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Improving image analysis in 2DGE-based redox proteomics by labeling protein carbonyl with fluorescent hydroxylamine

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Abbreviations: FHA, fluorescent hydroxylamine; IEF, isoelectric focusing; MALDI, Matrix-assisted laser desorption/ionization

ABSTRACT

Recent advances in redox proteomics have provided significant insight into the role of oxidative modifications in cellular signalling and metabolism. At present, these techniques rely heavily on Western blots to visualize the oxidative modification and corresponding two dimensional (2D) gels for detection of total protein levels, resulting in the duplication of efforts. A major limitation associated with this methodology includes problematic matching up of gels and blots due to the differences in processing and/or image acquisition. In this study, we present a new method which allows detection of protein oxidation and total protein on the same gel to improve matching in image analysis. Furthermore, the digested protein spots are compatible with standard MALDI mass spectrometry protein identification. The methodology highlighted here may be useful in facilitating the development of biomarkers, assessing potential therapeutic targets and elucidating new mechanisms of redox signalling in redox-related conditions.

INTRODUCTION

A large body of experimental evidence supports the role of oxidative stress and subsequent cellular damage in aging and neurodegenerative diseases (1-3). Oxidative modifications such as protein carbonylation are considered markers of protein damage (Reviewed in (3, 4)). However, recent advances in redox signaling have revealed that oxidative modification plays a significant role in the regulation of protein synthesis and degradation (5-7). This role of protein regulation by its oxidation has led to the re-defining of oxidative stress from redox state imbalance to a disruption of redox signaling and regulation (8), attributed in large part to recent advances in redox proteomics.

Two dimensional gel electrophoresis (2DGE)-based redox proteomics approaches are commonly used to identify

oxidized proteins. They typically include parallel matching between immunochemical detection of dinitrophenol hydrazine (DNPH) derivatized carbonylated protein on a Western blot, and total protein detected on corresponding 2D gels. A total of two 2D gels are used (Fig. 1A) (9, 10). However, matching gels to their respective Western blots from typical 2DGE methods is extremely challenging to computational geometric algorithms (11). Therefore, it is imperative that a reliable method is used to ensure appropriate matching between the 2D patterns of carbonylated protein and total protein.

Various attempts have been made, including post-IEF (12) and post-electrophoretic DNPH derivatization (12, 13), but these methodologies have not shown improved matching between the 2D pattern of carbonylated protein and total protein. Thus, the aim of our current study was to develop a method to improve the matching in 2DGE redox

proteomics studies by assessing carbonylation and total protein in a single gel (Fig. 1B).

MATERIALS AND METHODS

Sample Preparation

Brains from 3 month old mice ($n = 5$) were homogenized using previously established methods (14). Whole brains were sonicated briefly in lysis buffer (10mM HEPES pH 7.4, 137 mM NaCl, 4.6mM KCl, 1.1mM KH_2PO_4 , 0.6mM MgSO_4) containing protease inhibitor cocktail (Invitrogen). Homogenates were centrifuged at $20,000 \times g$ for 10 min to remove debris. The supernatant was extracted and total protein concentration was determined using the BCA method (Pierce).

SDS-PAGE

Egg albumin and bovine serum albumin (BSA) (Sigma) were oxidized in FeSO_4 (200mg/L) and H_2O_2 (1000mg/L) mixture for 30 minutes in room temperature. For immunochemical detection of the carbonyl groups, the oxidized proteins were labeled using the Oxyblot[®] kit (Chemicon) as previously described (15). Briefly, 200mM of DNPH was added to carbonylated protein. Mixtures were reacted in room temperature for 20 minutes. The proteins were then separated by electrophoreses using precast criterion Tris-HCl gels (Bio-Rad). The DNP-protein derivatives after electrophoresis were transferred onto nitrocellulose paper using a Transblot-Blot[®] SD Semi-Dry Transfer Cell (Bio-Rad) at 15V for 2 hrs. DNP adducts were detected using a primary rabbit antibody (Chemicon) specific for DNP-protein adducts (1:100) followed by a secondary goat anti-rabbit IgG (Sigma) antibody (1:8000). Colorimetric detection was carried out with application of Sigma-Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets. The image was obtained using a ChemiDoc XRS imaging system (Bio-Rad).

For fluorescent detection of carbonyl, the oxidized proteins were labeled by adding 1 mg Alexa 488 Fluorescent Hydroxylamine (FHA, Invitrogen) to 20 mg of the oxidized proteins. The solution was allowed to react at room temperature for 2 h. FHA-labeled egg albumin and BSA were separated by SDS-PAGE electrophoresis and subsequently detected by fluorescent imaging immediately using an FX imager (Absorption at 495nm,

emission at 519 nm, BioRad).

Additional controls included samples of BSA reduced with NaBH_4 (100 μM , dissolved in 0.1M NaOH), oxidized with 20mM H_2O_2 , or incubated with H_2O for 1 h. BSA samples were then treated with 1M HCl, incubated for 1 h with FHA, and separated and visualized as described above. A set of BSA samples were also treated with various concentrations of H_2O_2 (0-160mM) and processed as previously described to determine a relationship between carbonylation and fluorescent signal.

Two Dimensional Gel Electrophoresis (2DGE)

Alexa 488 Fluorescent Hydroxylamine (FHA, Invitrogen) was added to mouse brain protein extracts at 1 mg of FHA for 20 mg of protein. The solution was allowed to react at room temperature for 2 h. 2DGE of the FHA-labeled proteins from whole brains was performed as previously described (16) with the exception that experimental procedures were conducted in the dark. Briefly, FHA-labeled proteins were precipitated with ice-cold trichloroacetic acid. The protein pellets were washed with ethyl acetate/ethanol (1:1) solution three times. The protein pellets from the brains of five mice were each applied separately to pH 3-10 ReadyStrip[™] IPG strips (Bio-Rad) for isoelectric focusing (IEF). After focusing, linear gradient (10-20%) precast criterion Tris-HCl gels (Bio-Rad) were used to separate proteins according to their molecular weight (MrW). Precision Protein[™] Standards (Bio-Rad) were run along with the samples. After electrophoresis, the carbonylated proteins on the 2D gels were detected as described above. Once the 2D images of carbonylated proteins were obtained, the gels were stained with Bio-safe Coomassie blue stain (Bio-Rad) as per manufacturer's instructions. The total protein levels were detected by visible wavelength using an FX imager. A total of five gels were used but ten images were generated for these experiments.

Western blotting of the 2D gels was performed as previously described (16). 200mg of soluble protein extract from five mice brains was separately incubated with 10mM 2,4-dinitrophenyl hydrazine (DNPH) solution in 2N HCl at room temperature for 20 min. Additionally, a duplicate set of protein extract was incubated only in 2N HCl at room temperature. 2DGE was performed on five DNP-derivatized protein samples and five non-derivatized protein samples as detailed above. The

detection of DNP- protein derivatives on 2D gels were the same as the immunochemical detection described above. A total of ten 2D gel images were created (five 2D gels for total protein levels and five 2D Western Blots for protein carbonyl levels).

Image Analysis

PDQuest software (Bio-Rad) was used for matching of and analysis of visualized protein spots among different gels and 2D Western blots. The linear mode of background subtraction was used to normalize intensity values, representing either the total protein on gel or oxidized protein on 2D Western blot per spot. After completion of spot detection, matching of the carbonylated protein 2D Western blot images and total protein 2D gel images, and matching of the FHA labeled carbonylated protein 2D gel images and total protein 2D gels were performed by the same software. The match rates were reported by the software indicating the percent matching agreement between two groups of images. Additional spots were manually matched and used as landmark reference points to improve image matching. The geometric algorithms for matching 2DGE images used in this study were previously described (11).

Trypsin digestion and MALDI-TOF-MS analysis

Selected protein spots on the FHA-labeled gels were excised using an ExQuest[®] spot cutter. Samples were digested using previously described techniques (16). Briefly, excised spots were washed with 50mM ammonium bicarbonate, followed by 100% acetonitrile at room temperature. The samples were reduced with 10mM dithiothreitol and then alkylated with 50mM iodoacetamide solutions. Proteins were digested with 20 μ L of 5ng/ μ L modified porcine trypsin (Promega) for 16hr at 37°C. Digested peptides were extracted from the gel pieces in 150 μ L of 50% acetonitrile and 5% formic acid. The samples were concentrated to approximately 10 μ L under vacuum at room temperature, and further concentrated and de-salted in C-18 reverse phase Zip Tip columns (Millipore). Peptides were eluted from the columns with 2.5 μ L of 50% acetonitrile, 0.1% trifluoroacetic acid and 1mg/mL alpha-hydroxycinnamic acid, and spotted directly to a steel MALDI chip.

Mass spectra were acquired in positive ion, reflectron

mode in a MALDI TOF-MS (Waters). Peaks were obtained from the summed spectra of 20 combined spectra per sample. Each summed spectrum was smoothed, subtracted and centroided prior to obtaining m/z values for peptide mass fingerprinting. Peaks derived from keratin or autocatalytic trypsin activities were manually subtracted from the monoisotope peak list. The protein identifications were obtained by searching the peak lists against UniProt Database and Random UniProt Database (last updated 3/21/2006) using Protein Prospector v 4.0.7 MS-FIT (17).

Statistical Analysis

Linear regression was used to analyze the correlation of the signal intensity to carbonylated protein content. The Student's t-test was used to analyze the match rate data.

RESULTS AND DISCUSSION

In our current study, we used hydroxylamine chemistry in 2DGE-based protein carbonyl group detection and quantification (Fig. 1B). Protein carbonyl groups were modified by FHA through a reaction that is similar to the carbonyl-hydrazide Schiff base reaction (Fig. 1B insert), which utilizes fluoresceinamine to label protein carbonyls (18). Detecting biomolecular carbonyls by hydroxylamine were well characterized and used in many oxidation studies (19-23). The hydroxylamine approach is similar to hydrazine labeling approaches, except it does not require acidic conditions which are not amenable to 2DGE. FHA readily reacts with protein carbonyl groups in neutral conditions and shows a linear increase in the signal intensity with increasing amounts of carbonylated protein (Fig. 2). The fluorescence at 519nm indicated the oxidative state of proteins (Fig. 2A), and is proportional to the amount of carbonylated proteins on a SDS-PAGE gel (Fig. 2B, C). This linear increase shows similar dynamic range as the immunochemical detection used in the current study (Fig. 3).

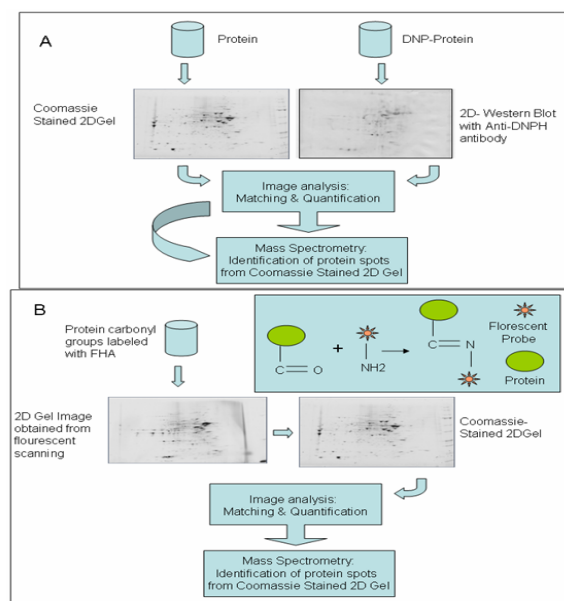


Fig. 1: Schematic Representation of two 2DGE-based redox proteomics. (A) Traditional parallel 2DGE-based redox proteomics methodology to identify carbonylated protein. (B) Fluorescent hydroxylamine labeling of protein carbonyls prior to 2DGE-based redox proteomics. (Insert) Fluorescent hydroxylamine covalently modifies protein carbonyl groups, leading to fluorescently labeled carbonylated proteins.

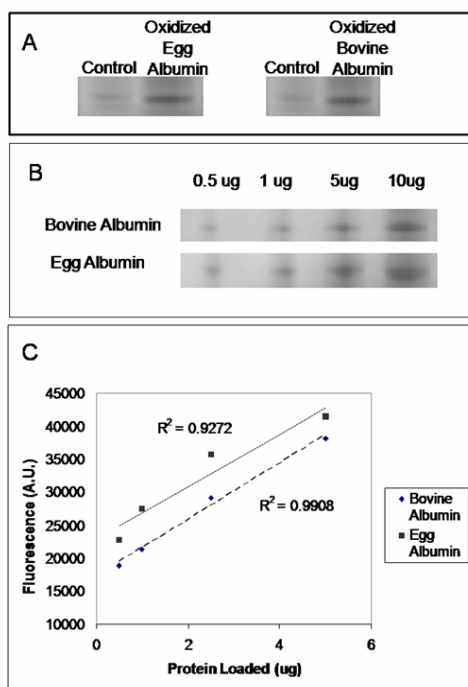


Fig. 2: In-gel quantification of FHA-labeled oxidized bovine serum albumin (BSA) and egg albumin. (A) Oxidized and Unoxidized BSA and EA. (B) SDS-PAGE gels of various amounts of oxidized BSA and EA. (C). Linear increase in fluorescence as a function of oxidized protein loaded.

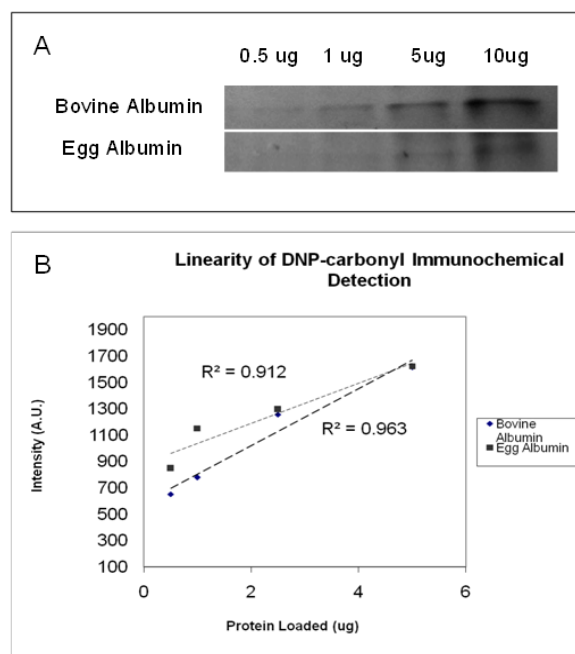


Fig. 3: (A) Western blot of various amounts of oxidized BSA and EA. **(B)** Linear increase in colorimetric density as a function of oxidized protein loaded.

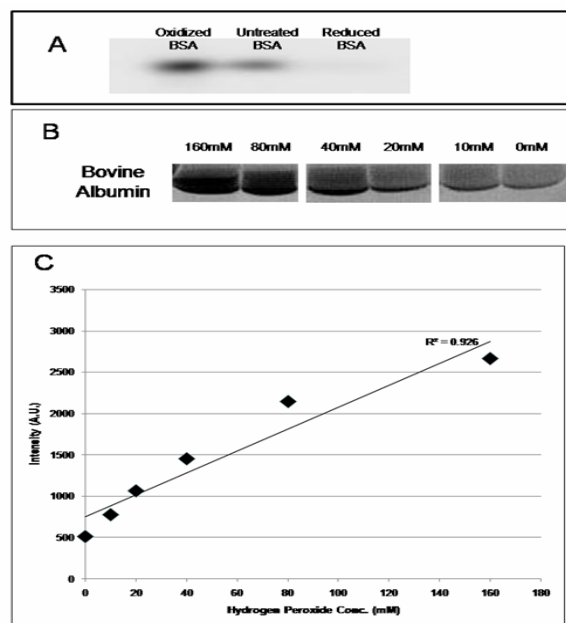


Fig. 4: Detection of oxidized bovine serum albumin (BSA) and egg albumin using FHA. (A) SDS-PAGE of oxidized, reduced, and control BSA samples incubated with FHA. (B) Gel showing BSA treated with various concentrations of H₂O₂. (C) Linear increase in fluorescence as a function of concentration of H₂O₂ added.

The specificity of FHA binding to protein carbonyl groups was tested by determining the fluorescent signal from oxidized, reduced, and control samples of BSA. Insignificant signal was detected from the reduced BSA,

whereas greater signal was seen from oxidized sample when compared to the control (Fig. 4A). Additional control samples of BSA were treated with various concentrations of H_2O_2 to determine a linear correlation between level of protein carbonylation and signal intensity (Fig. 4B, C). These results indicate that FHA binding is specific to protein carbonyls.

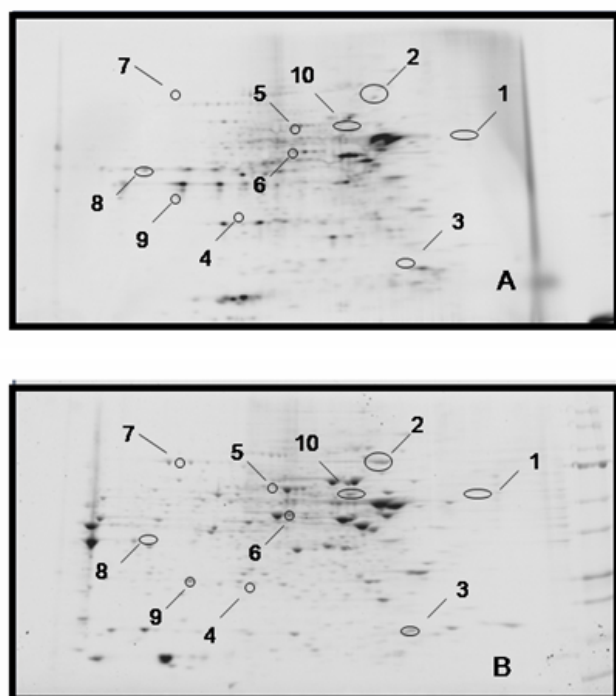


Fig. 5: Representative 2D gel Images. (A) Representative 2D gel image of protein carbonyl groups detected by fluorescent hydroxylamine labeling. (B) Representative 2D gel image of protein stained with Coomassie Stain. Numbered spots indicated protein spots used as landmarks for image matching analysis and protein identification by MALDI-MS.

The resolution of our 2D images is comparable to studies of other laboratories (10, 24-26). Representative 2D images, generated using FHA labeled proteins, are shown in Fig. 5. Match rates of these images are presented in Fig. 6. Using PDQuest software, the match rate between FHA labeled carbonylated proteins and Coomassie stained 2D gel images is higher than of those between 2D Western blot images of carbonylated proteins and Coomassie stained 2D gels. The match rate for FHA labeled 2DGE is significantly increased with and without using manual landmark referencing assistance (Fig. 6).

These results indicate that using FHA labeling versus DNPH labeling of protein carbonyl groups improves the

matching during image analysis of 2DGE. We speculate that the improved matching of FHA-labeled protein to total protein constellation is due to the fact that the images were both generated in gels, thus producing the same image size and resolution. These parameters are critical to the geometric algorithms used in image matching (11). Moreover, elimination of non-specific binding of antibodies and background (i.e. streaking) may also contribute to the improved matching in image analysis.

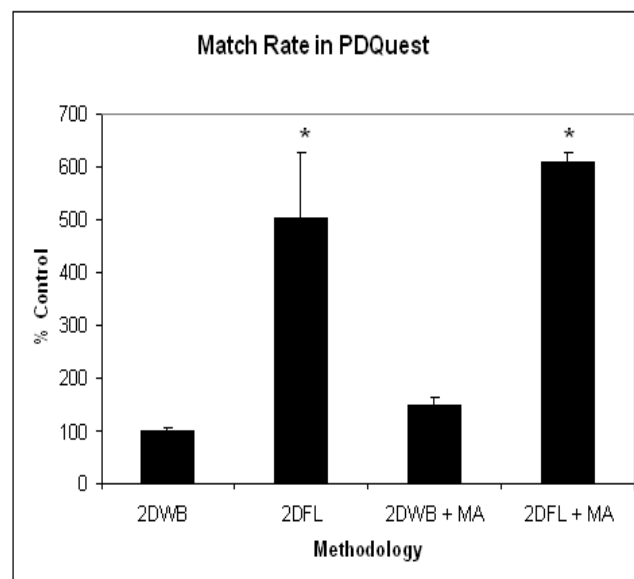


Fig. 6: Match rates of two 2D gel-based redox proteomics methodologies. Match rates of 2D Western blot (2DWB), 2D FHA labeling (2DFL), 2DWB + manual assisted matching (MA) and 2DFL + MA using PDQuest. Data represent the average matching rates of 2DWB, 2DFL, 2DWB + MA and 2DFL + MA. Measured values are normalized to the 2DWB values. Error bar indicate the SEM of the match rate, * $p < 0.005$. 2DWB, match rates between 2D Western blot of protein carbonyl and Coomassie gel; 2DFL, match rates between 2D gel of fluorescently labeled carbonyl and Coomassie gel; 2DWB + MA, match rates between 2D Western blot of protein carbonyl and Coomassie gel after manual assistance using spots from Fig. 3B as landmark references; 2DFL + MA, Match rates between 2D gel of fluorescently labeled carbonyl and Coomassie gel after manually assistance using the spot in Fig. 4A as landmark references.

The spots in Fig. 7A were excised and digested for MALDI mass spectrometry analysis to examine the compatibility of the FHA labeling protein with mass spectrometry. MALDI mass spectra were successfully generated which is consistent with previous studies that labeling carbonylated proteins with FHA does not affect tryptic digestion (18). A representative MALDI mass spectrum is shown in Fig. 7B.

The identifications of oxidized protein spots are summarized in Table 1. These results indicate that the

Table 1: Protein Identification by MALDI-TOF mass spectrometry.

Spot	Asscession No.	Protein	% Coverage	Peptide Matched	Predicted Mw(kDa)/ pI	Observed Mw(kDa)/pI	Mowse Score
a	Q6P5D0	Dihydropyrimidinase-like 2	35.3	14	62.3/6.0	61.3/5.8	2.33x10 ⁶
b	P17751	Triosephosphate isomerase	33.9	8	26.6/7.1	24.9/7.8	3.35x10 ³
c	Q3TVP6	Beta actin	32.8	11	41.8/5.3	39.1/4.7	1.70x10 ⁷
d	Q9CZW9	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase	21.1	8	61.9/6.7	62.3/6.5	1.64x10 ⁴
e	P17182	Enolase 1	24	8	47.0/6.4	44.5/6.0	4.67x10 ³
f	P05063	Fructose-bisphosphate aldolase C	36.2	9	39.2/6.8	37.5/6.0	8.62x10 ³
g	P08551	Neurofilament light polypeptide	26	10	61.4/4.6	66.9/4.0	9.74x10 ³

FHA labeling does not noticeably interfere with tryptic digestion or MALDI MS identification. One could speculate that the FHA labeled carbonyl groups result in a mass shift of the digested peptide. However, the concentration of carbonylated protein in normal cells is approximately 0.3 nmol per mg of protein (27). Since we have used wild-type mice, the majority of the peptides detected here by the MALDI mass spectrometry are probably “uncarbonylated”, thus making ID by protein mass finger printing possible. However, in certain disease states where larger amounts of a specific protein may be carbonylated, it should be noted that this mass shift should be obvious and may affect database searches. Hence, the search parameters should take these oxidative modifications into account while performing database searches for protein identification.

Previous efforts aimed at improving detection of carbonylated protein have resulted in development of immunochemical-based detection of DNP-derivatized carbonylated protein on Western blots, but requires parallel detection of total proteins on separate gels (9). This methodology currently forms the basis of most 2DGE redox proteomic studies (Reviewed in (10)).

However, use of this technique results in increased cost associated with duplication of gels and blots. More importantly, while the DNP-labeled proteins on Western blots produce similar patterns to the 2D gels (26), matching the two is usually challenging to geometric algorithms, due to previously listed reasons. Therefore, subsequent manual applications of landmark reference points are necessary for improving the image-matching process (Reviewed in (10)). A technique has been

developed which detected protein levels using Sypro stain with subsequent immunochemical detection of carbonylated protein detection on PVDF and nitrocellulose membranes (13). Unfortunately, this technique results in increased background on the 2D Western blot (13), again making it difficult to match the blots with the 2D gels during image analysis. The current methodology we described allows detection of protein carbonylation and total protein within a single gel, eliminating problems with alignment typically encountered during image analyses involved in matching two different detection systems. However, the application of the FHA-labeling method to redox proteomics of diseased states will require the matching between 4 images for each samples (control gel image of protein carbonyl groups, control gel image of protein, disease/treated gel image of protein carbonyl groups, disease/treated gel image of protein disease state protein expression, and disease state oxidation). Although we showed that FHA labeling technique improve the matching between control gel image of protein carbonyl groups and gel image of protein, the improvement of matching between the control and disease state may not be obvious since it is disease/treatment dependent. Nevertheless, the improvement of the matching of the carbonylated protein gel image and the protein level gel images resulted in better computer assisted matching 2DGE images, as well as increased efficiency and reduced cost. Moreover, protein spots excised from these gels are compatible with typical MALDI-TOF mass spectrometry protein identification. Therefore, we conclude that using this method for 2DGE base proteomic study could facilitate the development of biomarkers, assessment of potential therapeutics and the mechanism of redox signaling (3, 14, 26, 28).

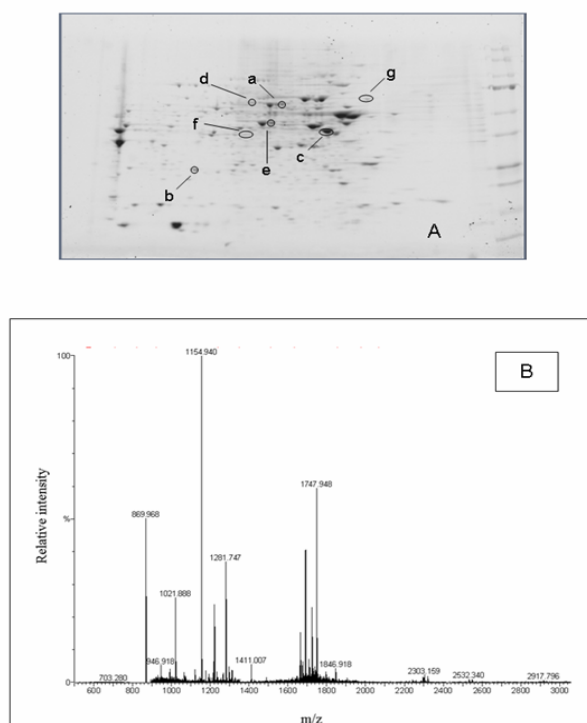


Fig. 7: (A) FLA labeled protein spots excised for MALDI-TOF mass spectrometry. **(B)** Representative MALDI Spectrum from spot g (Neurofilament -L).

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