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Research Paper

Validation of protein carbonyl measurement: A multi-centre study

Edyta Augustyniak [a], Aisha Adam [a], Katarzyna Wojdyla [b], Adelina Rogowska-Wrzesinska [b], Rachel Willetts [a], Ayhan Korkmaz [h], Mustafa Atalay [c], Daniela Weber [d], Tilman Grune [d], Claudia Borsa [e], Daniela Gradinaru [f], Ravi Chand Bollineni [g,1], Maria Fedorova [g], Helen R. Griffiths [a,*]

[a] Life & Health Sciences, Aston University, Birmingham B4 7ET, UK
[b] Department of Biochemistry and Molecular Biology, University of Southern Denmark, Denmark
[c] Institute of Biomedicine, Physiology, University of Kuopio, Kuopio, Finland
[d] German Institute of Human Nutrition, DIfE, Nuthetal 14558, Germany
[e] Ana Aslan - National Institute of Gerontology and Geriatrics, PO Box 2-4, Bucharest 011241, Romania
[f] Faculty of Pharmacy, Department of Biochemistry, Carol Davila - University of Medicine and Pharmacy, Bucharest 020956, Romania
[g] Faculty of Chemistry and Mineralogy, Center for Biotechnology and Biomedicine, Institute of Bioanalytical Chemistry, Universität Leipzig, Leipzig, Germany
[h] University of Eastern Finland, Institute of Biomedicine, Physiology, Finland

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ABSTRACT

Protein carbonyls are widely analysed as a measure of protein oxidation. Several different methods exist for their determination. A previous study had described orders of magnitude variance that existed when protein carbonyls were analysed in a single laboratory by ELISA using different commercial kits. We have further explored the potential causes of variance in carbonyl analysis in a ring study. A soluble protein fraction was prepared from rat liver and exposed to 0, 5 and 15 min of UV irradiation. Lyophilised preparations were distributed to six different laboratories that routinely undertook protein carbonyl analysis across Europe. ELISA and Western blotting techniques detected an increase in protein carbonyl formation between 0 and 5 min of UV irradiation irrespective of method used. After irradiation for 15 min, less oxidation was detected by half of the laboratories than after 5 min irradiation. Three of the four ELISA carbonyl results fell within 95% confidence intervals. Likely errors in calculating absolute carbonyl values may be attributed to differences in standardisation. Out of up to 88 proteins identified as containing carbonyl groups after tryptic cleavage of irradiated and control liver proteins, only seven were common in all three liver preparations. Lysine and arginine residues modified by carbonyls are likely to be resistant to tryptic proteolysis. Use of a cocktail of proteases may increase the recovery of oxidised peptides. In conclusion, standardisation is critical for carbonyl analysis and heavily oxidised proteins may not be effectively analysed by any existing technique.

Introduction

Non-enzymatic, free radical-mediated oxidation of proteins is common in biological systems. Some of the earliest work exploring the effects of chemical modification to proteins on function examined the oxidative damage induced by selected radicals on lysozyme, α-1-antitrypsin, and apolipoprotein B in LDL [1,2]. In each of these cases, there was an associated loss of function or null effect. However, the effects of oxidation are not always deleterious. Indeed, the chemical nature of oxidation and the biological consequences of this oxidation are dependent on (1) the primary sequence (2) whether or not the oxidant can gain access to susceptible amino acid residues within that protein i.e. three dimensional structure constraints and (3) the oxidising species [3–5].

Discrete chemical reactions can induce patterns of oxidation that vary according to the initiating radical species. For example, radiolytically-generated hydroxyl radicals will favour oxidation of aromatic amino acids such as tryptophan and tyrosine whereas peroxy radicals favour formation of hydroperoxides and hydroxides on aliphatic amino acids [6–8]. However, protein carbonyls on both aliphatic and aromatic amino acids are commonly produced by a range of oxidising species [5]. In addition, secondary oxidation of proteins to yield carbonyls is also common. During protein glycation and following lipid peroxidation, aldehydes are formed e.g. glyoxal and 4-hydroxynonenal. These aldehydes form Schiff's base adducts with primary amine groups present on lysine

* Corresponding author.
E-mail address: h.r.griffiths@aston.ac.uk (H.R. Griffiths).
[1] Current address: The Biotechnology Center of Oslo, University of Oslo, Gaustadalleen 21, 0349 Oslo, Norway.

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and in the case of dicarbonyls such as glyoxal and malondialdehyde, when cross-linking has not occurred and a free aldehyde group remains, contribute to total carbonylation [9,10]. Protein carbonyl measurements are used to provide an index of global protein oxidation irrespective of the initiating radical species [11].

Recently we have reviewed the existing methods for determining protein carbonyls, considering their strengths and weaknesses [12]. The majority of methods rely on derivatisation of the carbonyl group, most commonly with di-nitrophenol hydrazine (DNPH; Fig. 1) [13,14]. Dinitrophenol hydrazone (DNP)-carbonyl can be detected quantitatively by immunoassay using high specificity antibodies against DNP or absolutely by spectrophotometric calculation from the absorption measured at 360 nm relative to the extinction coefficient for DNP [15,16]. Mass spectrophotometric methods can also be used for identification as well as relative quantification of carbonylated peptides by label free techniques or using isotopically labelled derivatisation reagents.

Despite the use of common platforms such as ELISA for determination of protein carbonyls, orders of magnitude difference have been reported between different commercial kits [17]. For this reason, it has been hard to compare the data reported in different papers. The analysis of protein oxidation is further complicated by the complexity of tissue matrix which can contribute to differences in oxidised protein extraction according to the methods used.

In order to understand the reasons underlying the differences between laboratories and methods, we have surveyed the methods used in six different laboratories across Europe. We then undertook a ring study to compare the carbonyl content reported by different methods using a homogenised liver extract with and without UV radiation-induced oxidation. Each participating laboratory received blinded, lyophilised samples and was invited to process according to their protocol and to report their findings. In this manuscript, we compare the results of carbonyl analysis of the same tissue samples that were achieved by each method and highlight the need for improved reference standardisation.

Materials and methods

Preparation of liver lysate samples

Rat liver tissue (20 g) was homogenised using rotor stator homogeniser in ice cold 1/3 strength phosphate buffered saline (PBS; 45.6 mM NaCl, 0.9 mM KCl, 2.7 mM Na_2HPO_4 and 0.48 mM KH_2PO_4 in distilled water, pH adjusted to 7.4 with HCl), in ratio 2:1 (PBS:tissue). Immediately before homogenisation, protease inhibitor phenylmethane-sulphonyl fluoride (Sigma) was added to a final concentration of 1 mM. Homogenate was then centrifuged using a bench top centrifuge for 5 min at 600*g*. The supernatant was collected and then re-centrifuged for 20 min at 3000*g*. The supernatant was collected again and was finally re-centrifuged using an ultracentrifuge at 100,000*g* for 4 h after which clear supernatant containing soluble proteins was collected.

Protein content was measured using bicinchoninic acid (BCA) assay. Protein content of samples was adjusted to 1 mg/ml prior to irradiation. Samples (10 ml aliquots) were irradiated at a distance of approximately 15 cm from the UV lamp ($I = 1.74 \times 20$ mW/cm^2, $P = 250$ W, UV range 280–315 nm, IUV250 UV Curing Flood Lamp 230 V/50 Hz) for 0, 5 and 15 min respectively. After irradiation, protein damage was detected using carbonyl ELISA. Irradiated protein solutions (1 ml) were dried under vacuum centrifuge for 8 h with desferrioxamine added (5 mM) and stored at −80 °C.

Carbonyl analyses

Spectrophotometry method

Each laboratory that calibrated standards in their laboratory used a spectrophotometric method to ascertain absolute carbonyl

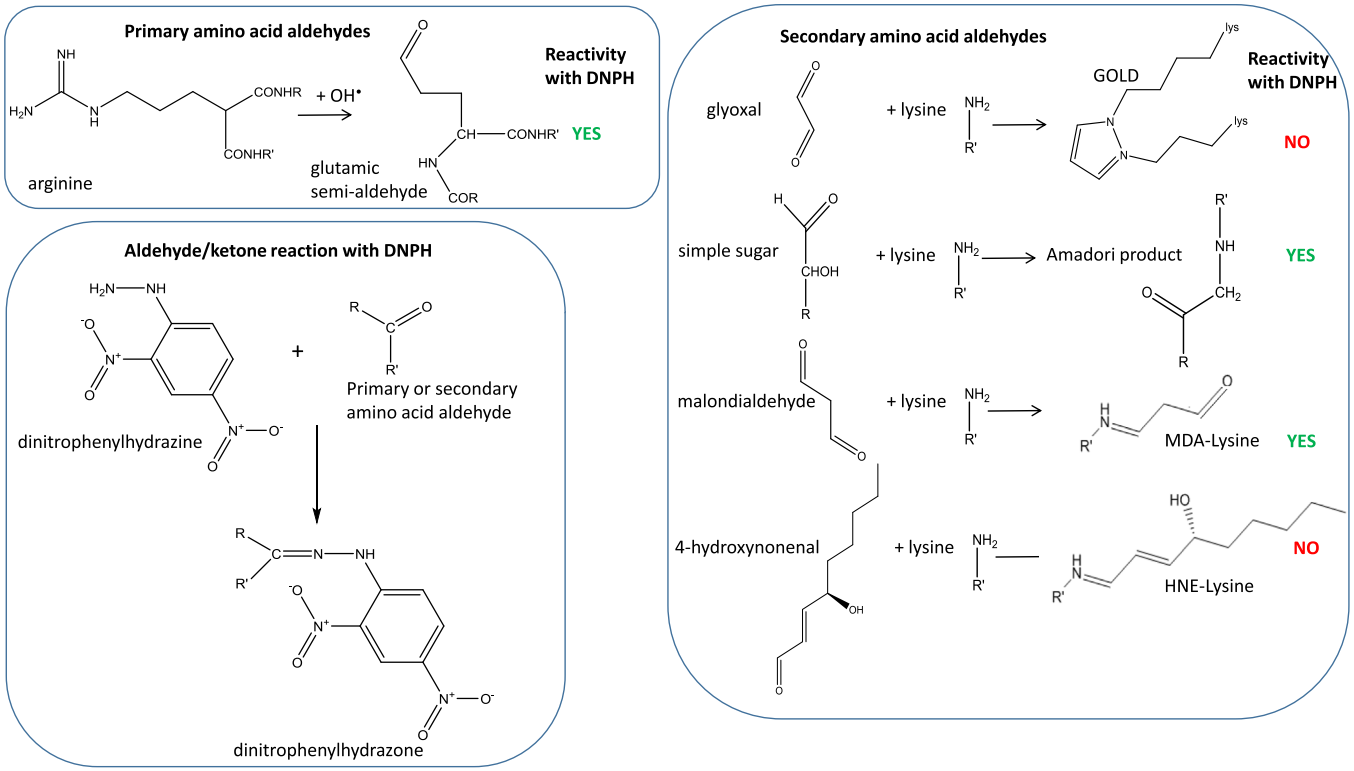

Fig. 1. Primary and secondary protein carbonyls and their derivatisation by DNPH.