PS2.07 Quantification of protein carbonyls in meat by DNPH-ELISA analysis 407.00

Sisse Jongberg (1) jongberg@life.ku.dk, Michael Rasmussen(1), Marianne N. Lund (1), Leif H. Skibsted (1)
(1)University of Copenhagen

Abstract **Protein carbonyl compounds are formed in meat during storage under oxygen, and the concentration of carbonyls is often used as a measure of the extent of protein oxidation. A DNPH-ELISA analysis was optimized for evaluation of protein oxidation in meat. The method is based on the traditional colorimetric carbonyl analysis but has a higher sample through-put. Carbonylated protein was adsorbed to the well of an ELISA (enzyme-linked immunosorbent assay) microplate, and unspecific binding sites were blocked with Tween 20 before derivatisation with 2,4-dinitrophenylhydrazine (DNPH). An anti-DNP polyclonal antibody from rabbit was used as primary antibody, and an anti-rabbit antibody from goat was used as secondary antibody. The secondary antibody was conjugated with horse-radish peroxidase (HRP), which can be quantified spectrophotometrically after reaction with the substrate TMB One (3,3',5,5'-tetramethylbenzidine). Oxidized BSA was used as an internal standard, and a calibration curve ranging from 1-10 nmol/mg protein was established.**

S. Jongberg is with the Department of Food Science, Faculty of Life Science, University of Copenhagen, DK-1958 Frederiksberg, Denmark (corresponding author: phone: +45 35 33 32 90; fax: +45 35 33 33 44; e-mail: jongberg@life.ku.dk).

M. Rasmussen is with the Department of Food Science, Faculty of Life Science, University of Copenhagen, DK-1958 Frederiksberg, Denmark (e-mail: micr@life.ku.dk).

M. N. Lund is with the Department of Food Science, Faculty of Life Science, University of Copenhagen, DK-1958 Frederiksberg, Denmark (e-mail: mnl@life.ku.dk).

L. H. Skibsted is with the Department of Food Science, Faculty of Life Science, University of Copenhagen, DK-1958 Frederiksberg, Denmark (e-mail: ls@life.ku.dk).

Index Terms**—protein oxidation, carbonyl formation, DNPH, ELISA.**

I. INTRODUCTION

PROTEIN oxidation decreases the eating quality of meat products. Packaging in high oxygen atmosphere accelerates the oxidation processes of both lipid and protein. Lipid oxidation products are responsible for the development of off-flavours in meat [1], and protein oxidation has been shown to affect both tenderness and the water holding capacity [2].

Proteins oxidize by several mechanisms, and many of these protein oxidation products have been found in meat products, e.g. cross-linking [2] and carbonyl formation [3]. Carbonyl formation is a precursor for both fragmentation and cross-linking, and especially the latter has great impact on the tenderness and juiciness of the meat.

Quantification of protein carbonyl groups is one of the most frequently used indicative of protein oxidation in various biological systems [4]. Carbonyl groups (CO), ketones and aldehydes, are introduced on different moieties of the protein, both at amino acid side chains and the backbone. The colorimetric DNPH carbonyl analysis is the most frequently used method for determination of carbonyl compounds in biological systems [4-6], however, there are certain disadvantages related to the method, especially when working with non-purified proteins, which often results in very low repeatability [7].

The aim of this study is to optimize the DNPH-ELISA method in order to determine the carbonyl concentration in myofibrillar protein extracts.

II. MATERIALS AND METHODS

A. Immunologic carbonyl determination – DNPH-ELISA

Protein carbonyl groups were quantified as described by Alamdari et al. [8] with some modifications. Triplicate 100 µl protein containing 10 µg/ml protein diluted in coating buffer (100 mM carbonate buffer, pH 9.6) was added to the wells of a microplate. The plate was incubated over night at 4 °C, and subsequently washed three times with 300 µl phosphate buffered saline (PBS). An aliquot of 300 µl blocking solution, PBS with 0.05 % Tween 20 (PBST), was added to the wells. The plate was incubated for 1.5 hours at room temperature, and washed three times with 300 µl PBST. For derivatization, 1.2 µM DNPH dissolved in 0.6 % phosphoric acid (pH 6.2) was freshly prepared. The DNPH solution was filtered with a 0.22 µm filter before use. DNPH solution (100 µl) was added to the wells, incubated for 45 minutes at room temperature in the dark, and then washed five times with 300 µl PBS:ethanol (1:1) and one time with 300 µl PBST. The polyclonal antibody, anti-DNP (100 µl) was diluted 1:10000 in 0.5 % (w/v) BSA (bovine serum albumin) diluted in PBST, and was added to the wells, incubated for 1 hour at room temperature, and washed five times with PBST. Secondary antibody (100 µl), anti-rabbit-HRP diluted 1:10000 in 0.5 % (w/v) BSA diluted in PBST was added to the wells, incubated for 1 hour at room temperature and washed five times with

PBST. TMB-One (3,3',5,5'-tetramethylbenzidine) (100 µl/well) was added and the reaction was stopped after 3-5 minutes by addition of 100 µl 0.3 M H_2SO_4. The absorbance was read at 450 nm.

A calibration range of oxidized BSA prepared from a stock-solution with a known concentration of carbonyls (determined by the colorimetric carbonyl determination assay) was used as internal standard. The calibration range consisted of 9 points ranging from 30 nmol/mg protein to <0.01 nmol/mg protein. The calibration range was a consecutive factor √10-dilution of oxidized BSA using reduced BSA as diluent in order to keep a constant protein concentration in the wells (10 µg/ml or 1 µg/well). The standard curve was measured in triplicate, and included in each ELISA microplate assay.

B. Internal standard preparation

Reduced BSA was prepared by mixing 2 ml 10 mg/ml BSA, 8 ml 0.2 M borate buffer, and 500 µl 3.7 % (w/v) $NaBH_4$. After 30 min at room temperature 6 drops of 12 M HCl was added to neutralize the solution. Oxidized BSA was prepared as described by Alamdari et al. [8]. BSA (50 mg) was dissolved in 1 ml PBS, 20 µl 100 mM EDTA, 57 µl 833 mM ascorbic acid, and 2 µl 100 mM ferrous ammonium sulphate. The solution was incubated at 37 °C for 1.5 h.

The volume of the reduced and oxidized BSA was reduced to 1 ml by using spin filter centrifugation (5 kDa cut-off) at 3000 rpm for 2 hours. Subsequently 10 ml PBS was added, and the volume was once again reduced to 1 ml by centrifugation. This procedure was repeated three times. The protein concentration was determined as described below, and the internal standards were stored at 4ºC until use.

C. Protein determination

The protein concentration was determined by Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using BSA as standard. The assay was carried out in a microplate using 5 µl sample or standard in triplicate and 250 µl 1x dye reagent (Coomassie Brilliant Blue G-250), which was let to react for 10 min before spectrophotometric determination at 595 nm.

D. Colorimetric carbonyl determination

The internal standard preparations were derivatized as described by Levine et al. [9]. Aliquots of 500 µl sample (reduced or oxidized BSA) containing 1 mg protein, was mixed with 500 µl 10.0 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.0 M HCl. Blank samples were prepared by mixing 500 µl sample and 500 µl 2.0 M HCl without DNPH. After mixing, the samples were placed in a water bath at 37 °C for 1 hour to derivatize. All samples were vortexed every 10 min. Subsequently, the samples were added 325 µl 50 % (w/v) trichloroacetic acid (TCA), vortexed, placed on ice for 10 min, and spun in a Microcentrifuge 154 (Ole Dich Instrumentmakers ApS, Hvidovre, Denmark) for 15 min at 11 000 g after which the supernatant was decanted. The protein pellet was washed as decribed by Fagan et al. [7] by adding 5.0 ml ethanol:ethylacetate (1:1), vortexing, letting react for 10 min, and then spun for 10 min at 11,000 g. This wash procedure was repeated three times. After the final wash, the pellet was dissolved in 1.0 ml 6.0 M guanidine hydrochloride dissolved in a 20 mM potassium dihydrogen phosphate buffer (pH 2.3), placed in water bath at 37 °C for 30 min, and finally centrifuged to remove insoluble material. The carbonyl concentration in the samples was determined spectrophotometrically by measuring the absorbance at 375 nm and 280 nm on a Cary 3 UV-Visible Spectrophotometer (Varian, Herlev, Denmark). The concentration of protein carbonyl (nmol carbonyl per mg protein) was calculated according to the method by Levine et al. [10]:

$$\frac{c_{hydrazone}}{c_{protein}} = \frac{A_{370}}{\varepsilon_{hydrazone,370} \cdot (A_{280} - A_{370} \cdot 0.43)} \cdot 10^6 \left[mol / g \right]$$

The blank value was subtracted from the corresponding sample value. Triplicate measurements were made for reduced and oxidized BSA.

III. RESULTS AND DISCUSSION

The DNPH-ELISA analysis is a merger between the colorimetric carbonyl determination using DNPH as derivatizing agent, and the immunologic technique of ELISA. Figure 1 shows a schematic overview of the reactants involved in the DNPH-ELISA. Carbonylated protein is adsorbed to the well of a microplate. Unspecific binding sites are blocked by PBST, and the protein carbonyls are derivatized with DNPH. A primary antibody, anti-DNP antibody from rabbit is added to the well, following wash and subsequent addition of a secondary antibody, anti-rabbit antibody from goat conjugated with horse-radish-peroxidase (HRP). Reaction with the substrate TMB-One results in a colored compound. The intensity of the color is proportional to the amount of protein carbonyls in the well, and can be determined spectrophotometrically.

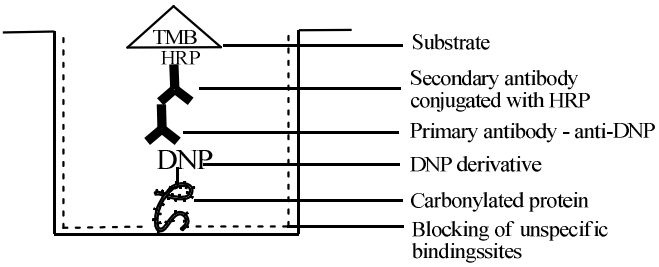

Figure 1. A schematic overview of compounds and reagents in the DNPH-ELISA analysis.

The concentration of DNPH, which was used to derivatize the protein carbonyls, was adjusted, as it was observed that DNPH binds unspecifically in the well resulting in an artificial high signal for reduced BSA (which should contain no carbonyl groups). As seen in Figure 2, a 100-fold dilution of the 1.2 mM DNPH concentration used by Alamdari et al. [8] resulted in a signal of reduced BSA similar to the background signal.

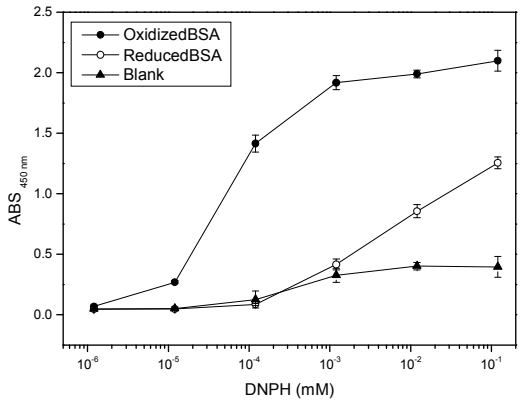

Figure 2. Determination of required DNPH concentration in DNPH-ELISA. Analysis of oxidized BSA, reduced BSA or a blank by varying DNPH concentrations.

A 1000-fold dilution ($1.2 \cdot 10^{-4}$ mM DNPH) resulted in minimum background signal, however, unfortunately the protein carbonyl groups were not saturated with DNPH at $1.2 \cdot 10^{-4}$ mM DNPH, as they were at $1.2 \cdot 10^{-3}$ mM DNPH (Figure 3).

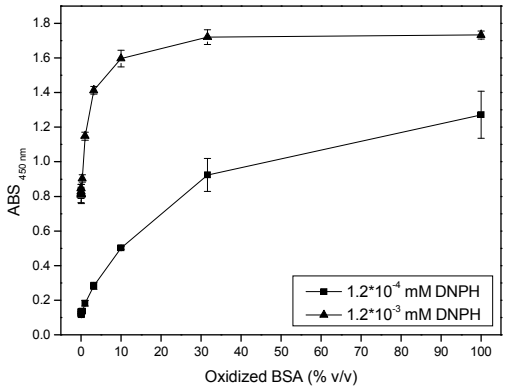

Figure 3. The signal after derivatization with $1.2 \cdot 10^{-4}$ mM DNPH or $1.2 \cdot 10^{-3}$ mM DNPH by increasing levels of oxidized BSA. The concentration of carbonylated protein is varied, while the protein concentration is maintained constant by diluting with reduced BSA.

In order to minimize the background signal caused by unspecific binding of DNPH, the microplate was blocked with PBST before derivatization instead of blocking after derivatization, which was originally done by Alamdari et al. [8]. The blocking of unspecific binding sites in the well was successful because PBST contains the detergent Tween 20, which has no reactivity towards DNPH.

The internal standard curve was prepared by dilution of oxidized BSA with a known carbonyl concentration. The internal standard curve is seen in Figure 4.

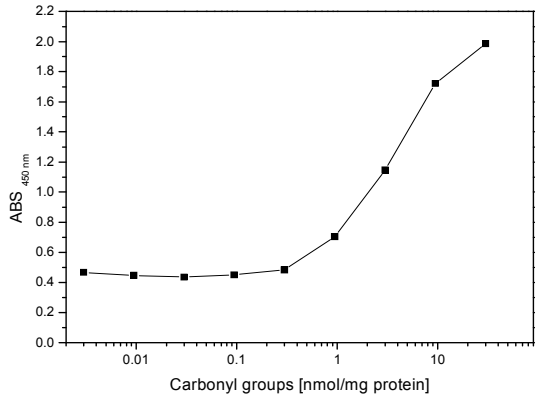

Figure 4. Internal standard curve of oxidized BSA for the DNPH-ELISA analysis. The carbonyl concentration in a meat sample can be quantified within the linear area of the curve ranging from 1-10 nmol/mg protein.

This preliminary study forms the basis of evaluating the protein carbonyl concentration in meat. The

optimized DNPH-ELISA method is suitable for quantification of carbonyls in meat, and ongoing research will fully implement DNPH-ELISA for quantification of protein carbonyls in myofibrillar protein extracts from beef. Further studies is however necessary in order to validate the capability of the method in relation to meat protein.

IV. CONCLUSION

An optimized DNPH-ELISA analysis for evaluation of carbonyl groups in meat was obtained. The method by Alamdari et al. [8] was modified by decreasing the DNPH concentration to 1.2 μM, and by blocking unspecific binding sites with PBST before derivatization in order to reduce the background signal. Ongoing research will determine the protein carbonyl concentration in myofibrillar protein extracts from beef.

REFERENCES

[1] Skibsted, L.H., Mikkelsen, A., & Bertelsen, G. (1998). Lipid-derived off-flavours in meat. In F. Shahidi, Flavour of meat, meat products and seafoods, 2 ed. (pp. 217-256)

[2] Lund, M.N. (2007). Protein oxidation in meat during chill storage. PhD thesis, Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Denmark.

[3] Martinaud, a., Mercier, Y., Marinova, P., Tassy, C., Gatellier, P., & Renerre, M. (1997). Comparison of oxidative processes on myofibrillar proteins from beef during maturation and by different model oxidation systems. Journal of Agricultural and Food Chemistry, 45, 2481-2487.

[4] Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. Clinica Chimica Acta, 329 (1-2), 23-38.

[5] Yuan, Q., Zhu, X., & Sayre, L.M. (2007). Chemical nature of stochastic generation of protein-based carbonyls: Metal-catalyzed oxidation versus modification by products of lipid oxidation. Chemical Research in Toxicology, 20, 129-139.

[6] Astruc, T., Marinova, P., Labas, R., Gattellier, P., & Sante-Lhoutellier, V. (2007). Detection and localization of oxidized proteins in muscle cells by fluorescence microscopy. Journal of Agricultural and Food Chemistry, 55, 9554-9558.

[7] Fagan, J.M., Sleczka, B.G., & Sohar, I. (1999). Quantification of oxidative damage to tissue proteins. International Journal of Biochemistry and Cell Biology, 31, 751-757.

[8] Alamdari, D.H., Kostidou, E., Paletas, K., Sarigianni, M., Konstas, A.G.P., Karapiperidou, A., & Koliakos, G. (2005). High sensitive enzyme-linked immunosorbent assay (ELISA) methd for measuring protein carbonyl in samples with low amounts of protein. Free Radical Biology and Medicine, 39, 1362-1367.

[9] Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., & Stadtman E.R. (1990). Determination of carbonyl content in oxidatively modified proteins. Methods in Enzymology, 186, 464-478.

[10] Levine, R.L., Williams, J.A., Stadtman, E.R., & Shacter, E. (1994). Carbonyl assays for determination of oxidatively modified proteins. Methods in Enzymology, 233, 346-357.