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One-Dimensional Western Blotting Coupled to LC-MS/MS Analysis to Identify Chemical-Adducted Proteins in Rat Urine

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Abstract

The environmental toxicant hydroquinone (HQ) and its glutathione conjugates (GSHQs) cause renal cell necrosis via a combination of redox cycling and the covalent adduction of proteins within the S₃ segment of the renal proximal tubules in the outer stripe of the outer medulla (OSOM). Following administration of 2-(glutathion-S-yl)HQ (MGHQ) (400 μmol/kg, i.v., 2 h) to Long Evans (wild-type Eker) rats, Western analysis utilizing an antibody specific for quinol–thioether metabolites of HQ revealed the presence of large amounts of chemical–protein adducts in both the OSOM and urine. By aligning the Western blot film with a parallel gel stained for protein, we can isolate the adducted proteins for LC-MS/MS analysis. Subsequent database searching can identify the specific site(s) of chemical adduction within these proteins. Finally, a combination of software programs can validate the identity of the adducted peptides. The site-specific identification of covalently adducted and oxidized proteins is a prerequisite for understanding the biological significance of chemical-induced posttranslational modifications (PTMs) and their toxicological significance.

Keywords

LC-MS/MS; Covalent modification; Quinones; Quinone–thioethers

1. Introduction

Hydroquinone (HQ) is an environmental toxicant found in some hair/skin products, as a food preservative in some countries, and as a byproduct of combustion, and it is a metabolite of both benzene and phenol. HQ is metabolized in the liver by CYP enzymes to 1,4-benzoquinone (1,4-BQ), followed by the reductive Michael addition of glutathione (GSH). Subsequent cycles of oxidation and GSH addition result in the formation of multi-GSH substituted HQ–GSH conjugates, including 2,3,5-tris-(glutathion-S-yl)HQ (TGHQ), which is the most potent nephrotoxic metabolite of HQ. Upon uptake by the epithelial cells lining the S₃ segment of the outer stripe of the outer medulla (OSOM), metabolites of TGHQ remain capable of redox cycling and of covalently adducting proteins, the combination of which results in proximal tubular necrosis (1). Because the dead and dying cells detach from the proximal tubule and the intracellular contents leak into the tubular lumen, the urine becomes semi-enriched of adducted proteins. Therefore, Long Evans rats treated with MGHQ were placed in metabolic cages and urine collected for a period of 2 h. Western blotting was

performed on urinary proteins using an antibody specific for the quinol–thioether protein adducts (2). Because Western blot analysis revealed that urine contained more adducted proteins than tissue extracts from the OSOM (based on milligram total protein), urine was selected as the source for initially identifying protein targets of MGHQ and for searching specific amino acids within the protein that become covalently modified.

Using an antibody raised against 2-bromo-(*N*-acetylcystein-*S*-yl)HQ (BrHQ-NAC) protein adducts, we are able to align immunopositive bands from the Western blot film with protein-stained bands from gel electrophoresis to identify adducted proteins from urine of MGHQ-treated rats (3). Representative blots are shown in Fig. 1. Once these bands have been identified, they are excised from the stained gel and promptly digested with trypsin. Peptides are then extracted from the gel matrix and analyzed by tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS). Analyzing the MS/MS data generated from the mass spectrometer requires at least one peptide matching program such as Sequest, X! Tandem, or MASCOT, but analysis with more than one of these programs is preferred. Such programs compare the various *b* and *y* ions generated from collision-induced dissociation of the peptide, to those predicted for known peptides from a user-specified database. A built-in feature of all of these programs is the ability to specify a mass addition on an amino acid; for example, +105 amu on Lys will prompt the program to search for native lysines as well as lysine +105 amu. This is generally used for common modifications such as oxidized methionines, but can also be used for chemical-mediated modifications such as a HQ adduct. Once the MS/MS-peptide matching software identifies a peptide as containing a chemical modification, that peptide must be validated both manually and by using supplementary software programs designed specifically for this purpose. By utilizing the above outlined strategy, it is possible to identify the specific site of the chemically mediated posttranslational modification. Complementary techniques, such as computer modeling, can then be employed to determine the structural consequences of the chemical adduction, and biochemical analyses used to determine whether any functional consequences arise as a consequence of the structural modification. This technique can also be applied to more complex matrices such as tissue homogenates or cell lysates by running the sample on a 2D gel instead of the 1D gel presented in this chapter. The general work flow for these studies is shown in Fig. 2.

2. Materials

2.1. Protein Band(s) from Gel Electrophoresis (In Vivo/Complex Sample)

2.1.1. One-Dimensional SDS–Polyacrylamide Gel Electrophoresis

1. Separating buffer: 1.5 M Tris–HCl, 14 mM sodium dodecyl sulfate, pH to 8.8 with HCl. Store at room temperature.
2. Stacking buffer: 0.5 M Tris–HCl, 14 mM sodium dodecyl sulfate, pH to 6.8 with HCl. Store at room temperature.
3. 40:1 Acrylamide:Bis solution (BioRad). Store at 4°C.
4. *N,N,N',N'*-Tetramethylethylenediamine (TEMED) (BioRad).

5. Ammonium persulfate (Fischer Scientific).
6. 50% Sucrose solution (Sigma).
7. Running buffer: 25 mM Tris-HCl (Sigma), 192 mM glycine (Sigma), 0.1% sodium dodecyl sulfate (SDS) (Sigma), pH to 8.3 with HCl. Store at room temperature.
8. Running unit: Hoefer SE 600.
9. Glass plates: 18 × 16 cm (Hoefer).
10. Spacers: 1.5 mm (Hoefer).
11. Comb: 1.5 mm, 10 lanes (Hoefer).
12. Sample buffer: Laemmli sample buffer 2× (Bio-Rad, Hercules, CA).

2.1.2. Western Blot for Protein Adducts

1. Polyvinylidene difluoride membrane (PVDF) (Pierce).
2. Methanol (Sigma).
3. Transfer buffer: 200 ml methanol, 700 ml MilliQ H₂O, 100 ml 10× Tris/glycine buffer (BioRad). Store at room temperature.
4. Sandwich cassette (BioRad).
5. Sponges (BioRad).
6. Filter paper (Biorad).
7. 0.02% Polyvinyl alcohol (PVA) (w/v) (Sigma). Prepare fresh with each use.
8. Tris-Saline buffer: 0.02 M Tris-HCl, 0.137 M NaCl, pH to 7.6 with HCl. Store at room temperature.
9. Block buffer: 5% non-fat milk in Tris-Saline buffer (Carnation). Prepare fresh with each use.
10. Wash buffer: 0.005% Casein, 0.154 M NaCl, 0.01 M Tris-HCl, 0.0005 M thimerosal, pH to 7.6 with HCl. Store at 4°C. Solution expires after 2 weeks.
11. Detergent buffer: use wash buffer with the addition of 0.02% sodium dodecyl sulfate (v/v) and 0.1% Triton X-100 (v/v) (Sigma). Store at 4°C. Solution expires after 2 weeks.
12. Primary antibody: generated in-house (3).
13. Secondary antibody: goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, SC-2030). Store at 4°C.
14. Electro-chemiluminescence Western Blotting Detecting Reagents (GE Health sciences). Store at 4°C.

2.1.3. Gel Staining

1. Stain: 45% deionized H₂O, 45% methanol, 10% acetic acid, 0.04% Coomassie blue G-250 (Sigma).
2. Wash: 45% deionized H₂O, 45% methanol, 10% acetic acid.

2.1.4. In-Gel Digestion Sample Preparation

1. Acetonitrile (ACN)/deionized water.
2. Digestion buffer: 100 mM NH₄HCO₃.
3. Disulfide reduction: 10 mM DTT in 100 mM NH₄HCO₃.
4. Thiol alkylation: 55 mM iodoacetamide in 100 mM NH₄HCO₃.
5. Digestion: trypsin-modified sequencing grade (Promega), 20 µg lyophilized powder. Store at -20°C. Dilute to 0.1 µg/µl.
6. Digestion quench: 2% trifluoroacetic acid (TFA).
7. Peptide extraction solutions: 30% ACN 0.1% TFA and 60% ACN 0.1% TFA.

3. Methods

In order to fully benefit from this strategy, it is essential to understand what types of metabolites are formed from the parent chemical, the relative abundance of each metabolite, which of the metabolites are capable of protein adduction, what amino acids are potential targets, and the precise molecular weight of each modified amino acid. Some of the above variables may be determined by *in vitro* studies, as outlined in the previous chapters. Once the above variables have been addressed and a full understanding of the metabolic fate of the chemical is elucidated, one can then select the most abundant or likely toxic metabolite with which to raise an antibody against. Antibodies directed at chemical adducts are generally not commercially available and, therefore, must be created in-house or from an antibody contract laboratory. These antibodies typically have a high background and unique characteristics compared to the more common antibodies generated against a protein or peptide. For these reasons, the Western blot protocol described below requires a more stringent washing procedure and the use of a chemical (polyvinyl alcohol) and a protein (5% milk) block. Such protocols will necessarily vary depending upon the nature of the raised antibody, the protocol described below being the best for the antibody generated against BrHQ-NAC, and purified against 2-(*N*-acetylcystein-*S*-yl)HQ (NAC-HQ).

The most challenging part of this protocol is exactly how the investigator interprets the enormous amount of data generated by the mass spectrometer from either a simple, 1-protein analysis, or from the analysis of a complex sample that contains multiple proteins. To address this challenge, the use of software programs capable of matching MS/MS spectra to peptides is necessary. There are a number of software programs that can perform this task, including Sequest, X!Tandem, and MASCOT. These programs use different matching algorithms, and for the most accurate and rigorous results, it is recommended that more than one program be used to analyze the same set of data. Once the data have been analyzed with

these programs, and adducted peptides with reasonable scores have been identified, the data should next be validated to ensure that the identified adduct is a valid hit, and not an artifact of the analysis. There are several programs that can assist in this validation, including P-Mod and FindMod (<http://ca.expasy.org/tools/findmod/>). These programs will parse the raw data for the user-specified peptide and determine whether or not it is adducted. After the adducted peptide has been identified and validated, it is then reasonable to validate manually the peptide using programs such as Ionen (4) or Protein Prospector (<http://prospector.ucsf.edu>) to generate the theoretical ions that can be matched to the spectra of interest.

3.1. One-Dimensional SDS–Polyacrylamide Gel Electrophoresis

This section describes one-dimensional SDS–polyacrylamide gel electrophoresis (1D-GE) for the Hoeffer SE-600 system with 11 × 14 cm gels, but these instructions can easily be modified to fit other electrophoresis systems.

1. Thoroughly clean two 16 × 20 cm glass plates and two 1.5-mm thick spacers (19 mm by 16 cm) with distilled water. Dry the equipment using wipes and check that there are no visible particles or lint on the glass plates or spacers. It is of extreme importance that the glass plates be as clean as possible. Once clean, assemble the plates in the gel pouring apparatus.
2. A 10%, 1.5-mm thick gel is typically used to maximize protein load and resolution for a broad range of molecular weights, which is critical for later mass spectrometric analysis. Prepare a 10% gel by mixing 8.8 ml of 4× separating buffer with 8.813 ml of 40:1 acrylamide:bis solution (BioRad), 18.21 ml of MiliQ H₂O, 3.76 ml of 50% sucrose, 352.5 µl of 10% ammonium persulfate, and 23.5 µl of TEMED (see Note 1). Pour the separating gel, leaving a 1-cm gap between the top of the separating gel and the bottom of the comb. Gently add isobutanol to the top of the gel and let the gel polymerize for 1 h.
3. Pour off the isobutanol and dry the space between the two glass plates. Prepare a 2× stacking gel by mixing 1.5 ml of 4× stacking buffer with 1 ml of 40:1 acrylamide:bis solution (BioRad), 6.4 ml of H₂O, 100 µl of 10% ammonium persulfate, and 20 µl of TEMED (see Note 1). Pour the stacking gel and insert a 10-well comb between the two glass plates. Let the stacking gel polymerize for 30 min. Carefully remove the comb and place the gel in the gel unit. Fill the unit with running buffer making sure to wash out each well thoroughly with running buffer.
4. Dilute samples with water to normalize volume and then add sample buffer in a 1:1 ratio. For the stained gel, 300 µg of protein will be loaded per lane. For the Western blot, 150 µg of protein will be loaded per lane (see Note 2).
5. Fill the gel running apparatus to fill line with running buffer and close the lid after connecting electrodes. Connect the electrode ends to power supply and run at 25 V

¹Do not add APS and TEMED until right before pouring the gel as the gel will start to polymerize as soon as they are added.

²Using the 2:1 ratio for loading protein for either the stained gel or Western blot, respectively, will ensure that immunopositive bands will be visible and comparable with the imperial blue stained gel, and that there will be enough protein present in each band for protein identification. If this experiment is run on a mini gel, protein concentrations must be adjusted to ensure good resolution.

constant till dye front runs through the stacking gel. Increase voltage to 100 V constant through the resolving gel and stop power once the dye front is at the bottom or just run off the bottom of the gel.

6. Remove gel from glass plates into a glass container and rinse with ddH₂O 3 × 5 min to remove excess SDS and other contaminants that will interfere with the staining process. Remove excess H₂O from the gel-containing glass container and cover gel with Imperial Blue Gel Stain and shake lightly for 1 h. Pour excess stain off and add water down the side of the container careful not to damage the gel, and shake lightly for 10 min. Continue this process until the background is sufficiently diminished. Bands of interest can now be excised in preparation of digest and LC-MS/MS analysis.

3.2. Western Blot

1. Cut PVDF and blotting paper to a size slightly larger than the gel. Immerse PVDF membrane into 100% methanol for 1 min to activate it, and then remove from methanol and place in milliQ water for 1 min, shaking by hand to remove excess methanol. Finally, place membrane, blotting paper, and two sponges in transfer buffer until ready to use (see Notes 3 and 4).
2. Open the cassette and place the black half down in the transfer buffer with the red part toward you. Place a soaked sponge on the black part of the cassette (see Note 5). Then place blotting paper followed by the gel. Quickly place the wet PVDF membrane over the gel and immediately add a wet blotting paper on top of the membrane. Add some transfer buffer to the top of the “sandwich” to ensure it remains soaked. Now add the final sponge to the sandwich and close the cassette. Leave submerged in transfer buffer until ready.
3. Transfer: Place cassette in transfer box and quickly add transfer buffer to the box at fill line. Connect to power supply and transfer for 1.5 h at 100 V constant (see Note 6).
4. Block: Once transfer is complete, remove cassette, open, remove PVDF membrane, quickly place it in 0.02% PVA, shake by hand for 1 min, pour off, and add 5% milk to cover the membrane (see Note 7). Incubate either at room temperature for 1 h, or at 4°C overnight.
5. Primary antibody: After blocking, pour off milk solution and quickly add antibody diluted to proper concentration to the membrane and incubate at room temperature for 90 min.

³It is extremely important not to let the PVDF membrane dry once it has been wet as it will interfere with the protein transfer. Drying of the membrane will appear as streaking or lines running across it. If it dries before the transfer has begun, repeat the activation step. If it dries after the transfer, it must be soaked in 1% (v/v) Tween20 in TBS for 1 h for mixing or until streaks disappear.

⁴This process should be carried out about 10 min before the gel electrophoresis is completed. This step should be continuous; where once the electrophoresis is completed, the transfer should begin immediately.

⁵When preparing for the transfer, everything including sponges and blotting paper must remain wet with transfer buffer and without the presence of air bubbles. Dryness and bubbles will interfere with the transfer.

⁶The voltage may never actually reach the 100 V it was set to.

⁷Be careful not to pour any liquid directly onto the membrane; instead, pour down the side of the container in which the membrane is contained in.

6. Wash: Pour off primary antibody solution and rinse the membrane with milliQ water three times, then add the detergent buffer to cover the membrane and rotate slowly for 5 min. Pour off detergent buffer and rinse the membrane three times with milliQ water. Then add washing buffer to the membrane and incubate for 5 min rotating slowly. Repeat the washing buffer and wash one more time.
7. Secondary antibody: After the final wash, add the secondary antibody after appropriate dilution in wash buffer. Incubate for 90 min at room temperature rotating slowly. At the end of this incubation, repeat wash from step 6.
8. Developing: Pick the membrane up with tweezers from a corner and dab on opposite corner on a paper towel to remove excess wash buffer. Place on Saran wrap with the protein side up and add the mixed ECL solution (1:1, Reagent A, Reagent B) and incubate for 1 min. Lift the membrane off the wrap with tweezers by the corner and dab corner on a paper towel to remove excess ECL solution and place on a clean Saran wrap. Fold the Saran wrap over to cover the protein side and place in a membrane cassette. Develop the film.
9. Using the ladder, line up stained gel and Western blot film to determine which bands are immunopositive and thus adducted. Cut out bands that are immunopositive.

3.3. In-Gel Digest of Protein Band (1D-GE)

1. Generally, adducted proteins are low in abundance and keratin (found in skin and hair) is a major contaminant capable of suppressing the signal of the protein contained within the band. In order to avoid this, bench top, tips, and tubes must be as clean as possible. Gloves and laboratory coat should always be worn while handling the samples and it may also be necessary to wear a hair cap and work under a hood.
2. Gel bands/spots are washed with 100 μ l of ddH₂O 2 $\times$ 10 min (see Note 8). After incubation, ddH₂O is removed, and 40 μ l of 50% ACN is added, the tube incubated for 15 min, and removed. Then 40 μ l of 100% ACN is added to the tube containing the band/spot and incubated for 15 min or until band becomes white and sticky. Remove ACN, add 40 μ l of 100 mM ammonium bicarbonate, and incubate for 5 min. Then add 40 μ l of 100% ACN and incubate for an additional 15 min. Pull off excess solution and dry gel pieces under vacuum centrifugation until bands are very dry (see Note 9).
3. Let samples return to room temperature after drying. Then add 40 μ l of 10 mM DTT solution to each band/spot and incubate in 55°C water bath for 45 min (see Note 10). After incubation, remove from the water bath and allow samples to return

⁸For all of the steps presented in Subheading 3.32, the band/spot should be submerged completely and volumes can be adjusted accordingly.

⁹It is critical that the gel bands are completely dry. This usually takes around 15 min of vacuum centrifugation is obvious when the band/spot is no longer stuck to the side of the tube, and makes a “rattle” sound when shaken.

¹⁰Both the DTT and IAA solutions and incubation of band/spots with these solutions should be protected from the light. This can be achieved by simply covering with aluminum foil.

to room temperature. Remove excess solution, add 40 μ l of 55 mM IAA solution, and incubate at room temperature in the dark for 30 min. Remove excess solution, add 40 μ l of 100 mM ammonium bicarbonate, incubate for 5 min, and then add 40 μ l of 100% ACN and incubate for an additional 15 min. Pull off excess solution and dry as described above.

4. Add 40 μ l of trypsin containing digestion solution and incubate on ice for 45 min (see Note 11). Remove excess trypsin containing digestion solution, add 40–60 μ l of digestion buffer (without trypsin), and incubate at 37°C for 12–16 h.
5. After incubation, add 10 μ l of 2% TFA to the digestion mixture to acidify reaction and thereby quenching the trypsin, and remove supernatant to a clean tube. Cover gel band/spot with 0.1% TFA in water and place the tube in a floating rack in a sonicating bath for 30 min to begin peptide extraction. Remove the supernatant and combine with previous solution. Repeat previous step by adding 30% ACN 0.1% TFA, sonicating for 30 min, and combining the supernatants. Repeat the above step for a final time with 60% ACN 1% TFA. Pool all supernatants for each sample and dry volume down to ~10 μ l in preparation for mass spectrometric analysis. The samples can be stored at –20°C for short term or at –80°C for long term.

3.4. LC-MS/MS Analysis

1. LC-MS/MS analyses of protein digests are carried out using a linear quadrupole ion trap ThermoFinnigan LTQ mass spectrometer (San Jose, CA) equipped with a Michrom Paradigm MS4 HPLC, a SpectraSystems AS3000 autosampler, and a nanoelectrospray source (5).
2. Peptides are eluted from a 15-cm pulled tip capillary column (100 μ m I.D. $\times$ 360 μ m O.D; 3–5 μ m tip opening) packed with 7-cm Vydac C18 (Hesperia, CA) material (5 μ m, 300 Å pore size), using a gradient of 0–65% solvent B (98% methanol/2% water/0.5% formic acid/0.01% trifluoroacetic acid) over a 60-min period at a flow rate of 350 nl/min. The LTQ electrospray positive mode spray voltage is set at 1.6 kV and the capillary temperature at 180°C.
3. Dependent data scanning is performed using the Xcalibur v 1.4 software (6) with a default charge of 2, an isolation width of 1.5 amu, an activation amplitude of 35%, an activation time of 30 ms, and a minimal signal of 100 ion counts. Global dependent data settings are as follows: reject mass width of 1.5 amu, dynamic exclusion enabled, exclusion mass width of 1.5 amu, repeat count of 1, repeat duration of 1 min, and exclusion duration of 5 min. Scan event series include one full scan with mass range 350–2,000 Da, followed by three dependent MS/MS scans of the most intense ion.

3.5. Data Analysis

1. Tandem MS spectra of peptides are analyzed with TurboSEQUENT™ v3.1 (7). The peak list (data files) for the search is generated by Bioworks 3.1. Parent peptide

¹¹If band/spot absorbs entire 40 μ l of trypsin containing digestion solution, add an additional 20–40 μ l.

mass error tolerance is set at 1.5 amu and fragment ion mass tolerance is set at 0.5 amu during the search. The criteria that are used for a preliminary positive peptide identification are the same as previously described, namely, peptide precursor ions with a +1 charge having a Xcorr > 1.8, +2 Xcorr > 2.5, and +3 Xcorr > 3.5. A dCn score > 0.08 and a fragment ion ratio of experimental/theoretical >50% were also used as filtering criteria for reliable matched peptide identification (8).

2. All matched peptides are confirmed by visual examination of the spectra. All spectra are searched against the ipiRAT v3.22 database from EMBL downloaded on October 06, 2006. At the time of the search, the ipiRAT protein database from EMBL contained 41,336 proteins. Tandem MS spectra of peptides are also analyzed with X!Tandem (<http://www.thegpm.org>; version 2007.01.01.1). X!Tandem is set up to search a subset of the ipi.RAT.v3.31 database also assuming trypsin. Sequest is set up to search the ipi.RAT.v3.31.fasta database (41,251 protein entries), assuming the digestion enzyme trypsin. Sequest and X!Tandem are searched with a fragment ion mass tolerance of 0.5 Da and a parent ion tolerance of 1.5 Da. Oxidation of methionine, iodoacetamide derivative of cysteine, +105 (HQ), +268 (NAC-BQ), +226 (CSHQ) at Lys, Arg, and Glu are specified in Sequest and X!Tandem as variable modifications.

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References

1. Lau SS, Monks TJ, Everitt JI, Kleymenova E, Walker CL. Carcinogenicity of a nephrotoxic metabolite of the “nongenotoxic” carcinogen hydroquinone. *Chem Res Toxicol*. 2001; 14:25–33. [PubMed: 11170505]
2. Kleiner HE, Jones TW, Monks TJ, Lau SS. Immunochemical analysis of quinol–thioether-derived covalent protein adducts in rodent species sensitive and resistant to quinol–thioether-mediated nephrotoxicity. *Chem Res Toxicol*. 1998; 11:1291–1300. [PubMed: 9815189]
3. Kleiner HE, Rivera MI, Pumford NR, Monks TJ, Lau SS. Immunochemical detection of quinol–thioether-derived protein adducts. *Chem Res Toxicol*. 1998; 11:1283–1290. [PubMed: 9815188]
4. Fisher AA, Labenski MT, Malladi S, Gokhale V, Bowen ME, Milleron RS, Bratton SB, Monks TJ, Lau SS. Quinone electrophiles selectively adduct “electrophile binding motifs” within cytochrome c. *Biochemistry*. 2007; 46:11090–11100. [PubMed: 17824617]
5. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem*. 1996; 68:850–858. [PubMed: 8779443]
6. Andon NL, Hollingworth S, Koller A, Greenland AJ, Yates JR III, Haynes PA. Proteomic characterization of wheat amyloplasts using identification of proteins by tandem mass spectrometry. *Proteomics*. 2002; 2:1156–1168. [PubMed: 12362334]
7. Yates JR III, Eng JK, McCormack AL, Schieltz D. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal Chem*. 1995; 67:1426–1436. [PubMed: 7741214]
8. Cooper B, Eckert D, Andon NL, Yates JR, Haynes PA. Investigative proteomics: identification of an unknown plant virus from infected plants using mass spectrometry. *J Am Soc Mass Spectrom*. 2003; 14:736–741. [PubMed: 12837595]

Fig. 1.

Alignment of protein-stained gel with Western blot film. Parallel gels were performed; one for protein staining and the other for Western blotting. The first gel was stained for 1 h with Coomassie blue protein stain. The second gel was transferred to a PVDF membrane and then probed with an antibody specific to protein adducts that occur as a result of MGHQ treatment, anti-BrHQ-NAC. Both the stained protein gel and the Western blot film were aligned, and stained protein bands that correspond to immunopositive bands on the film were excised and digested with trypsin.

Fig. 2.

General schematic for site-specific identification of immunopositive protein adducts excreted in urine of rats treated i.v. with MGHQ. Immediately after i.v. treatment with MGHQ, the Long Evans rats were placed in metabolism cages and urine was collected for 2 h. Urine was spun at $14,000 \times g$ for 10 min to pellet urinary particulates. Urinary proteins were then diluted in sample buffer. Parallel gels were then run; one for Western blotting with anti-BrHQ-NAC and the other for protein staining with Imperial or Coomassie blue stain. The film from the Western blot is then aligned with the stained gel, immunopositive protein bands are excised, and the protein digested with trypsin. The peptides are then analyzed via MS/MS. Raw data from mass spectrometry are then analyzed using different peptide matching software programs searching for adducts on specific types of amino acids, i.e., +105 (HQ) on Lys, Arg, and Cys. Peptides found to be adducted are then validated manually using longen and Protein Prospector, and with other software programs, such as P-Mod and FindMod.