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Original Contribution

PROTEIN CARBONYL MEASUREMENT BY A SENSITIVE ELISA METHOD

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Abstract—We describe a new immunoassay for measuring protein carbonyls as an index of oxidative injury. Protein samples were reacted with dinitrophenylhydrazine then adsorbed to wells of an ELISA plate before probing with a commercial antibody raised against protein-conjugated dinitrophenylhydrazine. The biotin-conjugated primary antibody was then reacted with streptavidin-biotinylated horseradish peroxidase for quantification. The method was calibrated using oxidized albumin and results correlated well with the colorimetric carbonyl assay. The method required only 60 μg protein and was used to analyze the amount of protein carbonyls in plasma and lung aspirate samples. It was sensitive in the 0–2.5 nmol/mg protein range within which clinical samples fell and was linear up to 10 nmol/mg protein. The ELISA method for protein carbonyls is more sensitive and discriminatory than the colorimetric assay and should have wide application for analysing experimental and clinical samples, especially where concentrations are low and where only small amounts of sample are available. © 1997 Elsevier Science Inc.

Keywords—Oxidative injury, Protein carbonyls, ELISA method.

INTRODUCTION

Assessment of the importance of oxidative injury in pathologic processes requires reliable and sensitive assays for oxidatively modified products. Oxidative stress can give rise to protein carbonyl derivatives, via a variety of mechanisms that include fragmentation and amine oxidation either due to metal catalysis or by hypochlorous acid,[1–4] and measurement of protein carbonyls as markers of injury has become popular. The carbonyl assay has been applied to experimental studies and clinical samples.[4–8] Carbonyl levels have been shown to be elevated in some pathologies[9,10] and to increase with age.[11,12] However, questions have been raised about the validity of some of these conclusions and the specificity of the assay.[13]

The conventional assay for protein carbonyls is a colorimetric procedure that measures binding of dinitrophenylhydrazine (DNP).[14–17] It requires more protein than may be available from clinical samples, it is labor intensive, and washing steps can give rise to variability.[17,18] Some of these issues can be overcome by using HPLC, which also increases sensitivity.[7,16] A Western blot procedure also increases sensitivity and selectivity,[19] but this has not been developed as a quantitative assay. Anti-DNP antibodies have also been used to detect carbonyls in tissue sections.[20] We have extended the immunological approach by developing an ELISA method based on recognition of protein-bound DNP with an anti-DNP antibody. The protein from any sample is nonspecifically adsorbed to an ELISA plate following reaction with DNP, probed with a commercial biotinylated anti-DNP antibody followed by streptavidin-linked horseradish peroxidase. This provides a quantitative assay that correlates well with the colorimetric assay. It requires only micrograms of protein and is, therefore, applicable to small samples.

MATERIALS AND METHODS

Materials

Unless noted otherwise, biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). 2,4-Dinitrophenylhydrazine was from Riedel-de-Haen (Seelze-Hannover, Germany), biotin-conjugated rabbit IgG

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polyclonal antibody raised against a DNP conjugate of keyhole limpet hemocyanin (anti-DNP), was from Molecular Probes Inc. (Eugene, OR), and streptavidin-biotinylated horseradish peroxidase was from Amersham International (Buckinghamshire, UK). Phosphate-buffered saline (PBS) contained 10 mM sodium phosphate buffer, pH 7.4, in 0.14 M sodium chloride.

Bovine serum albumin (BSA) as purchased already contained carbonyls that could be reduced by sodium borohydride. Fully reduced BSA was prepared by reacting a 1 g/100 ml solution in PBS with 2 g/100 ml sodium borohydride for 30 min, followed by neutralizing with HCl. After overnight dialysis against PBS, protein concentration was checked by A_{280} and was adjusted to 4 g/l for use in the standard curve or to 0.1% for use as blocking solution. Oxidized BSA containing additional carbonyls was prepared for use as a reference by reacting (at 50 mg/ml in PBS) with hypochlorous acid (final concentration 5 mM). Albumin solutions were stored at −80°C, or when used for blocking, at 4°C for up to a week. No changes in carbonyl content were observed under these conditions. BSA (40 mg/ml) was also oxidized with 25 mM ascorbic acid and 0.1 mM ferrous ammonium sulphate in PBS containing 1 mM EDTA.

Standard curves and calibration of the assay

Standard curves were constructed by mixing varying proportions (0–100%) of HOCl-oxidized BSA with fully reduced BSA while maintaining a constant total protein concentration. The carbonyl content of the oxidized BSA was determined from A_{375} in the colorimetric assay performed as described below. The fully reduced BSA consistently gave an A_{375} of about 0.13 per 10 mg, corresponding to the equivalent of 0.6 nmol carbonyls/mg. Further treatment with sodium borohydride did not decrease this absorbance. It was assumed to arise predominantly from free DNP or other non-specific effects rather than carbonyl derivatives and was subtracted from all readings.

Sample preparation

Plasma samples were obtained from normal individuals and from patients in intensive care whose next of kin had consented to their entering a clinical trial. Lung aspirates were also obtained from these patients. Samples were used fresh or stored at −80°C. A total protein assay was performed using the BioRad assay (BioRad laboratories, Richmond, CA) on all standards, plasmas, and aspirates, which were then diluted in PBS to a protein concentration of 4 mg/ml. Because aspirates generally contained lower protein concentrations than this, they were concentrated by mixing a volume containing 60 μg protein with 0.8 vol of 28% TCA, centrifuging at 10,000 × *g* and discarding the supernatant. PBS (15 μl) was added to the protein precipitate, which dissolved completely after adding the DNP-reagent (see below).

ELISA assay

Protein derivatization was carried out in 1.5 ml reaction tubes, with 45 μl of DNP solution (10 mM in 6 M guanidine hydrochloride, 0.5 M potassium phosphate buffer, pH 2.5; ref 16) added to 15 μl of sample (4 mg/ml) to give a final protein concentration of 1 mg/ml. Samples were incubated at room temperature for 45 min, vortexing every 10–15 min. Then 5 μl of each solution was added to 1 ml coating buffer (10 mM sodium phosphate buffer containing 140 mM NaCl, pH 7.0). Triplicate 200 μl aliquots (containing 1 μg protein) were added to wells of a Nunc Immuno Plate Maxisorp. Plates were incubated overnight at 4°C, then washed five times with PBS using a Dynatech AM60 washer between each of the following steps: blocking the wells with 0.1% reduced BSA in PBS (250 μl/well) for 1.5 h at room temperature; addition of 200 μl/well of biotinylated anti-DNP antibody (1:1000 dilution in 0.1% reduced BSA, 0.1% Tween 20 solution) and incubation for 1 h at 37°C; incubation for a further hour at room temperature with 200 μl of streptavidin-biotinylated horseradish peroxidase (1:3000 in 0.1% reduced BSA, 0.1% Tween 20 solution); addition of 200 μl of a solution containing *o*-phenylenediamine (0.6 mg/ml) and hydrogen peroxide (stock diluted 1:2500) in 50 mM Na_2HPO_4 plus 24 mM citric acid and development of color for 25 min before stopping with 100 μl of 2.5 M sulfuric acid. Absorbances were read with a 490 nm filter using a Dynatech microplate reader. A six-point standard curve of reduced and oxidized BSA was included with each plate. A blank for DNP reagent in PBS without protein was subtracted from all other absorbances.

Colorimetric carbonyl assay

To calibrate the carbonyl content of the BSA and to compare with results from the ELISA assay, colorimetric analysis of protein carbonyls was carried out by a modification of the standard method[15] in which 10 mg protein in 250 μl PBS was reacted with 1 ml 10 mM DNP in 2 M HCl for 45 min with occasional mixing, and TCA precipitates were washed three times with 2.5 ml ethanol/ethyl acetate (1:1). Pellets were broken up mechanically and with sonication. The final pellet was dissolved in 1 ml 6 M guanidine hydrochlo-

ride, 0.5 M potassium phosphate, pH 2.5, and A_{375} was measured. A blank with the protein reacted with 2 M HCl containing no DNP was carried through the procedure for each sample and its absorbance was subtracted after adjusting for the protein loss that occurs with this method.[17] Protein concentrations were determined on final extracts either (for albumin) by diluting 1:5 and measuring A_{280} (ϵ_{280} 5.31 for a 1 g/100 ml solution) or, for biological samples, by the BioRad assay. Losses were of the order of 10% and absorbances were adjusted to the equivalent of a 10 mg/ml solution. Carbonyl content was determined as nmol/mg protein using ϵ_{375} 22,000 M^{-1} cm^{-1} (ref 15) and subtracting the value for the reduced albumin.

RESULTS

Comparison of ELISA and colorimetric assay

Standard curves for a mixture of HOCl-oxidized and reduced BSA containing varying amounts of carbonyls gave linear responses in both the colorimetric (Fig. 1a) and ELISA assays (Fig. 1b). Linearity extended up to 10 nmol/mg protein. Linear responses were also obtained when increasing amounts of HOCl were added to a fixed amount of BSA and the protein was analysed directly (not shown). Treatment of the HOCl-oxidized protein with sodium borohydride resulted in a decrease in its absorbance to that of the reduced protein in both assays. To determine whether the relative response in the two assays depended on how the carbonyls were generated, HOCl-oxidized BSA was compared with BSA oxidized with iron and ascorbate. For an equivalent number of carbonyls measured colorimetrically, the response in the ELISA to iron/ascorbate-oxidised BSA was 65 ± 5% (n = 3) of that for HOCl-treated protein.

Validation of ELISA method

A key requirement for sensitivity is a low reagent blank (DNP reagent without protein). This was achieved by blocking the ELISA plate with borohydride-reduced BSA, which gave an absorbance for the reagent blank of only 0.03–0.04 absorbance units above coating buffer (~0.04). Although reduced BSA was selected on the basis that preexisting carbonyls in the blocking reagent could be problematic, limited experiments suggested that blocking with gelatin may also be satisfactory. BSA that had been reduced with sodium borohydride gave an absorbance on average 0.025 units higher than a reagent blank consisting of DNP reagent in PBS without protein. Nonderivatized protein and derivatized protein without anti-DNP treatment had the same absorbance as coating buffer alone.

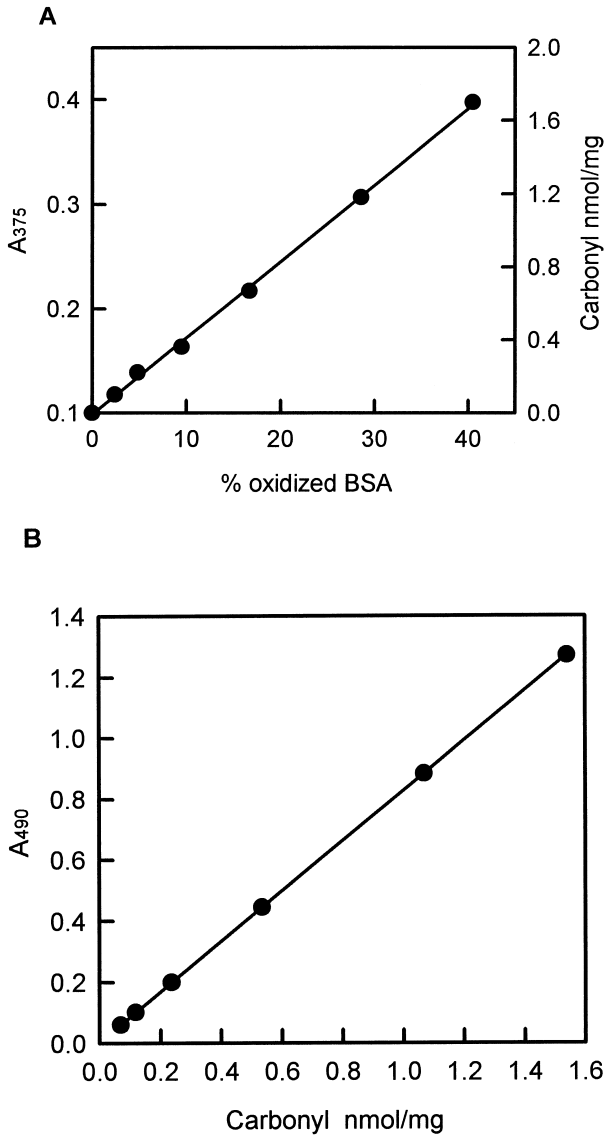

Fig. 1. Standard curves for oxidized BSA determined (A) by the colorimetric method and (B) by ELISA. Samples for the standard curves were prepared by mixing borohydride-reduced and HOCl-oxidized BSA in differing proportions to maintain a constant total protein concentration. The HOCl-oxidized BSA contained 4.2 nmol carbonyl/mg protein measured colorimetrically. Carbonyl concentrations in the BSA solutions used in the ELISA were calculated using this value and the proportion of oxidized BSA in each sample. In A, carbonyl content (right axis) was calculated from A 375 (left axis) then subtracting the reduced albumin blank. In B, the blank for fully reduced BSA has been subtracted from all absorbances.

Specificity of the DNP antibody was established by showing that concurrent addition of ≥10 μM DNP to wells coated with oxidized protein decreased absorbances to baseline levels. There was no nonspecific binding of the streptavidin-biotinylated horseradish peroxidase, as omission of the primary antibody, with BSA or plasma, decreased A_{490} to that of coating buffer.

When the amount of derivatized protein added to the

wells as coating antigen was increased, there was a linear increase in absorbance up to 0.25 μg/well, above which the increase was slight. Standard conditions were selected with 1 μg protein in 200 μl, as this gave a linear standard curve with maximal difference in response between the oxidized and reduced BSA standards. Under these conditions, minor variations in protein concentration have limited influence.

Assays were also performed with a nonbiotinylated anti-DNP antibody (rabbit IgG, raised against DNP-conjugated keyhole limpet hemocyanin, Molecular Probes) and antirabbit IgG peroxidase conjugate (Sigma A6154) as second antibody. Analyses of plasma samples using these antibodies correlated well with results using the biotin system ($r^2 = 0.79$, $n = 10$), but the biotin system was preferred because it gave a lower background and greater absorbance range for oxidized BSA. The nonbiotinylated antibody may have a use if the method is applied to tissue samples, where interference by other biotinylated proteins may be a complication. Nunc plates were selected after comparison with Falcon (3912 micro test III) plates, which showed greater interassay variability and lower absorbances for the albumin standard curve.

Reproducibility

Repeat analyses of 10 different plasma samples, carried out on three separate occasions, gave variations of 1.7–21.4% with a mean variation of ±8.8%. Most of this variation arose from analyses at the lower end of the standard curve (Table 1). Intraassay variation for nine samples analyzed in triplicate was 0.27–2.53%, with a mean of 1.4%.

Effects of DNA and RNA

It has been reported[13] that nucleic acids in tissue extracts can contribute to carbonyl measurements. To test whether this was the case for the ELISA assay, we mixed RNA or DNA in varying amounts with reduced and oxidized BSA prior to derivatization. DNA had no effect on carbonyl measurements. RNA added at 3–30 μg (RNA:protein mol ratio 0.05–0.5) to reduced BSA progressively increased the carbonyls detected from zero to 0.6 nmol/mg protein. Increases were seen, although of lesser magnitude, when the RNA was added to oxidized BSA. Thus removal of RNA from tissue extracts may still be necessary for the ELISA assay.

Analysis of plasma and lung aspirate samples

The ELISA was performed on 33 plasma samples obtained from healthy individuals and critically ill patients. Absorbances after subtraction of the reduced BSA blank were generally in the 0.01–0.5 range and when related to the HOCl-oxidized BSA standard curve gave carbonyl values of 0.01–2.14 nmol/mg protein (mean 0.54). Values were significantly lower in normal plasmas (mean 0.06, SD 0.05 nmol/mg, $n = 10$) than in patients (mean 0.75, SD 0.65 nmol/mg, $n = 23$, $p = .002$). Pretreatment of four plasma samples with sodium borohydride decreased their absorbances by 96 ± 2%, indicating a good specificity for carbonyls.

Table 1. Interassay Variability for Plasma Samples Analysed by ELISA

Carbonyl Content	*n*	Mean	% SD
<0.08 nmol/mg	3	0.06	16.7
0.3–0.7 nmol/mg	4	0.49	6.0
>0.8 nmol/mg	3	1.06	4.5

There was good correlation between the ELISA and colorimetric methods for the plasma samples (Fig. 2, $r^2 = 0.70$, $n = 26$), although absolute levels were lower for the colorimetric assay (mean 0.12 nmol/mg protein). Absorbances of some samples in the colorimetric assay were lower than that of reduced albumin, and therefore gave negative carbonyl values. This is an inherent problem of operating at the lower limit of the assay where the values and variability of the blanks are critical. The mean absorbance for the plasma samples analysed by the colorimetric assay was only 0.03 after subtraction of individual sample and reduced albumin blanks of up to about 0.25. This was less of a problem in the ELISA where plasma absorbances were on average several times higher than the blank.

It was possible to measure protein carbonyls by ELISA in lung aspirates obtained from critically ill patients. The aspirates were collected by a standard suctioning procedure and washing out the suction tubes with saline, and had protein concentrations between 0.21 and 16.4 mg/ml. The carbonyl content ranged from 0.09–1.61 (mean 0.57) nmol/mg protein ($n = 13$). Protein from these samples was concentrated by TCA-precipitation before the DNP derivatization step of the assay. Prior TCA precipitation did not alter the standard curve for BSA, but for eight aspirates with high enough protein concentrations to analyse directly, direct analyses gave results that were 20% lower than with TCA precipitation ($p = .0011$).

DISCUSSION

We have described an ELISA method for measuring protein carbonyls that is sensitive and reproducible. Because it involves adsorption of the protein to the plastic ELISA plate, unconjugated DNP and nonprotein con-

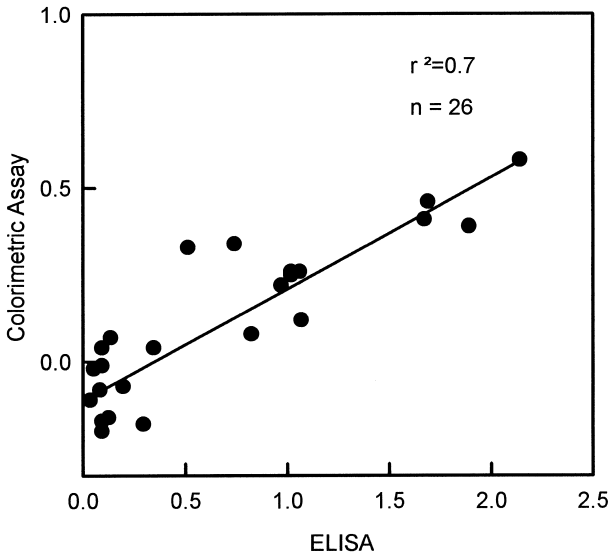

Fig. 2. Correlation between carbonyl concentrations, expressed as nmol/mg protein in plasma samples measured by ELISA and colorimetric methods.

stituents are easily washed away and give minimal interference. This allows much greater sensitivity and accuracy at the lower end of the range than for the colorimetric assay. The ELISA requires only 60 μg protein, which is similar to the requirement for the HPLC method,[16] compared with the 10 mg required for optimal results with clinical samples using the colorimetric assay.[6]

The ELISA was calibrated against BSA treated with hypochlorous acid, the carbonyl content of which was measured colorimetrically. Oxidation by HOCl proved to be a simple way of generating stable DNP-reactive carbonyls, all of which were reducible with sodium borohydride. We found, however, that the method of generating carbonyls had some effect on the ELISA response, with those generated with ascorbate and iron giving 65% of the response seen with HOCl. Thus, absolute carbonyl values will depend on the oxidized protein standard used. HOCl and ascorbate/iron will not necessarily generate carbonyls on the same protein residues, and a possible reason for the different responses is that DNP conjugates at different sites do not react uniformly with the anti-DNP antibody.

Others have also questioned absolute values from the colorimetric assay after finding that different versions gave different results.[18] The assay is highly dependent on blank values, which vary with different washing procedures and how much free DNP is retained.[13] Protein loss during washing also needs to be taken into account. As others have noted with human albumin,[8] we found that commercial BSA already contained carbonyl groups. We took the absorbance of fully reduced albumin as blank, on the assumption that it was nonspecific and due mainly to free DNP. Absolute results are higher if this blank is not subtracted, although comparative values are not affected. However, it is possible that the amounts of DNP retained by protein precipitates of pure BSA and plasma differ, and this may explain why subtraction of the blank sometimes gave negative values.

The ELISA was readily applicable to the analysis of plasma. We selected samples from healthy and critically ill subjects, so as to test sensitivity over a range of carbonyl values. The values extended over the full 0–2 nmol/mg standard curve, with results for healthy individuals grouped in the lower quarter. This allowed ample sensitivity for detecting abnormalities. The method was also used successfully with lung aspirates, where often there would be too little protein to perform a colorimetric assay. A protein concentration step using TCA precipitation was generally required. Carbonyl values were about 20% higher than if the samples were analyzed directly, perhaps because TCA modifies the adsorption characteristics of the protein (see below). The aspirates were not paired with plasma samples to allow direct comparison, but the levels of carbonylation were of the same order as in plasma. Gladstone et al.[7] measured carbonyl levels by HPLC in infant aspirates of up to 4 nmol/mg protein (using an average protein molecular weight), which is in a similar range to ours.

Of considerable interest is our finding that carbonyl values for critically ill patients were significantly elevated. Higher values in limited numbers of adults with respiratory distress syndrome[6] and ventilated premature infants with high inspired oxygen[7] have been reported previously. The relationship of our results to clinical outcome is the subject of an ongoing investigation that will be published in detail elsewhere.

There was a good correlation between the ELISA and colorimetric assays for plasma samples. The absolute values differ, but this is not surprising in view of the uncertainties affecting absolute values in both assays. With a mixture such as plasma, it is possible that some proteins would adhere better to the ELISA plate than others and, therefore, be selected for in the assay. Because some plasma proteins, for example, fibrinogen, are more carbonylated than others,[19] results could differ from assays that analyze total protein. Such selection might explain variations between different ELISA plates. However, the good correlation with the colorimetric assay suggests that this is a minor factor. Carbonyls arise directly from protein oxidation and are secondary conjugates of aldehydes formed, for example, by lipid peroxidation.[8,9] Neither method can distinguish between these sources.

The ELISA method is a highly sensitive assay for

protein carbonyls that requires only microgram amounts of protein. As with the colorimetric assay, absolute values are subject to some uncertainty, and the method is best for comparing samples analyzed using a standard system. It should have wide application for measuring protein oxidation both experimentally and clinically, especially where only small amounts of protein can be obtained.

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ABBREVIATIONS

BSA—bovine serum albumin
DNP—dinitrophenylhydrazine
ELISA—enzyme linked immunoassay
PBS—phosphate-buffered saline

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Erratum

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Buss, H.; Chan, T. P.; Sluis, K. B.; Domigan, N. M.; Winterbourn, C. C. Protein carbonyl measurement by a sensitive ELISA method. *Free Radic. Biol. Med.* **23:**361–366; 1997.

The method described in the above article for reducing existing carbonyls in bovine serum albumin contained an error in the amount of sodium borohydride to be added. The result is excessive hydrogen evolution and foaming. The following method should be followed:

Preparation of reduced BSA:
Add 0.1 g solid $NaBH_4$ to a solution of 0.5 g BSA in 100 ml PBS. After 30 min, bring to neutral pH with 2 M HCl, added slowly. Since this reaction produces hydrogen, it should be carried out in a fume hood. Then dialyse and adjust protein concentration as required.

Blocking and antibody solutions:
PBS containing 0.1% Tween 20 can be used with similar results instead of 0.1% reduced BSA with 0.1% Tween 20, thereby avoiding the preparation of bulk amounts of reduced BSA.

The authors, editors, and publisher regret any inconvenience this error may have caused.