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Protein Carbonyl Levels— An Assessment of Protein Oxidation

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1. INTRODUCTION

Oxidative stress may be a hallmark of several neurodegenerative disorders, including Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson's disease (PD), as well as Creutzfeldt-Jakob disease (CJD), frontotemporal dementia, and amyotrophic lateral sclerosis (ALS) *(1)*. Oxidative stress occurs when the formation of reactive oxygen species (ROS) increases, or when scavenging of ROS or repair of oxidatively modified molecules decreases *(2,3)*. ROS are highly reactive, toxic oxygen moieties, such as hydroxyl radical, peroxyl radical, superoxide anion, and hydrogen peroxide. Collectively, ROS can lead to oxidation of proteins and DNA, peroxidation of lipids, and, ultimately, cell death. To counteract these damaging radicals, antioxidant systems have been developed. Among these are enzymes, such as glutathione peroxidase, glutathione reductase, superoxide dismutase (SOD), and catalase, among others; and small, nonprotein, cellular antioxidants such as, glutathione, vitamin C, vitamin E, and uric acid.

Oxidative stress has been shown to increase protein oxidation *(2–6)*, and has been reported in AD *(7)*, animal models of AD *(8)* and HD *(9,10)*, rheumatoid arthritis (RA) *(11)*, respiratory distress syndrome *(12)*, PD *(13)*, atherosclerosis *(14)*, and accelerated aging *(15)*. One way to measure protein oxidation is to determine levels of protein carbonyls. Protein carbonyls can result from oxidative cleavage of the protein backbone, direct oxidation of amino acids such as lysine, arginine, histidine, proline, glutamic acid, and threonine, or by the binding of aldehydes produced from lipid peroxidation

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such as 4-hydroxynonenal (HNE) or acrolein *(2,3)* (Fig. 1). In addition, carbonyl groups may be introduced into proteins by reactions with reactive carbonyl derivatives generated as a consequence of the reaction of reducing sugars or their oxidation products with lysine residues of proteins, glycation, or glycoxidation reactions *(16,17)*.

The assay of carbonyl groups in proteins provides a convenient technique for detecting and quantifying oxidative modification of proteins. 2,4-dinitrophenylhyrdazine (DNPH) reacts with protein carbonyls to produce hydrazones (Fig. 2). Hydrazones can be detected spectrophotometrically at an absorbance of 370 nm or by fluorescence (18). Western- or slot-blotting techniques are also used for sensitive and specific detection of the protein-2,4-dinitrophenyl hydrazone moiety *(19)*.

2. MATERIALS

1. 0.2% (w/v) DNPH prepared in 2 *N* HCl.
2. 6 *M* guanidine hydrochloride dissolved in 20 m*M* sodium phosphate buffer with a pH of 6.5.
3. 100% (w/v) tricholoroacetic acid.
4. Ethanol/ethyl acetate solution (1:1 v/v).
5. 200 m*M* DNPH stock solution prepared in 100% trifluroacetic acid (TFA).
6. 12% (w/v) Sodium dodecyl sulfate (SDS).
7. Neutralization solution (2 *M* Tris/30% glycerol/ 19% 2-mercaptoethanol).
8. Primary antibody to the 2,4-dinitrophenylhydrazone protein moiety, available from Sigma (St. Louis, MO, D-8406) or Intergen (Purchase, NY, OxyBlot Kit #S7150).
9. Labeled secondary antibody to the species of the primary antibody appropriate for the method of detection (chemiluminescence, fluorescence, colorimetry).
10. Biological sample to be assayed for protein carbonyl determination. Protein concentration is determined by the Pierce BCA method.

3. METHODS

3.1. Spectrophotometric Assay

1. Sample derivatization. Two 1-mg aliquots are needed for each sample to be assayed. Samples are extracted in a final concentration of 10% (w/v) TCA. The precipitates are treated with 500 μL of 0.2% DNPH or 500 μL of 2 *N* HCl. Samples are incubated at room temperature for 1 h with vortexing at 5-min intervals. The proteins are then precipitated by adding 55 μL of 100% TCA. The pellets are centrifuged and washed three times with 500 μL of the ethanol:ethyl acetate mixture. The pellet is then dissolved in 600 μL of 6 *M* guanidine hydrochloride.

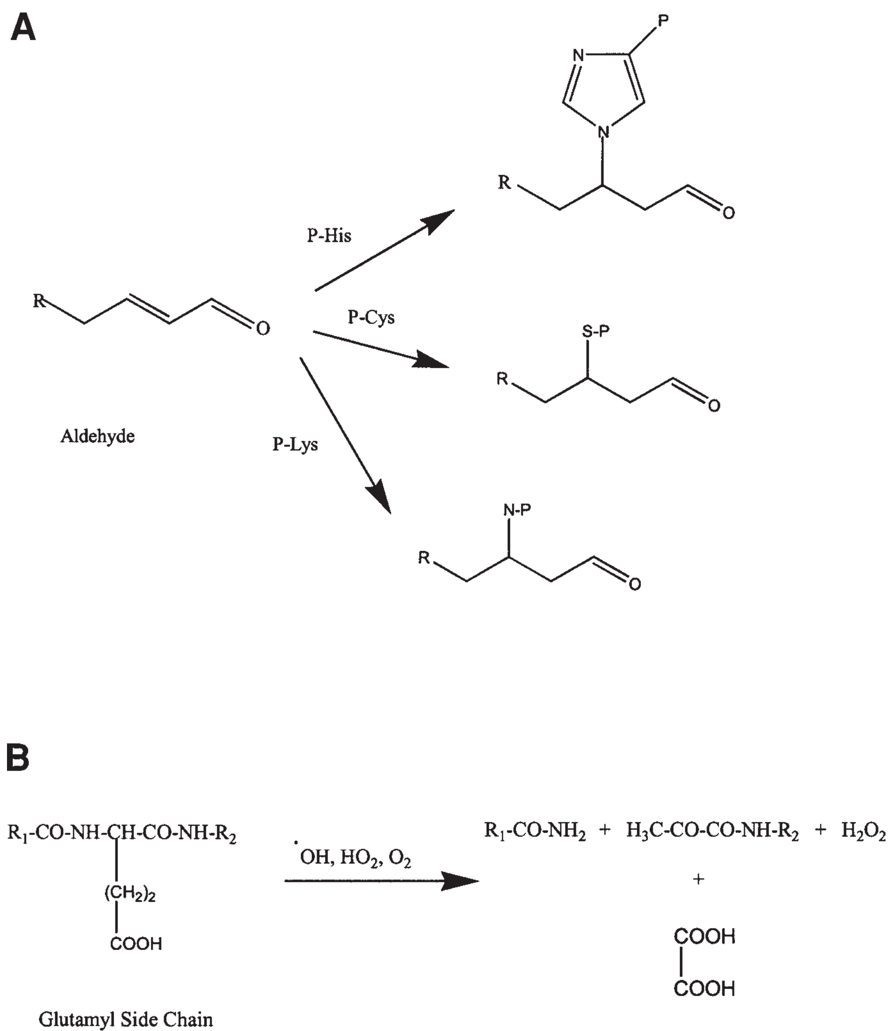
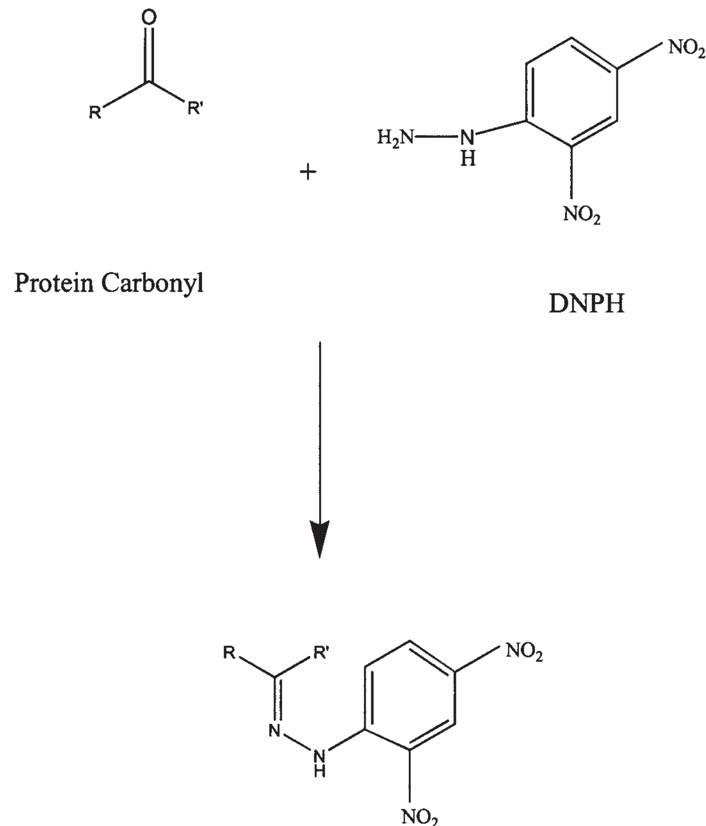


Fig. 1. (**A**) Michael addition of an aldehyde to protein lysine (P-lys), histidine (P-His), or cysteine (P-Cys) residues resulting in the addition of carbonyl groups to the protein. (**B**) Oxidation of the glutamyl side chain leads to formation of a peptide in which the N-terminal amino acid is blocked by an α-ketoacyl derivative.

2. The carbonyl content is determined by reading the absorbance at the optimum wavelength (λ = 360–390 nm) of each sample against its appropriate blank

3.2. Immunodetection Methods

1. Sample derivatization. 200 m*M* DNPH stock solution is diluted 10 times with water. Five μL of sample is incubated at room temperature with 5 μL of 12%



DNP Hydrazone Protein Carbonyl Moeity

Fig. 2. Dinitrophenylhydrazine (DNPH) reacts with protein carbonyl groups producing a protein-DNP hydrazone moiety that can be detected spectrophotometrically or by immunochemical techniques.

SDS and 10 μL of the diluted DNPH for 20 min with vortexing. The samples are neutralized with 7.5 μL of the neutralization solution.

2. Western blotting. 5–15 μg of the sample solution is loaded onto a gel and electrophoresed according to the Laemmli method *(20)*. The proteins on the gel are then transferred to nitrocellulose paper and standard immunodetection techniques are performed *(21)*.
3. Slot blotting. 250 ng of the sample solution is loaded into the wells of the slot blot apparatus. Proteins are transferred directly to the nitrocellulose paper under vacuum pressure and standard immunodetection techniques are performed.

4. ANALYSIS

For the spectrophotometric assay, 2,4-dinitrophenylhydrazone protein adducts are calculated using the millimolar adsorptivity of 21.0 mM^{-1} cm^{-1} for aliphatic hydrazones. Results are reported as nmol of DNPH incorporated per mg of protein. The carbonyls detected in the immunodetection methods can be reported either by comparing to a standard with an amount of 2,4-dinitrophenylhydrazone protein adducts as determined by the spectrophotometric method or by comparing the density to the control sample density employing suitable imaging software.

5. DISCUSSION

The method chosen for analysis depends on the sample and the needs of the researcher. The spectrophotometric method actually quantitates the 2,4-dinitrophenylhydrazone protein adducts and is therefore useful for comparing samples that will be collected and quantitated at different times. However, because a large amount of sample is required for analysis, it may be inappropriate for samples such as cerebrospinal fluid (CSF) where protein concentration is limited. For samples with limited protein concentration, the slot blot technique is appropriate, because for as little as 0.5 nanograms of protein can be loaded (Fig. 3). Slot-blot analyzes the total protein oxidation whereas Western-blot analysis allows for the comparison of individual proteins that may be oxidized. Two-dimensional (2D) gel electrophoresis followed by identification of the position of an individual protein with specific antibodies and immunoblot analysis for protein carbonyls have been shown to be a potential method to identify specifically oxidized proteins in situ *(22,23)*.

Proteomics may also offer tools to perform such identification in an automated procedure. By observing the total protein profile as well as the oxidized protein profile with 2D-gel electrophoresis, it is possible to select proteins showing higher levels of carbonyls vs control. The spot corresponding to the protein of interest is then excised and submitted for proteomics analysis. Chemical modification and protein digestion with a sequence-specific protease precede the extraction of the proteolytic digestion and the mass spectrometric (MS) analysis of the peptides. On-line HPLC-MS connection offers the possibility of direct mass detection during HPLC separation of the peptides. The masses of a proteolytic digestion are highly characteristic of a protein and represent an important means to identify unknown oxidized proteins utilizing suitable protein databases.

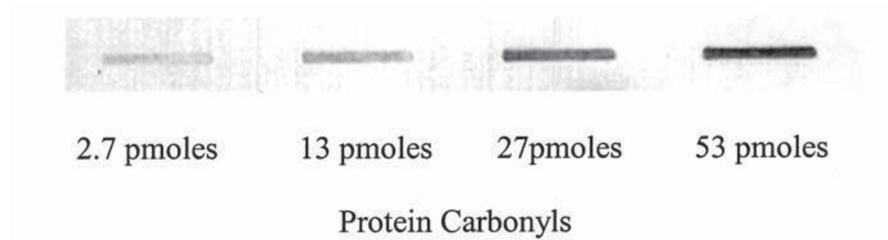


Fig. 3. Sample slot blot of bovine serum albumin (BSA) oxidized by hydroxyl radicals, produced by Fe^{2+} and H_2O_2, and reacted with DNPH, demonstrating sensitivity as low as picomolar levels of protein carbonyls.

The whole procedure potentially allows automated identification of several oxidized proteins at the time, yet some problems can occur. First, reproducibility of 2D-gel analysis, which is crucial for control-sample spot comparison, might be reduced by the instability of pH-gradients in the first dimension (isoelectric focusing; IEF). To circumvent this limitation, the use of immobilized pH gradients (IpHGs) may be suitable *(24)*. IpHGs are formed by copolymerization of buffering and titrant groups of acrylamido-derivatives into a polyacrylamide gel matrix, assuring a steady-state focusing and a consequent high reproducibility of the spot position.

In addition, oxidative modification may result in altering, even slightly, the electrophoretic properties of a protein and its migration with respect to the unmodified one. This problem can be overcome *(22,25)*, especially with the help of specific 2D-gel softwares, which increase the resolution and the possibility of spot crossmatching.

This automated method of proteomic analysis is in its infancy with respect to neurodegenerative diseases, but is under investigation in our laboratory *(26,27)*.

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