EFFECT OF SODIUM NITRITE ON PROTEIN OXIDATION IN PORK MYOFIBRILLAR PROTEINS

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Abstract – This study focuses on the effect of $NaNO_2$ on induced protein oxidation in isolated pork myofibrillar proteins and the possible use of 3-nitrotyrosine, a marker for oxidative stress *in vivo*, as a specific marker for reactive nitrogen species mediated nitration in processed muscle foods.

Higher protein carbonyls and lower thiol concentrations were found in the $NaNO_2$ treated samples immediately after addition of oxidants and $NaNO_2$, suggesting an initial pro-oxidative effect of $NaNO_2$. No effect of $NaNO_2$ was observed at later stages of oxidation. 3-Nitrotyrosine was present in all samples, but no clear effect of $NaNO_2$ addition was observed on this protein modification.

Key Words – 3-nitrotyrosine, Meat proteins, Protein carbonyls, Thiol groups

I. INTRODUCTION

Nowadays, protein oxidation in meat products receives increasing research interest. Protein oxidation is defined as the covalent modification of a protein, induced by reactions with reactive oxygen and nitrogen species or secondary oxidation products. As many other proteins, myofibrillar proteins are susceptible to oxidative reactions with myosin being the most sensitive [1]. Oxidative modifications of proteins can change their physical and chemical properties, which can be involved in the regulation of fresh meat quality and influence the processing properties of meat products [2].

Strangely, the effect of $NaNO_2$ on protein oxidation in meat products has not yet been investigated, although it is commonly used when curing meat and it has several properties affecting proteins. Different biomarkers such as the formation of protein carbonyls, loss of thiol groups, protein fragmentation and aggregation are commonly used to quantify or characterize protein oxidation processes in muscle foods [1]. However, for this study, utilization of more specific markers that could directly evaluate the role of $NaNO_2$ in the oxidative stability of food products was desirable. In this respect, 3-nitrotyrosine could be an interesting marker. It is formed by nitration of tyrosine via the peroxynitrite radical, which is formed by the reaction of nitric oxide and superoxide [3]. 3-Nitrotyrosine was previously found in oxidatively modified chicken muscles [4], but it has to our knowledge not yet been used as marker in cured and uncured processed meats.

This study focuses on the effect of $NaNO_2$ on induced protein oxidation in isolated myofibrillar proteins and the potential use of 3-nitrotyrosine as a specific marker for protein oxidation in combination with $NaNO_2$.

II. MATERIALS AND METHODS

Myofibrillar protein isolates (MPI) of pig *longissimus dorsi* were prepared according to Park *et al.* [5]. The MPI was oxidized under meat processing conditions according to Estevez *et al.* [6]. Briefly, MPI (20 mg protein/mL) was suspended in 15 mM piperazine-N,N bis(2-ethane sulfonic acid buffer (pH 6.0) containing 0.6 M NaCl. The MPI suspensions were oxidized with 0.01 mM $FeCl_3$, 0.1 mM ascorbic acid and 1 mM H_2O_2 at 37°C for 10 days under constant stirring. Three concentrations of $NaNO_2$ (0, 100 or 1000 mg $NaNO_2$/kg protein) were added. At sampling days 0, 4, 7 and 10, suspensions were divided in aliquots of 1.0 ml and stored at -80°C until analysis. MPI

suspended in PIPES buffer without oxidants and $NaNO_2$ and stored at day 0 was used as negative control.

The thiol and protein carbonyl concentration was determined spectro-photometrically after derivatisation with 5,5'-dithiobis(2-nitrobenzoic acid) and 2,4-dinitrophenyl hydrazine respectively. Both methods are described by Jongberg *et al.* [7].

3-Nitrotyrosine and actin were detected by western immunoblotting (n=2) using respectively a mouse monoclonal anti-nitrotyrosine antibody (Abcam, UK, 1:500 dilution) and rabbit anti-actin antibody (Sigma-Aldrich, Belgium, 1:2000 dilution) as primary antibody and IRDye 800 goat anti-mouse IgG (Li-COR, USA, 1:5000 dilution) and IRDye 680 Goat anti-Rabbit IgG (Li-COR, USA, 1:5000 dilution) as secondary antibody. Gels were scanned and quantified with an Odyssey infrared fluorescence detection system.

Statistical analysis was performed using SPSS 21.0. Carbonyl and thiol concentrations were analysed by ANOVA with incubation time (days) and $NaNO_2$ concentration as fixed variables. As the interaction term days×$NaNO_2$ was significant, a new variable 'treatment' combining 'day' and '$NaNO_2$' was used. Mean differences between treatments were tested using the Duncan *post-hoc* test operating at a 5% level of significance.

III. RESULTS AND DISCUSSION

Induced *in vitro* oxidation of MPI resulted in a significant increase in protein carbonyl concentrations (Figure 1). The values and curves obtained for the progress of protein carbonyl formation compare well with the observations of [8]. The concentrations found in the MPI samples are higher compared to those usually found in real meat products, as the concentrations of oxidants applied in a model system are designed to stimulate oxidation.

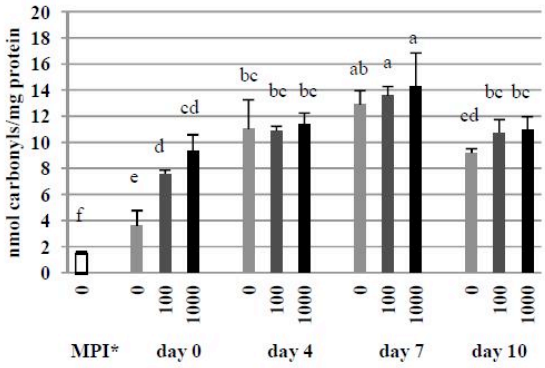

Figure 1 Protein carbonyl concentration (mean±SD, n=3) during induced *in vitro* oxidation of myofibrillar protein isolates with different $NaNO_2$ levels (0, 100 and 1000 mg/kg) . *MPI samples at day 0 suspended in PIPES buffer without pro-oxidants and $NaNO_2$.

A pro-oxidative effect of $NaNO_2$ was seen at day 0, immediately after the addition of the oxidants $FeCl_2$, H_2O_2 and ascorbic acid, but before incubation at 37°C. No dose effect was found as no significantly different carbonyl concentrations were found between 100 and 1000 mg/kg $NaNO_2$ at day 0. No differences between $NaNO_2$ treatments were found within the other sampling days.

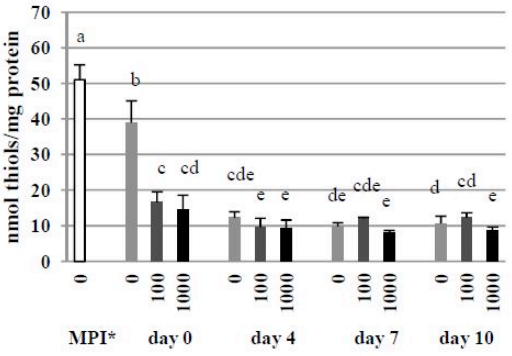

Figure 2 Protein thiol concentration (mean±SD, n=3) during induced *in vitro* oxidation of myofibrillar protein isolates with different $NaNO_2$ levels (0, 100 and 1000 mg/kg). *MPI samples at day 0 suspended in PIPES buffer without pro-oxidants and $NaNO_2$.

The thiol groups of the MPI were significantly affected by the induced oxidation (Figure 2). From the moment the oxidants were added, the thiol content decreased 5-fold compared to the untreated MPI and remained steady onwards.
Like for the protein carbonyl content, only at day 0 a significant effect of $NaNO_2$ was observed. Also for this marker $NaNO_2$ acted as a pro-oxidant as no addition of $NaNO_2$ resulted in twofold higher thiol concentrations compared to the addition of both 100 and 1000 mg/kg $NaNO_2$ at day 0.

$NaNO_2$ has antioxidant activity against lipid oxidation, which is explained by its ability to break the radical chain processes after its conversion to NO• and by chelating iron, a known oxidation promoter [9]. As the mechanism behind the oxidation of proteins is believed to proceed via a free radical chain reaction similar to that of lipid oxidation [1], it was expected that $NaNO_2$ would have had a positive effect against protein oxidation. However, $NaNO_2$ could also act as pro-oxidant. After its reduction to NO•, peroxynitrite ($ONOO^-$) is formed by reacting with $O_2•^-$. Peroxynitrite can induce lipid oxidation in food systems [10], protein oxidation *in vivo* [11] and cause discolouration of muscle food by oxidizing oxymyoglobine [12].
According to Rubbo *et al.* [13], the pro-oxidant versus antioxidant outcome critically depends on the relative concentrations of individual reactive species such as $O_2•^-$, H_2O_2 and OH•, which are abundantly present in *in vitro* oxidation systems. It should therefore be further explored how $NaNO_2$ would react in real meat products, instead of in isolated myofibrillar proteins subjected to induced oxidation.

Immunoblotting using an antibody against 3-nitrotyrosine was performed to investigate the occurrence of 3-nitrotyrosine in the oxidized myofibrils. Figure 3 confirms the presence of 3-nitrotyrosine in all samples, independent of the $NaNO_2$ treatments. As a loss of actin was seen during oxidation (data not shown), the band intensity of 3-nitrotyrosine in actin (~40 kDa) was only compared within each incubation day.

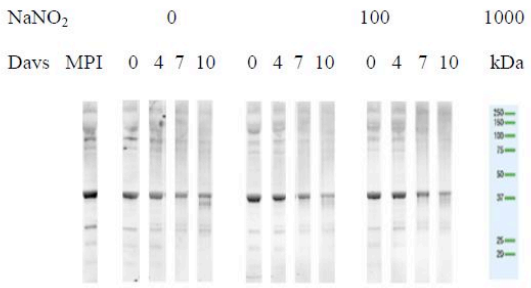

Figure 3 Representative Western blot for protein tyrosine nitration during induced *in vitro* oxidation of myofibrillar protein isolates with different $NaNO_2$ levels. *MPI samples at day 0 suspended in PIPES buffer without oxidants.

In this study, no clear effect of the addition of $NaNO_2$ on the 3-nitrotyrosine content in actin could be found, however, further investigations are necessary to explore the occurrence of 3-nitrotyrosine in muscle foods and its potential influence on their quality. Only one study was found on this topic [4], in which significantly higher concentrations of 3-nitrotyrosine in meat of chickens fed a low antioxidant diet were measured compared to chickens fed a low antioxidant diet in combination with corn. These authors suggested that the nitration of actin could have implications on the texture and water holding capacity of fresh meat. Still, this does not imply that the 3-nitrotyrosine concentration is affected *post mortem* and that this is a good marker for protein oxidation in relation to the quality of muscle foods.

IV. CONCLUSION

$NaNO_2$ showed pro-oxidative activity in isolated myofibrillar proteins. Since oxidation processes in food systems depend on several factors, it should be further investigated how $NaNO_2$ may affect protein oxidation in whole muscle foods. 3-Nitrotyrosine is abundantly present in untreated myofibrillar proteins, but whether other compounds, such as antioxidants, can influence its occurrence *post mortem* and whether this is a good marker for protein oxidation in processed meats, remains to be elucidated.

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REFERENCES

1. Lund, M.N., Heinonen, M., Baron, C.P., & Estevez, M. (2011) Protein oxidation in muscle foods: a review. Mol. Nutr. Food Res. 55: 83-95.
2. Zhang, W., Xiao, S., & Ahn, D. U. (2012) Protein oxidation: basic principles and implications for meat quality. Critical Reviews in Food Science and Nutrition accepted.
3. Ischiropoulos, H., Zhu, L., Chen, J., Tsai,M., Martin, J.C., Smith,C.D., & Beckman, J.S. (1992) Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Archives of Biochemistry and Biophysics 298: 431-437.
4. Stagsted, J., Bendixen, E.k., & Andersen, H.J. (2004) Identification of Specific Oxidatively Modified Proteins in Chicken Muscles Using a Combined Immunologic and Proteomic Approach. J. Agric. Food Chem. 52: 3967-3974.
5. Park, D., Xiong, Y.L., & Alderton, A.L. (2006) Concentration effects of hydroxyl radical oxidizing systems on biochemical properties of porcine muscle myofibrillar protein. Food Chemistry 101: 1239-1246.
6. Estévez, M., Ollilainen, V., & Heinonen, M. (2009) Analysis of protein oxidation markers alpha-aminoadipic and gamma-glutamic semialdehydes in food proteins using liquid chromatography (LC)-electrospray ionization (ESI)-multistage tandem mass spectrometry (MS). J. Agric. Food Chem. 57: 3901-3910.
7. Jongberg, S., Torngren, M.A., Gunvig, A., Skibsted, L.H., & Lund,M.N. (2013) Effect of green tea or rosemary extract on protein oxidation in Bologna type sausages prepared from oxidatively stressed pork. Meat Science 93: 538-546.
8. Estévez, M. & Heinonen, M. (2010) Effect of phenolic compounds on the formation of alpha-aminoadipic and gamma-glutamic semialdehydes from myofibrillar proteins oxidized by copper, iron, and myoglobin. J. Agric. Food Chem. 58: 4448-4455.
9. Skibsted, L.H. (2011) Nitric oxide and quality and safety of muscle based foods. Nitric Oxide 24: 176-183.
10. Brannan, R.G., Connolly, B.J., & Decker, E.A. (2001) Peroxynitrite: a potential initiator of lipid oxidation in food. Trends in Food Science & Technology 12: 164-173.
11. Tiago, T., Aureliano, M., & Gutiérrez-Merino, C. (2008) Effects of reactive oxygen and nitrogen species on actomyosin and their implications for muscle contractility. In Free Radicals in Biology and Medicine (Gutiérrez-Merino,C. & Leeuwenburgh,C., eds), pp. 1-19.
12. Connolly, B.J. & Decker, E.A. (2004) Peroxynitrite induced discoloration of muscle foods. Meat Science 66: 499-505.
13. Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., & Freeman, B.A. (1994) Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. Journal of Biological Chemistry 269: 26066-26075.