[13] Protein Carbonyl Measurement by Enzyme-Linked Immunosorbent Assay

By Christine C. Winterbourn and I. Hendrikje Buss

Introduction

Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indices of oxidative injury.[1] The conventional assay for protein carbonyls is a colorimetric procedure that measures binding of dinitrophenylhydrazine (DNP).[2–4] Protein-bound DNP can also be measured, with increased sensitivity, either by HPLC,[3] or using an anti-DNP antibody with Western blots[5] or tissue sections.[6] We have adapted the immunological approach to an ELISA method that enables carbonyls to be measured quantitatively.[7] The advantages of this method are that it requires only microgram quantities of protein and it avoids the high and sometimes variable blanks due to unbound DNP that are limitations for the colorimetric method.[4,8] We have found it to be highly sensitive in analyzing plasma and lung aspirates from both critically ill adult patients and premature infants. Results correlate well with the colorimetric assay but are more discriminatory. Absolute values in both assays are subject to some uncertainty, and the method is best for comparing samples analyzed using a standard system.

Principle

Samples containing protein are reacted with DNP, then the protein is nonspecifically adsorbed to an ELISA plate. Unconjugated DNP and nonprotein constituents are easily washed away and give minimal interfer-

[1] E. R. Stadtman, *Free Rad. Biol. Med.* **9,** 315 (1990).
[2] R. L. Levine, D. Garland, C. N. Oliver, A. Amici, I. Climent, A. Lenz, B. Ahn, S. Shalteil, and E. R. Stadtman, *Methods Enzymol.* **186,** 464 (1990).
[3] R. L. Levine, J. A. Williams, E. R. Stadtman, and E. Shacter, *Methods in Enzymol.* **233,** 346 (1994).
[4] A. Z. Reznick and L. Packer, *Methods Enzymol.* **233,** 357 (1994).
[5] E. Shacter, J. A. Williams, M. Lim, and R. L. Levine, *Free Rad. Biol. Med.* **17,** 429 (1994).
[6] M. A. Smith, G. Perry, L. M. Sayre, V. E. Anderson, M. F. Beal, and N. Kowall, *Nature* **382,** 120 (1996).
[7] H. Buss, T. P. Chan, K. B. Sluis, N. M. Domigan, and C. C. Winterbourn, *Free Rad. Biol. Med.* **23,** 361 (1997).
[8] L. Lyras, P. J. Evans, P. J. Shaw, P. G. Ince, and B. Halliwell, *Free Rad. Res.* **24,** 397 (1996).

Copyright © 1999 by Academic Press
All rights of reproduction in any form reserved.

0076-6879/99 $30.00

ence. The adsorbed protein is probed with a commercial biotinylated anti-DNP antibody followed by streptavidin-linked horseradish peroxidase. Absorbances are related to a standard curve prepared for bovine serum albumin (BSA) containing increasing proportions of HOCl-oxidized protein that is calibrated colorimetrically.

Procedure

Reagents

2,4-Dinitrophenylhydrazine (DNP; Riedel-de-Haen, Seelze-Hannover, Germany). Solution: 10 m*M* DNP in 6 *M* guanidine hydrochloride, 0.5 *M* potassium phosphate buffer, pH 2.5

Biotin-conjugated rabbit immunoglobulin G (IgG) polyclonal antibody raised against a DNP conjugate of keyhole limpet hemocyanin (anti-DNP) (Molecular Probes, Eugene, OR); 1:1000 dilution in PBS, 0.1% (w/v) Tween 20 solution

Streptavidin-biotinylated horseradish peroxidase (Amersham International, Buckinghamshire, UK); 1:3000 in PBS, 0.1% Tween 20 solution

Phosphate-buffered saline (PBS): 10 m*M* sodium phosphate buffer, pH 7.4, in 0.14 *M* sodium chloride

Blocking solution: PBS containing 0.1% Tween 20

o-Phenylenediamine (0.6 mg/ml) and hydrogen peroxide (30% stock diluted 1:2500) in 50 m*M* Na_2HPO_4 plus 24 m*M* citric acid, mixed directly before use

A microplate reader and washer are required. We use a Dynatech reader and AM60 washer. Nunc Immuno Plate Maxisorp ELISA plates.

Unless noted otherwise, biochemicals are obtainable from Sigma Chemical Co. (St. Louis, MO).

Albumin Standards

Bovine serum albumin (BSA) as purchased already contains carbonyls. Fully reduced BSA is prepared by reacting a 0.5 g/100 ml solution in PBS with 0.1 g sodium borohydride for 30 min, followed by neutralizing with 2 *M* HCl, added slowly. Since this reaction produces hydrogen, it should be carried out in a fume hood. After overnight dialysis against PBS, the protein concentration is checked by measuring A_{280} and adjusted to 4 g/liter. Oxidized BSA containing additional carbonyls is prepared for use as a reference by reacting (at 50 mg/ml in PBS) with hypochlorous acid (final concentration 5 m*M*). Albumin solutions do not change in carbonyl content when stored at −80°.

Standard Curves and Calibration of Assay

Standard curves are constructed by mixing varying proportions of HOCl-oxidized BSA with fully reduced BSA at a constant total protein concentration. A range of 0–2 nmol carbonyl/mg protein is appropriate for most purposes. The carbonyl content of the oxidized BSA is determined from A_{375} in the colorimetric assay performed as described below. In our hands the fully reduced BSA consistently gives an A_{375} of about 0.13 per 10 mg (equivalent to 0.6 nmol carbonyls/mg), which was unaffected by further treatment with sodium borohydride. It is assumed to be nonspecific and not due to carbonyls.

Analysis of Plasma and Lung Aspirates

Samples can be used fresh or stored at −80°. A total protein assay is performed using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) on all standards, plasmas, and aspirates, which are then diluted in PBS to a protein concentration of 4 mg/ml. Because aspirates generally contain lower protein concentrations than this, they need to be concentrated by mixing a volume containing 60 μg protein with 0.8 volume of 28% (w/v) trichloroacetic acid (TCA), centrifuging at 10,000*g* for 2 min and discarding the supernatant. PBS (15 μl) is added to the protein precipitate, which dissolves completely after addition of the DNP reagent (see below).

ELISA Assay

Protein derivatization is carried out with 45 μl of DNP solution added to 15 μl of sample (4 mg/ml) to give a final protein concentration of 1 mg/ml. The blank consists of DNP reagent added to PBS without protein. After vortex mixing and incubating at room temperature for 45 min, 5 μl of each solution is added to 1 ml PBS. Triplicate 200 μl aliquots (containing 1 μg protein) are added to wells of the ELISA plate which is incubated overnight at 4°, then washed 5 times with PBS before each of the following incubations:

Blocking solution (250 μl/well) for 1.5 h at room temperature
Biotinylated anti-DNP antibody (200 μl/well) for 1 h at 37°
Streptavidin-biotinylated horseradish peroxidase (200 μl) for 1 h at room temperature

o-Phenylenediamine/peroxide solution (200 μl) is then added and the color allowed to develop for 25 min before stopping with 100 μl of 2.5 *M* sulfuric acid and reading absorbances with a 490 nm filter. A six point standard curve of reduced and oxidized BSA is included with each plate. The background absorbance for DNP reagent (typically about 0.08) is subtracted from all other absorbances.

Colorimetric Assay for Calibration of Standard

The carbonyl content of the stock oxidized BSA is calibrated using a modification of the standard colorimetric method[2] in which 10 mg protein in 250 μl PBS is reacted with 1 ml 10 mM DNP in 2 M HCl for 45 min with occasional mixing, precipitated with 1 ml 28% TCA and washed three times with 2.5 ml ethanol/ethyl acetate (1:1, v/v). Pellets should be broken up mechanically and by sonicating during the washing steps, and dissolved at 37° in 1 ml 6 M guanidine hydrochloride, 0.5 M potassium phosphate, pH 2.5, to measure A_{375}. The protein concentration of the final extract is determined by diluting 1:5 and measuring A_{280} (ε_{280} 5.31 for a 1 g/100 ml solution) and the absorbance adjusted for the protein loss (about 10%) that occurs with this method.[4] A blank with the protein reacted with 2 M HCl containing no DNP is carried through the procedure and its absorbance is subtracted. Carbonyl content is determined as nmol/mg protein using ε_{375} 22,000 M^{-1} cm^{-1} after subtracting the value for reduced albumin.

Comments

Sensitivity and Reproducibility

Standard curves for the ELISA are linear up to at least 10 nmol carbonyl/mg protein. With the development time described, 1.0 nmol/mg gives a net absorbance of about 0.8 above the blank. Repeat analyses of 10 different plasma samples, carried out on three separate occasions, gave variations of 1.7–21.4% with a mean of ±8.8%.[7] Most of this variation arose from analyses at the lower end of the standard curve (mean 17% for values <0.06 nmol/mg compared with a mean of 4.5% for values over 0.8 nmol/mg). Intraassay variation for 9 samples analyzed in triplicate was 0.27–2.53% with a mean of 1.4%.

Blanks

A key requirement for sensitivity is a low reagent blank. This is typically about 0.04 absorbance units above PBS alone. We originally used reduced BSA in the blocking solution to achieve this, but have subsequently found that PBS containing 0.1% Tween 20 is also satisfactory. There are minor differences in sensitivity and absolute values for plasma samples, but results with both solutions correlate closely. Reduced BSA typically gives an absorbance about 0.02 units above the reagent blank.

Validation

As described elsewhere,[7] the conditions described for the assay have been chosen after testing different ELISA plates, protein concentrations,

and antibodies. Reactivity can be outcompeted by free DNP, it requires protein, DNP, and the primary antibody, and it decreases to baseline if the albumin or plasma is pretreated with sodium borohydride.

Clinical Samples

Plasmas from normal adults give a mean protein carbonyl of about 0.1 nmol/mg protein by the ELISA method. Higher levels (up to 4 nmol/mg) have been measured in plasma from critically ill patients and lung aspirates from adults and premature infants. The TCA-precipitation step before DNP derivatization that is generally necessary to concentrate the protein from aspirates causes a small (20%) increase in response.

Correlation with Colorimetric Assay

A good correlation has been found between the ELISA and colorimetric methods for plasma samples over a range of 0.01–2.2 nmol/mg ($r^2 = 0.70$, $n = 26$).

Absolute V Relative Values

We use BSA treated with hypochlorous acid to generate stable DNP-reactive carbonyls to calibrate the ELISA. Carbonyls can also be generated with iron and ascorbate. For an equivalent number of carbonyls measured colorimetrically, the response in the ELISA to iron/ascorbate-oxidized BSA was 65% of that for HOCl-treated protein.[7] Thus, carbonyl values are not absolute and depend to some extent on the oxidized protein standard used. Absolute values are also affected by whether the blank is taken as the reagents without protein, or fully reduced BSA. The absorbances of these solutions are normally within 0.01 absorbance units, but this can make a difference for samples with low carbonyl content.

Although results correlate well with the colorimetric assay, absolute values for plasma samples in the two assays differ.[7] This is partly due to the standards and blanks described above. However, absolute values from the colorimetric assay are also subject to uncertainties, with different versions giving different results.[8] The assay is highly dependent on blank values and protein loss, which vary with different washing procedures.[9] With a mixture such as plasma, it is possible that some proteins adhere better to the ELISA plate than others and therefore are selected for in the assay. The main strength of the assay, therefore, is for comparing samples analyzed using a standard system.

[9] G. Cao and R. G. Cutler, *Arch. Biochem. Biophys.* **320,** 106 (1995).

Effects of DNA and RNA

Although we have not used the ELISA for tissue extracts, it should be suitable for this purpose. However, it has been reported[9] that nucleic acids in tissue extracts can contribute to carbonyl measurements so that the assay is not specific for protein carbonyls. We found that adding 3–30 μg RNA to 30 μg reduced BSA progressively increased carbonyls detected by ELISA (from zero to 0.6 nmol/mg protein), whereas DNA at the same concentration had no effect.[7] Removal of RNA before analysis of tissue extracts may therefore be necessary.

Acknowledgments

This work was supported by the Health Research Council of New Zealand.

[14] Detection of 3-Chlorotyrosine in Proteins Exposed to Neutrophil Oxidants

By ANTHONY J. KETTLE

Introduction

Neutrophils have the greatest potential of any cell type to inflict oxidant-dependent tissue injury. These phagocytic white blood cells accumulate in substantial numbers at sites of inflammation where they generate an array of destructive oxidants.[1] When stimulated, they assemble an oxidase in their cell membrane which produces superoxide by using NADPH to reduce molecular oxygen.[2] The superoxide can give rise to a number of reactive oxidants, including hydrogen peroxide, hypochlorous acid, peroxynitrite, hydroxyl radical, and singlet oxygen.[3] Neutrophil oxidants are strong contenders for contributing to the pathophysiology of inflammatory diseases. However, their involvement in inflammation remains equivocal. This is primarily due to the difficulty of detecting short-lived oxidants, and the lack of availability of unique markers for these reactive intermediates.

[1] S. J. Weiss, *N. Engl. J. Med.* **320,** 365 (1989).
[2] A. W. Segal and A. Abo, *TIBS* **18,** 43 (1993).
[3] A. J. Kettle and C. C. Winterbourn, *Redox Report* **3,** 3 (1997).

Copyright © 1999 by Academic Press
All rights of reproduction in any form reserved.

0076-6879/99 $30.00