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Thermally enhanced enzymatic proteolysis for rapid ^{18}O labeling in proteomics

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This paper is dedicated to Dr. Catherine Fenselau, in recognition of her pioneering role and seminal contributions in the development of biological mass spectrometry and its applications.

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ABSTRACT

To streamline protocols for protein identification and better understand the contributions of purely thermal versus non-thermal effects, we utilize a microwave oven and a PCR thermocycler for rapid heating to accelerate enzymatic digestion of proteins. When performed in $H_2{}^{18}O$, rapid heating results in efficient C-terminal ^{18}O atom labeling of the proteolytic peptides. The approach is illustrated on the example of several pure proteins using trypsin and other proteases for rapid digestion. MALDI TOF/TOF MS provides unambiguous identification of the individual tryptic peptides. We have performed a time-course study on the degree of ^{18}O incorporation by varying the irradiation/heating times for each method. In order to gain insights into the mechanism of thermally enhanced trypsin digestion and ^{18}O labeling we carry out experiments in which the two events – lysis and labeling – are decoupled. We also study the rates of ^{18}O incorporation as a function of tryptic peptide C-terminal amino acid type and peptide length. Both heating methods are very rapid – in most cases digestion and incorporation of two ^{18}O atoms into R-terminated tryptic peptides is completed in less than 5 min, thus considerably reducing the time for bottom-up proteomics including quantitation by ^{18}O labeling.

1. Introduction

The increased risk of biological terrorism in recent years has highlighted the necessity for more efficient, rapid and robust methods to detect and identify bio-threat agents. Mass spectrometry, including bottom-up and top-down proteomics, is becoming a method of choice to successfully achieve this goal [1]. Currently, most strategies for bottom-up proteomics rely on chemical or enzymatic digestion, while quantitative proteomics encompasses metabolic, enzymatic or chemical incorporation of stable-isotope labels resulting in discernible mass shifts between corresponding peptides and proteins in the two sample pools [2–8]. Isotope labeling with ^{18}O atoms during proteolysis is one such qualitative and quantitative bottom-up proteomics strategy [9–14]. It is based on the incorporation of one or two ^{18}O atoms during N- or C-terminal specific endoprotease digestion of a protein in $H_2{}^{18}O$ [15–18]. Since incorporation of two ^{18}O atoms results in a more easily measured 4 Da shift between corresponding peptide pairs from the two samples (control versus labeled), C-terminal specific proteases (*e.g.*, trypsin) are most often used. The proteolysis and ^{18}O labeling can be successfully decoupled to achieve optimal conditions for each step, *e.g.*, different enzymes, pH, solvents, temperatures, times, etc. [7,8,19,20]. For quantification, the relative ion abundances of the control versus isotopically shifted peptides are measured and correlated to the respective precursor proteins. The relative merits and draw-backs of the ^{18}O labeling technique have been discussed in a number of reviews [4–7,9,20]. Proteolytic ^{18}O incorporation has been demonstrated to be compatible with shotgun proteomics [21] and this labeling technique has been developed into a useful tool in the relative quantification of complex protein mixtures, determination of post-translational modifications, intra-molecular protein cross-linking, and protein–protein interactions and complexes [8,12,19]. Specific software algorithms for estimates of ^{18}O incorporation efficiencies and peptide pair ratios [22,23] have been combined with traditional proteomics tools to fully exploit the advantages of this technique.

Typically, in a laboratory setting, when trypsin is used for proteolysis and combined with ^{18}O labeling, the reaction duration is between 12 and 24 h in order for complete digestion and incorporation of two ^{18}O atoms to occur. In order to devise a rapid ^{18}O labeling method for quantitative applications in proteomics for on-site screening, trypsin proteolysis [24,25] of model proteins in either ^{16}O or ^{18}O water is performed using a microwave oven or a PCR thermocycler [26]. For proteins investigated, ranging from ubiquitin to bovine serum albumin (BSA), we observe very efficient proteolysis and ^{18}O incorporation on a time scale of less than 5 min in either heating device. MALDI TOF/TOF MS is employed to identify the individual tryptic peptides. We evaluate the ^{18}O incorporation

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rates as a function of tryptic peptide C-terminal amino acid type, peptide length, and microwave irradiation time. In order to gain insights into the mechanism of thermally enhanced trypsin digestion and ^{18}O labeling experiments are carried out in which the two events – lysis and labeling – are decoupled. Rates of ^{18}O incorporation as a function of tryptic peptide C-terminal amino acid type and peptide length are also investigated. Both heating methods are very rapid – in most cases significant digestion and incorporation of two ^{18}O atoms into R-terminated tryptic peptides is achieved in less than 5 min, thus considerably reducing the time for bottom-up proteomics including quantitation by ^{18}O labeling.

2. Experimental

2.1. Materials

Bovine ubiquitin, equine cytochrome C, carbonic anhydrase, bovine serum albumin (BSA) and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co. (St. Louis, MO). MALDI matrices – α-cyano-4-hydroxycinnamic acid (CHCA) and 3,5-dimethoxycinnamic acid (SA) – were purchased from Laser BioLabs (Sophia-Antipolis Cedex, France) and were used without additional purification. Trypsin, TPCK treated (proteomics grade), was obtained from Worthington Biochemical (Lakewood, NJ) and endoproteinase Glu-C was from New England BioLabs (Ipswich, MA). Both proteases were reconstituted in either deionized water or ^{18}O water. ^{18}O water (97%) was obtained from Cambridge Isotope Laboratories (Andover, MA). Acetonitrile and water, both in HPLC grade (Burdick and Jackson), were purchased from VWR (West Chester, PA).

2.2. Thermally enhanced proteolysis

For all proteolytic digestion reactions, sample vials were filled with 150 μl of protein solution (30 μM in water, either $H_2{}^{16}O$ control or $H_2{}^{18}O$) followed by an aliquot of trypsin at an enzyme to substrate ratio of 1:100 (w/w) or for endoproteinase Glu-C 1:50 (w/w). For microwave-assisted proteolysis, glass sample vials containing the reactants were partially covered, and placed in a commercial microwave oven (Panasonic (900W), VWR) along with a 100 ml glass beaker partially filled with water. Microwave irradiation at the maximum oven power setting was done in intervals of 30 s to prevent solvent evaporation. In between irradiation times, sample vials were taken out of the microwave oven, capped and vortexed, then uncapped and returned to the oven for additional irradiation. Digestion was stopped after a pre-selected time, typically between 2 and 5 min, by immersion of the sample vial in an ice bath for rapid cooling. PCR thermocycler experiments were conducted using a Bio-RAD MJ Mini Personal Thermocycler (Hercules, CA). Reaction protocols were similar to those for the microwave reactions except volumes were reduced to accommodate the smaller size of the thermocycler tubes. Various cycling protocols were investigated and it was determined that a constant temperature of 65 °C for 5 min provided optimum protein digestion results. Conventional proteolytic digestions were conducted by placing reaction vials containing protein and enzyme in a water bath at 37 °C for 24 h. All reactions were stopped by placing sample vials in an ice bath after the designated reaction time for each method and reaction products were analyzed directly afterwards. For MALDI MS, 0.5 μl of the control and ^{18}O water-containing digests were each deposited in separate sample wells on the steel sample slide (Bruker Daltonics, Billerica, MA). The CHCA matrix solution (0.5 μl, 10 mg/ml in acetonitrile:water:TFA, 70:30:0.1) was then added to the wells and the sample was dried in air.

2.3. Mass spectrometry

Positive ion MALDI mass spectra were acquired on an Autoflex TOF/TOF instrument (Bruker Daltonics, Billerica, MA). Standard accelerating voltage (~20 kV) and delayed extraction (150 ns) conditions were used in linear or reflectron TOF mode. For tandem TOF/TOF MS, the LIFT method was employed, as previously described [27]. Briefly, ions were desorbed by a N_2 laser and delay-extracted at 6 kV in the first leg of the instrument (where the ions undergo unimolecular dissociation). The respective precursor ion and the fragment ions were then selected (isolated) in an ion gate, before re-acceleration in the second (reflectron) leg to 23 keV total energy for a singly charged precursor ion. Typically, several thousand single laser shot traces were summed for each MS/MS spectrum by manually scanning the laser beam across an individual sample spot. The MS and MS/MS peptide spectra were initially calibrated by using peptide and protein standards (average mass error in reflectron mode <0.5 Da across the entire mass/charge range below 3500). The linear and reflectron mode spectra were subsequently recalibrated with several tryptic peptide fragments used as internal mass standards (average mass error in reflectron mode <0.02 Da). Standard bioinformatics software (Bruker Biotools) was used to identify initially the proteolytic fragments observed. In order to assign sequences to unidentified fragments presumably resulting from disulfide bridges kept intact, the Web-accessible Links software (a part of the collaboration MS3D Portal – http://ms3d.org) was used [28].

Caution: Care should be exercised during microwave-assisted heating, *e.g.*, the sample vials should be uncapped in order to avoid explosion if a regular microwave oven is used.

3. Results and discussion

Several physical methods for accelerating enzymatic or chemical digestion in proteomics have been discussed in recent years [29,30]. Ultra-fast (<1 min) trypsin digestion of proteins by high intensity focused ultrasound has been demonstrated [31]. Similarly, high pressure cycling in the range of 5–35 kpsi has been introduced for in-solution digestions of single proteins and complex protein mixtures in about 1 min [32]. A rapid (<10 min) sample processing involves high intensity focused ultrasound to rapidly reduce and alkylate cysteines, digest proteins and then label peptides with ^{18}O [32]. Among the physical methods, microwave-assisted proteolysis – enzymatic [24,25] or chemical [33,34] – has become a very popular approach for reducing the overall sample preparation times in proteomics. Using water and/or other solvents, it has been shown that microwave-assisted digestion times for complex proteins and mixtures are markedly reduced from several hours to several minutes without reducing the tryptic enzyme efficiency or specificity as demonstrated with BSA (Fig. 1). By applying microwave irradiation in $H_2{}^{18}O$, both rapid trypsinization and efficient ^{18}O incorporation for proteins on a time scale of 2 min or less is achieved. We detect and identify by database searching more than 40 different tryptic peptides in the mass/charge range from 500 to 3000 (Fig. S1, Supplementary material). These all correspond to BSA-derived peptides with typically zero or one missed trypsin-specific cleavage at a K- or R-amino acid carboxyl terminal, thus providing more than 40% unique sequence coverage for BSA (^{16}O control sample, Table 1). No trypsin autolysis products were detected under our experimental conditions (MALDI spectra of the trypsin control reaction displayed an intense peak for the intact trypsin molecular ion, while no low mass ions were observed).

In order to minimize the overall experimental time, we have deliberately excluded Cys reduction and alkylation steps for disulfide-bond containing proteins in the current sample preparation protocols. Our premise is to develop protocols for rapid, on-site

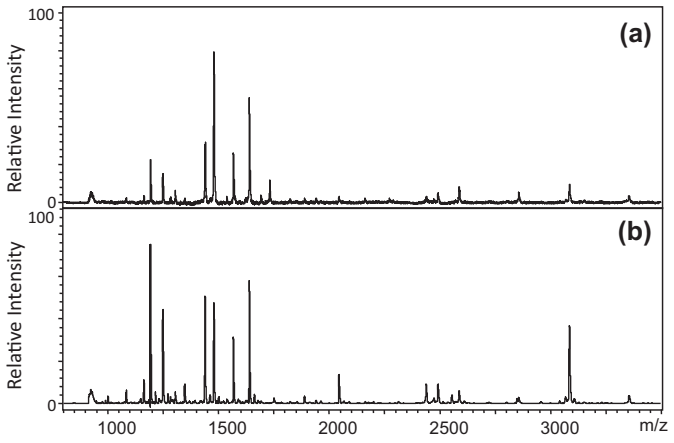

Fig. 1. Comparison of MALDI-TOF mass spectra of peptides resulting from trypsin-digested BSA: (a) Conventional overnight (24 h) reaction and (b) 5 min microwave-assisted digestion.

analysis in the event of a bio-threat response. For the last several years, our research team has investigated mass spectrometry-based methods for on-site field analysis of suspicious white powders, whereby analysis time is critical [35,36]. Based on our data for BSA, most of the 17 intra-molecular disulfide bridges are preserved intact during rapid thermally assisted proteolysis. Several tryptic peptide ions with masses reflecting the presence of intact disulfide bonds – either intra-fragment or disulfide-linked non-adjacent tryptic peptides are observed. These assignments have been confirmed also by ^{18}O labeling (vide infra). Evidence for cleaved disulfides (reduced Cys) was found in only two very low intensity peaks.

Comparing the baseline isotope-resolved spectra of the control and ^{18}O-labeled BSA digests, we observe a reproducible shift from 2 to 8 Da between the respective tryptic peptide masses in the two samples. The number of ^{18}O atoms incorporated during this short time proteolysis depends on the type of C-terminal amino acid (Fig. 2). Almost all K-terminated peptides incorporate exclusively one ^{18}O atom (Fig. 2a). The incorporation of the first ^{18}O atom occurs during the initial reaction step – cleavage of the amide bond (vide infra). After a reaction time of 90 s, only two out of fifteen K-terminated peptides have exchanged more than one ^{18}O atoms (Table 1). In contrast, the R-terminated peptides incorporate almost

Table 1
List of identified tryptic peptides from microwave-assisted trypsinization of BSA and degree of ^{18}O incorporation.

Experimental monoisotopic mass (Da)	Theoretical monoisotopic mass (Da)	Peptide sequence	Residue number[a]	Number of ^{18}O incorporated after 90 s digestion
508.38	508.25	FGER	229–232	2
572.46	572.36	QRLR	219–222	2
634.48	634.38	GVFRR	20–24	2
649.45	649.33	IETMR[b]	205–209	2
	649.33	CASIQK	223–228	
689.44	689.37	AWSVAR	236–241	2
712.43	712.37	SEIAHR	29–34	2
733.49	733.42	VLTSSAR	212–218	2
847.50	847.50	LSQKFPK	242–248	2
906.57	906.47	IETMREK	205–211	(n/a)[c]
918.55	918.52	LRCASIQK	221–228	1
922.48	922.49	AEFVEVTK	249–256	1
927.50	927.49	YLYEIAR	161–167	2
974.43	974.46	DLGEEHFK	37–44	1
977.53	977.45	NECFLSHK	123–130	1
990.59	990.56	EKVLTSSAR	210–218	2
1001.59	1001.59	ALKAWSVAR	233–241	2
1083.58	1083.60	YLYEIARR	161–168	2
1163.60	1163.63	LVNELTEFAK	66–75	1, 2
1193.60	1193.60	DTHKSEIAHR	25–34	2
1249.60	1249.62	FKDLGEEHFK	35–44	1
1283.71	1283.71	HPEYAVSVLLR	361–371	2
1305.65	1305.72	HLVDEPQNLIK	402–412	0, 1
1347.53	1347.55	TCVADESHAGCEK	76–88 + disulfide	1
1439.79	1439.81	RHPEYAVSVLLR	360–371	2
1479.79	1479.80	LGEYGFQNALIVR	421–433	2
1511.85	1511.84	VPQVSTPTLVEVSR[b]	438–451	1, 2
	1511.95	QIKKQTALVELLK	545–557	
1567.74	1567.74	DAFLGSFLYEYSR	347–359	2
1639.94	1639.94	KVPQVSTPTLVEVSR	437–451	2
1692.90	1692.94	AEFVEVTKLVTDLTK	249–263	1
1750.94	1750.97	LSQKFPKAEFVEVTK	242–256	1
1888.94	1889.00	SLHTLFGDELCKVASLR[b]	89–105	2
	1888.93	HPYFYAPELLYYANK	169–183	
1940.75	1940.82	VHKECCHGDLLECADDR	264–280 + disulfide	1
2044.99	2045.03	RHPYFYAPELLYYANK	168–183	1
2438.98	2439.10	VHKECCHGDLLECADDRADLAK	264–285 + disulfide	1
2492.02	2492.16	LVNELTEFAKTCVADESHAGCEK[d]	66–88 + disulfide	1
	2492.11	LRCASIQK-ECCHG DLLECADDR	221–228, 267–280 + disulfide	
2587.06	2587.15	CASIQK-VHKECCHGDLLECADDR	223–228, 264–280 + disulfide	3
3085.67	3085.43	CASIQK-VHKECCHGDLLECADDRADLAK	223–228, 264–285 + disulfide	3

[a] Residue numbering for P02726|ALBU_BOVIN including propeptide sequence (a.a. 1–24).
[b] Probable sequence assignment based on ^{18}O incorporation (not confirmed by LIFT MS/MS due to weak precursor ion signal).
[c] Signal too weak to accurately determine ^{18}O incorporation.
[d] Sequence assignment based on ^{18}O incorporation and confirmation by LIFT MS/MS.

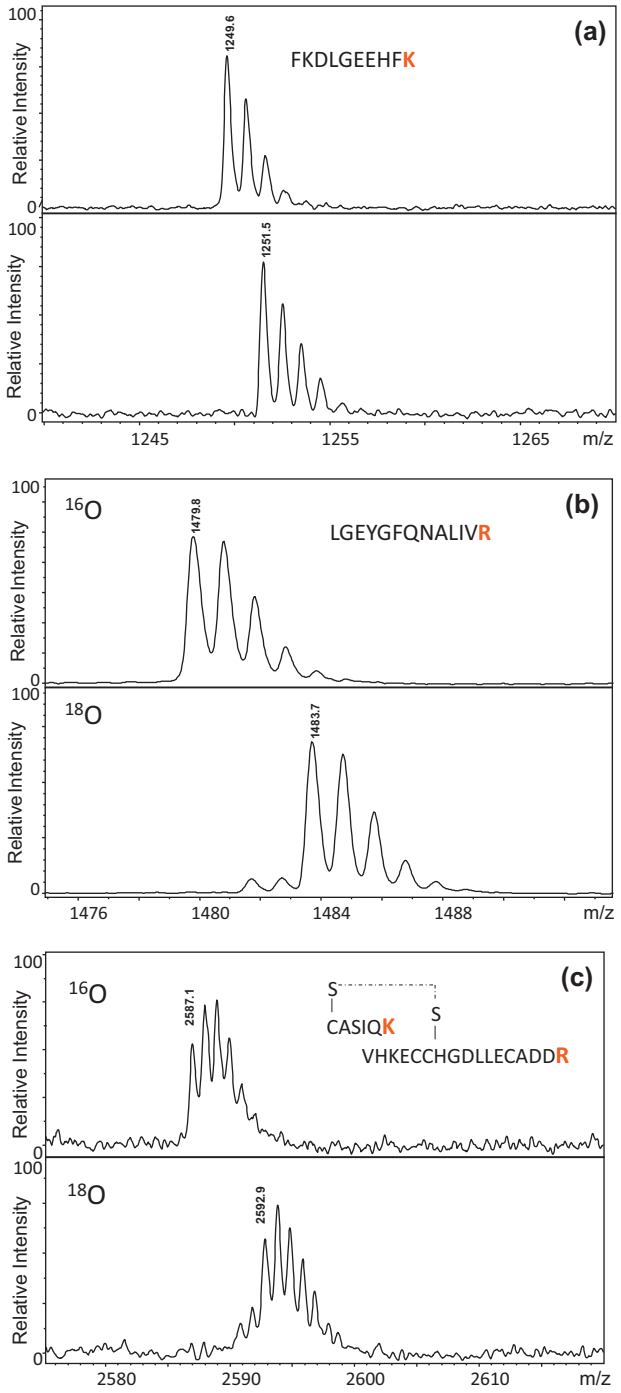

Fig. 2. Comparison of MALDI reflectron-TOF spectra (the resolved molecular ion region) of selected peptides resulting from a 90 s microwave-assisted tryptic digestion in $H_2{}^{16}O$ (top panel of each) or $H_2{}^{18}O$ (bottom panel of each): (a) Lys-terminated peptide, (b) Arg-terminated peptide, and (c) disulfide-linked fragment containing both Lys- and Arg-terminated peptides.

exclusively two ^{18}O atoms (Fig. 2b). No direct effects of trypsin cleaved peptide length with ^{18}O incorporation efficiency have been observed for either peptide type (Table 1). As already demonstrated [19,20], the mechanism explaining the proteolytic incorporation of two ^{18}O atoms in the C-terminal carboxyl group during proteolysis is a two-step reaction. In the first step, one ^{18}O atom is incorporated during peptide bond cleavage. The second step – the exchange of the second ^{18}O atom in the tryptic peptide – is the rate limiting. This exchange reaction proceeds through the formation of a

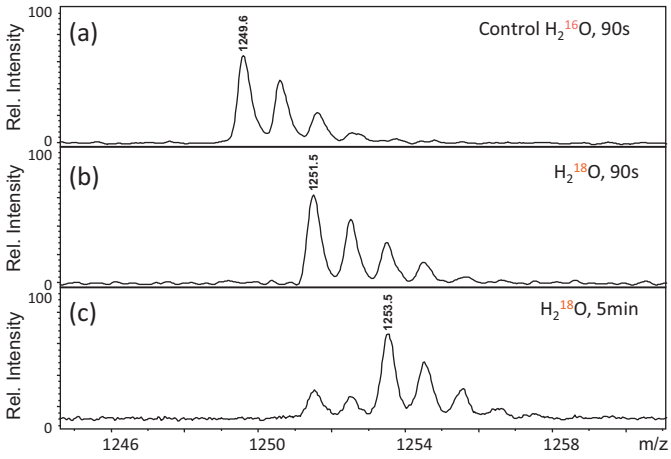

Fig. 3. Resolved molecular ion region spectra (MALDI reflectron-TOF) illustrating digestion/labeling time-course for a selected Lys-terminated peptide; control digestion for 90 s in $H_2{}^{16}O$ (top), 90 s digestion in $H_2{}^{18}O$ (middle), and 5 min digestion in $H_2{}^{18}O$ (bottom).

tetrahedral enzyme–peptide substrate complex intermediate followed by nucleophilic attack of $H_2{}^{18}O$ [19,20]. The complex formation and ultimately the completion of the reaction depend on a number of factors: the substrate–trypsin binding, enzyme concentration, peptide solubility, etc. [19]. On the timescale of this experiment for BSA (from 30 to 90 s for microwave digestion), we observe efficient incorporation of two ^{18}O atoms for R-terminated peptides, while longer microwave irradiation times (*e.g.*, 5 min) are needed to more efficiently incorporate the second ^{18}O atom in K-terminated peptides (Fig. 3). Microwave-assisted proteolytic digestion reactions in $H_2{}^{16}O$ and $H_2{}^{18}O$ were also conducted using endoproteinase Glu-C. Efficient fragmentation of BSA is observed after an irradiation time of 5 min as well as incorporation of one and two ^{18}O-atoms, depending on the C-terminal amino acid (Figs. S2 and S3, Supplementary material). Similar to the results using trypsin at this longer irradiation time, incorporation of two ^{18}O-atoms for lysing terminating peptides is observed. These results highlight the overall robustness of the rapid thermally assisted digestion approach, including its applicability to different proteases. When conducted in parallel, additional confirmation of protein identification can thus be achieved without sacrificing the rapid analysis time.

Additional experiments aimed at decoupling microwave-assisted trypsinization from microwave-assisted ^{18}O labeling were performed in order to better understand the mechanisms of both processes. Initially, microwave-assisted trypsin digestion of BSA was carried out in ^{16}O-water for 90 s. The sample was then dried, re-suspended in ^{18}O-water and subsequently irradiated again for additional 90 s. In this set of experiments, efficient incorporation of two ^{18}O atoms is observed for the enzymatically cleaved R-terminated peptides upon microwave irradiation in the presence of trypsin (Fig. 4). However, the time scale of this experiment has been insufficient for even one ^{18}O-atom exchange in most of the K-terminated peptides (Fig. 4). All K-terminated peptides incorporating only one ^{18}O atom during trypsinization in $H_2{}^{18}O$ (Table 1), have not exchanged any ^{18}O atoms in this decoupling experiment either. This observation demonstrates that the same two-step ^{18}O labeling reaction conducted using traditional methods also occurs during microwave-assisted trypsinization. In the first step, only one ^{18}O-atom is incorporated in the C-terminal upon amide bond cleavage. The second step, requiring formation of the serine protease–tryptic peptide substrate complex, is more efficient for the R-terminated peptides (compared to K-terminated peptides). It can proceed independently of peptide bond cleavage and thus

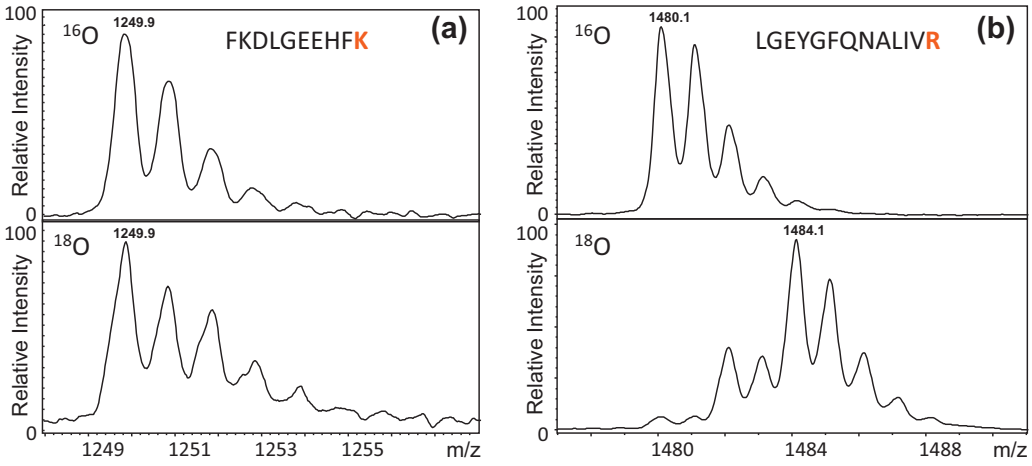

Fig. 4. Comparison of MALDI TOF spectra (the resolved molecular ion region) of selected peptides in the decoupling experiment: 90 s microwave-assisted tryptic digestion in $H_2{}^{16}O$ (top panel of each); subsequent drying, resuspension in $H_2{}^{18}O$ and 90 s irradiation (bottom panel of each): (a) Lys-terminated peptide – no exchange; and (b) Arg-terminated peptide – two ^{18}O atoms exchanged. For comparison purposes, the spectra of the same peptides as in Fig. 1(a) and (b) are plotted here as well.

results in substitution of up to two ^{18}O atoms for R-terminated peptides. We note that the time required for complete (two atom) ^{18}O exchange was more than an order of magnitude longer for K- versus R-terminated peptides, as reported for the original decoupling experiments [19]. It has been further argued by Yao et al. that the ^{18}O substitution reaction in the second step is somewhat similar to amide bond formation [19]. In this context, it should also be noted that microwave heating has been demonstrated to significantly reduce reaction times, improve yields of optically pure products and the overall reproducibility in solid state peptide synthesis [37].

In additional microwave trypsinization experiments, the overall microwave irradiation time is extended from 90 s to 5 min. This time was still not sufficient for complete incorporation of ^{18}O atoms in all K-peptides under these experimental conditions. Previous reports have also indicated variable rates of incorporation of ^{18}O atoms in labeled peptides, with the underlying cause directly attributable to the reaction conditions [7,8]. Thus it is anticipated that with additional optimization of reaction parameters reported herein, namely increasing reaction time, complete labeling of lysine terminating peptides is achievable [8]. We determine that trypsin was not only intact after prolonged microwave irradiation (no autolysis products observed) but it also retained its activity and specificity (as evidenced by the decoupling experiments). Since the reactions were performed at near neutral pH (pure water), no back exchange by ^{16}O is observed. The incorporation of one or two ^{18}O atoms on a short time scale is reproducible. Analogous results were obtained for other proteins investigated. This approach allows relative quantification studies to be performed, as well as K- versus R-terminated peptides to be discerned. In several instances, initial identification of BSA tryptic peptides in ^{16}O water very close in mass was not possible, since the observed peptide mass was close to more than one predicted peptides masses from *in silico* digestion (Table 1, peptide(s) with experimental *m*/*z* 649.45, 1511.85 and 2492.02). In these cases, when one of the predicted peptides terminates in K and the other in R, the number of experimentally incorporated ^{18}O atoms provides the correct assignment for the observed peptide. Some of these assignments are independently confirmed by MS/MS data of the respective unlabeled peptide (Fig. 5). We note that ^{18}O-specific software analysis algorithms [19] can be adapted to exploit the advantages of this rapid labeling technique. In some instances, mass shifts of 6 or 8 Da have been observed. These occur as a result of ^{18}O incorporation in the C-termini of two non-adjacent tryptic peptides held together

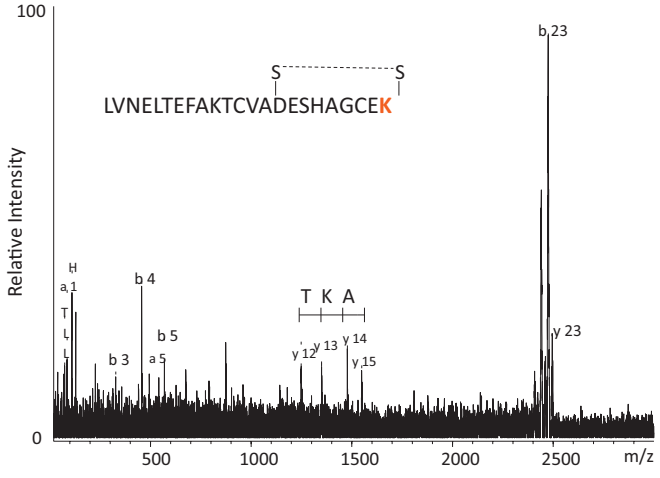

Fig. 5. MALDI MS/MS (LIFT) spectrum of the unlabeled tryptic peptide at *m*/*z* 2492.02. The sequence of the peptide inferred from this spectrum demonstrates that it is Lys-terminated (in agreement with the observed number of exchanged ^{18}O atoms – only one, Table 1).

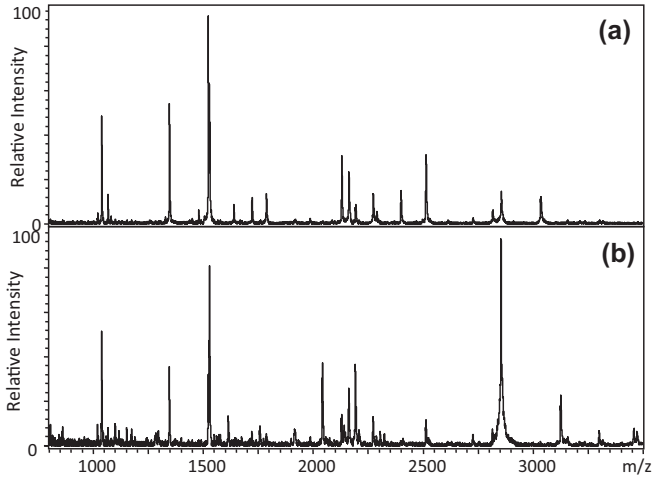

Fig. 6. MALDI TOF mass spectra of peptide fragments generated from the trypsin digestion of ubiquitin by (a) microwave-assisted digestion and (b) PCR thermocycler digestion.

by an intact disulfide bond. Therefore, the proposed rapid digestion and ^{18}O incorporation method can be implemented for identification of these and other types of cross-linked peptides in studies of protein–protein interactions [38].

Most of the microwave-assisted experiments, described above, have been repeated in the PCR thermocycler with very similar results. For the proteolytic digestion of ubiquitin using trypsin, a similar degree of protein cleavage is obtained, however, slight variations in relative ion abundances of cleaved fragments is observed (Fig. 6). This similarity in proteolysis products indicates that the same thermal activation mechanisms of protein digestion and labeling operate in both heating devices. The rate at which the reaction mixture is initially heated, as well as the final temperature of the reaction mixture affects digestion and ^{18}O labeling efficiency. One advantage of using a PCR thermocycler in bottom-up proteomics is its ubiquity and accessibility in multiple labs, and its affordability, compared to specialized microwave hydrolysis reactors.

4. Conclusions

Two rapid and efficient methods for protein digestion and ^{18}O incorporation during thermally enhanced trypsinization are compared. Both heating methods are rapid – in most cases significant digestion and incorporation of two ^{18}O atoms into R-terminated tryptic peptides is achieved in less than 5 min, thus considerably reducing the time for bottom-up proteomics including quantitation by ^{18}O labeling. The number of ^{18}O atoms incorporated correlates well with the type of C-terminal amino acid. This approach is not only useful for quantitative proteomics labeling but suggests a simple method for discerning K- from R-terminated tryptic peptides as well. These experiments provide additional insights into the mechanism of rapid thermally enhanced trypsin digestion of intact proteins. We demonstrate that up to two ^{18}O atoms can be efficiently exchanged in R-terminated peptides even when the exchange step is decoupled from the digestion step. The described rapid methods for incorporation of ^{18}O atoms can also be useful in approaches for rapid *de novo* peptide and protein sequencing. We plan to further optimize the methods and to test them with other endoproteases as well as different solvent systems. The methods are being modified with the aim to develop reliable protocols for rapid, high-throughput bottom up proteomics and accurate quantification of protein toxin bio-agents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2011.07.005.

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