

Applications of Microwave-Assisted Proteomics in Biotechnology

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Abstract: Biotechnology has recently celebrated 30 years both as a science and as a multi-billion dollar industry. One application of biotechnology is to use human genetic information to discover, develop, manufacture, and commercialize biotherapeutics. Recombinant proteins can be produced in large quantities at high purity. High-throughput proteomic analysis is at the heart of the biotechnology research and development process, and the industry is constantly striving to streamline and automate the analytical processes involved. Microwave-assisted proteomics has recently emerged as a tool for increasing the bio-catalysis of several processes including tryptic digestions [1-3] lipase selectivities [4], identification of metal-catalyzed oxidation sites on proteins [5], identification of protein N- and C-termini [6, 7] and enzyme catalyzed N-linked deglycosylation [8]. Here, we explore the above mentioned methods, and describe our experiences evaluating microwave-technology for other common proteomic protocols including: removal of N-terminal pyroglutamyl for antibody characterization, beta elimination and Michael addition for identification of phosphorylation sites on recombinant proteins and enzyme mediated O-linked deglycosylation.

Keywords: Proteomics, microwave-assisted, mass spectrometry, deglycosylation, PGAP.

1. INTRODUCTION

Biotechnology is a relatively new player in the drug industry, but, has rapidly become a multi-billion dollar market. There are currently more than 300 protein-based drugs on the market [9] and several of these, such as Bevacizumab (Avastin[®]) for curbing metastatic cancer growth, and Trastuzumab (Herceptin[®]) for interception of HER2 positive breast cancer, have recently had much publicity and success. During research and development of these bio-molecules, full protein characterization occurs. This typically involves a variety of analytical techniques such as: quantitation of material, intact molecular weight verification, proteolytic cleavage for peptide mass fingerprinting [10] or LC-MS/MS analysis [11] N-terminal sequencing [12, 13] to ensure correct translation and cellular processing and mapping and characterizing sites of posttranslational modification (PTMs) [14, 15]. Fig. 1 is an overview of the analytical processes typically employed during the early stage research characterization of a bio-therapeutic protein.

In the world of proteomics, many of the analytical instrumentation [16, 17] and bioinformatic applications [18] have evolved to increase sensitivity and throughput; however, traditional upfront biochemical approaches have not undergone such significant advances. Over the past couple of years however, researchers have been applying alternative energies to conventional heating protocols to increase catalysis of proteomic reactions; these have included ultrasound-assisted digestions [19] and microwave-assisted incubations. Microwave irradiation as a catalysis tool was first applied in the world of synthetic chemistry in the mid 1980's [20]. Later in the 1990's several publications using microwave irradiation for peptide synthesis [21] and other more

bio-molecular based protocols [22, 23] began to emerge. This trend has now included proteomics and several protocols have been described whereby microwave-assistance has provided decreased incubation times and more complete reactions (for a comprehensive review please refer to ref. [24]).

For over a decade, microwave-assisted protein hydrolysis for amino acid analysis for quantitation of proteins has been described [23, 25-27]. Other microwave-assisted proteomic techniques have included proteolytic digestions such as tryptic [1, 2, 28] and acid-mediated proteolysis, enabling more rapid sample preparation for bottom-up analysis [7, 29]. Also reported in the literature have been methods for C-terminal sequencing using a modified microwave-assisted Akabori reaction [30], and N- and C- terminal sequencing using microwave-assisted acid hydrolysis (MAAH) [6, 7]. Methods for removal of PTMs such as oligosaccharides, sphingoids and lipids [31, 33] as well as the rapid deglycosylation of specifically N-linked oligosaccharides [8] have also been described. PTM removal under microwave-assistance has been shown to facilitate protein characterization, simplifying analysis by reducing heterogeneity. Each of the above mentioned protocols has significantly reduced the amount of time traditionally spent on the biochemical and chemical preparation of samples for bio-analytical characterization.

Here we summarize the current literature and also describe our findings with regards to microwave-assisted proteomic techniques in the context of the characterization of therapeutic proteins.

2. METHODS

2.1. Microwave-Assisted Acid Hydrolysis

2.1.1. Microwave-Assisted Acid Hydrolysis for Protein Quantitation

Samples were hydrolyzed in a vacuum evacuated chamber with 6 M HCl with 0.1% phenol at 110°C for 24 h, 150°C for 1 h in a conventional convection oven, or at 175°C for 10 min

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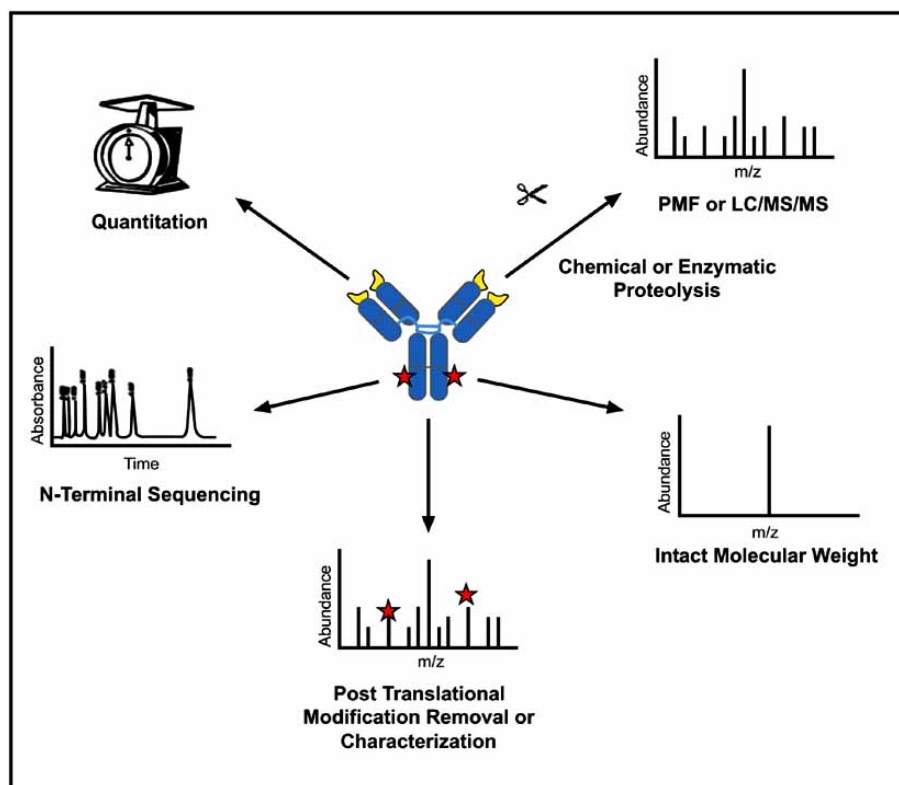


Fig. (1). An overview of the analytical processes typically employed for the characterization of a bio-therapeutic protein. These include quantitation, N-terminal sequence verification, characterization of post-translational modifications, intact molecular weight verification and either peptide mass fingerprinting (PMF) or LC-MS/MS analysis.

at 300 W in the Discover Microwave (CEM, Newark, CA). The hydrolyzed samples were then analyzed using the Hitachi L-8800 amino acid analyzer as previously described [24] (Hitachi High Technologies America, San Jose, CA).

2.1.2. Microwave-Assisted Acid Hydrolysis for N-Terminal Sequencing

Protein 10 μ L (0.1-1 mg/mL) in either water or Tris 0.1M or 20% acetonitrile (Burdick & Jackson, Muskegon, MI) was added to an equal volume of TFA (2 M) and placed into the Discover microwave (CEM, Newark, CA) at 100°C at 50 W for 2 min. Samples were filtered through 0.22 μ m filters (Agilent, Mountain view, CA) to remove precipitates and diluted 1:4 in water. 1 μ L of each sample was spotted onto a MALDI plate with 1 μ L of 5 mg/mL alpha-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile + 0.1% TFA. Samples were analyzed by matrix-assisted laser desorption Time-of-flight analysis (MALDI-TOF) in reflectron mode on a Voyager DE-STR TOF instrument (Applied Biosystems, Foster City, CA) between 600 and 4,000 Da.

2.2. Microwave-Assisted Removal of Post-Translational Modifications

2.2.1. Microwave-Assisted Enzyme Catalyzed Removal of N-Terminal Pyroglutamyl

Materials

Pre-cast 4-20% Tris-glycine gels were from Invitrogen (Carlsbad, CA). Dithiothreitol (DTT) was purchased from BioVectra (Oxford, CT), n-isopropyl iodoacetamide (NIPIA) [34] was synthesized in-house. ProBlot PVDF membrane

was purchased from Applied Biosystems (Foster City, CA). N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) and pf pyroglutamate amino peptidase (PGAP) were purchased from Sigma-Aldrich (St. Louis, MO). Zwittergent 3-16 was from Calbiochem (San Diego, CA). Anti OX40 ligand was purchased from R & D Systems (Minneapolis, MN).

SDS-PAGE and Electro-Blotting

Proteins were reduced with DTT (10 mM in 2 X sample buffer) at 95°C for 10 min. Alkylation was performed by addition of NIPIA (200 mM, 1/10 v/v) and incubated at room temperature (RT) for 20 min. Proteins were separated on a pre-cast 4-20% Tris-glycine gel (2 h, 120 V) and electro-blotted onto the PVDF membrane in CAPS buffer (10 mM, pH 11.0) (1 h, 250 mA). Protein bands were stained in Coomassie brilliant blue R250 for less than 5 min and destained in 50% MeOH/H₂O. The membrane was then rinsed extensively with MQ H₂O (10 X 1.0 mL) and air dried.

PGAP Digestion

The band corresponding to the heavy chain (HC) of anti OX40 ligand monoclonal antibody [35] was excised and treated with 100 μ L of 0.5% Zwittergent 3-16 in Tris (0.1 M, pH 8.0) at RT for 5 min as previously described [36]. The blocking solution was removed and the membrane was rinsed with MQ H₂O. Sodium phosphate buffer (30 μ L, 50 mM, pH 7.0) containing 10 mM DTT and 1 mM EDTA was added to the membrane. Lyophilized PGAP was dissolved in 100 μ L of sodium phosphate buffer, 10 μ L of PGAP solution was added to the membrane. Digestion was performed by incubating the membrane at 90°C for 1, 5, 10, 20, 30, and 60 min in the

thermocycler or in the Discover microwave (CEM Corporation, Newark CA) at 50 W and with the temperature reading at $90^{\circ}\text{C} \pm 3^{\circ}\text{C}$. After digestion, protein bands were rinsed with MQ H₂O (10 X 1.0 mL) and dried in the Speedvac.

PGAP-treated bands were subjected to N-terminal sequencing performed on a Procise 494 protein sequencer (Applied Biosystems, Foster City, CA) using 20 min Edman cycles [13]. Chromperfect software (Justice Innovation Laboratory, Denville, NJ) was used to integrate amino acid peaks. The initial yields of the de-blocked anti OX40 ligand HC were used to compare the efficiency of PGAP digestion performed in the thermocycler vs the microwave.

2.2.2. Microwave-Assisted N-Linked Deglycosylation

Materials

Vascular Endothelial Growth Factor Receptor-3 (VEGFR-3) variant was produced in-house at Genentech Inc. PNGase F, ethylenediaminetetraacetic acid (EDTA), HCl, NaCl and formic acid were purchased from Sigma-Aldrich. 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 Bio-Rad (Hercules, CA) and trifluoroacetic acid (TFA) from Applied Biosystems (Foster City, CA). The Milli-Q water system was from Millipore (Billerica, MA).

N-Linked Deglycosylation

Samples were prepared and analyzed as previously described [8]. In brief, proteins (10 μg each) were diluted in 0.1 M Tris containing 50 mM DTT to a final volume of 20 μL . 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 37°C using the microwave power settings of 1-20 W for 30 min. In all cases reactions were quenched with 5% TFA (2 μL). Samples were refrigerated until online LC-MS analysis commenced.

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 and loaded by auto-sampler onto a PLRP-S 300 $\text{\AA}$ reverse-phase micro-bore column (50 x 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 4,000 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 were collected in the mass range 300-3,000 Da using Xcalibur software and deconvolution was performed using ProMass.

2.2.3. Investigation into Microwave-Assisted O-Linked Deglycosylation

Materials

Bovine fetuin and PNGase-F (500,000 U/mL) were purchased from New England BioLabs (Ipswich, MA), sialidase A (5 U/mL) from Prozyme (San Leandro, CA) and O-

glycosidase (1.25 U/mL) was from Calbiochem (San Diego, CA).

Fetuin contains a mixture of both N- and O-linked oligosaccharides (typically 3 of each), therefore for assessment of O-linked oligosaccharide removal, the N-linked species were first removed. Removal of N-linked carbohydrate from Fetuin was performed according to the microwave-assisted protocol previously described [8].

Removal of O-Linked Carbohydrate

Sodium acetate (40 μL , 50 mM, pH 5.6), sialidase A (10 μL , 5 U/mL) and O-glycosidase (1.5 μL , 1.25 U/mL) were added to the PNGase-F treated Fetuin and the mixture was incubated at 37°C , 45°C and 60°C for 5 to 60 min in the thermocycler or in the microwave (each in duplicate). The deglycosylated material was then loaded onto a 4-20% Tris-glycine gel as described above.

2.2.4. Microwave-Assisted Dephosphorylation (β -Elimination and Michael Addition)

Materials

Phosphopeptide standard solution (20 pmol/ μL) was obtained from Molecular Probes (Eugene, OR), β -casein was from Sigma-Aldrich, barium hydroxide octahydrate, ethanethiol and 1-propanethiol were from Arcos Organics (Morris Plains, NJ), ethanol was from Burdick & Jackson (Muskegon, MI) and all sequencing reagents were from Applied Biosystems (Foster City, CA).

Method

Phosphopeptide standard solution (5 μL) was subjected to β -elimination alone or with subsequent nucleophilic addition in either the microwave or the water bath according to a modification of previously described procedures [37,38]. Samples were reacted with 50 μL of 0.05 M ethanethiol, 0.02 M $\text{Ba}(\text{OH})_2$ in 42% ethanol solution. Reaction mixtures were incubated in the water bath for 1 h at 60°C or in the microwave for 2 min at 100°C . Reactions were stopped by the addition of 2 μL of 5% TFA. 1 μL of solution was loaded onto a MALDI plate with 1 μL of CHCA matrix and analyzed by MALDI-TOF MS. For the control, i.e. β -elimination with no Michael addition, water was substituted for the volume of the nucleophile.

3. RESULTS

3.1. Microwave-Assisted Acid Hydrolysis

3.1.1. Microwave-Assisted Acid Hydrolysis for Protein Quantitation

Quantitation of recombinant protein is of paramount importance in any biotechnology research and development group as both *in vitro* and *in vivo* assays rely on the accuracy of this data. There are several methods that allow accurate quantitation of protein concentration including the Biocinchonic Acid (BCA) protein assay, the Bradford Protein assay, the Lowry Protein assay and the non-absorbant/colorimetric assay, amino acid analysis assay (AAA). The first step in AAA involves hydrolyzing the protein to its constituent amino acids typically by breaking the amide bonds in the presence of 6 M HCl at 110°C for 24 h [39]. Resultant amino acids are then subjected to derivatization and analysis. Protein hydrolysis for AAA was one of the preliminary pro-

tein chemistry methods shown to benefit from microwave-assistance and there are several comprehensive reports in the literature showing the benefits of microwave-assisted protein hydrolysis [23-27]. We employ microwave-assisted hydrolysis for protein quantitation and have found that results gained using this technique are comparable to much lengthier traditional hydrolysis protocols. Fig. 2 shows the recoveries of amino acids from hydrolyzed β -casein with theoretical amino acid recoveries. Overall, we observed comparable amino acid recoveries for proteins hydrolyzed by microwave irradiation at 175°C for 10 min compared to proteins that were hydrolyzed using two common protocols, i.e. at non-microwave mediated incubation at 110°C for 24 h or at 150°C for 1 h.

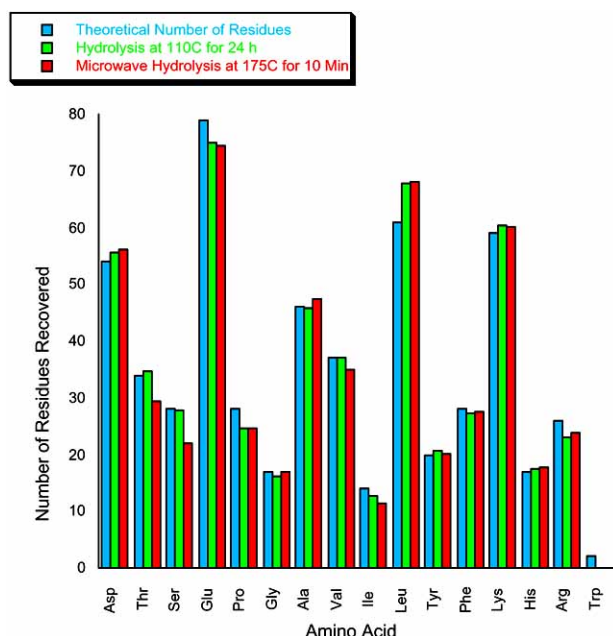


Fig. (2). A comparison of amino acid recoveries after hydrolysis using microwave-mediated hydrolysis and conventional-oven based hydrolysis methods.

3.1.2. Microwave-Assisted Acid Hydrolysis for “Bottom-Up” Protein Identification

Chemical proteolysis is often employed as an alternative proteolytic tool to biological enzymes in the mass spectrometric characterization of proteins. The advantages of acid hydrolysis are that alternative sites to conventional enzymes can be cleaved, for example, mild acid digestion can induce cleavage at Asp/Pro sites [40] and the digestion protocol is typically harsher and can work on proteins that have proven resistant to enzymatic degradation. In recent literature, Zhong *et al.* [6,7] used HCl and TFA for N-terminal identification, and Hua *et al.*, employed formic acid as a proteolysis tool for protein identification [29].

Our investigations also showed that by coupling acid cleavage with microwave irradiation, we were able to control the extent of proteolysis and recovered comparable peptide fragments to results achieved during typical enzymatic cleavages. Table 1 shows a comparison between the different acid-mediated proteolysis methods described in the literature. Acid hydrolysis can be controlled to give either N or C

terminal information, or, upon prolonged exposure, internal cleavage which can yield complete sequence coverage. Coupled with microwave-assistance, these acid-mediated cleavages can be complete in less than 5 min of exposure and offers another high-throughput tool for “bottom-up” protein characterization.

Table 1. A Comparison Between the Different Acid-Mediated Proteolysis Methods Described in the Literature

Conditions & Microwave Exposure	Cleavage Pattern
6 M HCl (30 sec - 2 min)	Predominantly N and C termini [6]
0.1 M HCl or 0.3 M TFA (1-2 min)	Predominantly N and C termini [7]
25% TFA (10 min)	Internal cleavage at predominantly acid labile sites [7]
1 M TFA (2 min)	Predominantly N and C termini ¹
2% Formic Acid (10 min)	Cleavage specifically at Aspartic Acid [29]
Formic or Acetic Acid	Cleavage at N termini but often see modification (Formylation/Acetylation) [7]

¹Our unpublished results.

3.1.3. Microwave-Assisted Acid Hydrolysis for N-Terminal Sequencing

N-terminal sequencing is routinely performed in most biotechnology research departments, with the principal aim being to verify that correct transcriptional and intracellular processing of a recombinant protein has occurred. It is also commonly employed to distinguish differences between the isobaric amino acids Ile and Leu in *de novo* sequencing projects [35] to assess the clonality of antibodies and to test for the presence of ragged N-termini, an indication that unpredicted intracellular proteolysis may have occurred. N-terminal sequencing can be performed using several chemistries including the Sanger method [41] and the Dansyl Chloride method [42] however, the most conventional protocol currently employs chemistries first described by Edman using derivatization of the free N-terminal amine with phenylisothiocyanate [12].

A variety of biochemical protocols followed by mass spectrometric analysis [43-46] and top down proteomic approaches [46] have recently been described as alternative methods for N-terminal characterization. To date however, these have not yet replaced Edman sequencing as the standard method for sequencing the N-termini of proteins, or for assessing clonality of antibodies which is performed by identifying Edman cycles containing more than one amino acid.

Microwave-assisted acid hydrolysis (MAAH) was also recently described as an alternative method for the N- and C-terminal characterization of proteins [6]. MAAH involves exposure of proteins to high concentrations of acid under microwave irradiation, resulting in the denaturation and cleavage of protein at acid labile sites, preferentially at the N- and C-termini. The initial protocol described the exposure of proteins in 6 M HCl to microwave irradiation for small

increments of time (30 sec to 2 min) with resultant hydrolysis products analyzed by MALDI-TOF MS. Ions corresponding to incremental ladders of amino acid chains from the N- and C-termini of the protein were observed. Fig. 3 is a reproduction from the work of Zhong *et al.* showing a schematic of the mass analysis of polypeptide-ladders generated from MAAH using 6 M HCl. Ions corresponding to truncations at each amino acid along the termini are observed.

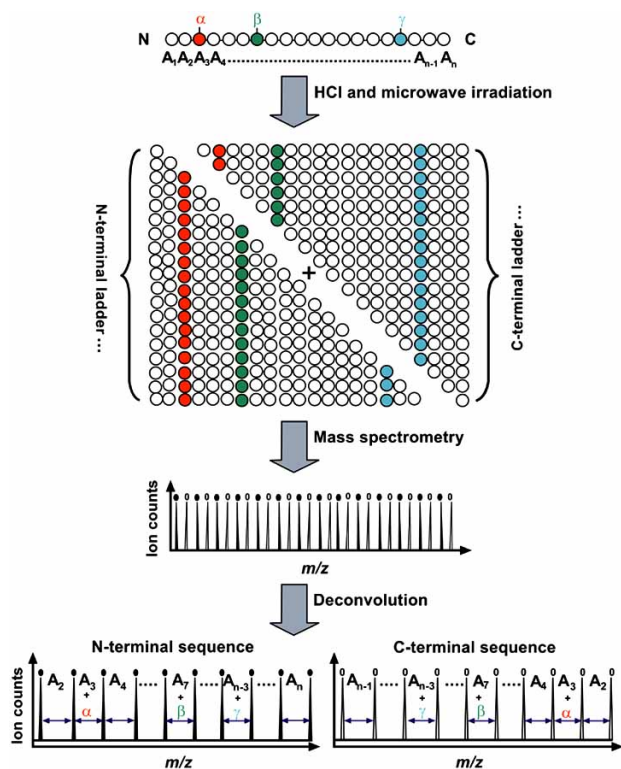


Fig. (3). Schematic of the Microwave-Assisted Acid Hydrolysis method (MAAH) for sequencing the N and C termini of proteins. The mass spectrum produced consists of peaks exclusively from the N- and C-terminal polypeptides of the protein. Amino acid sequences and any modifications are read from the mass differences of adjacent peaks within the same series of the polypeptide ladder. Reproduced with kind permission from Dr. Liang Li from the University of Alberta [6].

In 2005, Zhong *et al.* published a further protocol for MAAH but this time the objective was to generate larger peptides by cleavage at acid labile bonds for bottom-up mass spectrometric analysis (4.1.2) [7]. It was observed during their investigation into the cleavage of proteins using TFA, that if proteins were exposed to 1.5 M TFA for a brief time then only the N- and C-terminal peaks were typically observed.

We investigated both the HCl and TFA hydrolysis methods as potential alternatives to Edman degradation for the high throughput N-terminal sequencing of proteins, and observed the same trends described by Zhong *et al.* Based on a modified version of the protocol proposed by Zhong *et al.*, we adopted the use of 1 M TFA with exposure for 2 min at 100°C at 50 W for N- and C-terminal sequence analysis of recombinant proteins. Fig. 4 shows an example where murine serum albumin (MSA) was subjected to MAAH using the protocol described above, with resultant peptides analyzed in full MS using

MALDI-TOF. Peaks corresponding to N-terminal truncations were observed as well as a C-terminal peptide ion. During our investigation into MAAH we discovered that one terminus from the protein was often preferentially observed in the MALDI-TOF data. For example, under the conditions described above, Lysozyme preferentially cleaves at the C-terminus (Fig. 5) whereas Myoglobin (Fig. 6) preferentially cleaves at the N-terminus. Zhong *et al.* hypothesized that the high abundance of N- and C-terminal truncations was primarily due to acid lability of the more highly exposed termini. We agree with this hypothesis and further postulate that conformation plays a significant role in accessibility of the termini, even under such harsh conditions. Fig. 7a,b show the PyMol X-ray crystallography structure for Lysozyme and Myoglobin respectively. In Fig. 7a the N-terminus is shown in red and the C-terminus in blue. The N-terminus is buried and the C-terminus is more exposed. In addition, Lysozyme contains disulphide bonds at the N-terminus, hence without reduction the molecule was more resistant to MAAH cleavage. In Fig. 7b the N-terminus of Myoglobin (again in red) is more exposed than the C-terminus (blue). Although these structures are mapped in the presence of water and not in concentrated acid, one can still hypothesize that conformation as well as acid lability dictates which terminus is preferentially observed after MAAH. We were able to manipulate which termini were hydrolyzed by the addition of organic solvent or salts and also reduction and alkylation. Fig. 8 shows Myoglobin after MAAH has been performed in the presence of (8a) 1M TFA, (8b) 1M TFA + 20% acetonitrile and (8c) 1M TFA +250 mM Tris pH 8. In Fig. (8a) only ions corresponding to the N-terminus are observed, however after exposure to organic or salt solutions as in Fig. 8b,c, ions corresponding to the C-terminus are also observed.

We performed an extensive evaluation of more than 150 recombinant proteins using the MAAH method with MALDI-TOF MS analysis (or LC-MS/MS analysis for sequence verification in many cases) in parallel with N-terminal sequencing using traditional Edman chemistries on a Procise Protein Sequencer. Overall we had comparable results between the two methods for >75% of proteins i.e. the N-termini we had previously identified from N-terminal sequencing could also be identified using MAAH. This excluded antibodies and other structurally complex molecules, or molecules which contained an abundance of Asp-Pro cleavage sites due to their lability, as these were found to give predominantly internal cleavage using either the 6 M HCl or the TFA mediated hydrolysis. In addition, the cLC Procise N-terminal sequencer is capable of sequencing low femtomole amounts of protein, and we concluded this to be a more sensitive and reliable method for analysis of the N-terminus of low level material when compared to MAAH analysis. In addition the MAAH method did not always reveal ragged N-termini or clonality issues. MAAH did however, prove to be an invaluable quick verification tool for N-terminal characterization, especially for blocked N-termini as demonstrated below.

3.1.4. Microwave-Assisted Acid Hydrolysis for Analysis of N-Terminally Blocked Proteins

After evaluation of routine high-throughput analysis of proteins using MAAH, we chose not to replace traditional

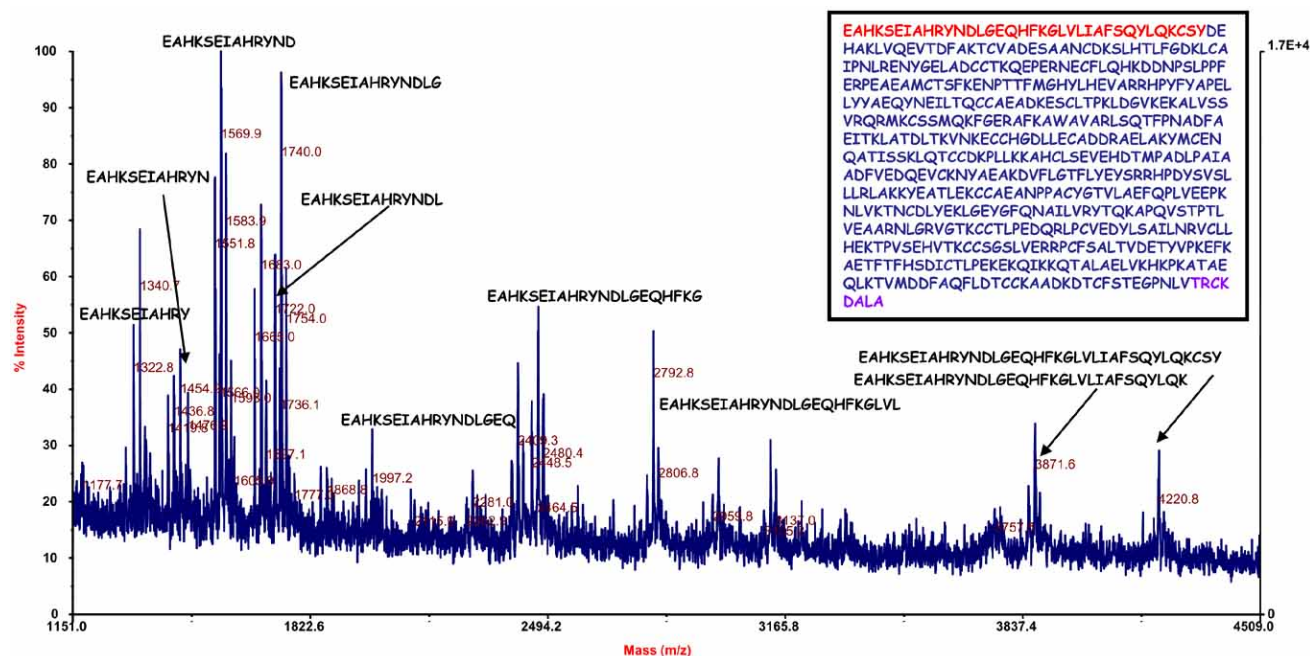


Fig. (4). A MALDI-TOF mass spectrum of peptides generated from mouse serum albumin after performing MAAH in 1M TFA at 50 W for 2 min in a CEM Discover Microwave.

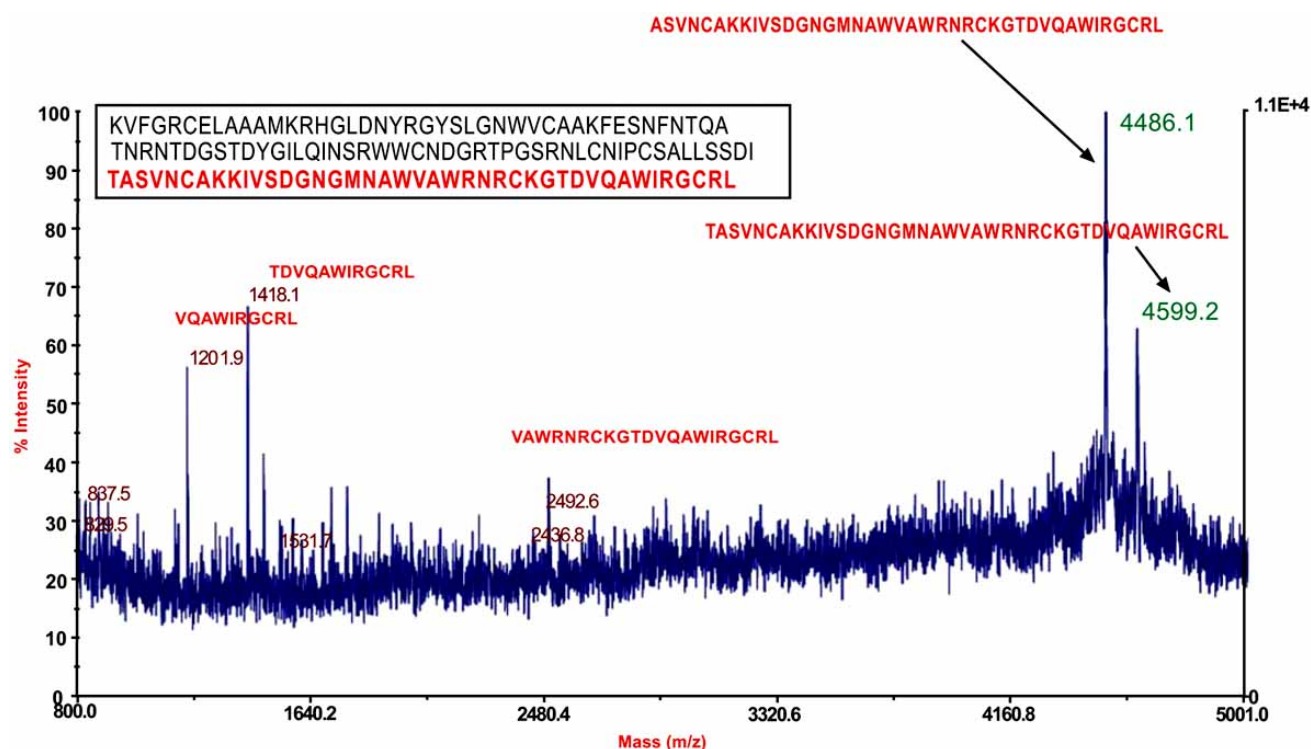


Fig. (5). A MALDI-TOF mass spectrum of peptides generated from lysozyme after performing MAAH in 1M TFA at 50 W for 2 min in a CEM Discover Microwave.

Edman sequencing with this method for standard analysis as discussed in section 4.1.3. We did, however, find a number of specific situations where the method has been of great value. One such instance occurs when a protein is N-terminally blocked and Edman sequencing is not possible.

One such modification is the presence of an N-terminal pyroglutamyl residue, however, this may be removed by the enzyme pyroglutamyl aminopeptidase (PGAP) (see 4.3.1). PGAP however, is ineffective when a proline residue follows the initial pyroglutamyl residue, due to conformational -

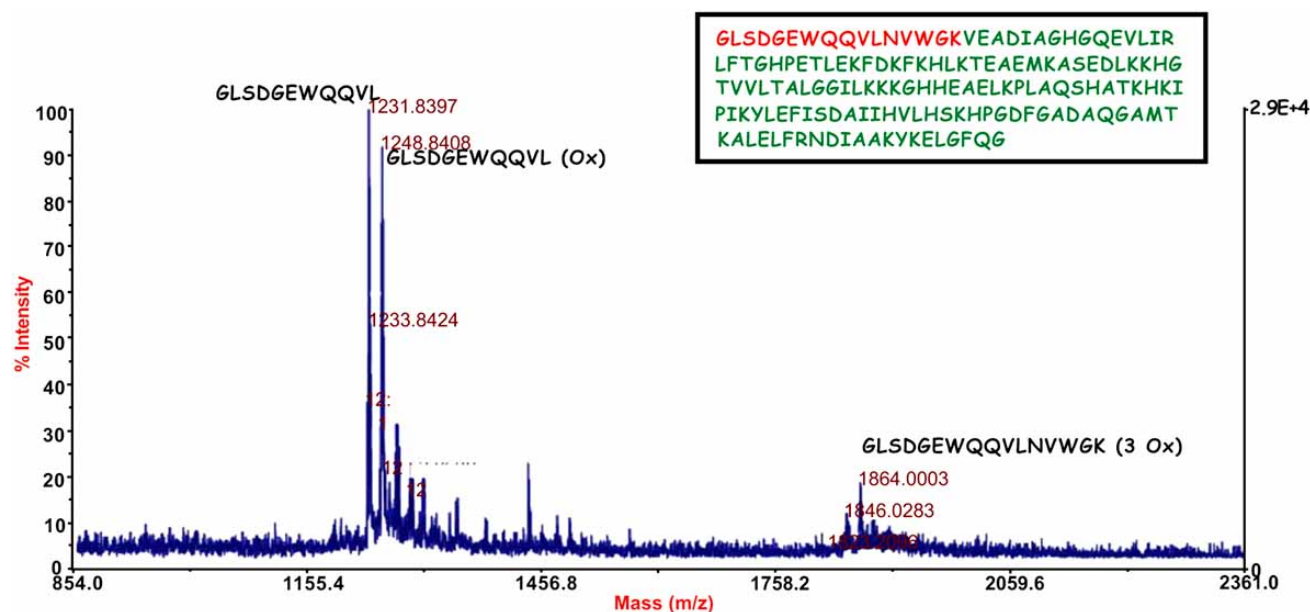


Fig. (6). A MALDI-TOF mass spectrum of peptides generated from horse myoglobin after performing MAAH in 1 M TFA at 50 W for 2 min in a CEM Discover Microwave.

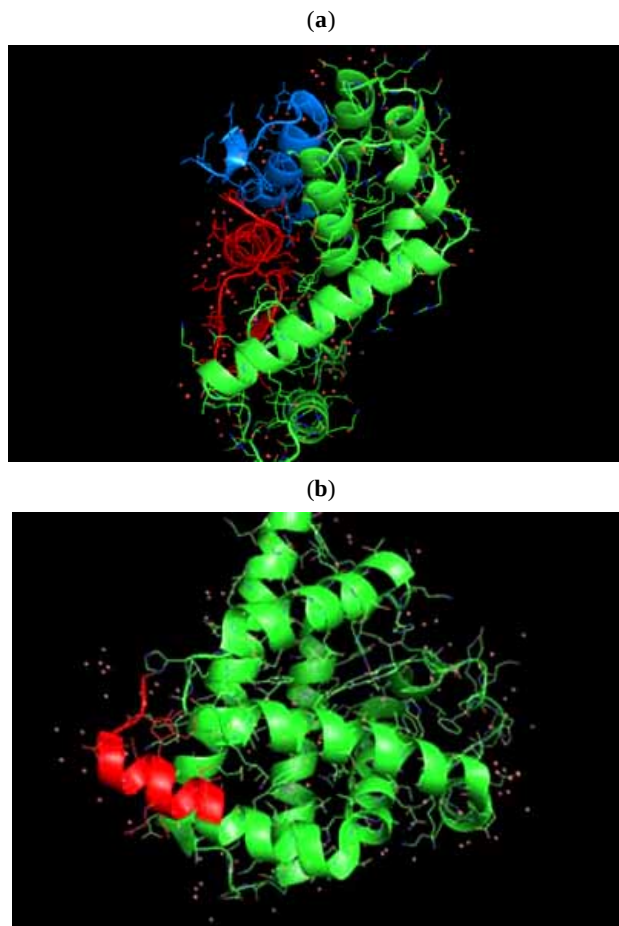


Fig. (7). PyMol X-ray crystallography structures for (a) lysozyme; the N-terminus is shown in red and the C-terminus in blue and (b) myoglobin; the N-terminus of myoglobin (again in red) is more exposed than the C-terminus (blue).

inaccessibility. Traditionally only internal sequence information is obtainable on QP blocked or other PGAP resistant proteins. Alternatively the biochemical capture methods described earlier may allow isolation and characterization of the N-terminal peptide [45], however, this involves several biochemical manipulations and can be time consuming. In addition, both of these methods require prior knowledge of sequence so that the correct proteolytic enzyme can be employed. In many cases, the N-terminus does not contain a convenient enzymatic cleavage site making the above mentioned methods more cumbersome.

Fig. 9 shows a MALDI-TOF mass spectrum of full length hepatocyte growth factor (HGF) after MAAH. The α -chain of HGF was N-terminally blocked with a pyroglutamate residue that, despite the absence of a Proline residue at position 2, was resistant to PGAP digestion. After 2 min incubation at 100°C in the microwave followed by MALDI MS, ions corresponding to the N-terminal sequence of HGF α -chain were observed, allowing fast and accurate sequence verification. We are currently working on a protocol that incorporates both MAAH and biochemical capture to allow a higher specificity of N-terminal capture than either method alone. However, at present the employment of MAAH for characterization of blocked N-termini is proving highly successful and allows higher-throughput analysis than traditional de-blocking or “bottom-up” methods alone.

3.2. Microwave-Assisted Proteolytic Digestions

Protein characterization is typically performed using a “bottom-up” work flow whereby proteins are subjected to proteolytic cleavage and resultant peptides are analyzed using either peptide mass fingerprinting (PMF) [10] or tandem mass spectrometry [11]. Due to the high-throughput nature of most proteomic laboratories today, proteolytic digestions should occur in the minimal amount of time, however, despite significant research into increasing the speed and effi-

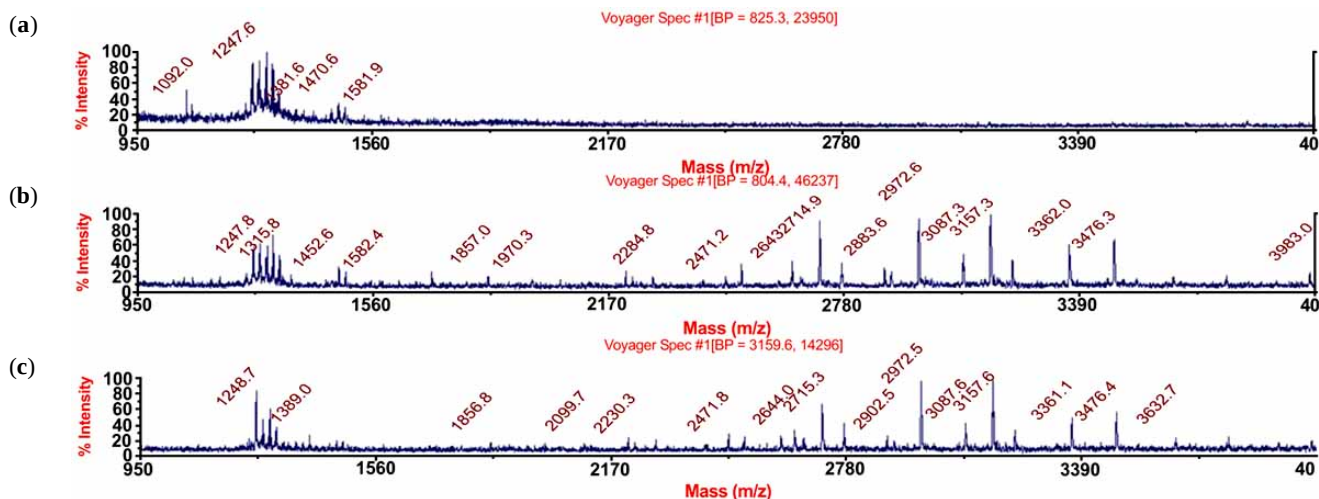


Fig. (8). MALDI-TOF mass spectra of peptides generated from myoglobin after performing MAAH in (a) 1 M TFA at 50 W for 2 min in a CEM Discover Microwave; (b) 1 M TFA with addition of 20% acetonitrile; and (c) 1 M TFA with addition of 250 mM Tris pH 8.

ciency of enzymatic reactions [21,47,48] typical digestions using trypsin and other common proteolytic enzymes still take several hours, or even overnight to perform.

Recently several publications have high-lighted the use of microwave-assisted proteolytic cleavage. Trypsin is currently the most commonly employed enzyme in “bottom-up” proteomic work-flows since it produces peptides of a convenient size for LC-MS/MS analysis and yields at least doubly charged ion species which leads to more easily interpret-

able MS/MS spectra. It is therefore not surprising that a lot of attention has been focused on trypsin with regards to microwave-assisted proteolysis. Microwave-assistance has been shown to increase the efficiency of tryptic digestions compared to conventional water-bath incubations in-solution and in-gel and in the presence of buffers containing organic solvents [1-3, 24, 28, 49]. A method incorporating the use of magnetite beads for the acceleration of microwave-assisted digestion reactions was described. Here it was demonstrated that microwave-assisted enzymatic digestions could be even

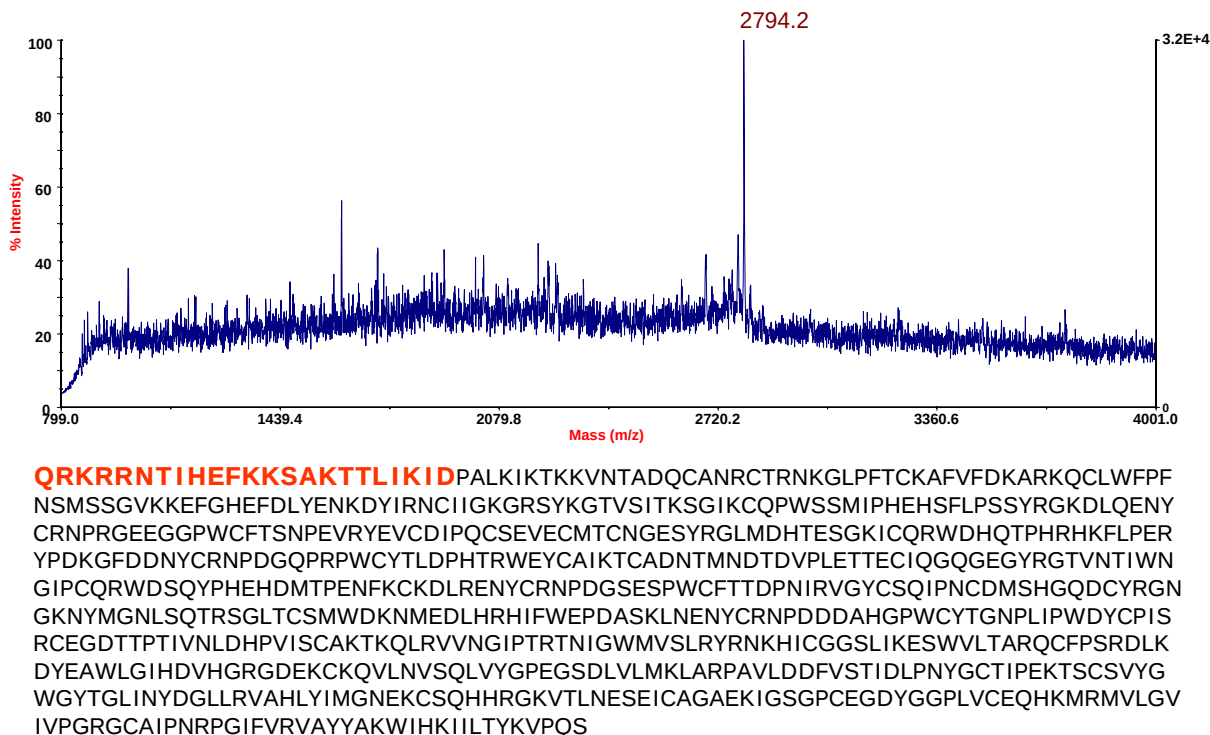


Fig. (9). MALDI-TOF mass spectrum after MAAH of a blocked protein, hepatocyte growth factor (HGF). The α -chain is blocked with a pyroglutamate residue. PGAP enzyme is ineffective on this protein and therefore, Edman sequencing is not possible. MAAH delivered sequence information from the N-terminus of the α -chain.

further accelerated by employing multifunctional magnetite beads. The beads improved the capacity of the reaction mixture to absorb microwave irradiation, and their negatively charged functionalities increases adsorption of proteins with opposite charges onto their surface by electrostatic interactions, leading to a concentration effect with the overall result being complete tryptic digestions within 1 min [50].

Microwave-assisted incubations using other proteolytic enzymes have also been explored, for example Glu-C [3] and Lys-C [1]. It was demonstrated that Glu-C digestions do not benefit from microwave exposure, as the enzyme is heat labile and susceptible to inactivation from microwave-induced denaturation [3]. On the other hand, Pramanik *et al.* demonstrated that endoproteinase Lys-C digestions, could benefit from microwave-assistance in an analogous manner to tryptic digestions [1].

In our laboratory, we repeated these experiments and agreed that tryptic digestion could indeed be catalyzed more rapidly in the presence of microwave exposure as compared to water-bath mediated incubations. For each of these experiments all apparatus, buffers and materials were pre-heated to 37°C or 60°C prior to incubation. This allowed one to accurately determine if increased proteolysis was due to optimal reaction temperatures being reached in a shorter time-frame, or if indeed, the increased catalysis was due to a true microwave-mediated effect. (For an in-depth discussion of microwave-assisted mechanisms please refer to ref. [24]). It must be noted that for many enzymatic proteolysis reactions as described above, that reaction times can be further decreased by elevating the temperature above the physiological temperature of 37°C towards 55-60°C in a non-microwave mediated environment [1].

After repeating the microwave-assisted experiments with the proteolytic enzyme endoproteinase Lys-C, we did not find any evidence that the reaction benefited from microwave exposure i.e. 37 or 60°C incubations in the microwave yielded similar results to the same temperature in a water-bath mediated incubation. We also tested endoproteinase Asp-N and found that although elevated temperatures could accelerate digestion (up to a maximum temperature of 60°C) microwave-assistance yielded little benefit compared to traditional water-bath mediated incubations at higher temperatures [24]. However, in a high-throughput laboratory setting, one would not typically pre-heat buffers and substrates to the required reaction temperature prior to incubation; therefore, having a mechanism such as microwave-assistance, which can “kick-start” a reaction, may be beneficial in itself, even if it does not increase reaction rates for the remainder of the incubation.

3.3. Microwave-Assisted Removal or Modification of Post-Translational Modifications

3.3.1. Microwave-Assisted Enzyme Catalyzed Removal of N-Terminal Pyroglutamyl

In the case of bio-therapeutic antibodies, assessment of monoclonality is of utmost importance as it is one way to measure the purity and specificity of an antibody. N-terminal sequencing using Edman degradation remains a key method for clonality determination, however some proteins, in particular the heavy chains of murine antibodies expressed in

mammalian cell lines, are frequently blocked with a pyroglutamyl group where the N-terminal glutamine residue is cyclized by the enzyme glutamine cyclase. This blocking group makes the N-terminal amino acid inaccessible to Edman sequencing. In such cases it is necessary to remove the pyroglutamyl group using PGAP prior to Edman degradation.

PGAP is a thermostable aminopeptidase used to remove the N-terminal pyroglutamic acid of proteins and peptides, active even at temperatures as high as 90°C. We previously reported an on-PVDF membrane PGAP digestion protocol that was performed in the thermocycler at 90°C for 1 h [36]. Taking advantage of the high thermal stability of this enzyme, we explored the potential of microwave irradiation to determine if microwave-assistance offers improved de-blocking efficiency over thermocycler mediated incubations.

To compare the efficiency of PGAP digestion performed in the thermocycler to that in the microwave, the heavy chain of anti OX40 ligand, which is blocked with a pyroglutamyl group, was employed. The heavy chain and light chain of this antibody were separated on a 4-20% Tris-glycine gel under reduced conditions. The band corresponding to the heavy chain was excised and treated with Zwittergent 3-16 solution, and the N-terminal pyroglutamic acid was removed by PGAP as described in the method section. DTT was added to the digestion buffer to keep the thiol group in the active site of PGAP under reduced conditions in order to maintain the activity of the enzyme. PGAP digestion was carried out at 90°C for 1 – 60 min in either the thermocycler or in the CEM Discover microwave system. The PGAP-treated heavy chain was then subjected to N-terminal sequencing. Initial yields of the de-blocked protein (based on the first five residues) obtained from sequencing data were calculated. The higher the initial yield at each time point indicates higher kinetics of the enzyme.

As seen in Fig. 10, at each time point past 10 min incubation, the microwave method produced higher initial yields and demonstrated a faster rate of reaction compared to the water bath or thermocycler mediated reaction. One hypothesis is that microwave irradiation helped denature the substrate and allowed more enzyme accessibility; however, as the protein is already immobilized in the PVDF membrane, accessibility due to protein conformation should not be an issue. Another hypothesis is that the microwave energy may have some effect on the activity of the enzyme. PGAP, unlike many other proteolytic enzymes, is heat stable as it is cloned from a thermophilic bacterial species. It is difficult to arrive at a conclusion from an experiment such as described above, however it can be ascertained that microwave-assisted enzyme mediated pyroglutamyl removal is more efficient than performing the same experiments using conventional heating methods.

3.3.2. Microwave-Assisted Removal of Other PTMs

The eukaryotic proteome owes its overwhelming diversity to a variety of genetic and proteomic events including, genomic splice variation, intracellular processing and the dynamic process of post-translational modification (PTM). Hundreds of unique PTMs exist and these facilitate the regulation of many cellular processes and intracellular signaling events. Two PTMs that are often complimentary in their functions are glycosylation and phosphorylation. Glycosyla-

tion sites are classified as either *N*-linked (via the amide nitrogen of asparagine) or *O*-linked via the hydroxyl group of serine, threonine and occasionally hydroxyproline and hydroxylysine. Analysis of glycosylation sites is often challenging due to the highly isobaric molecular weights of the component monosaccharides and the complex fragmentation patterns observed during MS/MS. As glycosylation is often highly heterogeneous, many protocols for both proteomic and glycomic analysis employ a deglycosylation step to simplify work flows. Previously both acid-catalyzed [32,33] and enzyme-mediated [8] microwave-assisted protocols have been described for the removal of oligosaccharides from glycosylated proteins and peptides.

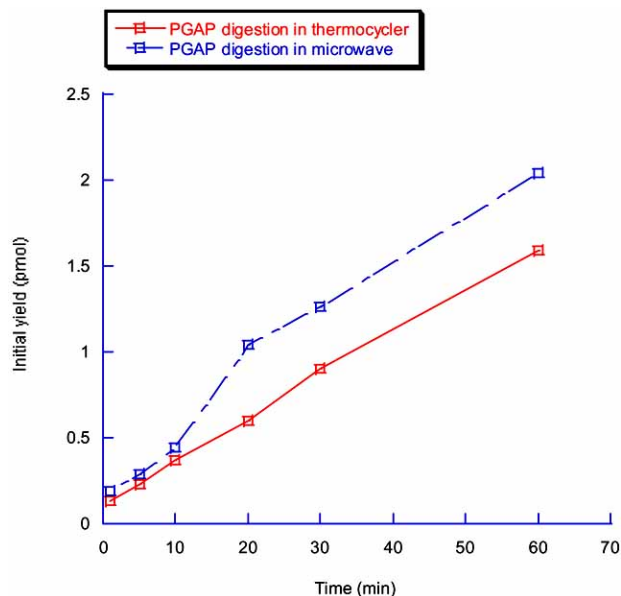


Fig. (10). A comparison of microwave-assisted vs thermocycler-mediated removal of N-terminal PyroGlu from the N-terminus of anti-OX40L antibody with PGAP enzyme. The initial yields (0-60 min) are taken as an average of the first 5 amino acids with each experiment analyzed in duplicate.

Phosphorylation is another common PTM which can direct the activity and function of a protein. The progression of many oncological pathways are dictated by kinase activity and phospho-specific events, hence phosphorylation mapping is currently one of the most active areas of proteomic research [14]. Many protocols for mapping phosphorylation sites involve either enrichment of phosphopeptides, or indeed modification with or without subsequent modification, for precise site mapping or characterization of recombinant proteins and phosphorylation sites.

Enzyme-mediated deglycosylation and removal of phosphate groups using beta-elimination, with or without subsequent nucleophilic addition, were investigated to see if they could benefit from microwave-assisted catalysis.

3.3.2.1. Microwave-Assisted N-Linked Deglycosylation

Deglycosylation is often carried out prior to proteomic analysis of recombinant proteins as it reduces the heterogeneity of the sample. Deglycosylation may be performed to reduce smearing during protein separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-

PAGE) or to facilitate ionization and interpretation of mass spectrometric data. Since the majority of therapeutic proteins to date are antibodies, the challenge of deglycosylation is common as immunoglobulins typically possess one or more *N*-linked glycosylation site on their heavy chains (most commonly on Asn 274). To measure the intact molecular weight of an antibody for monitoring characteristics such as the loss of a C-terminal lysine residue, or in the case of conjugated (magic-bullet) monoclonal antibodies [51], to monitor the number of small molecules coupled to the immunoglobulin, deglycosylation is routinely performed as it simplifies the mass spectrum allowing easier interpretation of data.

We routinely use microwave-assisted *N*-linked deglycosylation on therapeutic protein candidates prior to intact mass measurements [8] by mass spectrometry. Fig. 11 illustrates the utility of this method using recombinant VEGFR-3, a protein containing a known *N*-linked glycosylation site (N-X-S/T). The sample was diluted in 0.1% TFA and analyzed using a TSQ triple quadrupole mass spectrometer as described in the methods section. Due to the heterogeneity of the oligosaccharide on VEGFR-3, deconvolution of the mass spectrum for this protein was not possible prior to deglycosylation (Fig. 11a). However, after 30 min microwave-assisted deglycosylation followed by mass spectrometric analysis, the intact molecular weight of this recombinant protein was easily interpreted. Fig. 11b shows the deconvoluted mass spectrum of recombinant VEGFR-3 variant after microwave-assisted deglycosylation and analysis on a triple quadrupole mass spectrometer. As previously described [8] microwave-assisted *N*-linked deglycosylation allows removal of the oligosaccharide from monoclonal antibodies in less than 30 min. This is a >4- fold decrease in incubation time compared to traditional protocols for *N*-linked deglycosylation and this protocol has proved invaluable in our laboratory for the high-throughput intact molecular weight verification of monoclonal antibodies.

3.3.2.2. Investigation into Microwave-Assisted O-Linked Deglycosylation

It was shown that microwave-assisted *N*-linked oligosaccharide removal by PNGase-F was faster compared to water bath mediated incubation [8]. We therefore decided to continue this investigation to ascertain whether microwave irradiation had the same effect for the enzyme-mediated removal of *O*-linked carbohydrates. Bovine Fetuin was used as a model since this protein contains both *N*- and *O*-linked carbohydrates. Removal of *N*-linked sugar was performed as described in section 3.2.2. Fig. 12 shows Fetuin start material ~ 55 KDa (Lane 1) and Fetuin after complete removal of the branched *N*-linked carbohydrate content (lane 2) at MW ~45 KDa. For removal of the *O*-linked oligosaccharide, Fetuin was first treated with sialidase A. Sialidase A, cleaves sialic acid which typically end-caps the *O*-linked oligosaccharide chain, allowing access of the other *O*-linkage removing enzymes to the remaining oligosaccharide branches. To assess the completeness of the digestion, samples were analyzed by SDS-PAGE on a 4-20% Tris-glycine gel. Lanes 3 to 6 and lanes 7 to 10 of Fig. 12 show sialidase A & *O*-glycosidase - treated Fetuin at 37°C for 5, 10, 30, and 60 min in the thermocycler and in the microwave respectively. As can be seen, after 5 min incubation with sialidase A and *O*-glycosidase in the thermocycler there is a mass shift of 3

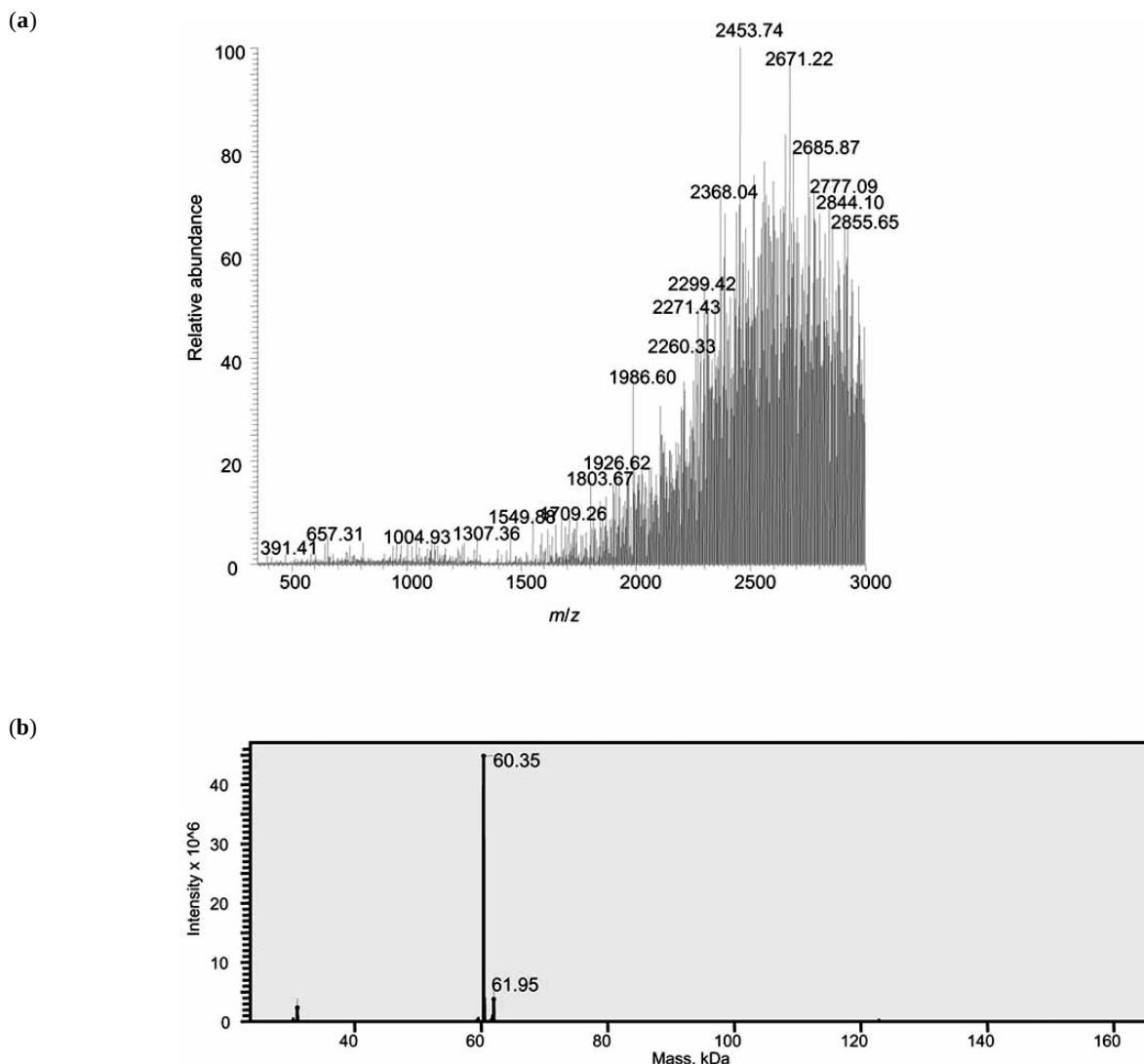


Fig. (11). (a) Mass spectrum of intact VEGFR-3. Deconvolution was not possible. (b) Deconvoluted mass spectrum of intact VEGFR-3 after deglycosylation in the microwave for 30 min with PNGaseF and DTT. After microwave-assisted deglycosylation, mass spectrometric analysis and data deconvolution of the spectrum resulted in one sharp peak corresponding to a mass of 60,354 Da.

KDa (lane 3) and the band at ~45 KDa (before removal of the *O*-linked sugar) was replaced with a band at ~42 KDa. This suggested that digestion was complete after 5 min incubation. Note that bands at ~88 and 36 KDa correspond to sialidase A and PNGase-F respectively. Results were identical at longer incubation times. 10, 30 and 60 min time points show that this reaction had reached completion. Microwave-assisted *O*-linked deglycosylation showed no advantage over thermocycler-assisted incubations as observed in lanes 7-10 of Fig. 12. Incubation was also performed at 45°C and 60°C at the same time points in thermocycler or microwave. Results were identical to the 37°C findings (data not shown). This experiment indicated that microwave irradiation did not show any advantage over thermocycler-assisted incubations for the removal of *O*-linked oligosaccharide. This was reproduced with similar results using a variety of other *O*-linked oligosaccharide removing enzymes. In addition, results showed that removal of *O*-linked sugar occurred almost instantly (only 5 min) in the thermocycler under these conditions. This is an example where a traditional incubation pro-

tol (of > 1 h incubation) may have been designed to cover all types of proteins and in many cases, especially for relatively simple recombinant proteins, short incubation periods using standard heating apparatus would suffice.

3.3.2.3. Microwave-Assisted Dephosphorylation (Beta-Elimination & Michael Addition)

Positional mapping of phosphoserine and phosphothreonine residues offers a challenge to the proteomic researcher due to the lability of these modifications, particularly under Edman sequencing or mass spectrometric analysis. Rapid phosphopeptide enrichment has recently been performed using tryptic digests of proteins with iron oxide nanocomposites of magnetic particles coated with zirconia in the presence of microwave irradiation [52]. We investigated an alternative method for precise mapping of phosphorylation sites involving microwave-assisted β -elimination and Michael addition. Selective modification of these residues to more stable S-ethylcysteine (phosphoserine) and β -methyl-S-ethylcysteine (phosphothreonine) derivatives is a useful tool

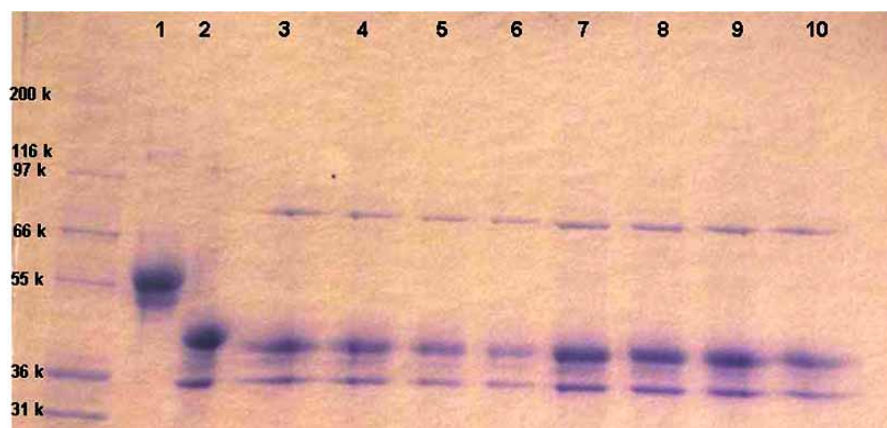


Fig. (12). Removal of O-linked carbohydrate at 37°C in the thermocycler vs the microwave, 10 µg/lane. Lane 1: Fetuin + DTT, Lane 2: Fetuin + DTT+ PNGase-F, Lanes 3, 4, 5, & 6: Fetuin +DTT+ PNGase-F + sialidase A + O-glycosidase in thermocycler for 5, 10, 30 & 60 min respectively. Lanes 7, 8, 9, and 10: Fetuin + DTT + PNGase-F + sialidase A + O-glycosidase in the microwave for 5, 10, 30, and 60 min, respectively.

for determining the exact location of a phosphorylation site, particularly when more than one Ser or Thr residue is present in close proximity.

At high pH, the phosphate ester of phosphoserine or phosphothreonine residues is compromised, resulting in an α,β -de-saturated moiety corresponding to a neutral loss of phosphoric acid (-98 Da). This process of β -elimination creates a Michael substrate that is susceptible to nucleophilic addition. Though a number of different nucleophiles have been reported [53,54] after evaluation of more than twenty compounds for completeness of reaction, derivative visibility by Edman sequencing and ease of use, our lab chose the alkyl thiols ethanethiol and 1-propanethiol, for these reactions. After β -elimination, addition of ethanethiol (62 Da) to the phosphopeptide or protein resulted in a net loss of 36 Da. 1-propanethiol (76 Da) shows a net change of -22 Da after successful nucleophile incorporation. Fig. 13 shows a step-wise schematic of the reaction.

Traditionally the nucleophile derivatization reaction is performed in the water bath for 1 to 3 h at 60°C. Phosphoserine residues readily undergo β -elimination within 1 h, but phosphothreonines generally require longer incubation times for a complete reaction. We have found that equivalent results may be obtained by microwave incubation for 2 min at 100°C to those achieved using traditional thermocycler-mediated incubations for 3 h (Fig. 14). The start material is shown in Fig. 14a and this was composed of a mixture of phosphorylated and non-phosphorylated peptides. Fig. 14b. shows the peptide solution after reaction at 60°C in the water bath for 1 h. Both the eliminated (-98 Da) and the ethanethiol modified moieties (-36 Da) of the phosphothreonine and phosphoserine peptides were observed. The non-phosphorylated peptide peaks (unlabeled) do not differ from the starting material. Fig. 14c shows the β -elimination/Michael addition reaction performed in the microwave for 2 min at 100°C. Data were comparable to the water bath results; Michael addition reaction was not complete under either sets of conditions (this is typically observed), however, equivalent results were obtained in a much shorter time using microwave-assistance.

A convenient sample format for Edman chemistries is to embed a protein in PVDF membrane. Therefore, to determine if the rate of reaction for β -elimination on-membrane could be improved, 50 pmol of β -casein was separated by SDS-PAGE and blotted onto a PVDF membrane. The band was excised from the membrane, wet with methanol, incubated in the reaction mixture as described above using 1-propanethiol as the nucleophile. Samples were incubated in the microwave for 2 min to 1 h or in the water bath at the same time points. The sample was then washed, dried and sequenced by Edman degradation on a Procise Sequencer (Applied Biosystems, Foster City, CA). After 2 min in the water bath the reaction had not yet commenced, however after 1 h in the water bath, results were found to be comparable to those achieved after just 2 min in the microwave (data not shown); peaks representing the modified Ser peaks eluted just after Leu on the PTH-chromatogram.

β -elimination as a tool for PTM characterization has been reported as far back as 1968 [55]. O-linked sugars from glycoproteins may also be selectively removed under alkali conditions from liquid samples or electro-blotted membranes [56]. Though we have not yet investigated this application, we believe that microwave-assistance could be applied to β -elimination of O-glycans for site determination. The incorporation of microwave-assistance into typical β -elimination work flows for the analysis of phosphorylation sites has shown a >30 fold decrease in reaction times compared to traditional methods employed thermocycler or water bath-mediated incubations.

4. SUMMARY

Microwave-assisted proteomics is a relatively new field and researchers are still exploring the possibilities this may offer to bio-analytical sciences. Currently our experience with microwave-assisted protein characterization has been limited to small scale reactions in the area of discovery research. However, we believe that the protocols investigated in this setting will also extend to larger scale operations for both characterization of recombinant proteins as well as for global proteomics. Microwave-assisted proteomics has been shown to enhance biochemical reactions at each stage of the

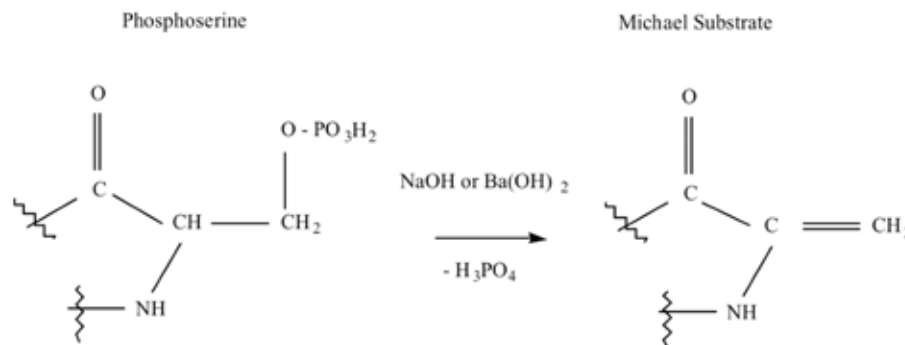
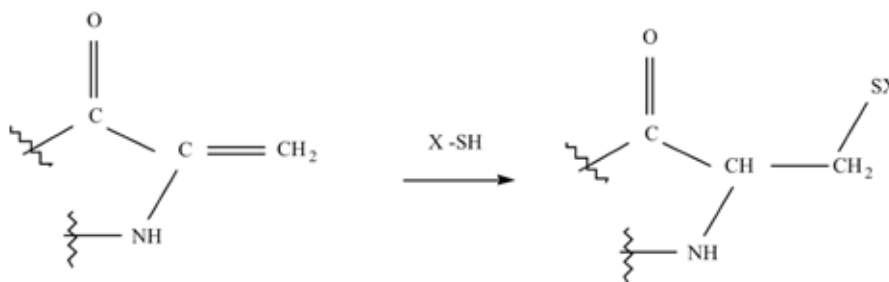
β - Elimination**Alkyl thiol Addition**

Fig. (13). A schematic of the β -elimination reaction of phosphoserine in base and Michael addition with an alkyl thiol (X-SH).

characterization of a therapeutic protein including its quantitation, sequence verification, termini characterization and removal and analysis of PTMs. Our experience has taught us that each microwave-assisted protocol should be compared directly to its counterpart experiment at identical non-microwave mediated incubation conditions, to ascertain if microwave-assistance is of true benefit, or if the reaction could simply be carried out using convection-based heating at a higher temperature than traditionally employed for higher yields and faster reaction time. In many cases reactions can indeed be catalyzed faster in the presence of increased temperature alone. However, it has been demonstrated for tryptic digestion [1-3, 24, 28, 49], enzyme mediated *N*-linked glycosylation removal [8], acid-mediated cleavage and *N*-terminal identification [6, 7, 29], protein hydrolysis for bottom-up analysis, protein quantitation, β -elimination and Michael addition and now PGAP mediated Pyro Glu removal that some reactions benefit specifically from microwave-assistance.

Although we have found that employing a specialized industrial microwave with a circular magnetron gives more control, flexibility and reproducibility compared to a domestic microwave oven, this does not mean that under well-executed conditions, a researcher cannot benefit from using a domestic microwave for testing microwave-assisted protocols if the specialized microwave is not available in their institution (for a discussion on this topic please refer to ref. [24]).

As the literature in this field expands, we should gain further understanding into the true mechanisms behind microwave-assisted proteomics, and better predict which biochemical processes truly benefit from microwave-exposure, which are detrimentally sensitive to exposure, and which experiments can be improved by being conducted in a non-microwave environment but at elevated temperatures than traditionally cited. The time-savings observed from the protocols described in the literature so far have demonstrated that microwave-assisted proteomics is definitely deserving of further investigation.

ABBREVIATIONS

AAA	= Amino Acid Analysis
CAPS	= <i>N</i> -cyclohexyl-3-aminopropanesulfonic acid
CHCA	= Alpha-cyano-4-hydroxycinnamic acid
DTT	= Dithiothreitol
HC	= Heavy Chain (in reference to an Antibody)
MAAH	= Microwave-assisted Acid Hydrolysis
MALDI-TOF	= Matrix-assisted Laser Desorption Ionization – Time of Flight
PGAP	= Pyroglutamyl Amino Peptidase
PMF	= Peptide Mass Fingerprinting

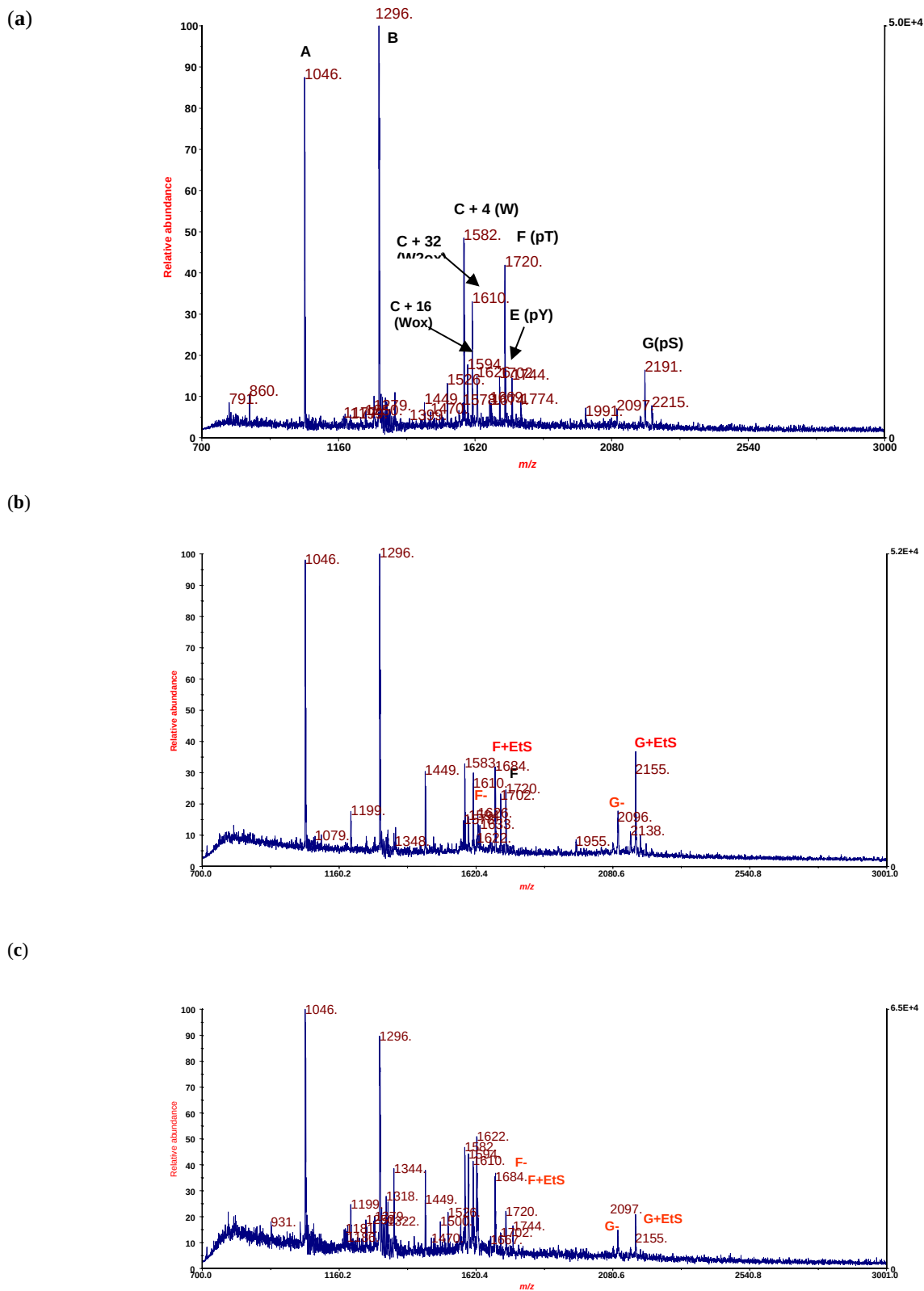


Fig. (14). MALDI-MS of a mixture of phosphorylated and non-phosphorylated peptide standards from Molecular Probes subjected to β -elimination/Michael addition. Peptides are represented by capital letters: A no phosphorylation DRVYIHPF (m/z 1046.5), B no phosphorylation DRVYIHPFHL (m/z 1296.7), C basic peptide GKGRGLSLSRFSWGA (m/z 1578.8), D phospho-Thr (pT) and phospho-Tyr (pY) DHTGFLpTEpYVATR (m/z 1669.7), E phospho-Tyr TRDIpYETDYYRK (m/z 1622.7), F phospho-Thr VPIpGRFDRRVpTVE (m/z 1640.9) and G phospho-Ser (pS) DLDVPIpGRFDRRVpSVAAE (m/z 2112.1). **(a)** Phosphopeptide start material, 500 fmol. The Tryptophan residue in peptide C is seen in oxidized forms (+4, +16 and +32). **(b)** Peptides after reaction in $Ba(OH)_2$ and Ethanethiol in a water bath at 60°C for 1 h. **(c)** Peptides after reaction in $Ba(OH)_2$ in a microwave for 2 min at 100°C.

PTM = Post-translational Modification
SDS-PAGE = Sodium Dodecyl Sulphate
Polyacrylamide Gel Electrophoresis
VEGFR-3 = Vascular Endothelial Growth Factor
Receptor-3

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