MEASUREMENT OF PROTEIN DISULFIDE CROSS-LINKING IN MEAT WITH 4,4'-DITHIODIPYRIDINE AND SDS-PAGE

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Abstract – Protein disulfide cross-linking due to thiol oxidation in minced beef stored in high oxygen modified atmosphere packaging (MAP) was evaluated by two methods. Thiols and disulfides were quantified spectrophotometrically with 4,4'-dithiodipyridine (4-DPS) after reduction with sodium borohydride, and myosin disulfide cross-links were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A significant increase in disulfide cross-links was found after nine days of storage at 4 °C by both methods. Separation by SDS-PAGE offers information about cross-linking on a molecular level, while the 4-DPS assay is an accurate method to quantify thiol groups and disulfides on a functional group level. Both methods contribute to the understanding of thiol-disulfide chemistry in meat and meat products.

I. INTRODUCTION

The impact of protein oxidation on sensory traits, protein functionality and the nutritional value of meat products has been an emerging topic over the last decade (1). Formation of disulfide cross-linking due to protein oxidation has shown to deteriorate texture properties such as meat tenderness and juiciness (2,3). Furthermore, cross-linked proteins might be less susceptible to proteolytic enzymes, which may have an impact on protein digestibility (1).

The general approach for the spectrophotometric quantification of protein disulfides is based on the reduction of disulfides, followed by detection of the newly formed thiols. The amount of disulfides can then be calculated by subtracting the amount of free thiols from total thiols (4). A method was developed for the quantification of protein disulfides in meat inspired by Hansen *et al.* (5), using sodium borohydride as a reducing agent and 4,4'-dithiodipyridine (4-DPS) for thiol detection. 4-DPS is an alternative for the more commonly used thiol detection agent 5,5'-dithio(2-nitrobenzoic acid) (DTNB or Ellman's reagent). 4-DPS is known to be a more sensitive detection agent and is stable in a wider pH range (4).

Protein cross-linking can also be evaluated by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a separation technique based on molecular weight. In oxidized, non-reduced proteins, a high molecular weight protein band at ~500 kDa has been identified as cross-linked myosin heavy chain (CL-MHC), whereas in reduced samples only a myosin heavy chain (MHC) band was visible (3,6). The pixel intensity of the bands on the SDS gel can be used as a semi-quantitative measure of the degree of disulfide cross-linking in MHC.

Although modified atmosphere packaging (MAP) is often applied on fresh meat to prevent discoloration and microbial growth, high levels of oxygen (70-80 %) have been shown to promote lipid, myoglobin and protein oxidation during MAP storage (3,7-9). The objective of this study was to compare the 4-DPS assay for disulfide quantification with the separation technique by SDS-PAGE, in order to accurately evaluate disulfide cross-link formation during storage of minced beef packed in high oxygen atmosphere.

II. MATERIALS AND METHODS

Sampling of MAP minced beef

Minced beef stored in high oxygen modified atmosphere packaging (MAP) was obtained from a local supermarket. At the day of purchase, three packages (replicate A, B, and C) were opened and the meat was vacuum

packed in portions of 50 g and stored at -80 °C until analysis. After nine days of storage at 4 °C, three more packages (replicate A, B, and C) were opened, vacuum packed, and frozen to -80 °C.

4-DPS assay

The meat was thawed and homogenized in 6 M guanidine hydrochloride in 0.1 M Tris buffer, followed by centrifugation and filtration. Protein concentration of the filtrates was determined spectrophotometrically at 280 nm using a standard curve prepared from bovine serum albumin (BSA). For the reduction, the filtrate was incubated at 50 °C in the presence of sodium borohydride, followed by acidification to remove the excess of reducing agent. Free and total thiols were determined with 4-DPS and thiol concentrations were calculated based on a standard curve from L-cysteine according to Hansen *et al.* (5). Thiol content was expressed as nmol thiols per mg protein and disulfide content was calculated as half of the difference between total and free thiols.

SDS-PAGE

Meat samples were thawed and homogenized in 5 % SDS in 0.1 M Tris buffer. After incubation at 80 °C, samples were centrifuged and filtrated. The protein concentration was determined spectrophotometrically at 280 nm using a standard curve prepared from BSA. The homogenates (non-reduced and reduced with dithiothreitol (DTT)) were analyzed by gel-electrophoresis using 3-8 % TRIS-acetate gels. After staining, band intensities were quantified from the pixel intensity using GelAnalyzer2010a©. To correct for variations in total protein for each sample, the intensities of CL-MHC and MHC were divided by the total protein pixel intensity of the corresponding sample. Levels of CL-MHC and MHC are thus given as percentages expressed relative to the total protein content in the sample, allowing to compare the samples loaded on the gel.

Statistical analysis

Data was analyzed using a linear regression model with storage time (days) as fixed effect (SAS 9.3). Significant difference was considered for $P < 0.05$.

III. RESULTS AND DISCUSSION

Thiol loss and disulfide formation quantified with 4-DPS

The levels of total thiols, free thiols and disulfides quantified with 4-DPS in minced beef stored in high oxygen atmosphere are shown in Figure 1. After 9 days of storage, a significant decrease in the amount of free thiols was measured. This observation is in agreement with previous studies, where a loss of free thiols during MAP storage of meat was measured with DTNB (3,9-11). The similar free thiol contents in the aforementioned studies and this paper suggests that 4-DPS is a reliable alternative for DTNB.

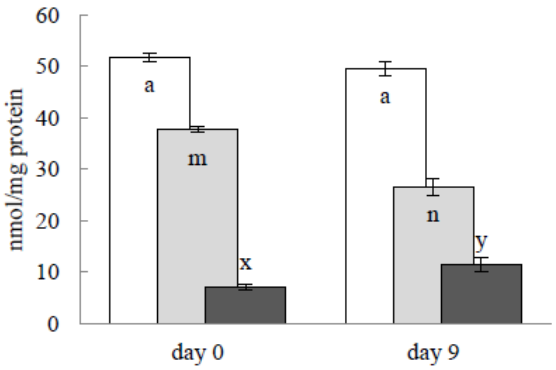

Figure 1: Total thiols (□), free thiols (□) and disulfides (■) in minced beef stored in high oxygen atmosphere packaging at 4 °C during 9 days. Results are shown as mean ± SD of three independent replicates. Means with different letters denote statistical differences between storage time ($P < 0.05$).

After reduction, thiol concentrations were significantly higher than in the non-reduced samples. Thus, during incubation with borohydride a higher concentration of thiol groups had become available for 4-DPS reaction, indicating that the reducing agent successfully cleaved disulfide bonds and, consequently, generated new thiols. When calculating the difference between free and total thiol content, a significant increase in disulfide concentration was found after storage during 9 days (Figure 1). There was no significant difference between the total thiol concentrations on day 0 and day 9, showing that borohydride was able to reduce all thiol

oxidation products that were formed during 9 days of storage. This suggests that no irreversible thiol oxidation took place during storage.

Disulfide cross-linking formation quantified with SDS-PAGE

Meat samples were subjected to SDS-PAGE under reduced and non-reduced conditions. In non-reduced samples, a protein band with a molecular weight at ~500 kDa was visualized. These high molecular weight protein bands have been identified as CL-MHC in previous studies using mass spectrometry (6,10). Band intensities were quantified and a significant increase of CL-MHC band intensities was found after 9 days of storage in high oxygen modified atmosphere packaging (Figure 2). No CL-MHC bands were detected in reduced samples (data not shown), indicating that cross-link formation is caused by reducible disulfide bonds between MHC monomers (6).

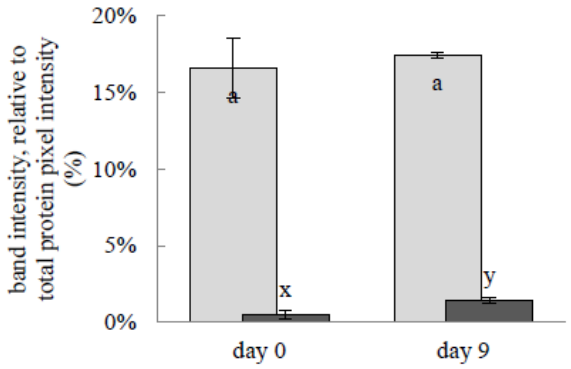

Figure 2: SDS-PAGE band intensities of myosin heavy chain (MHC: □) and cross-linked MHC (CL-MHC: ■) in non-reduced homogenates of minced beef stored in high oxygen atmosphere packaging at 4 °C during 9 days. Band intensities are expressed relative to the total protein pixel intensity. Results are shown as mean ± SD of three independent replicates. Means with different letters denote statistical differences between storage time ($P < 0.05$).

Comparison between disulfide quantification with 4-DPS and SDS-PAGE

In order to compare disulfide cross-links quantified with the 4-DPS assay and separation by SDS-PAGE, the cross-linking levels were expressed relatively to the reduced reference level on day 0 (total thiols$_{day\ 0}$ and MHC$_{day\ 0}$, respectively) (Figure 3).

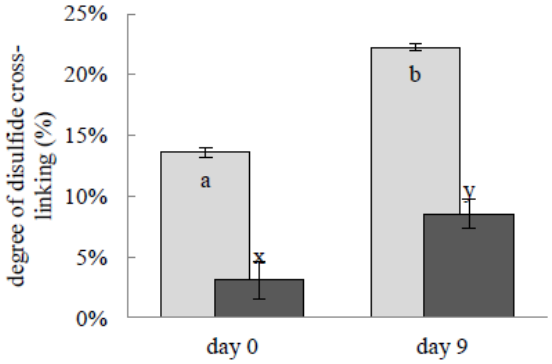

Figure 3: Degrees of cross-linking in minced beef stored in high oxygen atmosphere packaging at 4 °C during 9 days, measured with 4-DPS (□) and SDS-PAGE (■). Results are shown as mean ± SD of three independent replicates. Means with different letters denote statistical differences between storage time ($P < 0.05$).

The degree of disulfide cross-linking found with SDS-PAGE is clearly lower than with 4-DPS, meaning that relatively more disulfides could be quantified with 4-DPS. While the 4-DPS assay is based on a reaction with available thiol groups, SDS-PAGE is evaluating cross-linking by protein molecular weight. CL-MHC is separated from MHC, without providing information about the number of disulfide bonds between two cross-linked MHC molecules. Furthermore, intramolecular disulfides that are formed within MHC are quantified with 4-DPS, but will pass through the SDS gel as MHC monomers. It is also worth noting that only MHC monomers and dimers are visualized on the SDS gel. Severely cross-linked MHC polymers are most likely too big to enter the SDS gel (6), which leads to an underestimation of cross-linking quantification. Finally, although myosin is known to be the first target of protein oxidation in meat products (12,13), lower molecular weight protein are also susceptible to thiol oxidation, which may not be visualized with the applied SDS-PAGE technique. The 4-DPS assay thus offers a more sensitive and quantitative determination of protein thiols and disulfides.

IV. CONCLUSION

Disulfide cross-link formation in minced beef packed in high oxygen atmosphere stored at 4 °C during 9 days was evaluated with the spectrophotometric 4-DPS assay and

separation by SDS-PAGE. With both methods, a significant increase in disulfide cross-linking was found during storage. However, when comparing the cross-links quantified by the 4-DPS assay, a higher degree of cross-linking was measured. This can be ascribed to intramolecular cross-linking of MHC, and multiple disulfide bonds between two MHC monomers. Both methods offer valuable information about disulfide cross-link formation in meat and meat products. The 4-DPS assay provides accurate information about protein cross-linking on a functional group level, while SDS-PAGE visualizes cross-linking on a protein level. In order to establish reaction mechanisms for thiol oxidation in meat as well as evaluate the effect of antioxidants for protection of proteins against oxidation, quantitative methods are necessary such as the 4-DPS assay.

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