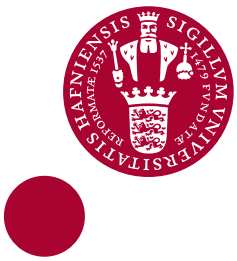

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Quantification of protein carbonyls in meat by DNPH-ELISA analysis

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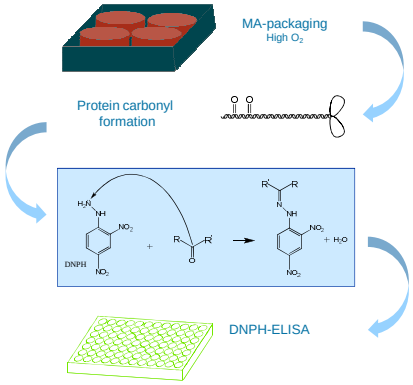
and Leif H. Skibsted

Aim

Development of a DNPH-ELISA analysis for detection of protein carbonyls formed during storage of meat in high oxygen atmosphere packaging.



Experimental

Immunological carbonyl determination - DNPH-ELISA
Protein carbonyl groups were quantified as described by Alamdari et al. [1] with some modifications. Triplicate 100 µl sample or standard containing 10 µg/ml protein diluted in coating buffer was added to the wells of a microplate. The plate was incubated over night at 4 ℃, and subsequently washed three times with PBS 0.05 % Tween 20 (PBST). The plate was blocked for 1.5 hour with blocking solution PBST, and washed. Each well was added 1.2 µM DNPH (2,4-dinitrophenylhydrazine) and incubated 45 min for derivatization. The polyclonal antibody, anti-DNP diluted 1:10000 in 0.5 % (w/v) BSA was used as primary antibody, and anti-rabbit-HRP diluted 1:10000 in 0.5 % (w/v) BSA was used as secondary antibody. Both antibodies was added in aliquots of 100 µl and incubated for 1 hour. TMB-One (3,3',5,5'-tetramethylbenzidine) was used as substrate, and the reaction was stopped after 10 minutes by addition of 0.3 M H_2SO_4. The absorbance was read spectrophotometrically at 450 nm. Figure 1 shows a schematic overview of the reactants involved in the DNPH-ELISA.

Carbonyl standard preparation
A carbonyl standard was prepared from oxidized BSA [1] with a known concentration of carbonyls, which was determined by the colorimetric carbonyl determination assay [2]. Oxidized BSA was diluted in reduced BSA [1] to maintain constant protein concentration (10 µg/ml or 1 µg/well). The carbonyl standard was included in each ELISA microplate assay, and measured in triplicate.

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

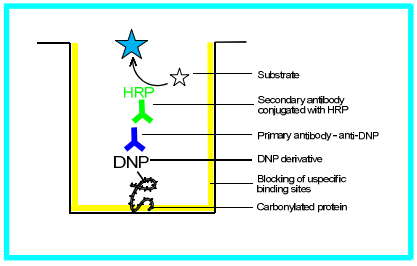
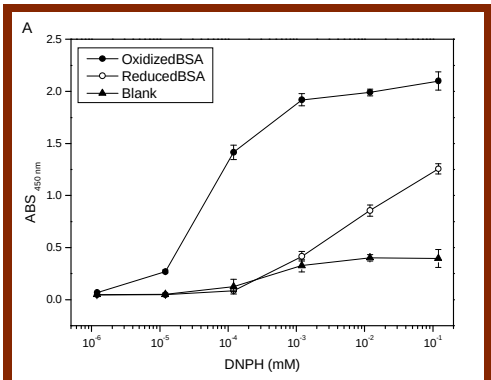
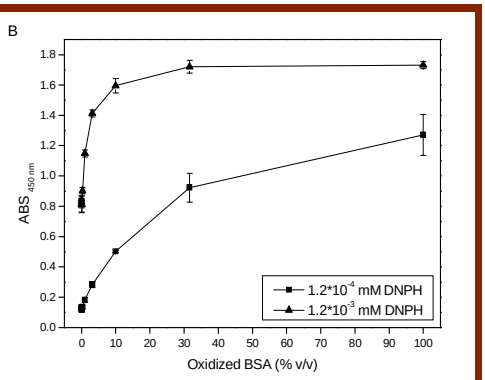


Figure 1.
A schematic overview of compounds and reagents in the DNPH-ELISA assay.





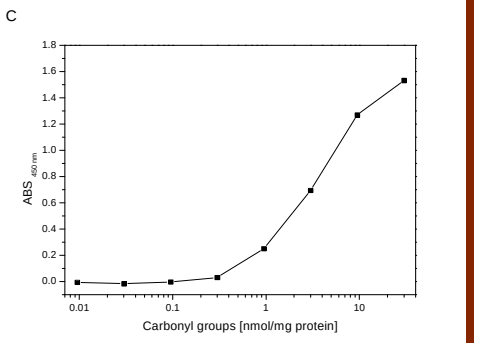


Figure 2.

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

B: The absorbance 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.

C: Dose-repsonse of carbonyl standard prepared from oxidized BSA diluted in reduced BSA. The carbonyl concentration in a meat sample can be quantified within the linear area of the curve ranging from ~1-10 nmol/mg protein.

Results and Discussion

Adjustment of the DNPH concentration
The concentration of DNPH, which was used to derivatize the protein carbonyls, was changed compared to the method by Alamdari et al. [1]. It was observed that DNPH binds unspecifically to the well resulting in an artificially high absorbance for reduced BSA (which contains no carbonyl groups) and blank samples (no protein). Figure 2A shows the absorbance of oxidized BSA, reduced BSA and a blank sample against the DNPH-concentration. The figure indicates that DNPH binds unspecifically to the well, and a lower DNPH concentration is preferable in order to reduce the signal to noise ratio.

Alamdari et al. [1] used 0.12 mM DNPH for derivatization. Our experiments showed that by reducing the concentration, a lower background signal was obtained, and thereby less unspecific binding of DNPH. A 1000-fold dilution to $1.2 \cdot 10^{-4}$ mM DNPH resulted in a minimum background signal. However, as shown in Figure 2B, a larger dynamic range at low carbonyl concentration is obtained at $1.2 \cdot 10^{-3}$ mM DNPH, hence, this concentration is preferable for carbonyl quantification in meat products

Blocking of unspecific binding sites
In order to minimize the background signal caused by unspecific binding of DNPH, the microplate was blocked with PBST before and after derivatization instead of only blocking after derivatization, which was originally done by Alamdari et al. [1].

Blocking before derivatization ensures that DNPH does not bind unspecifically to the well, while blocking after derivatization ensures that the blocking surface is maintained after the washing procedure, which contains ethanol. The dose-response of the carbonyl standard using the adjusted DNPH-concentration and the additional blocking step is shown in Figure 2C.

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.

Conclusion

An optimized DNPH-ELISA analysis for evaluation of carbonyl groups in meat was developed. The method by Alamdari et al. [1] was modified by the following steps:

- The DNPH-concentration was reduced to 1.2 µM for derivatization of protein carbonyls
- Unspecific binding sites were blocked with PBST both before and after derivatization

Ongoing research will determine the protein carbonyl concentrations in myofibrillar protein extracts from beef.

References

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