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Critical Methods in Free Radical Biology & Medicine

Quantification of protein modification by oxidants

Clare L. Hawkins [a], Philip E. Morgan [a], Michael J. Davies [a,b,*]

[a] The Heart Research Institute, 114 Pyrmont Bridge Road, Camperdown, NSW 2050, Australia
[b] Faculty of Medicine, University of Sydney, Sydney, NSW 2006, Australia

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ABSTRACT

Proteins are major targets for oxidative damage due to their abundance and rapid rates of reaction with a wide range of radicals and excited state species, such as singlet oxygen. Exposure of proteins to these oxidants results in loss of the parent amino acid residue, formation of unstable intermediates, and the generation of stable products. Each of these events can be used, to a greater or lesser extent, to quantify damage to proteins. In this review the advantages and disadvantages of a number of these approaches are discussed, with an emphasis on methods that yield absolute quantitative data on the extent of protein modification. Detailed methods sheets are provided for many of these techniques.

Introduction

Reactive oxidants are generated continually in biological systems (reviewed in [1]) via both endogenous processes and as a result of exposure to external factors (e.g., radiation, drugs, chemicals, pollutants), and this formation can be either deliberate or accidental. Thus oxidant formation plays a key role in the protection of tissues from invading pathogens (e.g., deliberate formation by activated leukocytes during the phagocytosis of exogenous particles) and in a number of human pathologies, as a result of inappropriate or misplaced generation. Radicals are also generated via the redox reactions of a wide range of enzymes, and this can result in the deliberate or accidental release of partially reduced species (e.g., superoxide radical, $O_2^{\bullet-}$, and hydrogen peroxide, H_2O_2) from enzyme active sites. Thus electron leakage from mitochondrial and endoplasmic reticulum electron transport chains is believed to be a major source of oxidants in vivo.

Damage has been reported to occur on all components of biological systems (e.g., DNA, RNA, lipids, proteins, carbohydrates, low-molecular-mass species, antioxidants) due to the high reactivity of many oxidants. Proteins are likely to be major targets, as a result of their abundance in cells (proteins compose ca. 70% of the dry mass of most cells), plasma, and most tissues, and their rapid rates of reaction both with many radicals and with other oxidants (e.g., peroxides, excited states, peroxynitrite, chloramines, ozone). It should be noted that the extent of damage to any particular component does not necessarily correlate with the importance of such damage. Thus a low level of damage to a critical species may be of much greater significance than massive damage to a nonessential target. However, it is currently unclear which are the critical causative events in many pathologies, and which are merely downstream effects of little importance.

Many of the techniques used to assess oxidative damage are based on simplicity and ease of use rather than unquestioned importance to the pathology in question. Furthermore, many of the methods employed to assess damage do not yield quantitative data, merely qualitative or semiquantitative assessments. This makes comparison between the data obtained in different laboratories on the same systems, or worse still, by different researchers using different

Abbreviations: apoAI, apolipoprotein A1; apoAII, apolipoprotein AII; 3Br-Tyr, 3-bromotyrosine; 3,5diBr-Tyr, 3,5-dibromotyrosine; BSA, bovine serum albumin; 3Cl-Tyr, 3-chlorotyrosine; 3,5diCl-Tyr, 3,5-dichlorotyrosine; DBNBS, 3,5-dibromo-4-nitrosobenzene sulfonic acid; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide; di-Tyr, dityrosine; DMPO, 5,5′-dimethyl-1-pyrroline-*N*-oxide; DNPH, 2,4-dinitrophenylhydrazine; DOPA, 3,4-dihydroxyphenylalanine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EPR, electron paramagnetic resonance; FAB, fast atom bombardment; FOX, ferrous oxidation-xylenol orange; GC, gas chromatography; GC/MS, gas chromatography coupled with mass spectrometry; GSH, reduced glutathione; GSSG, oxidized glutathione; HDL, high-density lipoprotein; HPLC, high-performance liquid chromatography; HSA, human serum albumin; IAF, 5-iodoacetamidofluorescein; LC/MS, HPLC coupled with mass spectrometry; LC/MS^n, HPLC coupled with multistage mass spectrometry; MetSO, methionine sulfoxide; MNP, 2-methyl-2-nitrosopropane; MS, mass spectrometry; MSA, methanesulfonic acid; *m*-Tyr, 3-hydroxytyrosine; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; NMR, nuclear magnetic resonance; $3NO_2$-Tyr, 3-nitrotyrosine; OPA, *o*-phthaldialdehyde; *o*-Tyr, 2-hydroxytyrosine; PBN, *N*-*tert*-butyl-α-phenylnitrone; PTQ, 9,10-phenanthrenequinone; SDS, sodium dodecyl sulfate; SIM, selective ion monitoring; TCA, trichloroacetic acid; TEMPO, 2,2′,6,6′,-tetramethylpiperidine-*N*-oxyl radical; TMB, 3,3′,5,5′-tetramethylbenzidine; TNB, 5-thio-2-nitrobenzoic acid; TPNH, triphosphopyridine nucleotide.

* Corresponding author. The Heart Research Institute, 114 Pyrmont Bridge Road, Camperdown, NSW 2050, Australia. Fax: +61 2 9565 5584.
E-mail address: daviesm@hri.org.au (M.J. Davies).

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methods on diverse systems, very difficult. For these reasons there is a critical need for well-validated techniques that yield absolute quantitative data that can be used on multiple systems. The widespread application of such techniques would provide valuable data on the relative importance of different oxidants, and information of the absolute importance of damage to different targets (e.g., DNA vs lipid vs protein; and different residues and alternative sites within each of these classes).

This article reviews a number of methods that have been developed to assess, and potentially quantify, damage to proteins and their components. This is by no means an exhaustive review, nor can it be taken as a definitive guide, as the nature of the systems under study may necessitate modification of the methods described. It is, however, hoped that the information provided will permit educated choices of systems to use, and potentially limit the inappropriate use and interpretation of data obtained from these techniques.

The sections that follow have been divided into three: (a) methods that examine loss of the parent material, (b) approaches that allow information to be obtained on the yields of various intermediate species, and (c) techniques that allow the quantification of products of oxidant reactions. Some methods are discussed in some depth and detailed "Methods Sheets" provided. Others are merely touched on, and references to further information provided.

Prevention of artifact generation during sample processing/handling

Inappropriate handling of samples before analysis is a critical and common source of artifacts. As such every effort needs to be made to ensure that this is minimized. The rates of oxidation reactions are critically dependent on the sample temperature, the presence of oxygen and catalysts (metal ions and light), and its physical form. Thus samples should, preferably, be kept frozen (usually the lower the temperature the better), in the dark, in the absence of oxygen and in the absence of metal ions. Addition of agents that slow or prevent oxidation can also be useful (e.g., presence of metal ion chelators, inhibitors of proteolytic and oxidative enzymes). It is important that the reagents and buffers used during the analysis are treated similarly; one of the most common sources of problems is trace transition metal ions in buffers. This can be minimized by the use of high purity water (e.g., Nanopure) and the treatment of prepared buffers with prerinsed Chelex resin [2]. Method Sheet 1 provides information on the preparation of Chelex-treated, 0.1 M sodium phosphate buffer, pH 7.4.

Changes in parent amino acid residues

Standard amino acid analysis by HPLC

Total amino acid analysis can be useful in examining poorly understood systems as it can yield data on the nature of oxidation reactions occurring by quantifying the loss of specific amino acids (e.g., [3–5]). The approach outlined here also allows the simultaneous quantification of some oxidation and modification products (e.g., methionine sulfoxide, MetSO [3–5], and the advanced glycation end product, *S*-(carboxymethyl)cysteine [6]).

In this method the protein(s) are hydrolyzed to their constituent amino acids, and then the resulting free amino groups are derivatized with *o*-phthaldialdehyde (OPA) or a related fluorescent tag, and separated by HPLC (Method Sheet 2). The OPA-amino acid adducts are quantified by fluorescence, relative to amino acid (or product) standards. This method is suitable for isolated or purified proteins and allows quantification all acid-stable amino acids (i.e., all common amino acids except Cys, cystine, Asn, and Gln; the former are completely destroyed, while the latter pair are converted to Asp and Glu, respectively). Methods suitable for the quantification of Cys and cystine are detailed below. Lys quantification can be variable with this method, due to the second free amino group on the side chain. Addition of an internal standard (e.g., homo-Arg) allows sample recovery and derivatization efficiency to be assessed. Methanesulfonic acid (MSA) is used in preference to other acids (e.g., gaseous HCl) as it preserves Met and Trp residues and gives quantitative amino acid release from the protein. Care must be taken to avoid artifactual oxidation of Trp and Met, with the hydrolysis carried out under vacuum (strictly O_2-free conditions). Lower than expected levels Met and Trp and high concentrations of MetSO, obtained from well-characterized standard proteins, are a sign of poor technique.

Enzymatic hydrolysis, which is less likely to result in artifactual oxidation due to the milder conditions employed (overnight incubation at 37 °C, pH 7.4), can also be used, but the release of free amino acids is often nonquantitative, and self-digestion of the protease results in the presence of additional amino acids. Self-digestion may also result in a lowering of the apparent relative concentration of oxidation products [7].

Some amino acid residues can be quantified by alternative methods. Trp residues can be determined by direct fluorescence measurements (λ_{EX} 280 nm, λ_{EM} 340–345 nm) (e.g., [8]), though it should be noted that the fluorescence of protein Trp residues is sensitive to the local environment, and changes in Trp fluorescence are routinely used to assess protein unfolding (reviewed in [9]). This method is difficult to use in the presence of other fluorophores, including heme groups, common photosensitizers, and any materials that have been subjected to glycation/glycoxidation (i.e., many clinical samples).

Other amino acid residues can be quantified by reaction with specific reagents that generate strongly fluorescent derivatives. Thus fluorescamine and 9,10-phenanthrenequinone (PTQ) can be used to quantify Lys [10,11] (Method Sheet 3) and Arg [12] (Method Sheet 4) residues, respectively. These methods have been used to assess the role of Lys and Arg residues in protein glycation and glycoxidation reactions (e.g., [8]) and enzymatic reactions (e.g., [13]). These methods are rapid, highly sensitive, and less prone to artifacts resulting from protein oxidation during sample preparation. However, fluorescamine and PTQ are not specific for protein-bound Lys and Arg, respectively, with the former also reacting readily with other primary amine groups, including those on free amino acids, and the *N*-terminus of proteins, and PTQ reacting with monosubstituted guanidines.

The modification or loss of specific (individual) residues present in a protein can be determined, on isolated proteins, by peptide mass mapping. The protein is generally digested using trypsin, and the resulting peptide fragments analyzed by LC/MS (e.g., [14,15]). The peptides detected are identified by database searches, such as the NCBI database (www.ncbi.nlm.nih.gov), using search engines (e.g., Sequest, Mascot, ProteinLynx). The loss of specific peptides in the oxidant-treated samples, relative to controls, together with the identification of new peptides, can be indicative of both the extent and the nature of the protein modification. Thus detection of additional ions in the oxidized samples with m/z of $+16$ or $+32$ is usually indicative of the addition of one and two oxygen atoms (alcohols and peroxides/diols, respectively). Subsequent data-dependent MS/MS analysis of the product peptides results in a series of b and y fragment ions, which correspond to cleavages along the peptide backbone (fragment peaks that extend from the N-terminus of a peptide are termed b ions, while peptide fragment ions extending from the C-terminus are termed y ions). Identification of the ions with additional mass provides data on the exact site of modification. This technique has been used, for example, to

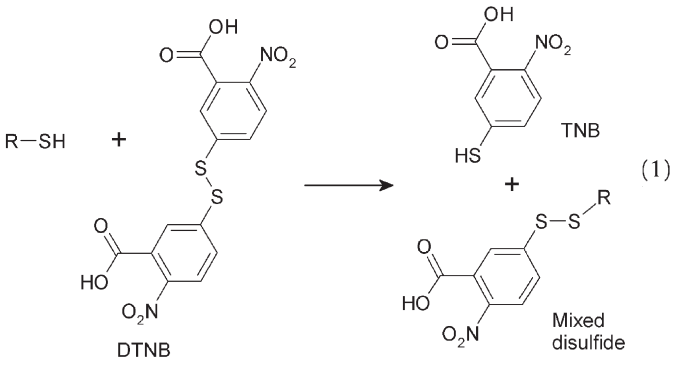
identify modifications to specific Tyr [16], Trp [17–19], Met [18,20], and Cys residues [14,15,21]. This method has also been used to identify sites of radical formation, by determining the site of addition of the spin trap 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) [22], and spin probe 2,2′,6,6′,-tetramethylpiperidine-*N*-oxyl (TEMPO) [23]. Although this approach is very powerful and provides very specific information, it is limited to isolated purified proteins (or well-defined mixtures) that are readily digested by trypsin or similar proteases. Quantification of damage using these MS approaches needs to carried out with care; this is reviewed in detail elsewhere [14].

Measurement of Cys residues

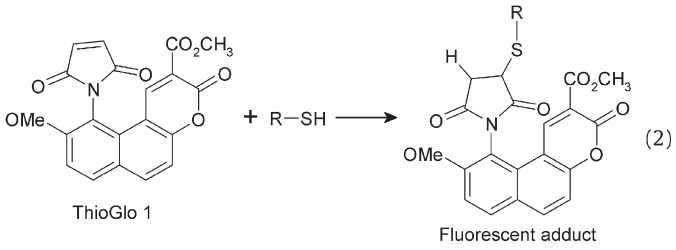
Oxidation of the thiol group of protein-bound Cys residues is a widely used nonspecific measure of protein modification. This reaction can be induced by many oxidants, including H_2O_2 and other peroxides [24], peroxynitrite [25], nitric oxide [26], singlet oxygen [27], HOCl [28], and many radicals. Multiple products can be formed, including cystine (disulfide), mixed disulfides (e.g., protein-glutathione species), nitroso adducts (RS-NO), and oxy acids (sulfenic, RSOH; sulfinic, RSO_2H; and sulfonic, RSO_3H) (reviewed in [29]). Disulfides, which are often the major product, can be repaired enzymatically (reviewed in [30]). However, when the rate of oxidation exceeds that of repair, a loss of thiols can be detected (e.g., in senile cataract, chronic renal failure, various liver diseases, systemic lupus erythematosus, and atherosclerosis [31,32]). Since a significant number of enzymes have active site, or other Cys residues, critical for activity, thiol loss can correlate with a loss of function.

Protein-bound thiols can be quantified by numerous methods; these include spectrophotometric (e.g., with 5,5′-dithiobis(2-nitrobenzoic acid), DTNB, [33]), fluorometric (e.g., ThioGlo 1), or MS [14]; these approaches have been extended to studies on complex samples where the proteins have been separated using 1D or 2D gels [34] or HPLC [14,15,21]. The use of isotope-coded affinity tagging combined with MS approaches, which is a powerful method for determining the sites and extent of Cys oxidation in complex biological mixtures, has been reviewed [35].

The oxidation of low-molecular-mass thiols (e.g., glutathione, GSH) has also been used as a quantitative marker of oxidative stress (e.g., [36]). GSH can be oxidized to multiple products including GSSG, mixed disulfides with other intracellular thiols, or other products (e.g., glutathione sulfonamide, oxy acids). Disulfide formation can be reversed by glutathione reductases (reviewed in [1]); this reaction can be exploited to determine levels of GSH and GSSG spectrophotometrically. Reduction of GSSG to GSH by glutathione reductase, and the subsequent reaction of GSH with DTNB, can be quantified at 412 nm [37]. Numerous kits that utilize this reaction are commercially available; thus a detailed method is not given here. It is also possible to quantify GSH by reaction with DTNB without the glutathione reductase system (e.g., [33]), though this method is typically less sensitive. In addition, several HPLC-based methods have been developed, for example, utilizing electrochemical detection methods (e.g., [38]), or derivatization with fluorescent moieties such as dansyl chloride (e.g., [39,40]). The GSH:GSSG ratio is commonly used to indicate the level of oxidative stress within a system (e.g., [41]), and when combined with measurement of protein thiols, can provide data on the overall thiol oxidation status of a system. The levels of Cys and cystine have been used similarly in plasma, with the development of rapid methods for their assessment and ratio (as an indicator of oxidative stress) in human plasma [42]. Method Sheet 5 details a method for quantifying protein thiols in human serum/plasma using DTNB (Ellman's reagent). DTNB reacts rapidly with reduced thiols resulting in the generation of 5-thio-2-nitrobenzoic acid (TNB), which is quantified at 412 nm by UV/Vis spectrophotometry (Reaction **1** [43]). This assay has a detection limit of ca. 0.3 μM [44].



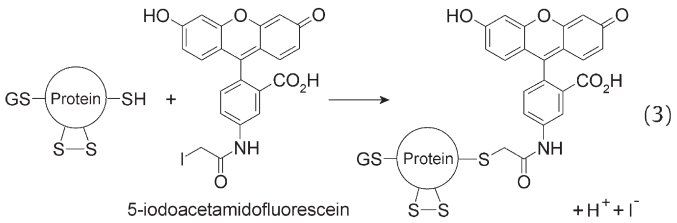
Method Sheet 6 outlines a protocol for the determination of thiols in cell lysates, using the fluorescent probe ThioGlo 1. ThioGlo 1 (10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-3*H*-naphthol [2,1-b]pyran-2-carboxylic acid methyl ester) is a maleimide derivative of a naphthopyranone fluorophore that yields a fluorescent adduct on reaction with thiols (Reaction **2**). ThioGlo 1 is considerably more sensitive than DTNB with a detection limit of ca. 10 nM [44], and does not appear to react readily with oxidants such as hypohalous acids and species derived from these that could confound measurements.



Protein-bound Cys and low-molecular-mass thiols can be individually assessed by a number of strategies. The latter are generally less stable than protein-bound thiols, and are readily lost on freeze-thawing and/or storage for prolonged periods [45]. In contrast, many protein-bound thiols are relatively robust; multiple freeze-thaw cycles appear to have negligible effects on serum protein thiol concentrations (P.E. Morgan, unpublished results). Protein thiol levels can be readily quantified after protein separation via trichloroacetic acid (TCA) precipitation. Simultaneous quantification of total levels gives the low-molecular-mass component by difference. In human serum/plasma the predominant component is the HSA Cys34 residue [33], due to the very low levels of low-molecular-mass thiols.

Reduced and oxidized thiols on proteins separated on 1D or 2D gels can be assessed by a number of methods (reviewed in [46]). 5-Iodoacetamidofluorescein (IAF, a fluorescent derivative of iodoacetamide) binds to protein thiol groups in the same manner as iodoacetamide (Reaction **3**), and has been used successfully to identify thiol oxidation in specific proteins (e.g., [47,48]). The presence of reversible thiol modifications can be examined by the addition of *N*-ethylmaleimide (NEM) after oxidant treatment to chemically modify any remaining reduced thiols, prior to reduction with DTT and derivatization with IAF (Reaction 4) [47]. It is also possible to determine the susceptibility of particular thiol proteins to oxidation

by examining their loss by IAF derivatization [48]; Method Sheet 7 details the use of this method to quantify protein thiols separated by 2D electrophoresis.



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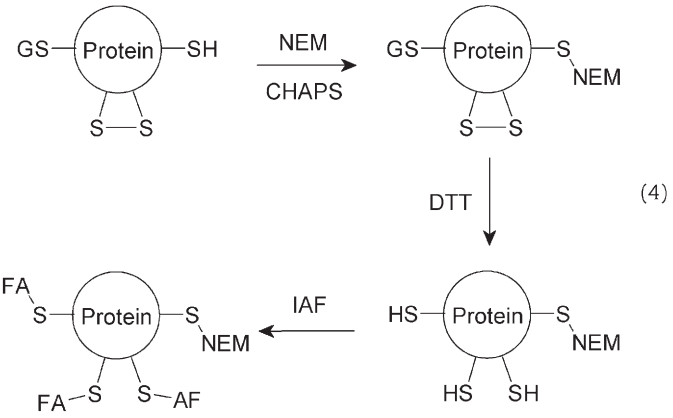


(4)

Measurement of cystine residues

Quantification of protein cystine residues can be achieved by careful protein digestion using chemical and enzymatic methods (e.g., cyanogen bromide or trypsin), which cleave the protein between the half-cystinyl residues, under conditions that minimize disulfide bond reduction and exchange. Early studies employed diagonal electrophoresis methods to determine disulfide bond positions (reviewed in [49]). These residues are now routinely studied using MS, particularly fast atom bombardment (FAB) techniques, after HPLC separation of the peptides resulting from partial digestion [50,51]. GSSG and other low-molecular-mass disulfides can be readily quantified by HPLC, after derivatization to introduce a highly sensitive chromophore or fluorophore (e.g., dansyl chloride), due to the absence of native chromophores (see above). MS methods have also been developed for cystine [42]. As low-molecular-mass disulfides are typically present at low levels in cells due to the presence of potent reductive systems, an increase in the levels of the oxidized material may be more readily discerned than a decrease in the level of the parent reduced thiol (e.g., GSH) due to the high background level of these materials. However this necessitates sensitive detection methods for the disulfide, and the formation of mixed disulfides may result in the presence of multiple products and poor overall (absolute) quantification.

Gross modification of the parent protein

Oxidative protein modification can be examined by loss of parent protein bands after separation by SDS-PAGE or HPLC. However, this approach is usually nonquantitative and has poor sensitivity, as extensive modification is typically required to detect changes. This is particularly the case in biological systems where there are very large numbers of components which may all be damaged to only a limited extent, especially with oxidants with limited selectivity (e.g., highly reactive radicals). However, this approach has been employed successfully with oxidants that are highly selective and induce damage only to particular residues. This is exemplified by the detection of specific changes on apolipoproteins AI (apoAI) and apoAII [20,52] where mild oxidation of these proteins in high-density lipoproteins (HDL) or plasma resulted in loss of the native isoforms, and the formation of new, oxidized species, as assessed by HPLC [20]. MS analysis of these products indicated loss of parent Met and generation of MetSO, which could be readily quantified. MetSO formation on soybean trypsin inhibitor treated with HOCl has also been detected using this approach (C.L. Hawkins, unpublished data).

Loss of recognition by specific antigens

Oxidation of protein side chains can result in structural changes including unfolding and/or aggregation (see above and below). This can result in altered recognition of antibodies raised against the undamaged protein. Thus reduced antibody recognition can be used as a method of assessing protein modification. However, this is typically nonquantitative, and changes are only usually detected after extensive protein modification. This approach can, however, be useful in localizing the site(s) of damage to particular regions of a protein if the epitope recognized by the antibody is known. Thus binding of three specific antibodies to subendothelial matrix (anti-fibronectin, anti-laminin, and anti-thrombospondin) is decreased on treatment with HOCl, implicating damage to these proteins in the matrix [53]. A similar approach has been used to probe damage to the protein and glycosaminoglycan components of matrix proteoglycans, such as perlecan, on treatment with HOCl and HOBr (M.D. Rees et al., submitted for publication); only recognition of the protein antibodies was modulated, implicating damage solely to these components of the complex.

Analysis of such data is complex, as some oxidative damage can also increase antibody binding. Thus low doses of HOCl increase the binding of anti-fibronectin antibodies to plasma fibronectin, whereas high levels of oxidant have the reverse effect [54]. This biphasic effect has been rationalized in terms of the fibronectin adopting an extended conformation at low HOCl doses, while at higher concentrations aggregation occurred. In all cases, knowledge of the specificities of the antibodies is critical to data analysis.

Detection of intermediates

Detection of radicals by direct spectrophotometric methods

Radicals can be detected directly by a number of spectroscopic and other methods (e.g., UV/visible, resonance Raman, conductivity, electron paramagnetic resonance (EPR)). This approach is only viable with techniques that have rapid response times due to the short half-lives of most radicals. As the majority of these techniques are not *specific* to radicals, their use in complex systems is limited due to background absorbances. Of these techniques, only EPR is specific for radicals and avoids this problem. Nonetheless some of these methods, particularly UV/visible spectroscopy when coupled to rapid radical generation techniques (e.g., pulse radiolysis, flash photolysis, stopped-flow) can provide very valuable data on the reaction kinetics (rate constants) of specific radicals.

EPR spectroscopy can be used to both identify protein radicals and determine the concentration of such species, in isolated and complex systems. Although this is a very powerful qualitative technique—and is often the *only* method that can provide unequivocable data on the nature of a radical—*direct* quantification is challenging due to the high reactivity/short lifetime of these species. This problem can be (partially) overcome by a number of ancillary techniques. Thus rapid-flow techniques, whereby a steady-state concentration of radicals is generated in the cavity of the spectrometer, can be used to provide data not only on radical identities (via their characteristic hyperfine coupling constants and g values), but also their relative and absolute concentrations (via spectral simulations and double integration of the

signal, respectively). This method has been employed successfully with amino acids and small peptides (e.g., [55]). However, the complexity of the EPR spectra, together with the large amounts of material required for rapid-flow measurements, limits the use of this approach. An alternative approach involves rapid freeze-quenching of the radicals of interest (e.g., by use of liquid N_2) to prolong their lifetime. This technique has been useful in the study of radicals derived from Tyr, Trp, Gly, and Cys residues among others (e.g., [56,57]). However, the EPR signals obtained using this method are often broad and poorly resolved (anisotropic) due to the slow molecular motion of the radical, which makes assignment complex. Thus, for many biological radicals, including those on proteins, indirect techniques are commonly used, the most prevalent of which is spin trapping.

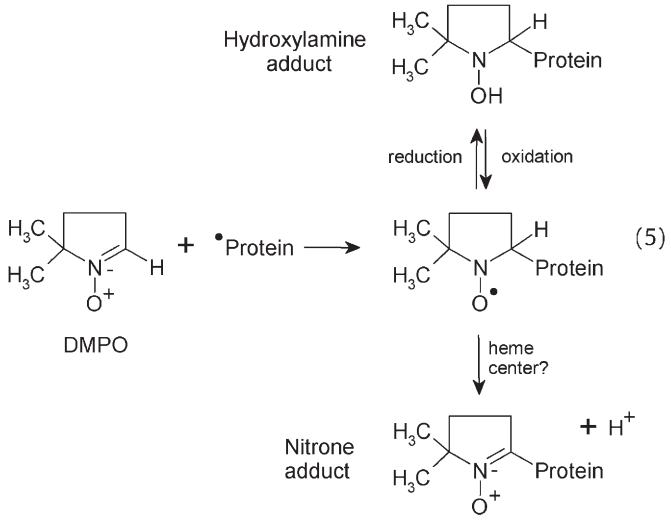
Indirect detection of radicals by EPR spin trapping

EPR spin trapping of radicals in biological systems has been reviewed extensively, and is therefore only discussed briefly here (e.g., [58–60]). In this technique, a spin trap (typically a nitrone or nitroso compound) is added to the system, which reacts rapidly with the radicals of interest, to give stable, readily detectable adducts. The latter, usually nitroxide radicals, are then detected by EPR spectroscopy. Nitroso spin traps (e.g., 2-methyl-2-nitrosopropane, MNP; 3,5-dibromo-4-nitrosobenzene sulfonic acid, DBNBS) have the advantage that the reactive radical attaches directly to the nitroso nitrogen atom, which often provides information about the added radical, due to the detection of additional distinctive hyperfine couplings from the magnetic nuclei present in the added radical (see [61] and the spin trap database at http://EPR.niehs.nih.gov). Nitroso traps are, however, usually only suitable for the analysis of carbon-centered radical adducts, due to the instability of other adducts, and many of these nitroso traps are thermally and photochemically labile, which can result in artifacts (reviewed in [62]). In contrast, nitrone spin traps (e.g., DMPO, *N*-*tert*-buytl-α-phenylnitrone, PBN; 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide, DEPMPO) form long-lived adducts with a wide range of different radicals (e.g., carbon-, nitrogen-, sulfur-, and oxygen-centered), but give more limited structural information, due to the lack of resolvable fine structure from the added radical.

Unlike amino acid and peptide radicals, which yield adducts that tumble rapidly in solution (and thereby provide considerable information on the nature of the original radical, e.g., [63]), the trapping of protein-derived radicals typically results in broad, partially anisotropic, EPR signals that lack resolvable hyperfine coupling constants (reviewed in [58]). Identification of such radicals is therefore challenging, but a number of approaches have been developed to circumvent this problem (reviewed in [58]). The broad nature of the spectral lines also makes the limit of detection of many protein radicals higher than those for low-molecular-mass radicals with sharp, well-defined EPR signals. The absolute concentrations of these species can be determined by double integration of the signal area (as EPR signals are usually recorded as the first derivative to maximize spectral resolution), and comparison with standard curves generated using EPR-active species of known concentration (e.g., stable nitroxides, metal ion complexes). It should be noted that spin trapping experiments usually do not yield data on the *initial* absolute protein radical concentrations—only relative data—as the kinetics of radical trapping and adduct decay are often unknown. These unknowns also cause problems in determining relative radical concentrations, as differential rates of radical trapping (or adduct decay) may make a major initial radical species appear to be a minor component, and vice versa. Thus the detection of radical species by EPR spin trapping can give information on the presence of a radical, but it does not usually provide information as to the quantitative importance of such intermediates. Such information can only be acquired from additional studies. Furthermore the *absence* of a radical adduct signal does *not* indicate that a particular radical is not generated—it may merely be formed, or trapped, at a rate that is too slow to give detectable adducts. Alternatively it may be generated and trapped rapidly, but the adduct may decay too rapidly for ready detection.

The observation that many protein radical adducts are long-lived, due to reduced rates of disproportionation and reduction as a result of steric and electronic constraints [64], has allowed EPR spin trapping of these species to be combined with other analytical techniques, including proteolytic digestion, modification of specific amino acid residues (site-directed mutagenesis, chemical blocking, and isotopic labeling), NMR, and LC/MS to provide detailed information on the identity of radicals (reviewed in [58]).

Immunospin trapping can also be used as a nonquantitative method for detecting protein radicals (reviewed in [65]). This method utilizes the reaction of radicals with the spin trap DMPO and subsequent decay of the nitroxide adduct to a nitrone to generate an antigen that is recognized by an antibody, that is in turn recognized by a secondary antibody linked to a detection system (typically chemiluminescence) [66] (Reaction **5**).



The majority of studies using this method in isolated protein systems have detected radicals generated on, or in the presence of, heme proteins, with oxidants including HOCl, peroxynitrite, and H_2O_2 ([22], also reviewed in [65,66]). There are few data available to date as to whether this approach is of use in detecting radicals such as hydroxyl, superoxide, peroxyl, and alkoxyl species. The presence of a heme center (or other powerful oxidant) may aid the detection of adducts, as these may help convert the initial protein-DMPO nitroxide adduct to its corresponding nitrone, which is the species detected [65,66]. The requirement for nitrone formation may limit the applicability of this technique, as many nitroxide radical adducts are converted in vivo to the corresponding reduced (hydroxylamine) species, in preference to the nitrone, as a result of the reducing environment of cells. Nonetheless, it has been demonstrated that protein-DMPO adducts are detectable both in cultured cells (e.g., [67,68] and in vivo [69,70]). A detailed protocol for immunospin trapping has been published [66].

Immunospin trapping alone is not able to pinpoint the exact sites of radical formation on proteins. However, loss of signal following modification of specific residues (e.g., iodination of Tyr, NEM, or DTNB blocking of Cys) can help to determine the identity of the amino acids on which the radicals are formed on oxidant addition and subsequent DMPO trapping [22]. The immunospin trapping system can also be used in connection with LC/MS/MS, with the anti-DMPO antibody used to screen HPLC fractions by ELISA to detect

peptides with bound DMPO; this has allowed specific amino acid residues on which radicals were present to be detected, as the mass alteration that occurs on covalent binding of DMPO can be readily detected [71].

Nonradical intermediates—Hydroperoxides

Protein hydroperoxides are a major product of protein damage in the presence of oxygen. These species can be formed, in high yield, on amino acids, peptides, and proteins by multiple oxidants, including singlet oxygen ($^1O_2$) [72], oxygen-derived radicals, activated white cells, peroxynitrite, and metal ion-mediated systems (reviewed in [73]). These long-lived, but reactive, species can decompose in the presence of metal ions to give further radicals (alkoxyl, peroxyl, and carbon-centered) that may damage other biomolecules [74]. Hydroperoxides can undergo two-electron reduction reactions (to yield the corresponding alcohols) with suitable reducing agents such as protein and low-molecular-mass thiols; reaction with protein thiols can result in inhibition of key intracellular thiol-dependent enzymes [21,24,75]. Decomposition of hydroperoxides formed at the α-carbon (backbone) site of proteins has been postulated to be a key step in protein fragmentation [76].

As hydroperoxides readily decompose, direct evidence for their formation in vivo is scarce. However, indirect evidence for their formation is strong. Markers consistent with the formation and subsequent decay of hydroperoxides have been detected in normal and pathological specimens, including human lens cataracts [77] and atherosclerotic lesions [78].

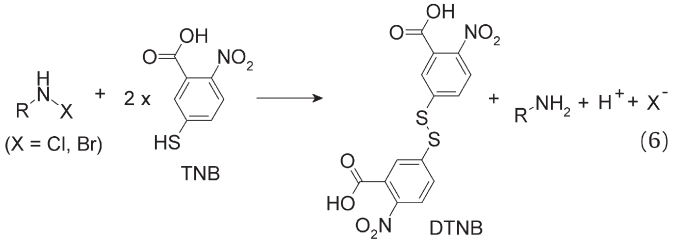
Method Sheet 8 provides a detailed procedure for the measurement of hydroperoxide concentrations on amino acid, peptide, and protein hydroperoxides using the FOX (*f*errous *o*xidation – *x*ylenol orange) assay. This method utilizes the hydroperoxide-mediated oxidation of a Fe(II)-xylenol orange to the Fe(III) form, with spectrophotometric quantification of the latter at 560 nm [79,80]. The FOX assay is suitable for the determination of total hydroperoxides and makes no distinction between protein- and lipid-derived hydroperoxides. It has been used to quantify amino acid, peptide, and protein hydroperoxides (e.g., [21,24,72,75,81]). For systems where other species are also present (e.g., lipid hydroperoxides), the FOX assay can be adapted and the lipid and proteins components separated before quantification [82]. Hydroperoxides may also be quantified using an iodometric method (reviewed in [83]). In this case, reaction of the hydroperoxide with iodide ions under acidic conditions generates triiodide (I_3^-), which is measured spectrophotometrically at 358 nm. The advantage of this method is that the reaction is quantitative, and occurs with a 1:1 stiochiometry, with hydroperoxides formed on different types of biological molecules. However, this assay must be performed under strict anaerobic conditions due to the sensitivity of acidified iodide solutions to oxygen [83]. In contrast, the iron-xylenol orange complex formed in the FOX assay shows minimal sensitivity to oxygen, making this assay much simpler to perform. Moreover, measurement of the absorbance at 560 nm is also minimally time critical once the reaction has proceeded to completion [80]. However, the FOX assay has the disadvantage of uncertain reaction stoichiometry (further details are given in Method Sheet 8), and care is required to ensure that false high readings are not obtained by contaminants in the system being studied. For example, unsaturated lipids can react with the Fe(II) [82]; the use of appropriate controls, or removal of lipids prior to analysis, can eliminate this source of error. Further comparison of the FOX and iodometric methods is reviewed in [84].

Nonradical intermediates—Chloramines/bromamines

The reaction of nitrogen-containing functional groups, including amines and amides, with HOCl and HOBr results in the formation of *N*-chlorinated and *N*-brominated species, respectively (reviewed in [85]). In proteins, chloramines and bromamines can be generated on the side chain of Lys and His residues, the *N*-terminal amine group, and to a more minor extent on the side chains of Arg, Asn, Gln, and backbone amides (peptide bonds). Rate constants for these reactions are collected in [86]. These materials retain the oxidizing equivalents of the initial HOCl/HOBr, and have been shown to be important intermediates in damage to host cells and invading pathogens during the inflammatory response [85,87].

Absolute quantification of these species can be obtained, for isolated systems, from their distinctive UV absorption bands. Thus, monochloramines (RNHCl) and monobromamines (RNHBr) have λ_{max} in the range 250–255 and 288–290 nm, respectively, and extinction coefficient between 350 and 450 M^{-1} cm^{-1} [88,89]. The di-halogenated materials arising from reaction with excess oxidants (dichloramines, $RNCl_2$; dibromamines, $RNBr_2$) typically display two absorbance bands at ca. 205/300 and 241/336 nm, respectively [88,89]. The UV spectra of chloramides and bromamides are less distinct with broad absorbances over the range 200–240 nm [90,91].

In more complex systems containing other chromophoric species that absorb over this region, quantification of chloramines and bromamines can be achieved via oxidation of TNB to the corresponding dimer (DTNB; Reaction **6**) [89], with this change accompanied by a loss of absorbance of the TNB at 412 nm, using an extinction coefficient of 14150 M^{-1} cm^{-1} [92] (Method Sheet 9). It should be noted that this assay is not specific for *N*-chloro and *N*-bromo species, and any species capable of oxidizing thiols (e.g., HOCl, HOBr, HOSCN, H_2O_2 and other peroxides, peroxynitrite, $^1O_2$, most radicals, and excited state species) can interfere by removing the TNB. Thus, such species need to be completely removed or consumed prior to assay. Moreover, chloramines/amides and bromamines/amides cannot be distinguished using this method, and knowledge of the approximate concentration of oxidant is required for accurate results.

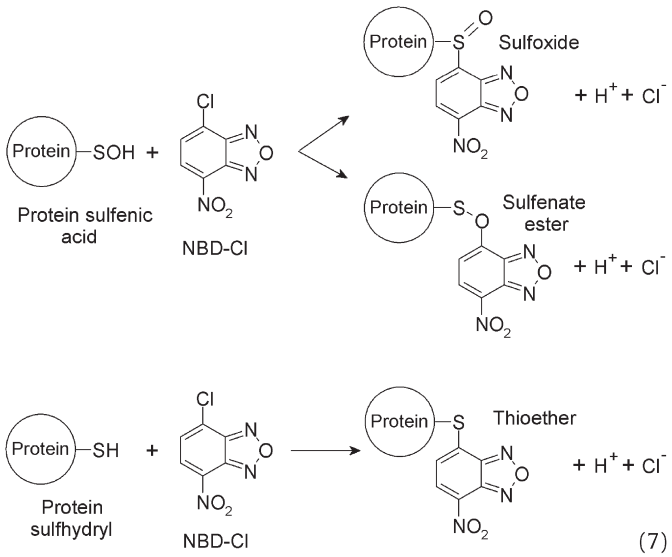


Iodometry has also been employed to quantify oxidants present in chlorinated water as HOCl and chloramines react with I^- to yield one molecule of I_2 per Cl^+, which can be quantified by titration or absorbance measurements (e.g., [93]). However, the rapid reaction of I_2 with proteins and other biomolecules (particularly lipids) makes this method less reliable for quantification of HOCl/HOBr/chloramines/bromamines) in complex systems [89]. Iodide has also been employed to catalyze chloramine-mediated oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) and dihydrorhodamine to chromophoric or fluorescent products [94]. This method can be used for some protein chloramines, though the rates of reaction are slow (ca. 30 min for BSA), which may result in an underestimation of yields due to fast competing side-reactions of these species. This method can distinguish between most chloramines and bromamines, as the *N*-bromo species react directly with TMB/dihydrorhodamine, whereas the *N*-chloro species require I^- as a catalyst [94].

N-Chloro formation on amino acids and peptides has also been investigated by LC/MS [95]. The utility of this method for protein-derived species remains to be established, but it is likely to be limited by the instability of protein chloramines and the potential requirement for digestion prior to LC/MS analysis.

Nonradical intermediates—Sulfenic acids

Sulfenic acids (RSOH) are reactive intermediates formed by two-electron oxidation of Cys (thiol) residues. These species are believed to play an important role in the catalytic and regulatory processes of some proteins and enzymes [96]. Sulfenic acids react readily with other thiols to give disulfides, with other sulfenic acids to give thiosulfinates and can be oxidized further to sulfinic (RSO_2H) or sulfonic acids (RSO_3H) [97]. These species lack characteristic UV-visible absorbances and fluorescence properties, and are unstable in the presence of acid. Quantification can be achieved by chemical derivatization or trapping methods. 5,5-Dimethyl-1,3-cyclohexanedione (dimedon) reacts in a highly specific manner with sulfenic acids to form stable thioether adducts [98], which can be quantified by MS using peptide mass-mapping techniques (e.g., [25]), or by proteomic analysis with modified dimedons that form fluorescent or biotinylated products (e.g., [99]). The electrophilic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) reacts with both sulfenic acids and thiols on proteins to give adduct species (Reaction **7**) that are distinguishable from each other by their UV-visible absorbances (λ_{max} 347 and 420 nm for Cys-S(O)-NBD and Cys-S-NBD, respectively [25,100]). This method has been employed to quantify both thiol loss and sulfenic acid formation on a range of proteins including peroxiredoxins, alkyl hydroperoxide reductase [99,100], tyrosine phosphatase [101], and HSA [25].



The extinction coefficient for the sulfenic acid-NBD product has not been determined, but has been assumed to be similar to that of the thiol product ($\varepsilon \approx 13{,}400$–$13{,}600$ M^{-1} cm^{-1}) [25,100,102]. This assumption does not appear to be completely valid, and can result in an overestimation of the sulfenic acid yield (M. Gracanin, unpublished data). Moreover, NBD-Cl can form noncovalent complexes with proteins which have chromophores that compromise the quantification of the sulfenic acid-NBD product [103]. Control experiments using proteins treated with a thiol-modifying/blocking reagents (e.g., *N*-ethylmaleimide or $HgCl_2$) can be used to clarify this point [103]. NBD-Cl also reacts with tyrosyl and amine residues under basic conditions; the resulting adducts have somewhat different UV-visible characteristics (λ_{max} 385 and 480 nm for tyrosyl and amines, respectively) [100], but the potential overlap of such absorptions with those from the thiyl and sulfenic acid adducts can complicate data analysis. Adequate pH control is therefore a necessity.

Sulfenic acid formation can also be assessed using TNB (e.g., [103]), although it is important that the TNB used for such assays is purified to prevent reaction of protein thiols with residual DTNB. This reaction is not specific for sulfenic acids (see above), but it may be useful in some situations. Oxidation of arsenite by sulfenic acids has been utilized to develop a biotin-switch method for labeling protein sulfenic acids [104]. In this assay, the sulfenic acids are specifically reduced by arsenite and subsequently labeled with biotin-maleimide. The labeled proteins are then visualized by Western blotting using streptavidin-horseradish peroxidase. The sulfenic acid-containing proteins can also be separated using streptavidin-agarose [104].

Nonradical intermediates—S-nitrosation

Oxidation of Cys residues by NO to give *S*-nitrosated (often misnamed as *S*-nitrosylated) products is believed to play an important role in cell signaling (reviewed in [105]). Protein *S*-nitrosation has also been implicated in multiple disease states, particularly those involving neurodegeneration and inflammation (reviewed in [106]). The chemistry of these reactions has yet to be fully elucidated, and widely varying basal levels of protein *S*-nitrosation have been reported (reviewed in [107]).

The biotin switch technique has been widely used to quantify protein *S*-nitrosation [108]. In this method, unmodified free thiols are first blocked (typically using *S*-methylmethanethiosulfonate, at 50 °C in the presence of SDS) and then following removal of excess alkylating reagent, ascorbate is added to selectively reduce any *S*-nitrosothiols (but not other modifications, including disulfides, *S*-glutathionylation, or sulfenic/sulfinic/sulfonic acids) to free thiols. The resultant reduced thiols are then labeled by *S*-biotinylation and detected by Western blotting following protein separation by 1D or 2D electrophoresis. Due to the complexity of this process and the use of Western blotting, the results are, at best, semiquantitative, and a number of shortcomings of this method have been reported. If the free thiols are incompletely blocked, false-positive signals result [109]; however, appropriate controls can minimize this effect. The ascorbate used to reduce the *S*-nitrosothiols (to the free thiol) has also been reported to accelerate the biotinylation reaction, thereby giving additional false-positives [110], as well as potentially reducing disulfide bonds, again resulting in erroneous data [111]. Inadvertent UV (or sunlight) exposure may also generate ascorbyl radicals that might reduce other modified thiols in addition to the expected *S*-nitrosothiols [112]. It has been noted that even if not all thiol sites are blocked, it is thermodynamically unfavorable for ascorbate itself to reduce sulfenic/sulfinic/sulfonic acids, unless the ascorbyl radical is formed. A detailed method for the biotin switch technique can be found in [113], and an adaptation of the original method, which uses a fluorescent labeling approach, has been reported, allowing comparison of the extent of *S*-nitrosation on 1D and 2D gels [114].

Formation of products

Detection and quantification of specific oxidation products

Modification of aromatic side chains can be used as a sensitive marker of protein oxidation, as these moieties are readily oxidized and often yield stable products that are readily quantified. Moreover, the nature of the products formed can be indicative of the species responsible for the modification. Thus, Tyr oxidation can yield 3,4-dihydroxyphenylalanine (DOPA), $3NO_2$-Tyr, 3Cl-Tyr, 3,5diCl-Tyr, 3Br-Tyr, 3,5diBr-Tyr, and the dimeric material, di-Tyr. Similarly, oxidation of Phe yields 2-hydroxytyrosine (*o*-Tyr) and 3-hydroxytyrosine (*m*-Tyr) (reviewed in [115]). The hydroxylated products can be formed by multiple mechanisms, including $HO^{\cdot}$ and peroxyl radical chemistry, peroxidase-mediated processes, and reactions mediated by HOCl and HOBr [115]. The majority of these materials are stable products. The exception is DOPA, which has a catechol structure that is readily

oxidized further to the quinone and cyclized products; these reactions can result in radical formation and damage to other biomolecules [116].

3Cl-Tyr, 3,5diCl-Tyr, 3Br-Tyr, and 3,5diBr-Tyr are generally regarded as specific markers of reactions mediated by the peroxidase-generated oxidants HOCl/chloramines and HOBr/bromamines, respectively. These materials have been employed extensively as markers of the involvement of myeloperoxidase/eosinophil peroxidase in disease (reviewed in [85,117,118]). Similarly, $3NO_2$-Tyr is commonly employed as a marker of the reactions of reactive nitrogen species generated from peroxynitrite, or peroxidase/H_2O_2/NO_2^- systems [119,120].

Three major methods have been developed to quantify these products: GC/MS, HPLC with various detectors, and immunological methods (Western blotting/ELISA). Protein-bound DOPA can also be detected (after separation of the proteins by SDS-PAGE) using a redox staining method [121]; this method has the advantage of allowing the detection of DOPA on intact proteins, but is only qualitative, due to the nonstoichiometric reaction of DOPA with the stain.

GC/MS, HPLC, and LC/MS techniques

Both the GC/MS and the HPLC methods require isolation and hydrolysis of the samples prior to product quantification (e.g., [7,115,122,123]). Most protocols use acid hydrolysis methods to achieve a high recovery of the parent amino acids and oxidized products; enzymatic methods (see earlier) are less quantitative in this respect. Studies have been carried out on tryptic digests of proteins, but care is needed; many of the problems associated with this approach are reviewed in [14]. Although a multiplicity of oxidation products can be formed on amino acids, most are not diagnostic of a particular oxidant, and a number are not stable enough to be analyzed (reviewed in [14,124]). For this reason Tyr oxidation products are some of the most commonly studied, and there are many reports on the quantification of these materials in biological systems using LC/MS (e.g., [7,122,123]).

Acid hydrolysis can generate potential artifacts as a result of the harsh conditions employed. For this reason, hydrolysis is typically performed under vacuum. Artifactual oxidation reactions may be further minimized by the addition of reductants (thioglycolic/mercaptoacetic acids) and/or phenols/tryptamine as sacrificial targets. Oxidation can also be minimized by using gas-phase rather than solution hydrolysis. The sensitivity of these methods is such that the use of HCl/HBr as the acid is unwise if quantification of chlorinated/brominated products is required, due to artifactual chlorination/bromination of Tyr. In this case, protein hydrolysis can be performed using MSA [7,123]. MSA hydrolysis can, however, result in high background signals in HPLC methods.

GC/MS can provide specific structural information, and has limited problems with coeluting peaks confounding accurate analysis, as can be the case with HPLC-based methods (reviewed in [7,122]). Moreover, these methods are highly sensitive and the run times are typically shorter than those of HPLC methods, allowing rapid analysis of multiple samples [125]. However, the preparation of samples is more complex than for HPLC, due to the need for multiple derivatization steps to make the materials of interest volatile, and the need for stable, isotopically labeled internal standards. The latter typically contain ^{13}C or ^{2}H isotopes, which can be costly and difficult to synthesize, although this approach can be a very convenient way of monitoring artifactual oxidation and sample loss during the extraction and sample preparation procedures.

The less complex sample handling required for most HPLC procedures lessens the risk of artifactual oxidation, and multiple products (together with the parent amino acids) can often be quantified in a single run with appropriate detectors (e.g., DOPA, di-Tyr, *o*-Tyr, *m*-Tyr, *p*-Tyr, 3Cl-Tyr, 3Br-Tyr, $3NO_2$-Tyr, 3,5diCl-Tyr, and 3,5diBr-Tyr), thereby minimizing intrarun variability. These HPLC methods have been used extensively to quantify protein oxidation products in a range of human tissues (reviewed in [115,126]). A detailed protocol is given in Method Sheet 10.

Oxidation of individual amino acids or small peptides can be detected without hydrolysis or enzymatic digestion, using GC/MS, or HPLC with various detectors. Such studies can provide data on the processes that may be occurring in more complex systems. MS analysis is particularly informative given the wealth of structural information that can be obtained from fragmentation patterns. As an example, direct LC/MS has been used to detect hydroperoxides formed on amino acids and peptides; these materials are too labile to survive acid, alkaline, or enzymatic digestion, thereby precluding the use of these approaches to detect these materials on intact proteins. The nature of the peroxides generated by γ-irradiation, or exposure to singlet oxygen, on a wide range of free amino acids and small peptides has been examined using this approach [81,127,128].

Another major advantage of MS approaches is the ability to screen for multiple products (if their masses are known or can be predicted) in single runs. Examples include the determination of the oxidation states of Cys and GSH in plasma and organ homogenates of Sprague-Dawley rats [129], Met oxidation on intact immunoglobulins [130], and multiple glycation and oxidation markers on free amino acids in plasma and urine [131]; in the last study enzymatic hydrolysis was also performed on intact proteins to give complementary data on free and protein-bound oxidation levels.

In some studies only the mass of the compound of interest has been monitored, which can result in potential artifacts, as molecules other than those of interest may have the same mass-to-charge ratio. For example, the addition of 32 mass units can be indicative of a hydroperoxide or two alcohols. This potential problem can be overcome with selective ion monitoring (SIM, e.g., [7,122,123,131]), whereby the ion of interest is fragmented by collision with an inert gas (e.g., He). Screening for (one or more) resulting fragments, particularly if they have been characterized previously for the oxidation product of interest, adds strength to the proposed assignment. This approach, although very powerful, will only detect known modifications that the system has been set up to examine; novel or unexpected materials can be easily missed. For this reason, databases of (electrospray ionization) fragmentation patterns of all amino acids and multiple oxidative modifications of these materials (e.g., DOPA, 4-hydroxyproline, MetSO, Met sulfone), in both positive and negative ion modes have been compiled [132]. Internet resources can also assist in interpretation of spectra (e.g., http://www.matrixscience.com/help/fragmentation_help.html). Once the masses of the products of interest are input, LC/MS^n can be a powerful tool to detect such modifications. Some guidelines for experiment planning are given in Method Sheet 11; a detailed protocol is not given as each system requires individual conditions.

Immunological methods

A number of antibodies, of varying degrees of specificity, have been developed for protein oxidation products; these theoretically allow the quantification of oxidant-derived damage by ELISA (reviewed in [133]). These methods are (at best) semiquantitative and do not yield absolute values, since different oxidized proteins may react with an antibody to lesser or greater extents than the standards to which they are being compared (if standards are available at all). Relative extents of modification are less problematic, but still suffer from some problems. Some commercial kits have undergone extensive validation, others much less so, and this information is not always easy to ascertain. Nonetheless, the determination of relative levels of oxidant damage by ELISA can still be a valuable tool.

ELISA methods have been developed to examine $3NO_2$-Tyr levels, and these have been employed to examine samples as diverse as

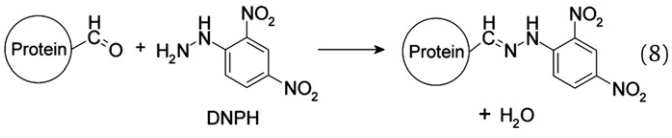
plasma from people with various inflammatory conditions compared to healthy controls [134], core proteins in proteoglycans in the cerebrospinal fluid of infants with hydrocephalus [135], and extracellular matrix treated with peroxynitrite/peroxynitrous acid [136]. The development of antibodies against 3Cl-Tyr, 3,5diCl-Tyr, 3Br-Tyr, and 3,5diBr-Tyr has been more problematic, and a number of commercial materials appear to have limited specificity or rather weak recognition. An antibody 2D10G9 (HOP-1) originally thought to be specific for HOCl-modified protein [137] is now known to also recognize HOBr-induced damage [138] and the exact epitope that this antibody recognizes remains to be determined. A similar situation exists with some commercial antibodies raised against advanced glycation end products; some clearly recognize multiple species (or even different products to those reported) as a result of the use of ill-defined materials as the original antigen.

As antibodies from different suppliers can have vastly different affinities for particular targets, considerable optimization is often necessary. Suggested dilutions can be a useful starting point, but appropriate dilutions still need to be determined by experiment. Control experiments should always be run to ensure that primary, secondary, or tertiary antibodies are not cross-reacting with other components of a system, and that blocking solutions and detection systems are not incompatible with each other (e.g., azide-containing solutions and horseradish peroxidase detection systems).

Generic markers of protein oxidation

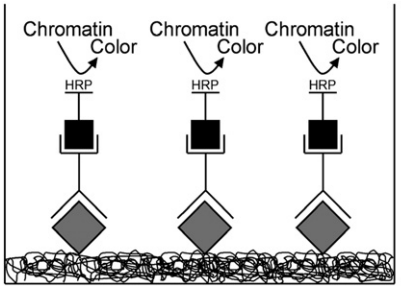
Protein carbonyls are a generic marker of protein oxidation that are generated by multiple radicals, excited state species, and singlet oxygen (as a result of secondary reactions). They can also arise from glycation/glycoxidation reactions, thus preventing the ready use of these products as a quantitative and exclusive marker of oxidation reactions in many human samples. Carbonyls can be formed on most amino acids (reviewed in [139]), though some are more prone to carbonyl formation than others; for example, metal-catalyzed protein oxidation results in the majority of carbonyls being formed on Arg, Pro, and Lys residues [140]. This is not, however, a universal phenomena and it has been shown that different oxidant systems give very different patterns of carbonyl formation, and that both free- and protein-bound carbonyls are generated on oxidation of proteins [141]. Protein-bound carbonyls have been shown to increase with age (reviewed in [142]), as well as in numerous diseases, including rheumatoid arthritis, adult respiratory syndrome pulmonary fibrosis, diabetes, Parkinson's disease, systemic lupus erythematosus, cystic fibrosis and Alzheimer's disease (reviewed in [142,143]).

Protein-bound carbonyl concentrations can be assessed via their reaction with 2,4-dinitrophenylhydrazine (DNPH) to give the corresponding hydrazone (Reaction **8**). This (yellow) product can be quantified spectrophotometrically at 370 nm [144]. While the absorbance values can be converted to absolute concentrations using published extinction coefficients, it should be noted that these vary with the material under study, so unless exact standards are used, errors are introduced in to the absolute values.



(8)

Protein carbonyl groups derivatized with DNPH can also be detected using antibodies to DNPH; these can be used in both ELISA [145] and Western blotting [146,147] experiments (Reaction **9**). Other methods to quantify include the reduction of carbonyl groups with tritiated borohydride, with the resulting radioactive incorporation quantified by radioactive counting [148]. This method needs to be used with caution, as it assumes that carbonyl groups are the only components that are reduced by borohydride to give radiolabel incorporation.



(9)

Detailed protocols are provided for the spectrophotometric determination of total protein carbonyl groups using DNPH (Method Sheet 12) and also for the use of a commercial kit (designed for ELISA) that has been adapted for use in determining the distribution of carbonyl groups on proteins separated on 2D gels. There are a variety of commercial ELISA kits (e.g., BioCell ALX-850-312-KI01, formerly manufactured by Zentech) available to quantify protein carbonyls (see [145,149]). Moreover, several commercial kits are also available for the detection of protein-bound carbonyls separated by 1D electrophoresis (e.g., Chemicon OxyBlot protein oxidation detection kit; Millipore S7150). Several studies have examined the distribution of carbonyls by 2D electrophoresis (e.g., [150,151]), which can be achieved by derivatizing the proteins with DNPH *after* isoelectric focusing [147]; the derivatization should not be carried out before isoelectric focusing as this can alter the isoelectric point of the proteins which hinders analysis [152].

In each of these cases removal of interfering substances can be critical to obtaining reproducible and accurate quantification. Protein precipitation is a useful technique; however, in some cases additional steps may be necessary, e.g., streptomycin treatment to remove nucleic acids [153]. Care should also be taken when using the ELISA method, as small molecules and DNA can bind to the ELISA plate, in addition to the desired protein(s). In contrast, during electrophoresis (1D or 2D) any nonprotein contaminants should be eliminated by the electrophoretic steps. Nonetheless, there are multiple cases where protein carbonyls cannot be quantified absolutely using these methods (e.g., samples from patients with diabetes, materials containing proteoglycans, or samples where adduction of carbonyl-containing lipids or sugars is likely to occur (reviewed in [154]). It should also be noted that these methods, which only measure protein-*bound* species, almost certainly underestimate the total yield of protein-*derived* carbonyls, as it has been shown that protein oxidation yields both protein-bound and low-molecular-mass, released, carbonyls [141,155].

Conclusions

There is a pressing need for reliable, robust methods for the absolute quantification of oxidation products formed from amino acids, peptides, and proteins, which can be applied to complex biological systems. While considerable progress has been made in the development of new techniques, it is clear that there is still much to be done. Only with the development of such methodologies will the relative importance of protein oxidation/modification become clear, when compared to other targets (e.g., to lipids, carbohydrates, and DNA), and whether protein alteration is a cause, or merely a consequence, of injurious processes.

Acknowledgments

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Appendix A. Method Sheet 1

Chelex-treated 100 mM sodium phosphate buffer, pH 7.4

Materials

1. Disodium hydrogen orthophosphate dodecahydrate (dibasic); Ajax Finechem; 478
2. Sodium dihydrogen orthophosphate monohydrate (monobasic); BDH; 10245
3. Chelex 100 resin (analytical grade); Bio-Rad; 142-2832
4. Whatman No.1 filter paper, 185 mm diameter; What-man Schleicher & Schuell; 1001185
5. Sintered glass funnel and side-arm conical flask capable of withstanding vacuum
6. Nanopure water

Protocol

The following protocol prepares approximately 500 mL of buffer. It can be scaled as required.

Chelex preparation

1. Using a plastic spoon, add approximately 25 g of Chelex resin to a sintered glass funnel.
2. Under vacuum, wash through approximately 250 mL of nanopure water. If a vacuum pump is not available, place the Chelex in filter paper, and wash with water.

Buffer preparation

1. Prepare a 100 mM solution of dibasic $Na_2HPO_412H_2O$ in nanopure water (12.53 g in 350 mL).
2. Prepare a 100 mM solution of monobasic $NaH_2PO_4H_2O$, in nanopure water (2.069 g in 150 mL).
3. Add the prewashed Chelex to each solution in proportion to its volume, and stir for 1 h.
4. Filter the Chelex from each solution—use either filter paper or a sintered glass funnel.
5. Place the pH probe in the dibasic solution. Add monobasic solution to this until the pH reaches 7.40 (most of the 150 mL is required).

Method Sheet 2

Amino acid analysis by HPLC with methanesulfonic acid hydrolysis and precolumn o-phthaldialdehyde derivatization

Materials

1. 4 M Methanesulfonic acid containing 0.2% w/v tryptamine (sealed ampoules under Ar); Sigma-Aldrich; M4141
2. *o*-Phthaldialdehyde (incomplete reagent); Sigma-Aldrich; P7914
3. 2-Mercaptoethanol; Fluka; 63689
4. Trichloroacetic acid (TCA); Sigma-Aldrich; T6399
5. Deoxycholic acid, sodium salt; Sigma-Aldrich; D6750
6. L-Homoarginine, hydrochloride salt; Fluka; 53460
7. Methionine sulfoxide (MetSO); Sigma-Aldrich; M1126
8. Amino acid standards (500 μM); Sigma-Aldrich; A9781
9. Sodium hydroxide, anhydrous; MP Biomedicals Inc.; 153495
10. Sodium acetate trihydrate; Fluka; 71190
11. Glacial acetic acid; Lab Scan Analytical Sciences; A8401
12. Methanol (HPLC grade); Mallinckrodt; 3041-68
13. Tetrahydrofuran (HPLC grade); Merck; 108107
14. Acetone (HPLC grade); Merck; 100020

Instrumentation

1. HPLC instrument with system controller (e.g., Shimadzu SCL-10Avp), binary pumps (e.g., Shimadzu LC-10ADvp) equipped with a gradient mixer, column oven able to maintain a temperature of 30 °C (e.g., Shimadzu CTO-10Avp), auto injector capable of performing precolumn derivatization equipped with a sample cooler (e.g., Shimadzu SIL-10A), and a fluorescence detector (e.g., Shimadzu RF-10AXL)
2. A microtube centrifuge (e.g., Eppendorf, 5415R)
3. PicoTag reaction vials (No. 3002EL, Alltech, Baulkham Hills, NSW, Australia)
4. Vacuum pump
5. Oven capable of maintaining a temperature of 110 °C
6. Diamond tip pen or engraver.

Protocol

Sample preparation for protein hydrolysis

1. Samples, typically with a final volume of 200 μL, are placed in a glass vial (8 × 40 mm, 1 mL, No. 98212, Alltech) labeled by etching with a diamond tipped pen or engraver.
2. Proteins (<0.5 mg in 200 μL) are delipidated and precipitated by the addition of 25 μL 0.3% (w/v) deoxycholic acid and 50 μL of 50% (w/v) TCA, with incubation on ice for 5 min.
3. Glass vials containing samples are placed in 1.5 mL centrifuge tubes (with caps removed) for 2 min at 9000 rpm at 5 °C (Eppendorf 5415R centrifuge) to pellet protein. Higher spin speeds may result in shattering of the glass vials.
4. Protein pellets are washed once with 5% (w/v) TCA, and twice with ice-cold acetone (stored in −20 °C freezer) with 2 min, 9000 rpm, spins between washes in each case to settle pellets. Pellets are dried by exposing to a gentle stream of N_2 to remove residual acetone.
5. Samples are resuspended in 150 μL of 4 M MSA containing 0.2% w/v tryptamine, before the addition of 5 μL of homo-Arg (10 mM) as an internal standard.
6. Transfer sample vials to PicoTag hydrolysis vessels using forceps. A PicoTag vessel typically holds 7 or 8 × 1 mL sample vials.
7. Evacuate PicoTag vessels using a vacuum pump, and regas with N_2 (back-flushing) at least 3 times to ensure all O_2 is removed. Place under vacuum one last time before placing PicoTag vessel containing samples in the oven at 110 °C.
8. Leave overnight, for 16–18 h. For greater sample reproducibility, keep the time constant.
9. Remove PicoTag vessels from oven and allow to cool before releasing vacuum. Remove vials using forceps, and rinse the outside of each vial with acetone to remove any acidic residue.
10. Samples are neutralized by the addition of 150 μL freshly prepared 4 M NaOH (0.8 g NaOH in 5 mL H_2O).
11. Transfer samples to centrifugal filtration devices (containing a PVDF 0.22 μm membrane, 0.5 mL volume, No. UFC30GVNB, Millipore), and centrifuge at 10,000 rpm for 2 min at 5 °C (Eppendorf 5415R centrifuge) to remove any insoluble precipitate. This step is very important to prevent column/HPLC blockages.
12. Dilute samples into water (typically 10- to 100-fold, depending on initial protein concentration) before transferring 40 μL to HPLC vials (12 × 32 mm clear screw-top vials with 8/425 thread, No. 32008-1232, Alltech, with polypropylene 8/425 thread vial caps, No. 24765, Supelco, containing red PTFE/silicone liners, No. 5128842, Alltech) containing 0.2 mL inserts (No. 200-228, Edwards). Samples are placed in the HPLC auto injector, and kept at 5 °C prior to derivatization and injection.

Preparation of OPA and amino acid standards

1. Incomplete OPA reagent (Sigma-Aldrich, P7914) should be opened and stored under N_2 or Ar to prolong the lifetime of the reagent. This is activated immediately before use by addition of 5 μl of 2-mercaptoethanol to 1 mL of OPA reagent in a HPLC vial (see above, no insert). The vial containing OPA should be placed in the auto injector in the position specified in the derivatization method.
2. In the derivatization method, 20 μL of activated OPA reagent is added per sample; ensure that sufficient OPA is prepared for all samples.
3. A solution of 5 μM standards is prepared by addition of 10 μL Sigma-Aldrich amino acid standards (A9781, 500 μM stock), 5 μL MetSO (1 mM stock), and 5 μL homo-Arg (1 mM stock) to 980 μL H_2O.
4. Prepare a series of dilutions of this standard solution (5 μM) to give 1, 2, 3, 4, and 5 μM standards. Transfer 40 μL of each standard to HPLC vials containing 0.2 mL inserts and place in the auto injector and kept at 5 °C as above. Standards should be prepared fresh for each experiment.

Preparation of HPLC mobile phase

1. Prepare a 1 M stock solution of sodium acetate trihydrate. Add 136.08 g of this compound to 900 mL of H_2O, then adjust the pH to 5.0 with glacial acetic acid (typically 29 mL) before addition of H_2O to a final volume of 1 L. NOTE: the pH of this solution is critical to the separation and retention times of the amino acids. Small variations across the pH range 5.0–5.6 have a dramatic effect on the elution order and separation.
2. Buffer A contains 400 mL methanol, 50 mL tetrahydrofuran, 1450 mL H_2O, and 100 mL of 1 M sodium acetate, pH 5.0 (to give 50 mM final).
3. Buffer B contains 1600 mL methanol, 50 mL tetrahydrofuran, 250 mL H_2O, and 100 mL of 1 M sodium acetate, pH 5.0 (to give 50 mM final).
4. Both buffer A and buffer B should be filtered through 0.2 μm membrane filters (e.g., VacuCap 90 filter unit with 0.2 μm Supor membrane, No. 4622, Pall Corporation), and degassed prior to running HPLC analysis.

HPLC precolumn derivatization method

1. The auto injector should be programmed to add 20 μL activated OPA reagent to the specified sample (40 μL), followed by 3 mixing cycles, and a 1 min incubation period. After the incubation step, 15 μL of the final reaction mixture should be injected.
2. For the standards (1, 2, 3, 4, and 5 μM) this gives 10, 20, 30, 40, and 50 pmol of each amino acid, respectively.

HPLC conditions and gradient

1. This method is optimized for separation of the amino acids on a Beckman Coulter Ultrasphere ODS, 4.6 mm × 25 cm, 5 μm pore size HPLC column (No. 235329), fitted with a Beckman Coulter Ultrasphere ODS, 4.6 mm × 4.5 cm (No. 243533) guard column.
2. Use a flow rate of 1 mL min^{-1}, with the column oven set at 30 °C.
3. Fluorescence detector should be set with λ_{EX} 340 nm, λ_{EM} 440 nm.
4. The amino acid derivatives are separated using the gradient shown below.

Time (min)	% Buffer B
0.1	5
7	5
17	25
27	50
35	50
40	100
45	100
46	5
54	5

Calculations and expected results

The concentration of each amino acid, homo-Arg, and MetSO in the unknown samples is determined from linear plots of the HPLC peak area vs concentration from the standards. Any variation in derivatization efficiency (usually minimal within a particular HPLC run) can be taken into account by expressing the results as a ratio with the internal standard homo-Arg. Any variation in the efficiency of hydrolysis or sample recovery after the precipitation and washing steps can be taken into account by expressing the concentration of the amino acids of interest (or MetSO) as a ratio with an amino acid that is not modified by the particular oxidant treatment.

Notes/caveats

1. The hydrolysis procedure above has been optimized for <0.5 mg protein per sample. Under these conditions the recovery of amino acids after hydrolysis is typically 80–90% with isolated proteins. If more protein is used, the amount of MSA added may need to be adjusted.
2. The Met/MetSO ratio is sensitive to MSA/tryptamine solution used. ACS reagent grade MSA (4 M) with tryptamine (0.2% w/v) added immediately prior to hydrolysis results in some reduction (ca. 10–20%) of any MetSO present back to Met [3,31]. This loss of MetSO can be minimized by use of high-purity MSA/tryptamine solution supplied in sealed ampoules (Sigma-Aldrich, No. M4141). The presence of reductants (e.g., $NaBH_4$, *N*-acetyl-Cys, DTT) in the reaction mixture can also affect Met/MetSO ratio, with a loss of MetSO observed in the presence of the thiol-containing reagents *N*-acetyl-Cys and DTT (C.L. Hawkins, unpublished data). This reaction is minimized by protein precipitation, and careful washing, but it is wise to avoid these reagents if possible during sample preparation.

Method Sheet 3

Quantification of protein Lys residues by fluorescence using fluorescamine

Materials

1. Fluorescamine, Sigma-Aldrich; F9015
2. Acetone (HPLC grade); Merck; 100020
3. Disodium tetraborate decahydrate ($Na_2B_4O_7{\cdot}10\ H_2O$); Merck; 1.06308
4. Boric acid (H_3BO_3); Sigma-Aldrich; B7660
5. 6-Aminocaproic acid [$H_2N(CH_2)_5CO_2H$], Sigma-Aldrich; A2504
6. Nitric acid (HNO_3); BDH; 10168-7F

Instrumentation

1. Fluorimeter (e.g., Perkin Elmer LS50B)
2. Vortex mixer
3. Multipipettor capable of dispensing 250 μL volumes (e.g., Eppendorf Multipette Plus)

Protocol

This method has been adapted from [11].

1. Prepare 0.1 M borate buffer, pH 9, as follows:
   a. Prepare 50 mL of 0.1 M boric acid (0.618 g in 50 mL of water).
   b. Prepare 100 mL of 0.025 M sodium tetraborate (0.954 g in 50 mL of water).
   c. Add (b) to (a) until the pH reaches 9.
2. Add 50 μL of protein sample (typically 0.1–0.5 mg mL^{-1}) to 750 μL of 0.1 M borate buffer, pH 9, in a glass test tube (e.g., Kimex disposable test/culture tubes made from borosilicate glass, No. 41098C).
3. If required for quantification, a standard curve may be prepared using 50 μL of 6-aminocaproic acid [$H_2N(CH_2)_5CO_2H$], 0–250 μM (see below).

4. 250 μL Fluorescamine solution (15 mg in 50 mL acetone) is added while vortex-mixing using a multipipettor (e.g., Eppendorf Multipette Plus No. 4980000.015, used with 10 mL Combitips Plus, No. 0030069.269). A multipipettor improves reproducibility due to the difficulty in accurately pipetting acetone with a standard pipette. Rapid mixing is essential for reproducible results due to rapid hydrolysis of the fluorescamine [11].
5. Samples are incubated at room temperature (21 °C) for 10 min.
6. The fluorescence of the samples is recorded in a fluorimeter (e.g., Perkin Elmer LS50B) at λ_{EX} 390 nm and λ_{EM} 475 nm.

Samples are contained in 1 mL quartz suprasil fluorescence cuvettes (Hellma 104F-QS with 10 mm path length) during fluorescence measurements. The cuvettes are cleaned prior to use by soaking in 6 M nitric acid for at least 30 min.

Calculations and expected results

The background fluorescence of the fluorescamine solution in pH 9 borate buffer should be very low (<10 fluorescence units on a Perkin Elmer LS50B). Results are generally expressed as a percentage of the fluorescence observed with nontreated control samples, as the relative fluorescence obtained in experiments with different proteins on adjustment to the molar equivalents of free amino groups present varies [11]. In general, the concentration of free amine groups on proteins correlates reasonably well with the standard curve obtained with 6-aminocaproic acid and related analogues of the Lys side chain [11].

Notes/caveats

The products formed from reaction of fluorescamine with Lys appear to change slowly over time, so measurements need to be taken after a fixed incubation time [11].

Method Sheet 4

Quantification of protein Arg residues by fluorescence using 9,10-phenanthrenequinone (PTQ)

Materials

1. 9,10-Phenanthrenequinone (*o*-phenanthroline monohydrate, PTQ); Fluka; 77500
2. L-Arginine; Sigma-Aldrich; A8094
3. Sodium hydroxide, anhydrous (NaOH); MP Biomedicals Inc.; 153495
4. Hydrochloric acid (HCl, 32% w/v); Chem Supply Pty; HA020
5. Ethanol (absolute); Labserve BSPEL9765

Instrumentation

1. Fluorimeter (e.g., Perkin Elmer LS50B)
2. Vortex mixer
3. Water bath or oven capable of maintaining a temperature of 60 °C

Protocol

This method has been adapted from [12].

1. Add 50 μL of protein sample (typically 0.5 mg mL^{-1}) or Arg standard (0–100 μM) to 100 μL of 1 M NaOH in a 1.5 mL centrifuge tube.
2. Add 300 μL of PTQ (120 μM in ethanol) to each sample standard using a multipipettor (e.g., Eppendorf Multipette Plus No. 4980000.015, used with 10 mL Combitips Plus, No. 0030069.269), followed by capping the tubes and vortex mixing. A multipipettor improves reproducibility due to the difficulty in accurately pipetting ethanol with a standard pipette.
3. Samples are incubated at 60 °C for 1 h. This time is dependent on the size/structure of the protein, and it may be necessary to increase the incubation time for large, globular proteins to ensure maximum fluorescence and complete reaction of the protein Arg residues with PTQ.
4. The reaction is quenched by the addition of 450 μL 1.2 M HCl before reading the fluorescence of the samples in a fluorimeter (e.g., Perkin Elmer LS50B) at λ_{EX} 312 nm and λ_{EM} 392 nm.
5. Samples are contained in 1 mL quartz suprasil fluorescence cuvettes (Hellma 104F-QS with 10 mm path length) during fluorescence measurements. The cuvettes are cleaned prior to use to remove traces of protein and detergent by soaking in 6 M nitric acid for at least 30 min.

Calculations and expected results

As the fluorescence from protein-bound Arg residues is comparable to that from free Arg [12], the concentration of protein residues is determined from a standard curve prepared with the free amino acid.

Notes/caveats

1. The derivatization reaction is generally slow, even at elevated temperatures. Thus maximum fluorescence is obtained with Arg residues on BSA after >3 h at 60 °C [12].
2. Care must be taken with PTQ to use only a small excess compared to the number of Arg residues to avoid unreacted reagent causing interference in the fluorescence measurements and a nonlinear response [12,156].

Method Sheet 5

Quantification of thiols using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB assay using Ellman's reagent)

Two methods are described: (a) a cuvette-based method for various protein-containing solutions and (b) a 96-well plate method optimised for serum/plasma. The cuvette-based method, with its defined path length, uses the extinction coefficient to quantify the released 5-thio-2-nitrobenzoic acid directly. In contrast, the 96-well plate method, which is ideal for small sample volumes, requires a standard curve owing to a nonstandard path length. The latter method can also be easily adapted to other protein-containing solutions, but it is important to ensure that the color change is complete at the end of the incubation. For cell lysates, longer incubation times may need to be employed (e.g., 60 min at 37 °C in the dark) [40].

Cuvette method

Materials

1. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB; MW = 396.35); Sigma-Aldrich; D8130
2. 100 mM sodium phosphate buffer, pH 7.4
3. Nanopure water
4. 50 mL volumetric flask
5. 0.5–2.0 mL disposable plastic cuvettes (1 cm path length); Greiner Bio-One; 613101
6. 1.5 mL centrifuge tubes

Instrumentation

1. Accurate balance (capable of 0.0001 g differences in mass)
2. Vortex mixer
3. Any dual-beam spectrophotometer capable of measuring absorbance at 412 nm.

Protocol for determination of thiols

1. Prepare 0.5 mM DTNB in 100 mM phosphate buffer, pH 7.4 (0.0099 g in a 50 mL volumetric flask). Make fresh each day.
2. Dilute protein samples to <1.5 mM with respect to the expected free thiol concentration. If unsure, prepare several dilutions.

3. Dispense 50 μL of each diluted protein solution into six 1.5 mL Eppendorf centrifuge tubes.
4. To three of the tubes, add 1 mL of DTNB solution.
5. To the other three tubes, add 1 mL of 100 mM phosphate buffer, pH 7.4.
6. Prepare in triplicate a DTNB blank, composed of 50 μL of water/buffer (the same solution as that used to dissolve the protein) plus 1 mL of DTNB solution.
7. Vortex all solutions, then incubate in the dark for 30 min at room temperature.
8. Measure absorbance at 412 nm for each solution. Use a new cuvette for each dilution.

Calculations and expected results

1. Calculate the average absorbance at 412 nm of the DTNB alone blank.
2. For each sample calculate the average absorbance at 412 nm of:
3. Protein + DTNB
4. Protein + phosphate buffer
5. Determine the thiol-derived change in absorbance as follows: Abs change = A_{412} (protein + DTNB) – A_{412} (protein + phosphate buffer) – A_{412} (DTNB alone blank)
6. Determine the thiol concentration by dividing the above absorbance change by the extinction coefficient at 412 nM (13,600 M^{-1} cm^{-1}).
7. Multiply by 21 to give the thiol concentration (in moles per liter) of the original solution. Remember to also allow for any additional protein dilutions that were necessary to give thiol concentrations in the appropriate range.

Notes/caveats

1. Protein solutions must be free of suspended matter. If turbidity is present, samples should be filtered or centrifuged prior to use.
2. If a DTNB solution is used over a number of hours, it is important to determine the absorbance of the DTNB alone blank at regular intervals, to compensate for any time-dependent changes in blank absorbance.
3. The pH of the reaction mixture strongly influences its rate of reaction with thiols; thus it is important to ensure that the final pH is 7–7.4 [33].
4. The presence of excess oxidants in the reaction mixture can lead to confounding results with this assay, resulting in an underestimation of thiol concentration due to the oxidation of TNB. This problem can be avoided by removing the oxidant by chemical quenching (e.g., addition of excess Met in experiments with HOCl), enzyme treatment (e.g., catalase), or gel filtration.

96-well plate method

Materials

1. Reduced glutathione (GSH; MW = 307.3); Sigma-Aldrich; G4251
2. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB; MW = 396.35); Sigma-Aldrich; D8130
3. 100 mM sodium phosphate buffer, pH 7.4
4. Nanopure water
5. 50 mL volumetric flasks
6. Clear, flat-bottomed 96-well plates (e.g., Corning Costar 3599)

Instrumentation

1. Accurate balance (capable of 0.0001 g differences in mass)
2. Plate shaker
3. 96-well plate reader capable of measuring absorbance at 412 nm.

Protocol for determination of human serum/plasma thiols

1. Prepare the following solutions fresh for each assay:
   a. 0.5 mM GSH in water (0.0077 g in a 50 mL volumetric flask)
   b. 0.5 mM DTNB in 100 mM phosphate buffer, pH 7.4 (0.0099 g in a 50 mL volumetric flask)
   c. Serum/plasma diluted 1:1 with water (e.g., 50 μL + 50 μL). Serum/plasma should ideally be fresh or thawed immediately prior to use.
2. Prepare 0.1, 0.2, 0.3, and 0.4 mM solutions from the 0.5 mM GSH stock, diluting with water.
3. In a 96-well plate prepare the following to a total volume of 10 μL (all in triplicate):
   a. 0–0.5 mM GSH standards (0 mM = 10 μL of water)
   b. Two different dilutions of serum/plasma:
      i. 5 μL of diluted serum/plasma (from 1 (c) above) + 5 μL water (total dilution 1 in 4)
      ii. 10 μL of diluted serum/plasma (from 1 (c) above; total dilution 1 in 2).
   c. A second set of the same dilutions of serum/plasma (needed for blanks).
   d. Ensure that some wells are left empty, as the absorbance of the plastic plate must be taken into account in the measurements.
4. To the GSH standards and the first set of dilutions of serum/plasma, add 200 μL of 0.5 mM DTNB solution using a multichannel pipette.
5. To the second set of dilutions of serum/plasma, add 200 μL of 100 mM phosphate buffer.
6. Mix well on a plate shaker.
7. Incubate in the dark for 30 min at room temperature.
8. Measure absorbance at 412 nm using a 96-well plate reader.

Calculations and expected results

1. Determine the average the absorbance of the empty wells on the plate.
2. For each sample dilution:
   a. Subtract the absorbance of the samples with phosphate buffer added (no DTNB) from the absorbance of the samples with DTNB. This accounts for any non-DTNB-derived absorbance at 412 nm (serum/plasma absorbs at this wavelength).
   b. Add the average absorbance of the empty wells. This is necessary as the GSH standards include the absorbance of the plate, but part (a) eliminated this absorbance.
3. Fit a straight line to the absorbance of the GSH standards (R^2 values of >0.995 should be routinely achieved with accurate pipetting).
4. Convert thiol concentrations to absolute values using the GSH standards. Serum/plasma from healthy humans typically contains 400–600 μM thiols [33]).

Notes/caveats

1. It is important to ensure that the GSH used for the standard curve is not oxidized. This can be checked in the following manner:
   a. Take 50 μL of the 0.5 mM GSH solution.
   b. Add 950 μL of the 0.5 mM DTNB solution, and mix well. This dilution gives a solution that is theoretically 25 μM with respect to GSH.
   c. Incubate in the dark for 30 min at room temperature.
   d. Measure absorbance at 412 nm using a dual-beam spectrophotometer (zero with 50 μL of water + 950 μL of 0.5 mM DTNB solution as the sample, with water as the reference).
   e. Using the molar extinction coefficient at 412 nm of 13,600 M^{-1} cm^{-1}, determine the concentration of the GSH. With a 1 cm path length cuvette, the absorbance of 25 μM GSH should be 0.34.
2. Protein solutions must be free of suspended matter. If turbidity is present, samples should be filtered or centrifuged prior to use.
3. The DTNB solution will slowly turn yellow with time. Thus it is important to run standards on every plate, in order to compensate for any changes in DTNB absorbance with time.
4. Ensure that there are no bubbles in the wells before determining the absorbance, as these will perturb the results. Any bubbles

should be popped using a needle before determining the absorbance. Dilution of serum/plasma largely eliminates this problem.
5. Thiol concentrations can be expressed as nmol/mg of protein, the protein concentration being determined by a suitable protein assay (e.g., BCA assay). In healthy people, where there is little change in protein concentrations, μM concentrations are usually acceptable.
6. Again, care must be taken to ensure that no residual oxidizing species are present to potentially react with the TNB generated in the assay.
7. This assay can be readily adapted to other systems. To do this, prepare a series of protein dilutions, and determine the protein thiol concentrations by reference to the GSH standard curve. Use a protein concentration that gives an absorbance at 412 nm in the midregion of the standard curve. Controls should be run to check for background absorbance at 412 nm. If there is no background absorbance in the absence of DTNB, this step can be omitted.

Method Sheet 6

Quantification of thiols using ThioGlo 1

Materials
1. ThioGlo 1 fluorescent thiol reagent (FW = 379.3); Calbiochem; 595501
2. Acetonitrile (HPLC grade); JT Baker; 9017-03
3. Phosphate-buffered saline (PBS), 20X concentrate, pH 7.5; Amresco; E703
4. Clear, flat bottomed 96-well plates; Corning Costar; 3599

Instrumentation
1. Accurate balance (capable of 0.0001 g differences in mass)
2. Plate shaker
3. Fluorescent plate reader capable of measuring in the region of λ_{EX} 384 nm, λ_{EM} 513 nm

Protocol for the determination of cell lysate thiols
1. Prepare an anhydrous 2.6 mM stock solution of ThioGlo 1 in acetonitrile (5 mg in 5.07 mL). This solution is stable in the dark (wrap in foil) at 4 °C for prolonged periods.
2. Each time the assay is performed, prepare a fresh 5 μM solution of GSH in water in 2 steps:
   a. 0.5 mM GSH in water (0.0077 g in a 50 mL volumetric flask)
   b. Dilute (a) 1:100 (500 μL in 50 mL water, use a volumetric flask) to give a 5 μM solution.
3. Use the 5 μM GSH solution to prepare a series of standards from 0 to 5 μM in 1 μM steps in a 96-well plate in triplicate. Make up to 50 μL with water (0 μM = 50 μL of water).
4. Transfer 50 μL of cell lysates (lysed at 1 or 2×10^5 cells/mL in water) for each condition to wells of a 96-well plate (in triplicate).
5. Add 50 μL of the freshly diluted ThioGlo 1 reagent in 1X PBS to standards and samples
6. Mix briefly using a plate shaker.
7. Incubate in the dark for 5 min at room temperature.
8. Measure λ_{EX} 384 nm, λ_{EM} 513 nm using a 96-well fluorescence plate reader.

Calculations and expected results
1. Fit a straight line or second order polynomial to the absorbance of the GSH standards (R^2 values of >0.995 should be routinely achieved with accurate pipetting).
2. If a sample gives a fluorescence reading of below 0.5 μM rerun the sample with a smaller dilution, as the quality of the standard curve deteriorates at low concentrations.
3. Convert thiol concentrations to absolute values by reference to the GSH standards.
4. Thiol concentrations in human coronary artery endothelial cell lysates are typically 1–2 μM when lysed at 1×10^5 cells/mL using cells plated down overnight at 2×10^5 cells/mL.

Notes/caveats
1. This assay can be easily adapted for other cells as long as the cells are appropriately diluted to give a fluorescence reading within the range of the standard curve.
2. ThioGlo 1 can bind in a nonspecific manner to some proteins (e.g., albumin in serum/plasma) resulting in significant fluorescence quenching and hence inaccurate data (F.A. Summers, unpublished data).
3. ThioGlo 1, being a maleimide derivative, can also react with other nucleophiles on proteins (e.g., terminal amino and ε-amino groups at pH>8); this reaction is, however, slower than with thiols, so is generally not significant, as long as the pH is controlled and the incubation time is short.

Method Sheet 7

Quantification of thiols on proteins separated by 1D gel electrophoresis using IAF fluorescence

Materials for serum/plasma and cell lysate methods
1. 5-Iodoacetamidofluorescein (IAF, MW = 515.26); Invitrogen/Molecular Probes; I-30451
2. Dimethyl sulfoxide (DMSO), ≥99.9%; Sigma-Aldrich; 154938
3. Glycerol (Biotechnology grade); Amresco; 0854
4. 0.5 M Tris-HCl, pH 6.8; Amresco; J832
5. Sodium dodecyl sulfate (SDS, for electrophoresis, approx 99%); Sigma-Aldrich; L3771
6. Bromophenol blue; ICN; 193990
7. Iodoacetamide (MW = 184.96); Sigma-Aldrich; I1149
8. 100 mM sodium phosphate buffer, pH 7.4
9. Broad range SDS-PAGE standards; Bio-Rad; 161-0317
10. Nanopure water
11. Materials for the pouring of 14% or 8–16% gradient SDS-PAGE gels
12. 1.5 mL centrifuge tubes

Additional materials for cell methods only
1. Clear, flat-bottomed 24-well plates (e.g., Corning Costar 3524)
2. Clear, flat-bottomed 96-well plates (e.g., Corning Costar 3599)
3. HBSS without calcium chloride, magnesium sulfate, phenol red, or sodium bicarbonate; Sigma-Aldrich; H4891
4. Sodium bicarbonate (≥99.5%, cell culture tested); Sigma-Aldrich; S5761
5. Hepes (sodium salt, >99.5%, MW = 260.29); Sigma-Aldrich; H7006
6. Triton X-100; Sigma-Aldrich; T9284
7. Complete Mini protease inhibitor tablets; Roche; 11 836 153 001
8. 1 mL syringe; Terumo; DVR-5175
9. Bio-Rad protein assay reagent with BSA standard; Bio-Rad; 500-0002
10. Trichloroacetic acid (TCA); Sigma-Aldrich; T6399
11. 1.5 mL centrifuge tubes
12. Acetone (HPLC grade, stored at -20 °C); Merck; 10020

Instrumentation
1. Vortex mixer
2. Refrigerated 1.5 mL tube microcentrifuge (4 °C); Eppendorf 5415R
3. Rotary shaker; Ratek OM5
4. Heating block capable of heating to 95 °C; Ratek DBH30
5. Electrophoresis equipment: Bio-Rad Protean II xi cell, 16×20 cm gels
6. Power pack capable of delivering 100 V constant voltage; Bio-Rad;164-5050

7. Flatbed scanner capable of transparency scanning (e.g., Umax PowerLook 1120 UDS scanner)
8. Fluorescence scanner capable of λ_{EX} 488 nm, λ_{EM} 530 nm (e.g., Bio-Rad Pharos FX Plus Molecular Imager)

Protocol

IAF preparation (both methods)

1. Prepare a 10 mM stock solution of IAF, by dissolving 25 mg in 4.85 mL of DMSO.
2. Store at -80 °C in small aliquots (e.g., 20 μL), protected from light at all times.
3. Dilute IAF immediately before use (it is more stable in DMSO than aqueous solution), and discard any unused IAF after thawing.

5X Gel loading buffer (both methods)

1. Thoroughly mix the following: 12.5 mL 0.5 M Tris (pH 6.8) with 2.0 mL 20% (w/v) aqueous SDS, 0.5 mL saturated aqueous bromophenol blue solution, 4.0 mL glycerol, and 1.0 mL water. Store at room temperature.
2. Just before use, add 25 μL of 2-mercaptoethanol to 475 μL of the above, and mix well.

50 mM Hepes + 0.15% (v/v) Triton X-100 (cell lysate method only)

1. Dissolve 1.30 g of Hepes sodium salt approx 95 mL of water, and adjust pH to 7.4 with NaOH.
2. Add 150 μL of Triton X-100.
3. Make up to 100 mL with water, and mix well. This solution can be kept at room temperature until required.

Complete protease inhibitors (cell lysate method only)

1. Prepare a 7X stock by dissolving one mini tablet in 1.5 mL of water.
2. Divide into aliquots, and freeze at -20 °C until required.

80% (v/v) acetone (cell lysate method only)

1. Mix 80 mL of acetone with 20 mL of water
2. Store in a 100 mL Schott bottle at -20 °C (required at this temperature)

Serum/plasma treatment protocol

1. Dilute the 10 mM IAF stock to 200 μM immediately before use with phosphate buffer (e.g., 20 μL of 10 mM IAF + 980 μL of buffer)
2. Dilute plasma samples to 1:25 of their original concentration with phosphate buffer. Remember to take into account any dilutions arising from treatments (e.g., with oxidants).
3. Mix 20 μL of 200 μM IAF solution with 20 μL of diluted plasma sample. This gives an ca. 10-fold excess of IAF over healthy plasma thiol concentrations (400–600 μM when undiluted [33])
4. Incubate for 15 min at room temperature in the dark with shaking (plate shaker).
5. Add 8 μL of freshly prepared 6 mM iodoacetamide, (1.1 mg/mL in phosphate buffer) to each sample, and vortex. Keep in the dark, as iodoacetamide is light sensitive. This minimizes nonspecific binding of IAF to HSA in serum/plasma.
6. Add 12 μL of gel loading buffer to each sample, and vortex briefly.
7. Heat at 95 °C for 5 min.
8. Place samples on ice to cool then centrifuge briefly to spin down the condensation on the lids of the tubes.
9. Load 40 μL of sample per well of an 8–16% gradient or 14% homogenous SDS-PAGE gel.
10. Molecular weight markers should also be run on each gel. To prepare these:
    a. Add 1 μL of standards to 40 μL of gel loading buffer.
    b. Heat at 95 °C for 5 min.
    c. Allow to cool, and centrifuge to spin down condensation.
    d. Load 10 μL.
11. Separate proteins overnight at 100 V in the dark (cover apparatus in aluminium foil, or with a cardboard box). Run until blue dye reaches the bottom of the gel.

Cell lysate treatment protocol

1. Plate cells down in 24-well plates at 0.2×10^6 cells/mL (0.5 mL/well), and allow to adhere overnight.
2. Expose cells to oxidants/treatment. Ensure that at the end of any treatment there is no unreacted oxidant remaining. Thus with chloramine treatment of cells, excess methionine is added to quench unreacted chloramines [48].
3. Gently wash cells with 0.5 mL of HBSS without calcium, magnesium, or phenol red.
4. Just before use, prepare lysis/labeling buffer containing 35 mM Hepes, 0.1% (v/v) Triton X-100, 1X Complete protease inhibitors, and 80 μM IAF. For 5 mL of this buffer (> 3 mL is required per 24-well plate) mix the following:
    a. 50 mM Hepes + 0.15% (v/v) Triton X-100: 3.5 mL
    b. 7X stock Complete protease inhibitors: 715 μL
    c. 10 mM IAF: 40 μL
    d. Water: 745 μL
5. Add 125 μL of lysis/labeling buffer to each well.
6. Lyse the cells fully using the plunger of a 1 mL syringe to detach the cells from the plate. Use a different plunger for each condition, and wash the plunger with water between wells.
7. Incubate the plate for 30 min with shaking (rotary shaker) at room temperature in the dark.
8. Remove an aliquot of lysate solution (10 μL) and determine the protein concentration using the Bio-Rad protein assay with BSA standards according to the manufacturer's specifications.
9. Remove the cell lysate solutions from the 24-well plate, and place in 1.5 mL centrifuge tubes
10. Precipitate the proteins by adding 32 μL of 50% (w/v) TCA to each tube (final TCA concentration = 10%). Mix well.
11. Incubate the tubes on ice for 15 min in the dark.
12. Centrifuge the tubes at 4 °C for 15 min at 6000*g*
13. Remove the supernatant, being careful not to disturb the protein pellet
14. Add 200 μL of 80% (v/v) acetone (stored at -20 °C) to each tube, and vortex to break up the protein pellet.
15. Freeze at -20 °C for at least 1 h, before centrifuging at 4 °C for 15 min at 16,000 *g*.
16. Carefully remove the supernatant, and allow the pellet to air-dry to remove any acetone. Do not allow the pellets to dry out completely as these then become difficult to redissolve.
17. Dilute the gel loading buffer to one-fifth of its original concentration with 0.5 M Tris, pH 6.8, and add sufficient diluted buffer to each sample to give 40 μg of protein in 40 μL.
18. Follow steps 7–11 of the serum/plasma treatment protocol.

Scanning protocol to detect IAF fluorescence (serum/plasma and cell lysates)

1. Following electrophoretic separation, remove the gels from the apparatus. All subsequent stages are light sensitive, so light exposure should be minimized.
2. Cut the blue front line off the bottom of the gels. If this is not done, non-protein-bound IAF will diffuse through the gel, and impair image acquisition.
3. Place the gels in water. Do NOT place in fixing solution, as its acidity will quench the pH-dependent fluorescein fluorescence signal.
4. Scan gels at λ_{EX} 488 nm, λ_{EM} 530 nm, using the Pharos FX Plus Molecular Imager. Use settings that give maximal signal without pixel saturation. Save the image and/or export in an appropriate format.

Protein quantification (serum/plasma and cell lysates)

1. Stain gels for protein using a stain at least as sensitive as colloidal Coomassie (e.g., "blue silver" as per [157] or silver staining [158]).
2. Scan the gels using a flatbed scanner with transparency capability. Ensure that all settings are set to their full linear range, with no corrections for brightness, contrast, etc. Save as a grayscale TIFF file at 300 dots per inch, acquiring 16 bits per channel. Some systems (e.g., Pharos FX Plus Molecular Imager) do not give scans compatible with image analysis software.

Notes/caveats

1. IAF binds nonspecifically with human serum albumin (HSA), so quantification of the Cys34 thiols on this protein is problematic.
2. If cells are lysed in water, changes in pH may affect the binding of the IAF.
3. The reaction of free thiols with IAF is not 100% efficient, which can result in the nondetection of some thiol-containing proteins. However, there is a lower incidence of false-positives, compared to the reducible thiol detection method [47], particularly if the pH is maintained at around 7.4 to ensure thiol specificity.
4. This method can be readily adapted to 2D gels. However, even with great care, 2D electrophoresis can be variable between experiments. Thus, wherever possible, control and treated samples should be run in parallel throughout, including sample preparation and derivatization, isoelectric focusing, second-dimension separation, and scanning.

Method Sheet 8

Quantification of amino acid/peptide/protein hydroperoxides using the FOX assay

Materials

1. Iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$; MW = 278.02); EMD/Merck; 1.03965
2. Xylenol orange tetrasodium salt, ACS reagent (MW = 760.58); Sigma-Aldrich; 398187
3. Sulfuric acid, 95–98% (approx 18 M); Ajax Finechem; A534
4. 0.45 μM syringe filter, 33 mm diameter; Sartorius; 16555
5. 0.5–2.0 mL disposable plastic cuvettes (1 cm path length); Greiner Bio-One; 613101
6. Hydrogen peroxide, 30% solution; Merck; 1.07209
7. 50 mL and 250 mL volumetric flasks
8. 1.5 mL centrifuge tubes

Instrumentation

Any dual-beam spectrophotometer capable of measuring absorbance at 560 nm.

Protocol

Stock solutions

Three stock solutions are required:

1. 2.5 M sulfuric acid (H_2SO_4). For 250 mL of solution add 34.7 mL of concentrated H_2SO_4 to ca. 200 mL of water in 250 mL volumetric flask. The acid must be added to the water and NOT the inverse, as the reaction is highly exothermic. Once the solution cools to room temperature, top up to 250 mL with water. Store at room temperature. Stable indefinitely.
2. Acidified iron(II) sulfate solution (25 mM). Dissolve 0.0695 g $FeSO_4.7H_2O$ in 10 mL of 2.5 M H_2SO_4. Stable at 4 °C for several months.
3. Xylenol orange solution (10 mM). Dissolve 0.1521 g xylenol orange in 20 mL of water. Stable at 4 °C for several months.

On the day of use, prepare the working solution. Mix the following components IN THIS ORDER: 1 part $FeSO_4$/acid solution, 2 parts water, 2 parts xylenol orange solution. Filter this mixture before use using a 0.45 μm syringe filter. Keep on ice in the dark until needed.

Preparation of hydrogen peroxide standards

The concentration of commercial H_2O_2 stock solutions (typically 30% (v/v) H_2O_2) should be determined accurately (typically 9–10.5 M) using a molar extinction coefficient (ε) of 39.4 M^{-1} cm^{-1} at 240 nm [159].

1. Prepare a 50 μM solution of H_2O_2 from the 30% (v/v) stock solution. Do this in at least two steps to ensure accuracy. Use immediately as dilute H_2O_2 solutions decompose rapidly.
2. Use the 50 μM solution to prepare a series of standards in 1.5 mL centrifuge tubes from 0 to 25 μM H_2O_2 in 5 μM steps. Make each up to 1 mL with water (0 μM = 1 mL of water). Prepare in triplicate, immediately before assay.

Protocol

Make up solutions at desired dilutions to a final volume of 1 mL in water. Prepare all solutions in triplicate.

Add 50 μL of FOX working solution to each sample and standard. Vortex.

Incubate in the dark for 30 min at room temperature.

Measure absorbance at 560 nm, using a new 0.5–2.0 mL disposable plastic cuvette for each standard concentration/sample dilution. The spectrophotometer should be zeroed with water at both positions in the sample cell.

Calculations and expected results

Fit a second-order polynomial curve to the absorbance of the H_2O_2 standards (R^2 values of >0.995 can be achieved with accurate pipetting). A straight line should not be used, as the values plateau at high concentrations of H_2O_2. Experimental values beyond the range of the standard curve should be diluted and rerun. Convert peroxide concentrations to "H_2O_2 equivalents" by use of the standard curve. If low peroxide concentrations are expected, the H_2O_2 standard curve can be prepared over a smaller range, e.g., 0–5 μM in 1 μM steps.

Notes/caveats

1. Solutions must be free of suspended matter. If turbidity is present, samples should be filtered or centrifuged prior to use. Different batches of xylenol orange have different levels of insoluble particulate matter, so the filtration step is usually required to remove these. If the xylenol orange dissolves completely in 2.5 M H_2SO_4, this step can be omitted.
2. It is possible to increase the absorbance for various peroxides by the addition of sorbitol that results in a chain reaction of unknown stoichiometry [160]. The use of this additive may exacerbate problems in absolute quantification. Perchloric acid has been proposed as a replacement for sulfuric acid [161]; however, the benefits of the latter are negligible in our hands.
3. Care should be taken if peroxides are assayed in buffer-containing solutions, as they can modulate the acidity of the final solutions (the reaction is pH sensitive), and hence the color change. Phosphate buffer is a particular problem, as the phosphate can promote oxidation of the Fe(II) complex to the Fe(III) form, turning the solutions purple almost instantly. If the use of buffers is unavoidable, check the performance of the assay by preparing a H_2O_2 standard curve in the buffer of interest before proceeding to assaying samples.
4. The stoichiometry of the reaction of the Fe(II) complex with hydroperoxides is variable (see, e.g., [80]) and hence in the absence of standard materials to allow the calculation of this factor, the assay can only give relative peroxide values. Typically peroxide

levels are reported as H_2O_2 equivalents with use of a standard curve obtained with this peroxide used for calibration. As the stoichiometry varies markedly, absolute values need to be treated with extreme caution as these may be erroneous by several fold. If absolute hydroperoxide concentrations are required, other more complex methods can be used, such as that involving triiodide [83]. The latter method is challenging, not least because of its requirement for complete oxygen exclusion for accurate quantification.
5. This method is not specific for peroxidic species, thus the presence of other oxidizing materials in the reaction mixture (e.g., HOCl, chloramines) can also induce oxidation of Fe(II) to Fe(III).
6. Compounds that absorb at 560 nm will raise the apparent hydroperoxide concentration. This can be corrected for by subtracting the absorbance at 560 nm of an identically diluted sample to which FOX reagent prepared without xylenol orange is added.
7. This assay can also be used as an on-line detection method for samples separated by HPLC [81]; although not strictly quantitative, it is as sensitive as, and cheaper than, chemiluminescence detection (which is also not strictly quantitative). However, attempts to adapt this method to a 96-well plate reader have proved to be unsatisfactory as the slope of the standard curve is insufficient to give reliable results.

Method Sheet 9

Quantification of protein chloramines/bromamines using 5-thio-2-nitrobenzoic acid (TNB)

Materials

1. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB; MW = 396.35); Sigma-Aldrich; D8130
2. Sodium hydroxide, anhydrous (NaOH; MW = 40.00); MP Biomedicals Inc.; 153495
3. 100 mM sodium phosphate buffer, pH 7.4, OR 1X phosphate buffered saline (PBS), prepared from a 20X concentrate, pH 7.5; Amresco; E703-1L
4. 0.5–2.0 mL disposable plastic cuvettes; Greiner Bio-one; 613101

Instrumentation

1. Any dual-beam spectrophotometer capable of measuring absorbance at 412 nm.
2. Magnetic stirrer

Protocol

1. Prepare a 50 mM solution of NaOH (e.g., 0.2 g in 100 mL of water).
2. Prepare the TNB reagent by alkaline hydrolysis of DTNB. Dissolve 1 mM DTNB in 50 mM NaOH (2.0 mg of DTNB in a final volume of 5 mL) in a sample tube and stir for 5 min to give a highly colored yellow/orange solution. This must be freshly prepared for each experiment.
3. Dilute the stock solution of TNB 40-fold into either 100 mM sodium phosphate buffer, pH 7.4, or 1X PBS. This should give a final concentration of TNB of ca. 35–50 μM with an absorbance at 412 nm of between 0.5 and 0.6, depending on the extent of hydrolysis.
4. Add 50 μL of chloramine/bromamine sample (<500 μM) to 1 mL of TNB reagent, and mix well. 1.5 mL centrifuge tubes are convenient for this assay. It is important to run a blank that contains the same volume of buffer or PBS at the same time as the samples, to allow the concentration of TNB consumed in each case to be determined accurately. Similarly, if quantifying protein chloramines/bromamines a control, nontreated protein sample should be prepared. If the solution becomes colorless, this indicates complete consumption of the TNB due to excess oxidant. In this case, the assay should be repeated with more dilute solutions of chloramine/bromamine.
5. Incubate the samples/buffer controls for 15 min in the dark (to avoid photodecomposition of the TNB) to ensure complete reaction of the chloramine/bromamine with the TNB.
6. Read the absorbance at 412 nm using 100 mM sodium phosphate buffer, pH 7.4, or 1X PBS as the reference (in a spectrometer with a reference beam, otherwise zero prior to reading samples with buffer or PBS).

Calculations and expected results

In this assay, 1 mol chloramine/bromamine reacts with 2 mol TNB to give 1 mol DTNB. The concentration of TNB consumed on reaction with chloramines/bromamines is calculated using an extinction coefficient ε 14,150 M^{-1} cm^{-1} [92] and the following equation: [chloramine] = ([TNB buffer blank] – [TNB remaining in sample])/2.

Notes/caveats

1. Care must be taken to ensure complete consumption of the oxidant, to prevent confounding reactions of the oxidant with TNB resulting in an overestimation of the concentration of chloramine or bromamine. Protein-derived species can be purified prior to assay by, for example, using a Sephadex PD-10 column. This method is not suitable for a large numbers of samples, and significant decomposition of the reactive protein-derived species will occur during separation, particularly in the case of bromamines.
2. The alkaline hydrolysis of DTNB to form TNB can be variable, and is generally 75–85% efficient. This can be a problem in experiments with thiol-containing proteins and biological samples such as plasma, due to reaction of the thiols with residual DTNB resulting in an increase in the concentration of TNB present. This can be checked by running a control with non-oxidant-treated protein. It is difficult to accurately compensate for this effect, as in the case of oxidant-treated samples, as it is likely that if chloramines/bromamines are present, then the thiols have been consumed, due to the greater reactivity of HOCl and HOBr with thiols compared with amines [90]; this may not be always be the case. Residual DTNB is a potential problem if this method is used to quantify HOCl or HOBr, due to the rapid rate of reaction of these oxidants with disulfides (e.g., DTNB) as well as free thiols (e.g., TNB) [90]. This is not a problem with chloramines/bromamines as the reaction of these species with disulfides is very slow [5]. If trace DTNB is a problem, the TNB can be purified after alkaline hydrolysis and reduction with 2-mercaptoethanol, by ion exchange chromatography or recrystalization (e.g., [103]).
3. Solutions must be freshly prepared to minimize the presence of DTNB and photodegradation products in the reaction mixtures.

Method Sheet 10

Analysis of Tyr- and Phe-derived oxidation products by HPLC

Materials

1. HCl (32% w/v); Chem Supply; HA020
2. Thioglycolic acid (mercaptoacetic acid); Sigma-Aldrich; T3758
3. Sodium perchlorate monohydrate, 98% (MW = 140.46); Sigma-Aldrich; 310514
4. Trichloroacetic acid (TCA); Sigma-Aldrich; T6399
5. Deoxycholic acid, sodium salt; Sigma-Aldrich; D6750
6. Sodium borohydride; Sigma-Aldrich; 21 346-2
7. Orthophosphoric acid (approx 85% w/v, i.e., 14.6 M); BDH; 101736U
8. Methanol (HPLC grade); Mallinckrodt; 3041-68
9. Acetone (HPLC grade); Merck; 100020
10. Phosphate buffered saline (PBS), 20X concentrate, pH 7.5; Amresco; E703-1L

11. 3,4-Dihydroxy-L-phenylalanine (DOPA); Sigma-Aldrich; D9628
12. D,L *o*-Tyrosine (*o*-Tyr); Fluka; 93851
13. D,L *m*-Tyrosine (*m*-Tyr); Sigma-Aldrich; T3629
14. L-Tyrosine (*p*-Tyr); Sigma-Aldrich; T3754
15. 3-Chloro-L-tyrosine (3Cl-Tyr); Sigma-Aldrich; 512443
16. 3-Nitro-L-tyrosine ($3NO_2$-Tyr); Sigma-Aldrich; N7389

Instrumentation

1. HPLC instrument with system controller (e.g., Shimadzu SCL-10Avp), binary pumps (e.g., Shimadzu LC-10ADvp) equipped with a gradient mixer, column oven able to maintain a temperature of 30 °C (e.g., Shimadzu CTO-10Avp), auto injector equipped with a sample cooler (e.g., Shimadzu SIL-10A), a UV-visible or photodiode array detector (e.g., Shimadzu SPD-M10Avp), a fluorescence detector (e.g., Shimadzu RF-10AXL), and an electrochemical detector (e.g., Antec Leyden Intro)
2. Diamond tip pen or engraver
3. Microtube centrifuge (e.g., Eppendorf, 5415R)
4. Vacuum concentrator/freeze-dryer (e.g., Christ RVC 2-33)
5. PicoTag reaction vials (No. 3002EL, Alltech, Baulkham Hills, NSW, Australia)
6. Oven capable of maintaining a temperature of 110 °C.
7. A fume hood is required for the addition of thioglycolic acid prior to protein hydrolysis, and venting the reaction vials following hydrolysis.

Protocol

Sample preparation for protein hydrolysis

1. Tissue samples are prepared for protein extraction by freezing in liquid N_2 and grinding to a fine powder with a mortar and pestle. Biological fluids including plasma and serum should be diluted with PBS to typically <5 mg mL^{-1} protein. Cells (ca. 2×10^6 cells) should be washed with PBS to remove media prior to analysis. Solutions containing purified or isolated proteins should be adjusted to give a final concentration of <5 mg mL^{-1}.
2. Samples (typically at a final volume of ca. 200 μL in PBS or H_2O) are placed in a glass vial (8×40 mm, 1 mL, No. 98212, Alltech) and labeled with a diamond-tipped pen or engraver.
3. 10 μL freshly prepared sodium borohydride ($NaBH_4$, 1 mg mL^{-1}) is added to each sample, and incubated for 5 min at 21 °C. This step minimizes oxidation mediated by reactive species such as hydroperoxides or chloramines during sample processing.
4. Samples are delipidated and precipitated by the addition of 25 μL of 0.3% (w/v) deoxycholic acid and 50 μL of 50% (w/v) TCA, with incubation on ice for 5 min. The deoxycholic acid prevents sample loss due to frothing during the hydrolysis procedure [115].
5. Glass vials containing samples are placed in 1.5 mL centrifuge tubes with caps removed prior to centrifugation for 2 min at 9000 rpm at 5 °C (Eppendorf 5415R centrifuge) to pellet protein. Spin speeds>9000 rpm may result in shattering of the vials.
6. The protein pellets are washed once with 5% (w/v) TCA, and twice with ice-cold acetone (stored in −20 °C freezer) with 2 min, 9000 rpm, spins between washes to settle pellets. Pellets are dried by exposing to a gentle stream of N_2 to evaporate any residual acetone.
7. Transfer sample vials to PicoTag hydrolysis vessels using forceps. These typically hold seven to eight 1 mL sample vials.
8. Pipette 1 mL of 6 M HCl and 50 μL of thioglycolic acid into the bottom of the PicoTag vessel, taking care not to get any HCl or thioglycolic acid into the individual sample vials. This step must be performed in a fume hood due to the volatile, corrosive, and pungent odor of these materials.
9. Evacuate PicoTag vessels using a vacuum pump, and regas with N_2 (back-flushing) at least twice to remove all O_2. Place under vacuum again, then place vessels in an oven at 110 °C.
10. Leave overnight (16–18 h). For greater sample reproducibility, ensure that the incubation time is consistent between experiments.
11. Remove PicoTag vessels from oven, then immediately release the vacuum in a fume hood. Extreme care should be taken to ensure that the released acid vapor does not come in contact with skin or eyes.
12. Remove individual vials using forceps and carefully rinse the outside of each vial with acetone to remove acidic residues.
13. Place the individual vials in 1.5 mL centrifuge tubes (without caps) and dry using a centrifugal vacuum concentrator (Christ RVC 2-33, John Morris, Australia, fitted with a Savant RT 490 refrigerated condensation trap, Savant vacuum gauge and LH Leybold Trivac D8A pump).
14. Resuspend samples in 200 μL of H_2O by vortex mixing.
15. Transfer samples to centrifugal filtration devices (containing a PVDF 0.22 μm membrane, 0.5 mL volume, No. UFC30GVNB, Millipore), and centrifuge at 10,000 rpm for 2 min at 5 °C (Eppendorf 5415R centrifuge) to remove the insoluble precipitate. This step is essential to prevent HPLC column blockages.
16. Transfer to HPLC vials (12×32 mm clear screw-top vials with 8/425 thread, No. 32008-1232, Alltech, with polypropylene 8/425 thread vial caps, No.24765, Supelco, containing red PTFE/silicone liners, No. 5128842, Alltech) containing 0.2 mL inserts (No.200-228, Edwards). Samples are placed in the HPLC auto injector, and kept at 5 °C prior to derivatization and injection (typically 20–50 μL).

Preparation of HPLC mobile phase and standards

1. Prepare a 1 M stock of orthophosphoric acid (H_3PO_4), by adding 68.5 mL of 85% acid to 900 mL of water. Do NOT add water to the acid as this reaction is highly exothermic. Once the solution cools to room temperature, top up to the 1000 mL mark with water. Store at room temperature. Stable indefinitely.
2. Prepare the following HPLC buffers:
   a. Buffer A : 100 mM sodium perchlorate (14.05 g L^{-1})/10 mM H_3PO_4 (10 mL of 1 M acid L^{-1})
   b. Buffer B : 80% (v/v) methanol
3. Both buffers should be filtered through 0.2 μm membrane filters (e.g., VacuCap 90 filter unit with 0.2 μm Supor membrane, No. 4622, Pall Corporation), and degassed prior to use.
4. Prepare a solution containing 50 μM final concentrations of parent Tyr and each oxidized derivative. Parent Tyr, DOPA, *o*-Tyr, *m*-Tyr, 3Cl-Tyr, and $3NO_2$-Tyr are available commercially. 3,5diCl-Tyr is synthesized by treating Tyr with HOCl as described in [162]. 3Br-Tyr and 3,5diBr-Tyr are synthesized by reaction of Tyr with HOBr as described in [163]. Di-Tyr is prepared from Tyr using horseradish peroxidase and H_2O_2 as detailed in [164]. In each case, the product is isolated by reversed-phase HPLC.
5. A separate solution of Tyr is prepared (1 mM, dissolved in buffer A), as the parent amino acid is generally present in higher concentrations compared to the oxidized products. Moreover, Tyr preparations usually contain some residual DOPA, which may be apparent at 1 mM concentrations of Tyr.

HPLC conditions and gradient

1. This method is optimized for separation of the Tyr derivatives on a Zorbax ODS column (4.6×250 mm, 5 μm pore size, No. 880952-702, Agilent) fitted with a LC-18 Pelliguard column (No. 59654, Supelco).
2. Use a flow rate of 1 mL min^{-1}, with the column maintained at 30 °C.
3. The detector settings are as follows:
   a. UV (PDA) detector at 280 nm
   b. Fluorescence detector set at λ_{EX} 280 nm, λ_{EM} 320 nm for DOPA, *o*-Tyr, *m*-Tyr, and *p*-Tyr with a change at ca. 30 min to λ_{EX} 280 nm, λ_{EM} 410 nm for di-Tyr analysis

c. Amperometric electrochemical detector at 1000 mV or a dual-channel coulometric electrochemical detector set at 400 mV and 800 mV.

4. The Tyr derivatives are separated using the gradient shown below.

Time (min)	% Buffer B
0.1	2
20	2
50	50
55	50
56	2
60	2

Calculations and expected results

The concentration of each oxidized derivative together with the parent Tyr in the samples is determined from linear plots of the HPLC peak area vs concentration from the known standards. The concentration of the oxidized material is usually expressed as a ratio with respect to the parent amino acid in biological samples. This compensates for any loss of material due to incomplete recovery of protein after hydrolysis, assuming that both parent and oxidized product are lost equally. However, this calculation is not recommended for experiments with oxidant-treated isolated protein where oxidation can lead to significant loss of parent Tyr. The low levels of products present in many samples preclude UV/visible detection, though this can be used for quantifying the parent amino acid (in the case of Tyr). DOPA can be quantified by fluorescence (λ_{EX} 280 nm, λ_{EM} 320 nm), electrochemical detection (e.g., with an electrode set at 400 mV), or MS. *o*-Tyr and *m*-Tyr can be quantified using these same methods, though electrochemical detection requires a higher potential (e.g., 800 mV). Di-Tyr can be detected by fluorescence (λ_{EX} 280 nm, λ_{EM} 410 nm), by MS or electrochemically. 3Cl-Tyr, 3,5diCl-Tyr, 3Br-Tyr, 3,5diBr-Tyr, and 3-NO_2-Tyr can be quantified by UV absorbance (at 280 nm, if high concentrations are present), by electrochemical oxidation at high potential (800 mV–1000 mV), or MS. Authentic standards of most products are commercially available. Sample recovery and artifactual oxidation during handling and processing can be assessed by the addition of isotopic (if MS is employed), or structurally related compounds, and monitoring of the recovery of these materials and their corresponding oxidation products.

Notes/caveats

1. Care must be taken to avoid artifactual oxidation of the samples during storage, handling, and sample processing. Freeze-thawing of samples after hydrolysis is best avoided, but it is possible to store protein precipitates at −80 °C prior to hydrolysis without an appreciable increase in the levels of the oxidation products.
2. Protein hydrolysis must be performed under strict O_2-free conditions to avoid artifactual oxidation. If the vacuum fails on a PicoTag vessel, discard these samples.
3. A common problem encountered with electrochemical detectors, particularly with samples of biological origin, is fouling of the electrodes resulting in a lower detector response and reduced sensitivity. This should be monitored during a run by placing standards at regular intervals between the samples. Amperometric detectors are easily cleaned manually, resulting in restoration of electrode response. Coulometric detectors must be cleaned electrochemically by switching between very high and very low potential, which is time consuming, and generally never completely restores electrode function, thereby requiring a replacement cell. Coulometric detectors have (traditionally) had a greater sensitivity, particularly those with multiple channels (e.g., Coularray detector, ESA) where specific potentials can be selected for the compounds of interest. However, recent amperometric detectors now have near comparable sensitivity, and the ease of cleaning usually outweighs the small differences in sensitivity and increased cost.
4. If MS detection is to be employed, the separation of materials needs to be optimized with MS-compatible buffers (see below).

Method Sheet 11

Guidelines for LC/MS studies of protein oxidation products

The following points are suggested for consideration in order to maximize meaningful data. They are not definitive, but are nonetheless a useful starting point:

1. As with any LC method used with MS, any salts needed to obtain satisfactory LC separation should be MS compatible (e.g., volatile ammonium salts). Avoid sodium or potassium salts, as they can form adducts with the ions of interest. Ion-pairing reagents, or volatile acids (e.g., formic or trifluoroacetic acid, TFA) can be used in place of buffers, but TFA can cause ion suppression [165].
2. The presence of volatile organic solvents in the LC mobile phase (e.g., methanol, acetonitrile) can improve signal strength, so LC gradient profiles that result in the peaks of interest eluting in a significant concentration of organic solvent are preferable to systems that result in elution of the material of interest in mobile phase that is mainly aqueous. Selection of an appropriate HPLC column can aid this process.
3. Screening of masses of interest should be performed before full-scale experiments are performed. This can be achieved by infusing nonfractionated samples into the mass spectrometer using a syringe pump:
   a. Dilute the oxidation product of interest in HPLC buffer (use a standard if available), and tune the mass spectrometer to maximize the signal obtained.
   b. If only a very small signal can be detected for an oxidation product, tune on an untreated control instead. For example, if an oxidized amino acid is to be examined, often the nonoxidized amino acid can be used to tune the mass spectrometer.
   c. Once the mass spectrometer is tuned, acquire full range scans of all possible masses of both the oxidized and the control samples, to look for possible mass modifications.
   d. Perform MS/MS of any peaks that are found in the oxidized sample, but not present in, or smaller than, the control sample. If the peak is real, the total ion count for the oxidized sample should be much greater than the control, which should give a negligible ion count. The fragment masses may give clues as to the identity of the product.
   e. If specific masses have been predicted, but are not visible in the full range scan, it may still be worth looking for them by MS/MS. This can be particularly true if the product being looked for is expected to be present in much lower abundance than the unmodified parent compound. Again, compare the oxidized and control samples; if fragments are detected for the oxidized sample, but not the control, assume that the mass is worth further investigation.
4. Once screening has been performed, LC/MS can be employed, with SIM to look for each of the specific masses of interest. SIM on LCQ Deca XP Max spectrometer (Thermo Electron Corporation, Rydalmere, NSW, Australia) with a 2 mass unit window for the precursor ion greatly increases the sensitivity of detection, compared to a 1 mass unit window (3 mass unit windows have also been used, e.g., [166]). However, it is important to be sure that the SIM fragments are unique to the species of interest when using such a wide window, or false-positive results may be obtained.

5. Care must be taken when quantifying products by mass spectrometry. It cannot be assumed that all products of a reaction will ionize equally. If they are available, internal standards consisting of known amounts of an isotopically labeled version of the compound of interest should be added to samples. Isotopically labeled derivatives can also be added before sample workup, to check for and/or quantify any artifactual oxidation.
6. Prediction of fragment mass structures can be achieved using fragmentation prediction software (e.g., Mass Frontier 4.0, HighChem Ltd, Slovak Republic). Such software can yield improbable or thermodynamically unfavorable products, so the resulting data need to be considered with care. Knowledge of the chemistry likely to be occurring in the system under study is very useful.

Method Sheet 12

Quantification of protein carbonyls: Total carbonyls on proteins

Materials

1. 2,4-Dinitrophenylhydrazine (DNPH; MW = 198.14); Sigma-Aldrich; D199303
2. Hydrochloric acid, 32% (w/v) (approx 10.2 M); Chem Supply; HA020
3. Ethanol (absolute); BioLab LabServ; BSPEL975
4. Ethyl acetate (ACS spectrophotometric grade); Sigma-Aldrich; 15,485-7
5. Trichloroacetic acid (TCA); Sigma-Aldrich; T6399
6. Guanidine hydrochloride (MW = 95.53); Sigma-Aldrich; G4630
7. BSA (essentially fatty acid free); Sigma-Aldrich; A6003
8. 1.5 mL centrifuge tubes
9. 0.5–2.0 mL disposable plastic cuvettes (1 cm path length); Greiner Bio-One; 613101
10. Matched 1.2 mL quartz cuvettes

Instrumentation

1. Vortex mixer
2. Freezer (-20 °C)
3. Refrigerated 1.5 mL microcentrifuge (4 °C): Eppendorf 5415R
4. Fume hood
5. Any dual-beam spectrophotometer with a 1 cm path length that is capable of measuring absorbance at 280 nm (for protein assay) and 370 nm (for carbonyl assay).

Protocol

Stock solutions

The following stock solutions are required:

1. 2.5 M HCl. For 100 mL of solution add 24.5 mL of concentrated HCl slowly to approx 60 mL of water in a 100 mL volumetric flask. Do NOT add water to the acid as this reaction is highly exothermic. Once the solution cools to room temperature, top up to the 100 mL mark with water. Store at room temperature. Stable indefinitely.
2. 10 mM DNPH in 2.5 M HCl. Dissolve 0.2 g DNPH in 100 mL of 2.5 M HCl. Even though the product contains approx 30% water, it is not necessary to take this into account, since the DNPH is in a large excess over the carbonyl concentration. Store in the dark. Discard after approx 1 month.
3. 50% (w/v) TCA. Dissolve 50 g of TCA in 100 mL of water (a measuring cylinder is accurate enough). Store at room temperature.
4. Ethanol/ethyl acetate (1/1). Combine in a 1/1 ratio in a measuring cylinder, and mix well. Store at 4 °C, as this solution is needed cold for the assay.
5. 6 M guanidine-HCl. Dissolve 57.32 g of guanidine-HCl in 100 mL of water. Some preparations of guanidine-HCl contain anticaking agent, which makes the solution cloudy and compromises absorbance readings. If this is the case, filter the solution through filter paper (e.g., Whatman No.1 filter paper, 185 mm diameter: Whatman Schleicher & Schuell 1001 185) before use. Store at room temperature.

Protein carbonyl determination method

1. Dilute samples (if necessary) in triplicate to a protein concentration of approx 1 mg mL^{-1}.
2. For each sample, pipette 250 μL of the above solution into each of two 1.5 mL Eppendorf centrifuge tubes.
3. To the first tube (labeled " + DNPH") add 250 μL of 10 mM DNPH in 2.5 M HCl. To the second tube (labeled "–DNPH") add 250 μL of 2.5 M HCl. Vortex both solutions.
4. Incubate in the dark for 15 min at room temperature, vortex mixing the samples every 5 min.
5. Add 125 μL of 50% (w/v) TCA to each tube (final concentration = 10% w/v), and vortex.
6. Incubate at -20 °C for at least 15 min, before centrifuging at 4 °C for 15 min at 9000*g*.
7. Remove and discard the supernatant from each tube, without disturbing the protein pellet.
8. Wash protein pellets three times with ice cold ethanol/ethyl acetate (1/1 mixture). Centrifuge for 2 min at 9000*g* between washes, and discard the supernatant each time, being careful not to disturb the protein pellet. This step removes excess DNPH and is best carried out in a fume hood, as the ethyl acetate has a strong odor.
9. Redissolve the protein pellets in 1 mL of 6 M guanidine-HCl. Mix well.
10. Once the protein pellets are fully dissolved, measure the absorbance at 370 nM of the solution that DNPH was added to (+ DNPH). Zero the UV/Vis spectrophotometer with 6 M guanidine-HCl.

Protein concentration determination

1. Prepare a 1 mg mL^{-1} BSA solution in 6 M guanidine-HCl, then dilute this in 6 M guanidine-HCl to give a series of standards from 0 to 1 mg mL^{-1} in 0.2 mg mL^{-1} steps. Prepare each concentration in triplicate.
2. Determine the absorbance at 280 nm of the BSA standards using matched quartz cuvettes. Zero the UV/Vis spectrophotometer with 6 M guanidine-HCl. Rinse the cuvettes with methanol and water between samples to minimize protein carryover.
3. Determine the absorbance at 280 nm of the corresponding solutions that were treated with HCl without added DNPH (– DNPH).

Calculations and expected results

1. Carbonyl concentration: the protein carbonyl concentration is determined using the extinction coefficient of DNPH at 370 nm (22,000 M^{-1} cm^{-1}), with the carbonyl concentration (in moles L^{-1}) = [(Abs at 370 nM)/22,000]. Multiply by 1×10^6 to convert to nmol mL^{-1}. Determine the average carbonyl concentration for the triplicate tubes for each sample.
2. Protein concentration: fit a straight line to the absorbance of the BSA standards (R^2 values of >0.995 should be routinely achieved). Determine the protein concentration of each sample (in mg mL^{-1}) by reference to this standard curve, then determine the average protein concentration for the triplicate tubes for each sample.
3. Carbonyl concentrations are often expressed as nmol of carbonyl per mg protein using the average values determined above: [carbonyls (nmol mL^{-1})]/[protein (mg mL^{-1})] = nmol carbonyl per mg protein
4. Since the solutions used to determine the carbonyl and protein concentrations were diluted identically, there is no need to take into account the original sample dilution.

Notes/caveats

1. Care needs to be taken at each step during the protein precipitation and supernatant removal. The DNPH is not compatible with protein assays; thus it is not possible to use the DNPH-containing sample to determine protein concentrations. Treating the solution without DNPH in an otherwise identical manner is the best compromise.

2. A sample with a starting protein concentration of 1 mg mL^{-1} will be within the range of the 0–1 mg mL^{-1} BSA standard curve, owing to loss of sample during the precipitation/washing steps.
3. Protein concentrations are determined by measuring absorbance at 280 nm rather than other protein assays, because most protein assays are not compatible with 6 M guanidine-HCl. These values will, however, be misleading if extensive oxidation of Trp residues in the samples has occurred as this amino acid, which is readily oxidized, is the major chromophore at this wavelength. Use of this approach on heavily oxidized samples can therefore yield misleading data.

Protein carbonyls by 2D electrophoresis

This method is an adaptation of the OxyBlot protein oxidation detection kit to allow its use in 2D electrophoresis, and is loosely based on the method of Reinheckel et al. [167]. Only the antibodies supplied with the OxyBlot kit are utilized.

Reagents

1. OxyBlot protein oxidation detection kit; Millipore; S7150
2. 10 mM DNPH in 2.5 M HCl; see previous carbonyl method
3. Urea (ultra pure grade); Amresco; 0568
4. Tris (ultrapure grade); Amresco; 0497
5. Sodium dodecyl sulfate (SDS, for electrophoresis, approx 99%); Sigma-Aldrich; L3771
6. Glycerol (Biotechnology grade); Amresco; 0854
7. Dithiothreitol (DTT; MW = 154.25); Astral; C-1029
8. Iodoacetamide (MW = 184.96); Sigma-Aldrich; I1149
9. Glycine; Amresco; 0167
10. Agarose (electrophoresis grade); BRL; 5510UB
11. Bromophenol blue; ICN; 193990
12. Precast gels; e.g., Bio-Rad Ready Gel Tris-HCl Gel, 8–16% linear gradient, 4% stacking gel, 7 cm IPG well, 8.6 × 6.8 cm; 161-1394
13. ECL detection reagents; Amersham; RPN2105
14. IPG strip rehydration/equilibration trays; Bio-Rad 165-4035 (7 cm trays), 165-4015 (17 cm trays)

Instrumentation

1. Rotary shaker; Ratek OM5
2. Gel electrophoresis equipment for separation of proteins in two dimensions
3. Western blotting apparatus, e.g., Invitrogen iBlot semidry blotting system

Protocol

1. Separate proteins of interest in the first dimension using 7 cm IPG strips.
2. Place each focused IPG strip into separate lanes of a 17 cm rehydration/equilibration tray, being careful to note down the identity of each IPG strip.
3. Add 5 mL of 10 mM DNPH in 2.5 M HCl to each lane containing an IPG strip, and incubate with shaking (rotary shaker; approx 50 rpm) for 20 min.
4. Transfer each IPG strip to a separate lane in a clean 7 cm rehydration/equilibration tray.
5. Add 2 mL of IPG strip equilibration solution to each lane, and incubate with shaking for 5 min.
6. Repeat step 5 for a total of 5 × 5 min washes.
7. Add 2 mL of equilibration solution with added 2% (w/v) DTT, and incubate with shaking for 10 min.
8. Add 2 mL of equilibration solution with added 2.5% (w/v) iodoacetamide, and incubate with shaking for 20 min.
9. Dip the IPG strips briefly in 1X running buffer (6 g L^{-1} Tris base, 28.8 g L^{-1} glycine, 1 g L^{-1} SDS; this can also be made up as a 5X concentrated solution and diluted with water to this concentration just prior to use), then place horizontally on the top of gels, with the plastic strip backing against the taller gel plate.
10. Place an IPG electrode wick (or a piece of filter paper cut to approx 4 × 8 mm) containing molecular weight markers on top of each gel, adjacent to one end of the IPG strip (ensure the wick is completely dry before placement, or the markers will spread across the gel)
11. Quickly seal the IPG strip and molecular weight markers in place with 1X running buffer solution with added agarose (0.6% w/v) and bromophenol blue (0.001% w/v); this should be briefly heated immediately prior to use to melt the agarose. Tap IPG strips with a spatula immediately following agarose addition, to ensure that no air bubbles are trapped between the IPG strips and the gel surface.
12. Separate proteins in the second dimension at 150 V. Run until the blue front reaches the bottom of the gel.
13. Remove the IPG strip and wick from the gel, and place in Western blotting apparatus. We use the Invitrogen iBlot semidry blotting system with PVDF membranes, as per the manufacturer's instructions (7 min transfer).
14. Carbonyls should be detected using the OxyBlot Protein Oxidation Detection Kit (Chemicon, Boronia, VIC, Australia), as per the manufacturers' instructions supplied with the kit.
15. Visualize protein spots by chemiluminescence, using ECL detection reagents, according to the instructions supplied with the reagents.
16. Record the resulting chemiluminescence with a chemiluminescence detector (e.g., Bio-Rad Chemidoc XRS). Start by acquiring an image every minute for 10 min; shorten or lengthen the exposure time as required.

Notes/caveats

1. The first time an experiment is performed using this method, a control should be prepared where the IPG strip is incubated in 2.5 M HCl without any added DNPH. This will show whether any non-DNPH-specific antibody binding is occurring.
2. Even with great care, 2D electrophoresis can be variable between experiments. Thus, wherever possible, control and treated samples should be run in parallel throughout the entire process from sample preparation, through isoelectric focusing, derivatization, second-dimension separation, and immunodetection. This makes comparison of immunoblots more meaningful.
3. If problems with signal strength are encountered, consider using film development (e.g., Amersham Hyperfilm ECL; Catalog No. RPN2103K) rather than a chemiluminescence detector (e.g., the Bio-Rad Chemidoc XRS). Film development is considerably more sensitive in our hands.

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