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Journal of Chromatography A, 1053 (2004) 133–142
JOURNAL OF CHROMATOGRAPHY A
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Use of two-dimensional liquid fractionation for separation of proteins from cell lysates without the presence of methionine oxidation

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Available online 17 September 2004

Abstract

A novel two-dimensional (2D) chromatographic method is developed to separate proteins from malignant breast cancer whole cell lysates. Protein mixtures are first separated according to their p*I* by chromatofocusing followed by an orthogonal non-porous reversed-phase separation. An important advantage of this 2D chromatographic method is that, unlike gel-based methods, it does not result in methionine oxidation. The lack of methionine oxidation during separation is demonstrated by the analysis of protein tryptic digests using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS. Our novel 2D chromatographic method used in combination with on-target light-induced methionine oxidation provides a means for studying methionine-containing peptides. Methionine residues in peptide sequences are partially oxidized with light exposure. Neither the location nor the modification of methionine in the peptide sequence affects the oxidation. As a result, multiple peaks are observed in MALDI-TOF-MS spectra after light exposure. Sequence information derived from light-induced methionine can be applied to enhance the database search results obtained through peptide mass fingerprinting.

Keywords: Reversed-phase separation; MALDI-TOF; 2D-LC; Methionine oxidation; Chromatofocusing

1. Introduction

Despite the popularity of the application of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in protein separation, several liquid phase methods have been developed [1–12] to overcome its limitations. A disadvantage for the gel-based method is that methionine residues in proteins are oxidized to methionine sulfoxide (MeSOX) during separation. Methionine oxidation is commonly attributed to gel-induced oxidation [13] because of residual oxidative polymerization enhancers in gels [14]. The oxidized methionine-containing peptide may have a hydrophilic shift of several minutes in reversed-phase separation [15] which may introduce additional complexity for the follow-up LC–MS or LC–MS–MS experiment. Moreover, multiple peaks resulting from partial methionine oxidation introduce errors in the isotope labeled quantitation method for all methionine-containing proteins. Further, gel-based methods are not routinely used to obtain MeSOX information, where the oxidation of methionine to MeSOX plays a key role in many processes [16,17]. As a result, new methods for protein profiling that do not induce methionine oxidation are needed.

A current problem with protein identification strategies is that the probability of obtaining unambiguous identifications by peptide mass fingerprinting (PMF) decreases continuously with the increase of database entries [18]. Use of improved mass accuracy on peptide mass measurements aids in improving the accuracy of such protein identifications [19]. Digestion of proteins with different enzymes provides additional specificity to increase the accuracy of identification [20]. Amino acid sequence and composition information obtained by isotopically labeled peptides [18,21–24] and mass tag methods [25–27] can also increase the confidence of protein identification. In the above methods, improved protein identification requires substantial chemical derivatization

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doi:10.1016/j.chroma.2004.08.080

and additional amounts of sample. Recently, a method for on-target oxidation of methionine using hydrogen peroxide [28] was introduced to improve database searching. On-target oxidation allows for minimal sample consumption prior to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS analysis. New convenient methods providing sequence and composition information without the need for chemical derivatization or large amounts of sample are needed to reduce ambiguous identifications and the need for further peptide characterization by sequencing methods.

In this work, a 2D liquid separation of proteins from cancer cell lysates using chromatofocusing (CF) as the first dimension followed by nonporous silica (NPS) RP-HPLC in the second dimension is used to produce proteins free of methionine oxidation. Tryptic digests of 2D-LC purified proteins with methionine-containing peptides remain in their reduced form. In contrast, when samples on MALDI target plates are exposed to UV light for three days, methionine-containing peptides are oxidized to sulfoxide. Furthermore, the light-induced methionine oxidation is not affected by acetylation on methionine or the location of methionine in the peptide sequence. Light-induced methionine oxidation can be used to reveal the presence of methionine in peptide sequences. Also, the number of methionine residues in sequences can be derived from the number of new peaks after light exposure. By including methionine sequence information in PMF database searches, obtained molecular weight search (MOWSE) scores and confidence levels of searches are improved. Moreover, the light-induction method does not require an extra modification step, sample handling, or extra sample consumption. In this work, we revealed the potential of using UV light as a "clean" modification method in a positive way for proteomics study. Our work also suggests the importance of preventing light exposure during sample preparation for isotope labeled quantitative study.

2. Experimental

2.1. MCF10CA1d.cl1 cell preparation and lysis

Fully malignant human breast cancer cells, MCF10CA1d clone 1 (CA1d) cells [29], were grown in monolayer on plastic in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium supplemented with 5% horse serum, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). Harvest was performed in the log phase (∼75–80% confluence). After the growth medium was aspirated, the cells were gently washed with sterile phosphate buffered saline (PBS) before they were scraped with a rubber policeman and stored at −80 °C.

Cell lysis is performed by adding 1.0 mL lysis buffer containing 6 M urea, 2 M thiourea, 0.5% (w/v) *n*-octyl-β-D-glucopyranoside (OG), 50 mM DTT (dithiothreitol), 2 mM Tris(2-carboxyethyl)phosphine (TCEP), 1 μL protease inhibitor (Sigma, St. Louis, MO, USA) and 10% (v/v) glycerol. The cell lysis buffer mixture was vortexed for two minutes and then left at room temperature for an hour. In order to eliminate cell debris, sample and buffer mixture were centrifuged at 20 000 × *g* for 20 min. The supernatant was collected and the protein content determined using a Bradford-based assay (Bio-Rad, Hercules, CA, USA).

2.2. Chromatofocusing

Chromatofocusing (CF) is a weak anion exchange technique that has the resolving power of isoelectric focusing and the simplicity of ion-exchange chromatography [30]. In order to minimize the effect of buffer differences, the lysis buffer in the whole cell lysate is exchanged with start buffer using a PD-10 column (Amersham Pharmacia, Piscataway, NJ, USA). The start buffer contains 25 mM bis-Tris propane (Sigma) and 6 M urea, where the pH is adjusted to 7.4 using saturated imminodiacetic acid (Sigma). Whole cell lysate is diluted to 2.5 mL with start buffer, and the diluted solution is introduced onto a PD-10 column. 3.5 mL of eluent is collected after the same amount of start buffer is loaded. Before sample introduction onto the HPCF column (Eprogen, Darien, IL, USA), the column is equilibrated with start buffer until a stable pH and UV (280 nm) absorbance are reached.

Approximately 7 mg of protein extracted from CA1d cells is loaded onto the CF column. During sample loading, proteins with a p*I* lower than 7.4 will be negatively charged and will bind to the stationary phase. During elution, the net charge on the protein is gradually neutralized so that decrease in solubility becomes a concern. In order to enhance solubility, a chaotropic reagent, 6 M urea, is added to both the start and elution buffer. Online pH measurement is performed off column before fraction collection using a pH electrode (Lazar Research, Los Angeles, CA, USA).

Separation is achieved by eluting the column with a buffer containing 10% (v/v) PolyBuffer 74, 6 M urea adjusted to pH 4 with saturated imminodiacetic acid. During CF, only part of a protein binds to the stationary phase through specific areas of the protein or individual charge sites. Therefore, elution of proteins at pH values higher than their isoelectric points (p*I*) is commonly observed. Thus, the pH value of the start buffer was set 0.4 pH unit higher than the desired pH range of 7.0–4.0. The PolyBuffer in the elution buffer is diluted to 1/10 from the original concentration to produce a shallow pH gradient which improves the resolution of the separation. The separation is monitored at 280 nm using a Beckman 126 model UV detector (Beckman-Coulter, Fullerton, CA, USA).

The eluent from CF separation is collected from pH 7.4 to 4.2 in 0.2 pH unit intervals. The collection process is controlled using software written in-house. The collected fractions are stored in a dry ice box right after the collection to minimize light exposure. For long term storage, the samples are stored at −80 °C.

2.3. Non-porous (NPS) reversed-phase liquid chromatography

The NPS RP-HPLC separation is performed at a flow rate of 0.5 mL/min on an ODS IIIE (33 mm × 4.6 mm) column packed with 1.5 μm non-porous silica beads derivatized with C18 (Eprogen Inc.). Approximately 180 μg of protein is collected from the CF fraction with pH of 4.6–4.8 which is further separated by NPS RP-HPLC. The column is maintained at 65 °C with a Timberline column heater (Boulder, CO, USA) in order to improve the resolution and speed of the separation. Elution is performed using a water (A)–acetonitrile (B) [0.1% (v/v) trifluoroacetic acid (TFA)] gradient. The gradient profile used is as follows: (1) 5–15% B in 1 min; (2) 15–25% B in 2 min; (3) 25–31% B in 3 min; (4) 31–41% B in 10 min; (5) 41–47% B in 3 min; (6) 47–67% in 4 min; (7) 67–100% in 1 min; (8) 100% B in 2 min; (9) 100–5% in 1 min. The acetonitrile is 99.93% HPLC grade (Sigma) and the TFA was from 1 mL sealed ampules (Sigma). The HPLC system was a Beckman Model 127 HPLC where the separation is monitored at 214 nm using a Model 166 detector. The proteins separated by NPS-HPLC are collected into 1.5 mL tubes using a Beckman SC-100 fraction collector controlled by software available in the laboratory. Purified samples are stored in dry ice after collection to prevent exposure to light and high temperature.

2.4. Peptide preparation for MS analysis

Prior to tryptic digestion, purified proteins are dried down to 20 μL using a CentriVap concentrator (Labconco, Kansas City, MO, USA) followed by the addition of 20 μL of 100 mM ammonium hydrogencarbonate (Sigma) to adjust the solution pH to ∼7.8. 0.5 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) modified sequencing grade trypsin (Promega, Madison, WI, USA) is added prior to vortexing. The mixture is stored in a warm room with a controlled temperature of 37 °C for 24 h where the samples are shielded from light. After digestion is halted by adding 1 μL 10% (v/v) TFA, the tryptic digest mixture is concentrated to 5 μL using a ZipTip (Millipore, Billerica, MA, USA). Again, light exposure is minimized by storing the peptide mixture in a dark box before and after concentration.

2.5. MALDI-TOF-MS

Sample spotting is performed by layering 1 μL of matrix on top of 1 μL of sample. The spot is allowed to dry in air but without light exposure. The MALDI matrix is prepared by diluting saturated α-cyano-4-hydroxycinnamic acid (α-CHCA) (Sigma) solution made in 50% (v/v) ACN and 1% (v/v) TFA with the same solution at a 1:4 ratio (v/v). Also, 50 fmol/mL Angiotensin I, Adrenocorticotropic hormone (ACTH) 1–17 and ACTH 18–39 (Sigma) are introduced in the matrix as internal standards prior to spotting.

Peptide masses are measured on a Micromass TofSpec2E (Micromass/Waters, Milford, MA, USA) with delayed extraction in the reflectron mode using a nitrogen laser (337 nm). Peptide mass spectra are internally calibrated using the three peaks from internal standards resulting in a mass accuracy of 50 ppm or less. The calibrated spectra are processed using PeptideAuto (Micromass MassLynx application) to obtain experimental masses that are submitted to MS-Fit [31] to search the SwissProt database for protein identity. The following parameters are used for database searching: maximum number of two missed cleavages, unmodified cysteine, Homo Sapiens for the species, no restriction on the pH range, monoisotopic mass. In terms of modification, acetylation is allowed. However, only when the methionine is oxidized, "methionine oxidation" is included in the database search.

2.6. MALDI plate light exposure

Following preparation and analysis under conditions where the sample was shielded from light, MALDI target plates are exposed to direct sunlight for approximately 20 h over 3 days by placing them on a sunny windowsill. During the light exposure, the plate was left by the window and exposed directly to sunlight through the windowpane. The oxidation process depended only on the light exposure and did not depend upon any other conditions. The room temperature and humidity were not found to be major factors affecting oxidation. Using the α-CHCA matrix as described in section e and the layering sample deposition method results in methionine oxidation upon light exposure. Using FA or TFA in the matrix does not affect the result. The particular time of exposure provided partial oxidation of the methionines in the sample as desired. The oxidation could be driven to completion by a much longer exposure time to sunlight, i.e. on the order of two weeks. After the light exposure, a second MALDI-TOF-MS spectrum is acquired to monitor the effects of exposure on peptide masses and intensities. For comparison, ACTH 1–17 standard is spotted on the MALDI plate and MALDI-TOF-MS spectra were acquired before and after the storage for three days in a box shielded from light. Oxidation is not induced under these conditions. No additional steps are required to study methionine oxidation.

2.7. MALDI-QTOF-MS

After analyzing the MALDI-TOF-MS data, the same MALDI plate is reanalyzed using a Micromass MALDI quadruple time-of-flight MS (MALDI-QTOF-MS) Ultima (Micromass/Waters) for selected peptides. The MS–MS spectra are acquired from 50 to 50+ precursor ion (*m*/*z*). The nitrogen laser (337 nm) is scanned over each sample spot in a raster pattern for a total of 1680 s, 560 s for each parent ion. The parent ion window is set at 5 Da. Spectra of ACTH 1–17 are acquired for 60 s after completing MS–MS on each sample spot. The product ion calibration is achieved by applying the lock mass calibration obtained from ACTH 1–17.

The calibrated MS–MS spectrum is subtracted, smoothed and centered before QTOF transformation, a process that transforms a spectrum with multiply-charged ions into a spectrum of singly-charged monoisotopic ions. Fragment ions are selected from the MS–MS spectrum manually and searched against SwissProt using MS-Fit and Mascot for the peptide sequence and methionine oxidation.

3. Results and discussion

3.1. Protein purification using chromatofocusing and non-porous reversed-phase liquid chromatography

Chromatofocusing is a chromatographic technique that has the capability of performing separations according to the p*I* of proteins. The technique finds its applications in proteomic studies as an alternative to IPG gels with the advantages of reproducibility, ease of use and automation [32]. In addition, gel-based separation methods are known for inducing modifications, such as carbamylation, β-elimination [33], Cys and Met oxidation. In order to circumvent gel-induced oxidation, Allmaier and co-workers [34] selectively purified the non-oxidized methionine-containing peptides from the methionine-sulfoxide-containing peptides using solid-phase isolation. In contrast, reversed-phase chromatographic methods have been widely used in studying the oxidation of various amino acid residues in peptides [35] and proteins [36] without induced oxidation [37].

In this work, one CF fraction with pH of 4.6–4.8 is separated by NPS RP-HPLC and eluent is collected for each peak. Fig. 1 displays the second dimension chromatogram of CF fraction 4.8–4.6. In a 30 min separation, ∼100 peaks are detected. The relatively clean samples obtained from the first

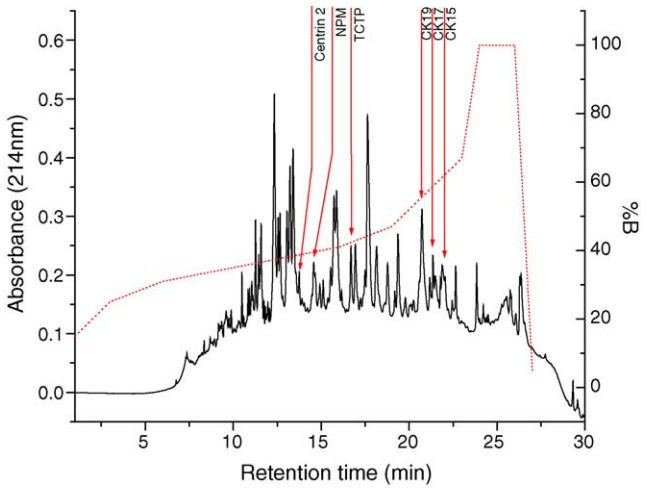

Fig. 1. The NPS RP-HPLC chromatogram of a fraction (pH 4.8–4.6) collected from the chromatofocusing. An ODSIIIE column (33 mm × 4.6 mm) packed with 1.5 μm non-porous silica beads is used for separation. Mobile phase A is D.I. water with 0.1% TFA and mobile phase B is acetonitrile with 0.1% TFA. Flow rate is controlled at 0.5 mL/min. Separation is monitored at 214 nm.

dimension CF fractionation contribute to the improved separation. In each of the dimensions of separation, the purified proteins were shielded from light exposure after collection. In order to check for the presence of oxidized methionines in the proteins separated in the liquid phase, MALDI-TOF-MS of the tryptic digests of several proteins were performed where one such example is shown in Fig. 3B. The methionine-containing peptide is from nucleophosmin, which contains eight methionines. The peptide is terminated with arginine and produces strong MALDI signals. As expected, no peaks corresponding to methionine oxidation, which should appear 16 u higher in the spectrum, are observed. This appears to be a general result where no induced methionine oxidation is observed in the liquid separation. Therefore, the combination of chromatofocusing, followed by NPS RP-HPLC is an ideal separation method when induced modification is not desired.

Given that the accumulation of oxidatively modified proteins during aging has been suggested to contribute to the loss of cellular function associated with a number of age-related diseases, such as Alzheimer's disease, muscular dystrophy, diabetes and Parkinson's disease [38–41], this liquid phase protein profiling method can be applied to investigate such diseases. Furthermore, it can shed light on regulatory functions of the reversible oxidation of methionine and its reduction catalyzed by methionine sulfoxide reductase [42], where the extent of cellular methionine oxidation has been underestimated partially due to the acid digestion-based amino acid analysis which is not suitable for detection of methionine sulfoxide [43].

3.2. Peptide standard experiment

It has been reported that methionine can be oxidized to sulfoxide in the solid phase and the oxidation process can be dramatically accelerated when exposed to UV light [44], in which case the methionine sulfur is excited and radicals are formed [45]. The oxidation process occurs in both solution and solid state [46]. However, in the proteomics community, methionine oxidation has been exclusively attributed to gel-induced modification. No research has been done on the effect of light on the methionine oxidation during MALDI sample preparation process.

In this study, we have used light to induce methionine oxidation for structural studies. Other methods could be used to induce oxidation of methionine; however, the light-induced method proved to be a convenient and simple means to induce oxidation. Moreover, no further cleanup steps are required, so light exposure is an ideal way of inducing modifications if needed. Further, either partial or complete oxidation of a sample can be performed as desired. In order to study the effect of light on methionine-containing peptides on target, ACTH 1–17 (2093.0867) Da, SYSMEHFRWGKPVGKKR), which contains one methionine residue, is freshly prepared and analyzed by MALDI-TOF-MS. As shown in Fig. 2B a peak is only detected at *m*/*z* 2093 in the sample not exposed to light. No peak appears at *m*/*z* 2109, which is 16 u higher

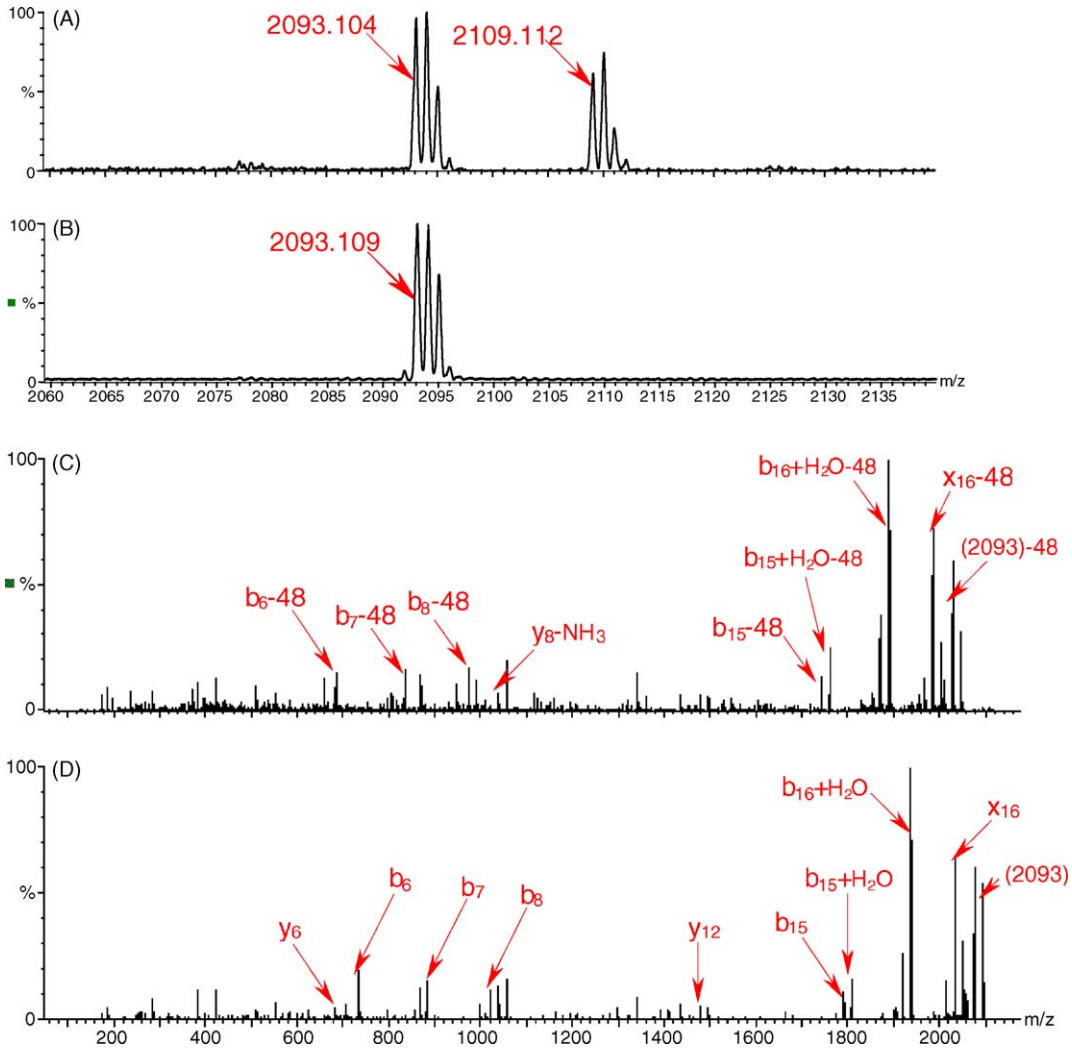

Fig. 2. (A) MALDI-TOF-MS spectrum ACTH 1–17 after it is exposed to light for three days. (B) MALDI-TOF-MS spectrum of freshly prepared ACTH 1–17. (C) MS–MS spectrum of methionine oxidized ACTH 1–17 obtained on MALDI-QTOF-MS. (D) MS–MS spectrum of ACTH 1–17 obtained on MALDI-QTOF-MS.

than the original peak and should be observed when the methionine in ACTH 1–17 is oxidized. The same result is obtained for the ACTH 1–17 that is stored in a box shielded from light for three days. However, after the same plate is exposed to light for 3 days, a new peak at *m*/*z* 2109 is detected for the same spot, as shown in Fig. 2A. In order to confirm that the new peak actually resulted from methionine oxidation, the MS–MS spectra of both *m*/*z* 2109 and 2093 are acquired by a MALDI-QTOF-MS spectrometer from the same spot as shown in Fig. 2C and D, respectively. In ACTH 1–17 fragments, all b ions contain methionine except for b_1, b_2 and b_3, while all detected y ions do not contain methionine. As reported [21,47,48], peptides containing oxidized methionine readily lose the methyl sulfoxide moiety, corresponding to a 64 u loss. Thus, corresponding peaks without a sulfoxide moiety will appear 48 u lower than their original non-oxidized peaks. Comparing Fig. 2C and D, all observed b ions in Fig. 2D have their corresponding methyl sulfoxide peaks eliminated in Fig. 2C. The *m*/*z* 2045 and 1985 peaks in Fig. 2C are such that their corresponding peaks are observed at *m*/*z* 2093 and *m*/*z* 2034 (x_{16}) in Fig. 2D, respectively, which narrows the oxidized amino acid residues into four amino acids–YSME, where only oxidized methionine has methyl sulfoxide moiety loss. The MS–MS data strongly supports that the peak at *m*/*z* 2109 in Fig. 2A results from the oxidation of methionine in ACTH 1–17. One significant difference of the MS–MS data presented in Fig. 2C and D compared to reported results using MALDI-ion trap MS–MS is that methionine-containing peptides almost exclusively lose the sulfoxide moiety where product ions containing the sulfoxide moiety are rarely observed.

3.3. Samples from 2D-LC

The oxidation of methionine in a peptide is not sequence dependent. The same effect is observed for the tryptic digest of nucleophosmin, a protein purified by 2D-LC using CF coupled with NPS RP-HPLC. Fig. 3A and B display the MALDI

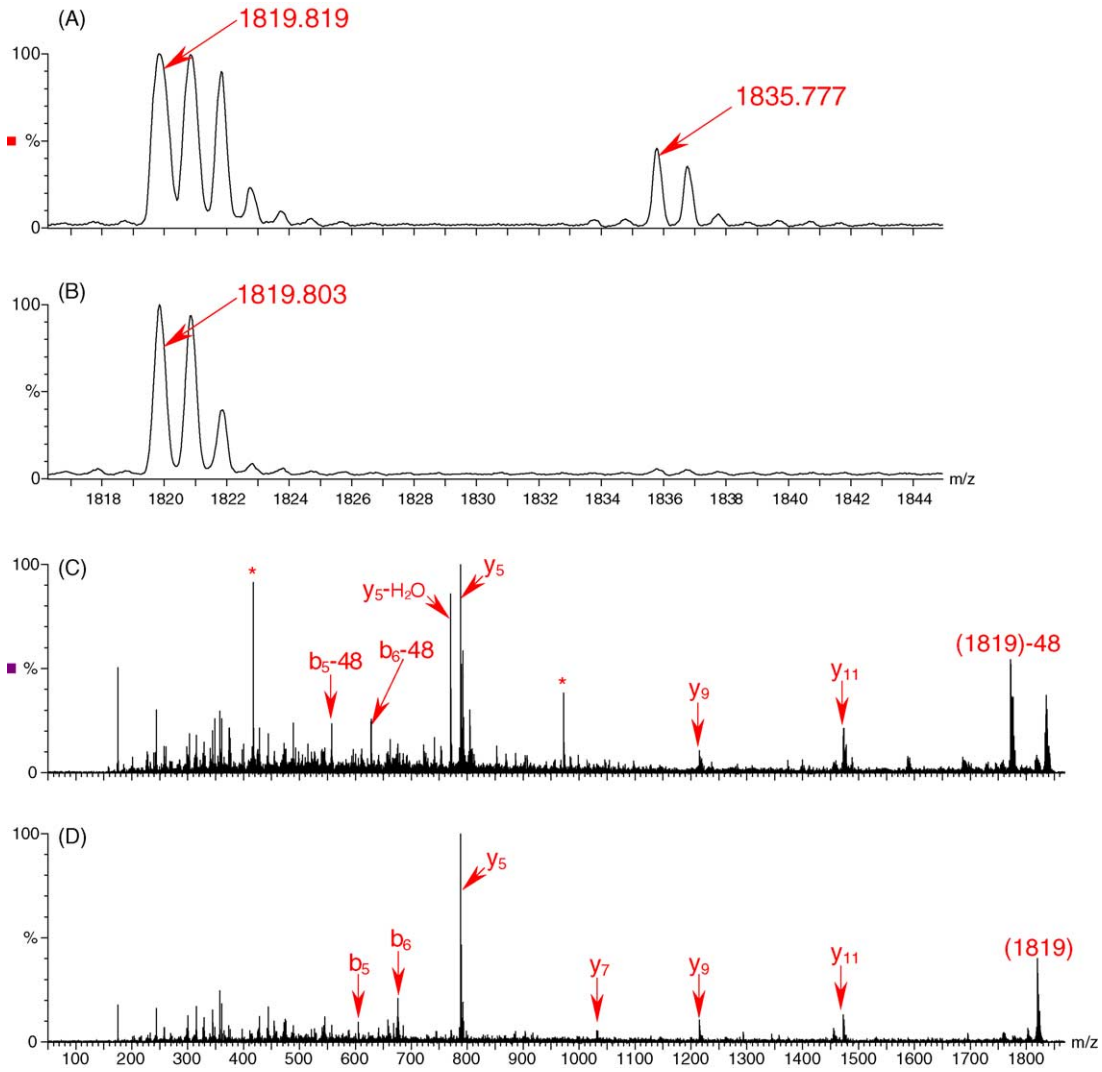

Fig. 3. (A) MALDI-TOF-MS spectrum of the tryptic digest of NPM zoomed in 1815–1844 after the sample is exposed to light for three days. (B) MALDI-TOF-MS spectrum of the freshly prepared tryptic digest of NPM zoomed in 1815–1844. (C) MALDI-QTOF-MS–MS spectrum of the oxidized peak (1835). (D) MALDI-QTOF-MS–MS spectrum of the non-oxidized peak (1819). Asterik (*) stands for new fragments in the oxidized peptide.

spectra of a tryptic digest of nucleophosmin after and before exposure to UV light. MS–MS spectra are obtained using a MALDI-QTOF mass spectrometer for the peptide at *m*/*z* 1819 and the peak 16 u higher (M_r 1835) resulting after light exposure. The result confirms that *m*/*z* 1819 corresponds to the sequence MTDQEAIQDLWQWR, where methionine is unmodified. The MS–MS spectrum for *m*/*z* 1835, as shown in Fig. 3C, is very similar to that of *m*/*z* 1819 in Fig. 3D. However, the b ions resulting from *m*/*z* 1819 in Fig. 3D (b_5, b_6, and parent ion 1819) are such that their corresponding peaks in Fig. 3C are 48 u lower in *m*/*z*, which is a signature for oxidized methionine. Two new peaks at *m*/*z* 417, 972, and y_5-18 are all strong in Fig. 3C, but not observed in Fig. 3D. The difference could result from the change in polarity after the methionine is oxidized. Another methionine-containing peptide (*m*/*z* 2227, MSVQPTVSLGGFEITPPVVLR) from nucleophosmin is also studied using MALDI-TOF-MS and MALDI-QTOF-MS–MS. The results agree with the results observed for *m*/*z* 1819. In both cases, the methionine is located at the N-terminal site. Another example, VVIGMDVAASEFFR is a methionine-containing peptide from α-enolase, where methionine is located in the middle of the sequence. Fig. 4 demonstrates that the location of methionine does not affect oxidation by light, where the two ions (1557 and 1541) readily lose the methyl sulfoxide moiety.

Compared with the MS–MS spectrum of ACTH 1–17, a significant difference in MS–MS spectra of sample ions is that a less facile loss of methyl sulfoxide moiety is observed in Figs. 3 and 4. The result can be explained by differences in peptide sequence. Both peptides are derived from purified samples and terminated with Arg. Moreover, they contain D, E or P, in their sequence. In low energy CID spectrum, dissociation of a protonated peptide is not always evenly distributed across the peptide backbone. Singly charged peptides generated in MALDI-TOF-MS preferentially cleave the amide bond C-terminal to aspartic acid (D) and glutamic acid (E)

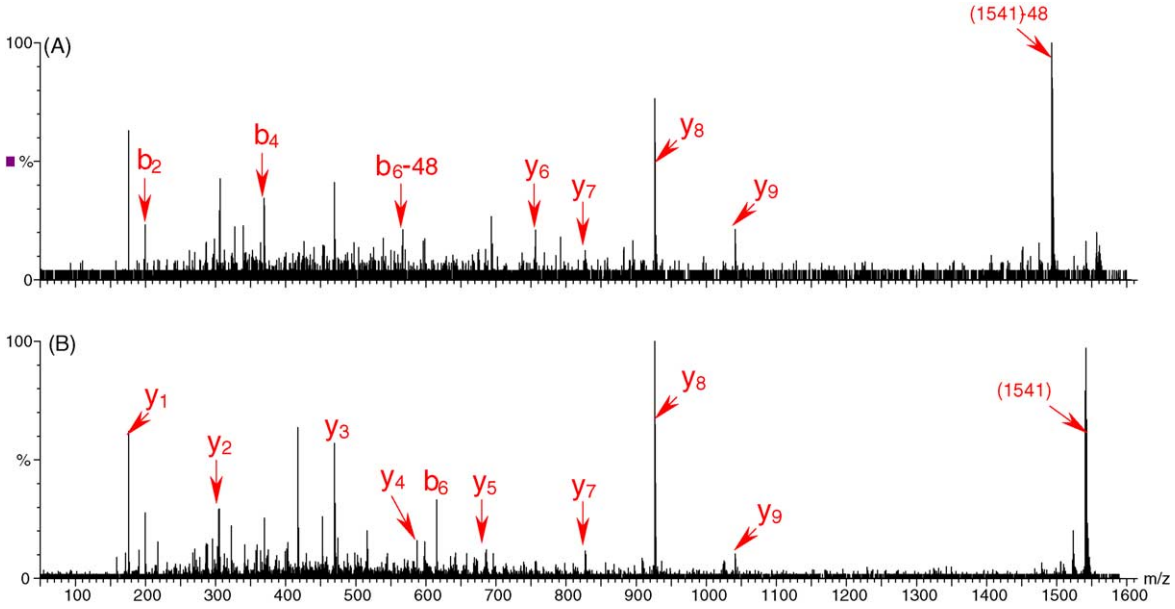

Fig. 4. MALDI-QTOF-MS spectra of (A) the oxidized peak (1557) and (B) the non-oxidized peak (1541) derived from α-enolase.

if the C-terminus is arginine [49–51]. Furthermore, cleavage N-terminal to proline (P) is enhanced [52]. y_5 in Fig. 3 and y_8 in Fig. 4 are the strongest peaks and support this theory. In the standard ACTH 1–17, there are several basic residues in the sequence; thus, selective cleavage is not significant. In the sample peptides, selective cleavage severely suppresses the formation of other product ions resulting in less structural information obtained.

3.4. Acetylated methionine oxidation

Methionine still experiences light-induced oxidation when acetylated. Differentiation factor precursor (8.2 K, human) is another protein purified from 2D-LC. One of its methionine-containing tryptic peptides, MQNDAGEFVDLYVPR (acetyl N-term, M_r 1794), is fragmented and the MS–MS spectra of the oxidized and non-oxidized peptides are shown in Fig. 5A and B. One can observe that more methionine-containing b ions are detected compared with the MS–MS result of 1819 and 1835 from nucleophosmin. The result is that more b-48 peaks are detected in the oxidized form of the peptide although the methionine is acetylated. The difference of the MS–MS spectra is related to the acetylation of the methionine in the *m/z* 1795 but not in *m/z* 1819 and *m/z* 2227. Acetylation of methionine makes the N-terminus of the peptide more acidic.

3.5. Peptides with multiple methionines

When there are more than one methionine residues in a peptide sequence, all methionines can be oxidized. The MALDI-TOF-MS spectrum of nucleophosmin is displayed

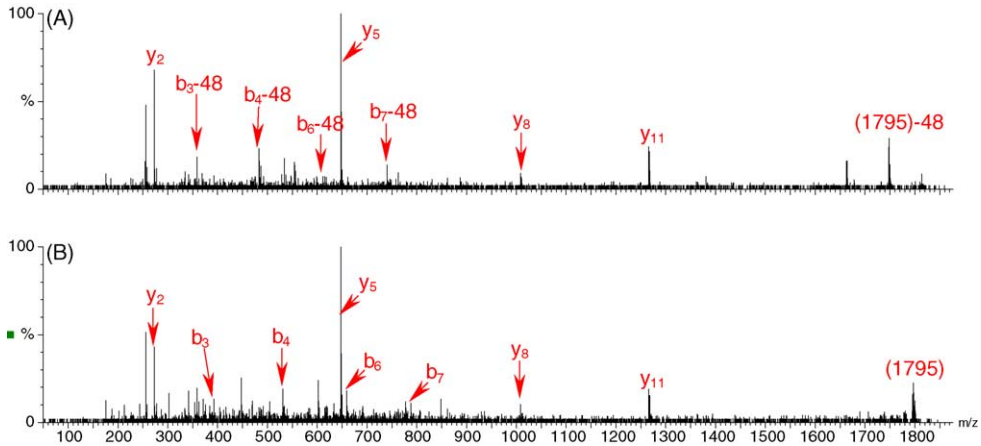

Fig. 5. MALDI-QTOF-MS spectra of (A) the oxidized peptide 1812 (MQNDAGEFVDLYVPR, acetyl N-term) and (B) the non-oxidized peptide (1794) derived from the differentiation factor (8.2 K, precursor, human).

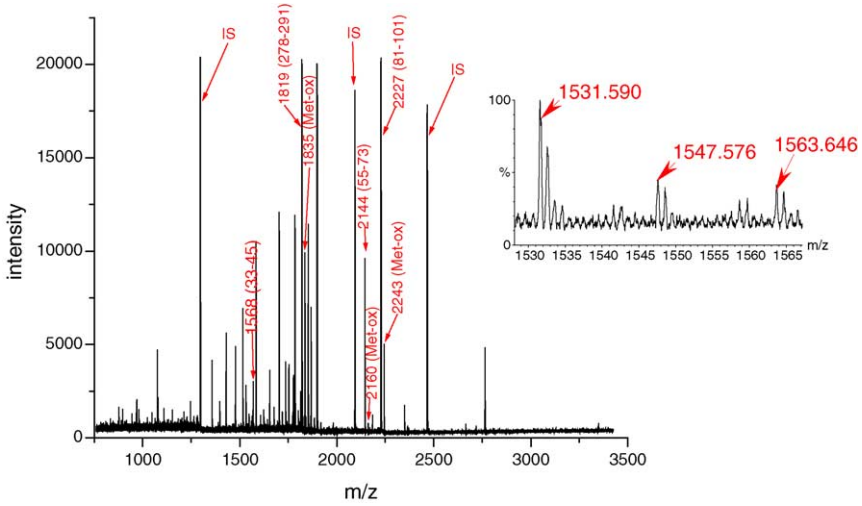

Fig. 6. MALDI-TOF-MS spectrum of the light exposed tryptic digest of nucleophosmin. The peptide masses and the corresponding sequence are denoted in the spectrum. Three standards were mixed with matrix and denoted with IS. The inset is an enlarged view of MNFGDFLTVMTQK from Centrin 2 (P41208) that contains two methionines in its sequence.

in Fig. 6. The unassigned peaks are from prohibitin (P35232) and CK6 (P48669) due to coelution in the liquid separation. The inset of Fig. 6 is a triplet of the sequence TMQALEIELQSQLSMK. The triplet peaks with an interval of 16 u indicate that there are two methionine residues in the peptide sequence. A 16 u peak interval is a mass tag indicating the presence of methionine residues, while the number of methionines can be determined by the number of peaks separated by 16 u where in this case there are two methionine residues.

3.6. Protein identification enhancement with methionine oxidation as a sequence tag

Various methods have been explored to extract sequence specific information to enhance protein identification using peptide mass fingerprinting. Such methods require substantial chemical derivatization or isotopic labeling; for example, cysteine alkylation, in vitro Met, Ser, Tyr isotopic labeling [22,53] and in vivo Leu [23] are reported for sequence information. Recently, another method based on methionine sequence information has been introduced [28]. In this method, sample spots are treated with hydrogen peroxide on the MALDI target plate and analyzed by MALDI-TOF-MS. Methionine is completely oxidized after the treatment. In comparison, light-induced methionine oxidation requires no chemical modification or extra steps after the induced oxidation. Sample consumption is the same. The light exposure simply provides extra sequence information. Multiple peaks with 16 u differences that are associated with partial oxidation are readily apparent.

The peptide mass fingerprint search results with Met oxidation and without Met oxidation are summarized in Table 1. In Table 1, proteins are categorized into different cases with either one peptide containing one methionine, with two single methionine-containing peptides, with three single methionine-containing peptides and with a single peptide containing two methionine residues. For example, after being exposed to light for three days, methionine was oxidized for the peptide (VALVYGQMNEPPGAR) of ATP synthase. When using the methionine content information in the database search, the MOWSE score is increased by more than five-fold. For proteins containing peptides with more than two methionine-containing peptides and for proteins with a single peptide but containing two methionines in the sequence, the increase in MOWSE score is about 50. In the MOWSE score algorithm, high mass peptides or peptides with low frequency in the database weigh more heavily in the scoring scheme. The two methionine peptides are both small, which explains the small increase in MOWSE score. The trend is such that with more methionine-containing peptides detected and with more sequence content information, the increase in MOWSE score is higher. This is further confirmed by proteins containing three methionine peptides. An exception is for CK15, which has three peptides containing methionine while one of the peptides has two methionines in the sequence. There should be a significant increase in the MOWSE score after specifying the methionine content in the database search, but there is only <10-fold increase.

There are three methionine-containing peptides observed for nucleophosmin (NPM) as shown in Table 1 each of which contains one methionine. Without methionine oxidation, the MOWSE score is 8995, whereas it is increased to 4.8×10^6 after methionine oxidation is included. In TCTP, NYQFFIGENMNPDGMVALLDYR contains two methionines, which result in a triplet. Only one methionine is present in the other peptide. The extra sequence information increased the MOWSE score from 876 to 7.1×10^5. The confidence level increased for all proteins listed in Table 1. The extra sequence tag information significantly improves pro-

Table 1
Database search enhancement with Met-oxidation

Protein	Observed Met-containing peptides	Database search result					
		Without Met-Ox			With Met-Ox		
		MOWSE score	Peptides matched	Coverage	MOWSE score	Peptides matched	Increase in MOWSE score
ATP synthase β chain (P06576)	VALVYGQMNEPPGAR	2.591×10^6	10	34	1.367×10^7	11	5.28
Centrin 2 (P41208)	MNFGDFLTVMTQK	1392	3	29	5.6×10^4	5	40.23
CK17 (Q04695)	TMQALEIELQSQLSMK	1.048×10^8	13	38	4.707×10^9	15	44.91
TCTP (P13693)	DLISHDEMFSDIYKIR, NYQFFIGENMNPDGMVALLDYR	876	3	22	7.1×10^5	6	810.5
NPM (P06748)	MTDQEAIQDLWQWR, DELHIVEAEAMNYEGSPIK, MSVQPTVSLGGFEITPPVVLR	8995	5	24	4.8×10^6	8	533.63
CK19 (P08727)	EVAGHTEQLQMSR, TLQGLEIELQSQLSMK, TDLEMQIEGLKEELAYLK	2.947×10^9	18	54	3.563×10^{11}	21	120.9
CK15 (P19012)	SLLEGQDAKMAGIGIR, TMQELEIELQSQLSMK, NHEEEMKEFSSQLAGQVNVEM DAAPGVDLTR	2.232×10^7	12	41	1.589×10^8	15	7.12

tein identification using PMF. The limitation of the method is that only methionine-containing peptides contribute to the enhancement in database searching.

4. Conclusion

The two-dimensional liquid separation used in this work prevents methionine oxidation during purification. This novel separation method provides oxidation-free proteins so that oxidation induced by light exposure on a MALDI target plate can be studied. The light-induced methionine oxidation is a general phenomenon regardless of the position and the modification of the methionine residue in the peptide sequence. In this light-induced oxidation, methionine is partially oxidized. The appearance of new peaks with 16 Da intervals relative to the original peak reveals the existence and the number of methionines in the peptide sequence. The methionine sequence information obtained can be used for the enhancement of protein identification with peptide mass fingerprinting without additional sample consumption and chemical derivatization steps. Although more sophisticated MALDI-TOF-MS–MS can be used to identify proteins, the method described herein provides a simple means for enhancing identification of protein in peptide mapping experiments.

Credit

This work was supported in part by the National Science Foundation under grant DBI 9987220 (D.M.L.), the National Institutes of Health under grant R01 GM 49500 (D.M.L.) and the National Cancer Institute under grants R21CA83808 (D.M.L., F.R.M.) and R01CA90503 (F.R.M., D.M.L.). Support was also generously provided by Eprogen Inc. The MALDI-TOF-MS instrument used in this work was funded by the National Science Foundation under grant DBI 99874.

Acknowledgment

We thank Dr. Phillip Andrews of the University of Michigan Medical Center Proteomics Facility for use of the Q-TOF Ultima.

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