POTENTIAL MECHANISMS OF PROTEIN OXIDATION IN COOKED SAUSAGE AS EFFECTED BY SODIUM ASCORBATE AND APPLE PHENOLICS

Tine Rysman[1], Thomas Van Hecke[2], Stephanie Van Weyenberg[1], Stefaan De Smet[2] and Geert Van Royen[1]

[1]Technology and Food Science Unit, Institute for Agricultural and Fisheries Research, Melle, Belgium

[2]Laboratory for Animal Nutrition and Animal Product Quality, Faculty of Bioscience Engineering, Ghent University, Belgium

Abstract – The effect of sodium ascorbate and apple phenolics on the oxidative stability of cooked sausage during chilled illuminated storage for 7 days was investigated. Protein oxidation was evaluated by means of loss of free thiols, total carbonyl formation, and formation of α-aminoadipic and γ-glutamic semialdehyde (AAS and GGS, respectively). During storage and compared with the control treatment, the addition of 0.05% sodium ascorbate resulted in a significant and irreversible decrease of free thiols, and a significant increase in total carbonyls. The amounts of GGS and AAS increased significantly during storage in the ascorbate treatment, however on day 7 no significant differences were found compared with the control treatment. The addition of 3% freeze dried apple pomace caused a significant and irreversible loss of free thiols, and no significant changes in GGS and AAS formation. During storage of the apple treatment, a small but significant increase in total carbonyls was found, though not significant from the control treatment on day 7. Several mechanisms involving protein-ascorbate and protein-phenol interactions are proposed.

Key Words – thiols, carbonyls, protein-ascorbate interactions, protein-phenol interactions

I. INTRODUCTION

It is well known that processing and storage of meat products promote the formation of radicals, causing both lipid and protein oxidation, which in turn leads to technological, nutritional and sensory deterioration [1]. The use of natural bioactive compounds as an alternative for conventionally used antioxidants such as butylated hydroxy-toluene, butylated hydroxyanisole and sodium ascorbate has been a rising topic in the research field of shelf life stability of meat and meat products [2]. Plant and fruit materials rich in phenolic compounds have been shown to have similar radical scavenging and metal chelating properties as synthetic antioxidants [1]. However, the dosage level of antioxidants must be chosen carefully, since either a too high or a too low concentration may work adversely and show pro-oxidative actions [3]. Furthermore, the optimal antioxidant dose against lipid oxidation may not be appropriate against protein oxidation [4]. Additionally, lipid-protein interactions as well as protein-phenolic interactions [5] may interfere with analytical techniques that are used to determine oxidation products, emphasizing the difficulty and importance of investigating antioxidant strategies.

The most commonly used method to measure protein oxidation is determination of total carbonyls, which is based on derivatization of the carbonyl group of oxidized amino acid side chains (mainly arginine, lysine and proline) with 2,4-dinitrophenylhydrazine (DNPH), forming a DNP hydrazone which can be measured spectrophotometrically at 370 nm [6]. In 2011, Utrera *et al.* [7] developed a HPLC method for the detection of two specific carbonylation products, namely α-aminoadipic semialdehyde (AAS, derived from lysine) and γ-glutamic semialdehyde (GGS, derived from arginine and proline), which make up 70% of the total carbonyl content determined with the DNPH method [8]. Next to carbonylation, thiol oxidation is often taken as a measure for protein oxidation. Oxidation of the thiol group on the cysteine residue in meat proteins can lead to the formation of disulfide bonds and other thiol oxidation products. Recently, Rysman *et al.* [9] developed a method for measuring the reversibility of thiol oxidation, which allows to examine the effects of antioxidative strategies more profoundly.

In this paper, the effects of sodium ascorbate and apple phenolics on the shelf life stability of cooked sausage are investigated in terms of total carbonyls, AAS and GGS formation, and thiol oxidation. Several mechanisms are proposed to

explain protein-ascorbate and protein-phenol interactions.

II. MATERIALS AND METHODS

Three batches of cooked sausage were prepared based on a commercial recipe. Two batters were enriched with 0.05% (w/w) of sodium ascorbate and 3% (w/w) of freeze dried apple pomace, respectively. A third batter without antioxidants served as a control treatment. Samples were taken after production and after 4 and 7 days of chilled illuminated storage.

Protein thiol oxidation (free and total thiol content) was measured according to the procedure of Rysman *et al.* [9], based on reduction with sodium borohydride and detection with 4,4'-dithiodipyridine (4-DPS). Protein carbonylation was measured as total carbonyls with 2,4-dinitrophenylhydrazine (DNPH) [4], and as AAS and GGS formation following determination by HPLC after derivatization with amino benzoic acid (ABA) [7]. Results are expressed as nmol of protein oxidation products per milligram of protein.

Free and total thiols were analyzed using a linear model with storage time (days), treatment (control, ascorbate, apple), reduction (with or without) and their interactions as fixed effect. Total carbonyls, AAS and GGS were analyzed using a linear model with storage time (days), treatment (control, ascorbate, apple) and their interaction as fixed effect. Significant difference was considered for $p < 0.05$ (SAS 9.4).

III. RESULTS AND DISCUSSION

The amount of free and total thiols in the three batches of cooked sausage (control, 0.05% sodium ascorbate, and 3% freeze dried apple pomace) are shown in Figure 1. During 7 days of chilled illuminated storage, a significant decrease in free thiols was found in all treatments, indicating that thiols were lost due to (oxidative) reactions. After 7 days of storage, the amount of free thiols was significantly lower in the presence of ascorbate and apple, suggesting that more reactions with thiols took place. Similarly, the free thiol content after production (day 0) was significantly lower in the apple treatment as compared to the control. This is in agreement with Jongberg *et al.* [10], who found significantly lower levels of free thiols in meat emulsions treated with high concentrations of green tea extract, and ascribed this to protein-phenol adducts and protein-phenol-protein cross-links. The more extensive loss of free thiols on day 7 in the presence of ascorbate compared with the control treatment can be ascribed to reactions with dehydroascorbate (DHA), an oxidized form of ascorbate. DHA can be reduced back to ascorbate by taking hydrogen atoms from thiol groups [11], resulting in the loss of measurable thiols. Furthermore, under severe oxidative stress, DHA can decompose into the reactive five-carbon intermediate DHA*. This degradation product has been shown to react with proteins via the thiol group, forming a protein-DHA* adduct [12]. Thus, free thiols may be lost because of reaction with degradation products of ascorbate or phenolics, and not only because of direct thiol oxidation reactions such as disulfide formation.

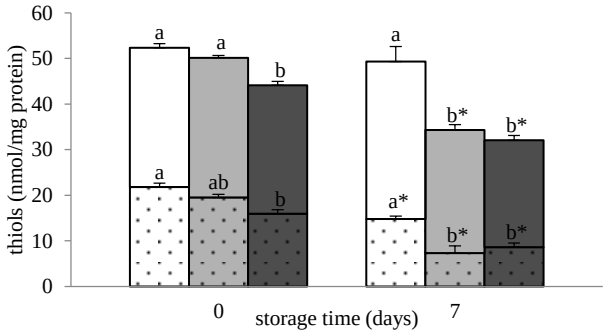

Figure 1: Free (dotted) and total (dotted + clear) thiols as determined with 4-DPS in cooked sausage (□: control, ▨: 0.05% ascorbate, and ■: 3% apple) during 7 days of chilled illuminated storage. [a-b] Significant differences between treatments within the same day. * Significantly different from day 0 ($p < 0.05$).

In the control treatment, no significant difference was found for total thiols between day 0 and 7 of chilled illuminated storage (Figure 1). This indicates that all reactions resulting in the loss of free thiols in the control treatment, were fully reversible. The cooked sausage treated with ascorbate and apple, however, showed a significant decrease of total thiols in time. This suggests that the thiol reactions that took place in cooked sausage treated with ascorbate and apple were irreversible. Although dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP) and sodium

sulfite were able to reduce protein-phenol bonds and protein-phenol-protein cross-links in model systems [13], it is not known whether the reducing agent sodium borohydride is able to brake such bonds in meat emulsions under the conditions used in the 4-DPS assay for total thiol determination. Furthermore, high concentrations of green tea extract have been shown to induce non-reducible cross-links in meat emulsions as determined with SDS-PAGE [10], emphasizing the importance of dose-dependent effects of phenolic compounds. Taking this into consideration, it is possible that the concentration of apple phenolics in the cooked sausage was too high and was causing irreversible loss of free thiols through protein-phenol interactions, instead of thiol protection against oxidation. As for the irreversible thiol oxidation in cooked sausage treated with ascorbate, Kay *et al.* [12] suggested that the reaction between DHA* and thiols involves a Michael addition. Since the reversibility of these type of adducts are dependent on temperature and structure [14], it is plausible that in this case the proposed protein-DHA* adducts are irreversible as determined with the 4-DPS assay.

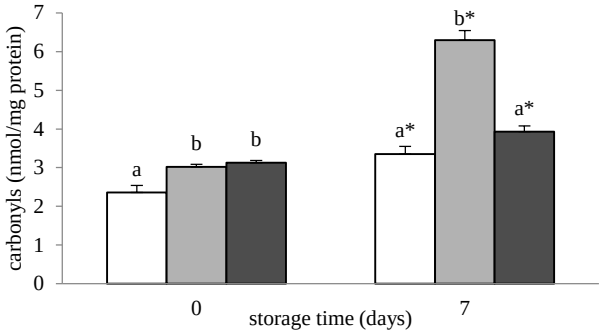

Figure 2: Total carbonyls as determined with DNPH in cooked sausage (□: control, ■: 0.05% ascorbate, and ■: 3% apple) during 7 days of chilled illuminated storage. [a-b] Significant differences between treatments within the same day. * Significantly different from day 0 ($p < 0.05$).

Total carbonyls were determined in all samples according to the DNPH method (Figure 2). During storage, total carbonyls increased significantly in all treatments. Although the initial carbonyl level (day 0) was significantly higher in the apple treatment, no difference was found between the control and the apple treatment after 7 days of chilled illuminated storage. This indicates that less carbonylation took place during storage in the presence of apple. The addition of ascorbate, however, led to a significantly higher total carbonyl level compared with the control and apple treatments after 7 days of storage. Since the DNPH method is based on a non-specific reaction with carbonyls, it is possible that the DNPH is reacting with free carbonyl groups on the DHA molecule. Thus, the DNPH results of the ascorbate treatment as shown in Figure 2, may be an overestimation of protein carbonylation. Similar non-protein carbonyl reactions could also take place with phenolics and their oxidized forms (quinones). However in the current meat matrix this seemed not to be as abundant as in the ascorbate treatment.

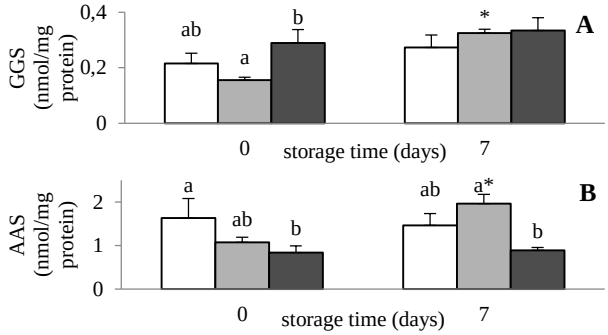

Figure 3: GGS (panel A) and AAS (panel B) formation as determined with HPLC in cooked sausage (□: control, ■: 0.05% ascorbate, and ■: 3% apple) during 7 days of chilled illuminated storage. [a-b] Significant differences between treatments within the same day. * Significantly different from day 0 ($p < 0.05$).

HPLC analysis of the carbonyl compounds GGS and AAS (Figure 3) revealed a significant increase of both compounds in the ascorbate treatment, however no significant differences were found between the ascorbate treatment and the control after 7 days of storage. The sum of GGS and AAS made up 80% of total carbonyls in the control treatment on day 0, whereas this was only 41% and 36% for the ascorbate and apple treatment, respectively. This suggests either that other carbonyls than GGS and AAS contributed to the total carbonyl level in the ascorbate and apple treatment as determined with DNPH, or that DNPH reacted with carbonyls on (degradation products) of ascorbate and phenols. The fact that the AAS content in the apple treatment was significantly lower than in the control treatment on

day 0, could suggest that the apple phenolics acted as radical scavengers, preventing oxidation of lysine into AAS during processing. However, this is unlikely because the thiol, total carbonyl and GGS concentrations indicate otherwise. It is plausible that certain apple phenols were directly bound to lysine, e.g. through ionic bonds between its positively charged ε-amino group and negatively charged hydroxyl groups from phenolics [5], and thus preventing it from further oxidation.

IV. CONCLUSION

Although the primary function of ascorbate and phenolics is to act as an antioxidant, no significant protection of proteins against oxidation was observed when adding 0.05% of sodium ascorbate or 3% of apple phenolics to cooked sausage. Both treatments showed significantly more thiol oxidation than the control treatment, and results for carbonyl formation were inconsistent. This suggests the presence of protein-ascorbate and protein-phenol interactions and their interference with spectrophotometric assays such as the DNPH assay. HPLC analysis and mass spectrometry of ascorbate, dehydroascorbate, DHA degradation products, and phenolics in protein model systems should give more insight into these potential mechanisms.

ACKNOWLEDGEMENTS

The authors thank the Institute for Agricultural and Fisheries Research (ILVO) for granting the project entitled: “Protein oxidation in meat products: Effects of natural bioactive compounds from side flows on quality and health aspects”.

REFERENCES

1. Hygreeva, D., Pandey, M. C. & Radhakrishna, K. (2014). Potential applications of plant based derivatives as fat replacers, antioxidants and antimicrobials in fresh and processed meat products. Meat Science 98: 47-57.

2. Shah, M. A., Bosco, S. J. D. & Mir, S. A. (2014). Plant extracts as natural antioxidants in meat and meat products. Meat Science 98: 21-33.

3. Falowo, A. B., Fayemi, P. O. & Muchenje, V. (2014). Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. Food Research International 64: 171-181.

4. Vossen, E. (2014). Strategies to reduce nitrite and to increase n-3 polyunsaturated fatty acids in meat and meat products: oxidative stability and nutritional quality. PhD thesis, Ghent University, Belgium, 283 p.

5. Le Bourvellec, C. & Renard, C. M. G. C. (2012). Interactions between Polyphenols and Macromolecules: Quantification Methods and Mechanisms. Critical Reviews in Food Science and Nutrition 52: 213-248.

6. Estévez, M., Morcuende, D. & Ventanas, S. (2008). Determination of Oxidation. In L. M. L. Nollet & F. Toldrá, Handbook of Processed Meats and Poultry Analysis (pp 141-162). CRC Press.

7. Utrera, M., Morcuende, D., Rodríguez-Carpena, J.-G. & Estévez, M. (2011). Fluorescent HPLC for the detection of specific protein oxidation carbonyls - α-aminoadipic and γ-glutamic semialdehydes - in meat systems. Meat Science 89: 500-506.

8. Estévez, M. (2011). Protein carbonyls in meat systems: A review. Meat Science 89: 259-279.

9. Rysman, T., Jongberg, S., Van Royen, G., Van Weyenberg, S., De Smet, S. & Lund, M. N. (2014). Protein thiols undergo reversible and irreversible oxidation during chill storage of ground beef as detected by 4,4'-dithiodipyridine. Journal of Agricultural and Food Chemistry 62: 12008-12014.

10. Jongberg, S., Terkelsen, L. d., Miklos, R. & Lund, M. N. (2015). Green tea extract impairs meat emulsion properties by disturbing protein disulfide cross-linking. Meat Science 100: 2-9.

11. Hatahet, F. & Ruddock, L. W. (2009). Protein Disulfide Isomerase: A Critical Evaluation of Its Function in Disulfide Bond Formation. Antioxidants & Redox Signaling 11: 2807-2850.

12. Kay, P., Wagner, J., Gagnon, H., Day, R. & Klarskov, K. (2013). Modification of Peptide and Protein Cysteine Thiol Groups by Conjugation with a Degradation Product of Ascorbate. Chemical Research in Toxicology 26: 1333-1339.

13. Jongberg, S., Lund, M. N. & Otte, J. (2015). Dissociation and reduction of covalent ß-lactoglobulin–quinone adducts by dithiothreitol, tris(2-carboxyethyl)phosphine, or sodium sulfite. Analytical Biochemistry 478: 40-48.

14. Allen, C. F. H. & Humphlett, W. J. (1966). The thermal reversibility of the Michael reaction: V. The effect of the structure of certain thiol adducts on cleavage. Canadian Journal of Chemistry 44: 2315-2321.