Meat Science 106 (2015) 78–84

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Protein oxidation affects proteolysis in a meat model system

Alberto Berardo [a], Erik Claeys [a], Els Vossen [a], Frédéric Leroy [b], Stefaan De Smet [a,*]

[a] Laboratory for Animal Nutrition and Animal Product Quality, Department of Animal Production, Ghent University, Melle, Belgium
[b] Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Faculty of Sciences and Bio-engineering Sciences, Vrije Universiteit Brussel, Brussels, Belgium

ARTICLE INFO

Article history:
Received 4 November 2014
Received in revised form 2 March 2015
Accepted 3 April 2015
Available online 11 April 2015

Keywords:
Protein oxidation
Dry fermented sausage
Proteolysis
Carbonyls
Thiols
Protein electrophoresis
Meat model

ABSTRACT

The effect of hydrogen peroxide-induced protein oxidation and pH (4.8 and 5.2) on meat proteolysis was investigated in a meat model system for dry fermented sausages. In oxidised samples, increased protein carbonyl contents and decreased thiol concentrations were found. The initial concentration of protein carbonyls was significantly lower in oxidised samples at pH 4.8 than in ones at pH 5.2, but after ten days comparable levels were reached. The inhibition of proteolysis by the addition of a protease inhibitor cocktail did not influence protein oxidation. Yet, proteolysis was negatively affected by low pH values as well as by oxidation, resulting in a reduced release of amino acids during ripening.

1. Introduction

Dry fermented sausages are widely consumed, often traditionally important cured meat products, which are characterized by a ripening period in which the desired texture and flavour develop (Leroy, Geyzen, Janssens, De Vuyst, & Scholliers, 2013). Ripening consists of a first fermentation step, usually lasting two to five days, followed by a drying phase that can last several weeks. Although many different types of dry fermented sausages exist in Europe, based on different recipes and processing methods, they can roughly be classified in two main groups, i.e. Northern-type and Southern-type sausages (Hui et al., 2004).

The fermentation phase differs markedly between European Northern-type and Southern-type sausages (Ravyts, De Vuyst, & Leroy, 2012). In the former, the pH drops rapidly below 5.0 due to the activity of lactic acid bacteria which convert the added sugars into lactic acid. In the latter, acidification is more moderate due to the lower amounts of fermentable sugars and the lower fermentation temperatures applied. As a result, the pH usually remains above 5.0, which may even increase during maturation due to the metabolic activity of moulds. The pH drop obtained during fermentation provokes protein denaturation and enhances the activity of some important proteolytic enzymes (Astiasaran, Villanueva, & Bello, 1990; Molly et al., 1997). Endogenous exopeptidases and endopeptidases are the main enzymes responsible for proteolysis in dry fermented sausages, while bacterial proteolytic enzymes seem to play a less pronounced role (Hierro, de la Hoz, & Ordonez, 1999; Toldrá, Aristoy, & Flores, 2000). Several authors have indicated cathepsins as the most active endopeptidases involved in proteolysis in cured meat products (Demeyer, Claeys, Ötles, Caron, & Verplaetse, 1992; Molly et al., 1997; Toldrá, Rico, & Flores, 1993; Verplaetse, Demeyer, Gerard, & Buys, 1992).

Besides the impact of acidification and proteolysis, fermented sausage production is affected by oxidation processes. Lipid oxidation might impair sensory quality since high levels of malondialdehyde correlate with rancid taste (Wood et al., 2008). The oxidative stability of dry fermented sausages is determined by the balance between pro-oxidant and antioxidant factors. Additives, like sodium chloride, exert pro-oxidant effects (Ruiz, 2007), whereas sodium ascorbate and nitrite might have either pro-oxidant or antioxidant activities. Myoglobin, abundantly present in meat, also exerts pro-oxidant effects (Carlsen & Skibsted, 2004). Moreover, some lactic acid bacteria used in fermented products produce hydrogen peroxide which is a strong oxidizer (O'Toole & Yuan, 2006). In contrast, meat-associated catalase-positive cocci, which are added as starter cultures or which are naturally present in the sausage batter, may neutralise peroxides (Ravyts et al., 2012). To a certain extent, protein breakdown taking place during the ripening period may improve the oxidative stability since small peptides present higher antioxidant properties than intact proteins (Freitas et al., 2013).

Whereas lipid oxidation has been extensively studied during the last decades, the impact of protein oxidation on the quality of dry fermented sausages has still to be elucidated. In meat and meat products, oxidation implies modifications at the protein level resulting in

* Corresponding author at: Proefhoevestraat 10, 9090 Melle, Belgium. Tel.: +32 9 264 90 01; fax: +32 9 264 90 99.
E-mail address: stefaan.desmet@ugent.be (S. De Smet).

http://dx.doi.org/10.1016/j.meatsci.2015.04.002
0309-1740/

protein carbonylation, breakdown, and aggregation (Lund, Heinonen, Baron, & Estevez, 2011). Those modifications involve changes in protein solubility and functionality, potentially leading to decreased digestibility, disturbance of gelation, emulsification, and water holding capacity, as well as having a potential impact on flavour due to the formation of certain carbonyls and Schiff bases (Lund et al., 2011). Contradictory effects of protein oxidation on proteolysis were reported. On the one hand, the increased hydrophobicity due to oxidation favours the recognition and the subsequent degradation of oxidised proteins by proteases (Davies, 2001; Pacifici, Kono, & Davies, 1993). This occurs in mild oxidative conditions, in which the proteolytic susceptibility of myosin heavy chain increases by the action of oxygen radicals (Xue, Huang, Huang, & Zhou, 2012). On the other hand, intense oxidative conditions generate cross-links between proteins so that the resulting aggregates are poor substrates for proteases (Pacifici et al., 1993). Moreover, the direct oxidation of proteolytic enzymes impairs their activity (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004), with cysteine proteases being highly susceptible (Lametsch, Lonergan, & Huff-Lonergan, 2008).

To the best of our knowledge, the influence of oxidation on proteolysis in dry fermented sausages has not been studied before. Sausage preparation processes, like meat grinding and the consequent exposure to oxygen as well as the addition of sodium chloride, might trigger protein oxidation and affect proteolysis. Yet, the understanding of how physico-chemical changes occurring in dry fermented sausages, including pH drop and proteolysis, interact with protein oxidation may enable strategies to control its negative effects. Therefore, the aim of this study was to investigate protein oxidation in a meat model system for dry fermented sausages and to assess its effect on proteolysis, and conversely the effects of pH and proteolysis on protein oxidation.

2. Materials and methods

2.1. Dry fermented sausage preparation

The experimental set-up was a 2 × 2 × 2 full factorial design with two pH values installed, induction or not of oxidation, and the addition or not of a protease inhibitor cocktail. The experimental set-up was repeated twice at two different days and each time a batch of sausage models was prepared. Lean pork from shoulder muscles, which contained 4.5% fat upon analysis by the ISO 1444-1973 method, was ground through a 3-mm plate and mixed with the curing agents sodium chloride (2.5%, m/m), sodium nitrite (0.015%, m/m), and sodium ascorbate (0.05%, m/m). The batch was subsequently divided into sub-batches for the different treatments. The batch was first divided into two equal parts and the pH was set at 5.20 ± 0.10 in the first batch and at 4.80 ± 0.10 in the second batch, in both cases by adding lactic acid. The pH remained in the ranges of 5.20 ± 0.10 and 4.80 ± 0.10 during ripening. The batches were further split into sub-batches for the oxidation treatment, control (C) versus oxidised (O), and for the addition or not of a protease inhibitor cocktail.

Oxidation was induced by adding hydrogen peroxide before the stuffing (12 μL/g meat of a 6% hydrogen peroxide solution). Based on a preliminary test, the concentration of hydrogen peroxide was chosen to ensure an increase of about 4 nmol carbonyl/mg of protein. The cocktail of protease inhibitors was made by mixing pepstatin A (60 μM) and E-64 (1.4 mM). Pepstatin A was dissolved in ethanol and subsequently mixed with E-64 which had been previously dissolved in water (1:1, v/v). The protease inhibitor cocktail was added at 0.02 mL per gram of meat. A solution containing ethanol and water (1:1, v/v) replaced the protease inhibitor cocktail in batches where it was not added.

The meat mixtures were stuffed into falcon tubes of 50 mL. Samples were taken after 0, 5 and 10 days of incubation at 22 °C. The length was chosen to allow sufficient proteolysis in order to mimic the first days of ripening.

2.2. Sarcoplasmic protein solubility

Sarcoplasmic protein solubility was measured in a low ionic strength solution (150 mM NaCl), as described previously (Claeys, De Vos, & De Smet, 2002), and was expressed in mg soluble protein/g of meat. Three grams of meat was homogenised in 30 mL of 150 mM NaCl and 0.01 mM iodo-acetic acid. The samples were centrifuged and filtered. The protein concentration of the supernatant, assumed to contain the soluble sarcoplasmic protein fraction, was determined using the biuret method.

2.3. Protein carbonyl content

The protein carbonyl content was determined by derivatization with DNPH (2,4-dinitrophenyl hydrazine) as described by Levine, Williams, Stadtman, and Shacter (1994) with some modifications. Three grams of meat with 30 mL of phosphate buffer (20 mM, pH 6.5 containing 0.6 M NaCl) was homogenised and four aliquots of 0.2 mL were treated with 1 mL ice-cold TCA (10%) to precipitate the proteins. After centrifugation the supernatant was discarded and two aliquots were treated with 0.5 mL of 10 mM DNPH dissolved in 2.0 M HCl and two aliquots were treated with 0.5 mL of 2.0 M HCl (blank). After 1 h of reaction, 0.5 mL of ice cold 20% TCA was added. The samples were then centrifuged and supernatant was discarded. Excess DNPH was removed by washing three times with 1 mL of ethanol:ethylacetate (1:1, v/v). The pellets were dissolved in 1 mL of 6.0 M guanidine hydrochloride in 20 mM phosphate buffer (pH 6.5). The carbonyl concentration (nmol/mg protein) was calculated from the absorbance at 280 nm and 370 nm of the samples using the following equation (Levine et al., 1994):

$$\frac{C_{hydrazone}}{C_{protein}} = \frac{A_{370}}{\varepsilon_{hydrazone,370} \times (A_{280} - A_{370} \times 0.43)} \times 10^6$$

where $\varepsilon_{hydrazone,370}$ is 22,000 M^{-1} cm^{-1} and the carbonyl concentrations obtained from the blanks were subtracted from the corresponding treated sample.

2.4. Thiol concentration

The thiol concentration was determined after derivatization by Ellman's reagent, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) adopted from Jongberg, Torngren, Gunvig, Skibsted, and Lund (2013). Two grams of frozen meat was homogenised in 50 mL of 5% SDS in TRIS buffer (pH 8.0) and incubated for 30 min in a water bath at 80 °C. The homogenate was centrifuged to eliminate insoluble particles. Two millilitres of 0.1 M TRIS buffer (pH 8) and 0.5 mL of 10 mM DTNB dissolved in TRIS buffer were added to 0.5 mL of supernatant. For each sample, a blank was included containing 0.5 mL of supernatant and 2.5 mL of TRIS buffer. A solution containing 0.5 mL of 5.0% SDS in TRIS buffer, 0.5 mL of 10 mM DTNB, and 2.0 mL of TRIS buffer was used as a reagent blank. All mixtures were protected against light and allowed to react for exactly 30 min. The absorbance was measured spectrophotometrically at 412 nm and the thiol concentration was calculated using the formula of Lambert–Beer (ε_{412} = 14,000 M^{-1} cm^{-1}) and expressed in nmol thiols/mg protein. The protein concentration of the blank was determined spectrophotometrically at 280 nm using a BSA standard curve.

2.5. Electrophoresis

About 2.5 g of meat was homogenised in 50 mL of 0.01 M imidazole buffer (pH 7.0), containing 2% SDS, and kept at 95 °C for 10 min to dissolve proteins. After cooling to room temperature, protein solutions were centrifuged and filtered. After determination of protein concentration (Kjeldahl), solutions were diluted to obtain 3 mg of crude protein