycosylation time (nor a decrease). Addition of Rapigest^{TM} did greatly decrease reaction time, however. Addition of 0.1% Rapigest decreased both the microwave-assisted and standard water bath reactions to under 10 min, but sample losses occurred and precipitation from the surfactant was deemed problematic for some of the analyses, in particular injection *via* the auto-sampler on the HPLC. For high recovery of low level material it was decided not to further pursue the use of surfactants.

It has long been a source of debate amongst synthetic chemists as to whether acceleration to the desired thermal reaction temperature, or the microwave energy itself, is the cause of the decreased reaction times [30,31]. Although knowledge of the exact mechanism is not necessary for exploiting the accelerated removal of N-linked oligosaccharides by PNGase F, a basic understanding of the forces influencing the catalysis could

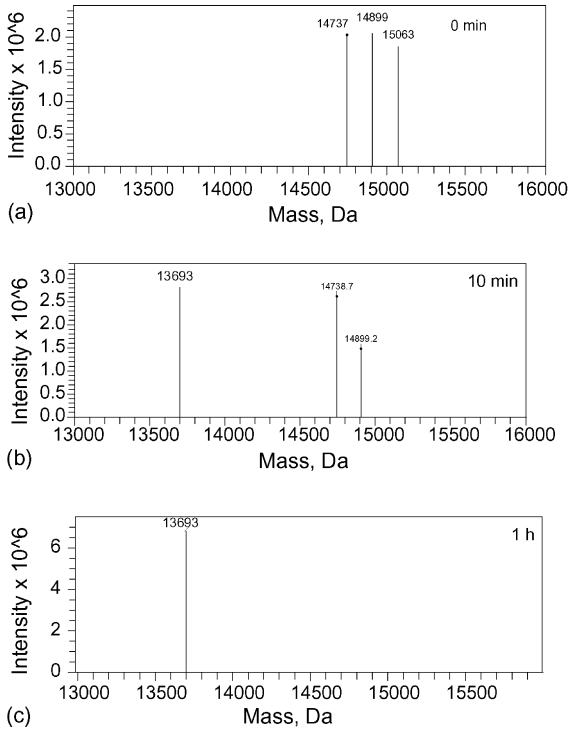

Fig. 6. (a) Deconvoluted mass spectrum of reduced RNase B prior to deglycosylation, (b) after 10 min incubation with PNGase F in the microwave at 37 °C, and (c) 37 °C for 1 h.

help in experimental design and optimizing conditions. A set of experiments was therefore performed whereby all vessels and reagents were preheated to 37 °C prior to the start of the experiment. This was done to gauge whether the increased speed of the microwave reaction was purely due to decreasing the lag time in achieving optimal reaction temperature or not. The results were mixed: although in some cases pre-heating the reagents and vessels prior to starting the reaction resulted in a decrease in deglycosylation times, this did not account for the marked overall decrease in reaction times demonstrated with microwave irradiation (e.g., see Figs. 6c and 7b where RNase B deglycosylation in the microwave is complete within 1 h, yet after an overnight incubation at the same temperature in the water bath the reaction is not yet complete). To quote Pramanik et al. [23] "no microwave-assisted reaction is slower than the corresponding conductivity/convection reaction", and in a real-life scenario, one is unlikely to take the time to heat all vessels and reagents in order to gain several minutes on reaction speeds for the enzymatic reaction.

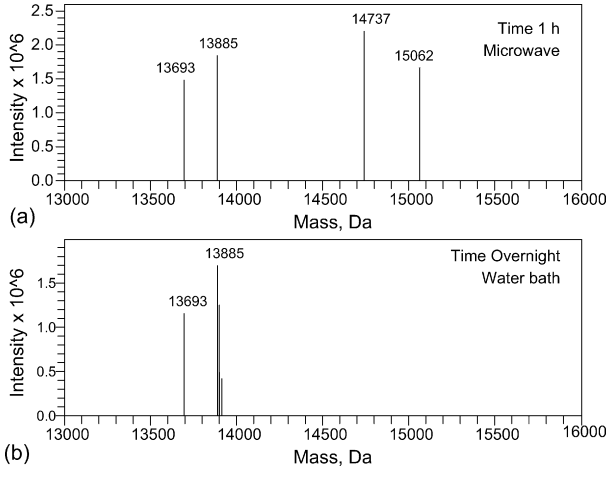

Fig. 7. (a) Deconvoluted mass spectrum of the same RNase B after 1 h incubation at 40 °C in the water bath and (b) overnight incubation.

For antibodies, and also the RNase B protein, the glycosylation sites were N-linked. Since many proteins are heterogeneously glycosylated with a mixture of both O- and N-linked sugars, the possibility of using microwave-assisted techniques for deglycosylating O-linked sugars is currently under investigation. Deglycosylation of O-linked sugars using beta elimination, and also *via* treatment of the glycosylated-protein with the enzymes sialidase (neuramidase) are some of the techniques being explored. It should be noted that not all enzymatic reactions are enhanced by microwave irradiation. Indeed, Vesper et al. [32] demonstrated that the relatively low thermal stability of the proteolytic enzyme Glu-C rendered it inactive in the presence of elevated energies and temperatures and thus proteolytic cleavage with this enzyme was not accelerated under microwave conditions.

4. Conclusions

An investigation into microwave-assisted deglycosylation using PNGase F is described. This microwave-assisted N-linked deglycosylation technique has now been employed on a range of antibodies and proteins, and so far no detrimental effects such as poor recovery, deamidation (apart from the deglycosylated Asn residue which is always observed) or cleavage at the protein backbone have been observed. For naked MAb's complete N-linked deglycosylation is typically observed in less than 10 min, significantly faster than literature cited conventional deglycosylation protocols.

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