Meat Science 93 (2013) 538–546

Contents lists available at SciVerse ScienceDirect

Meat Science

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

Effect of green tea or rosemary extract on protein oxidation in Bologna type sausages prepared from oxidatively stressed pork

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ARTICLE INFO

Article history:
Received 12 July 2012
Received in revised form 4 September 2012
Accepted 10 November 2012

Keywords:
Protein oxidation
Bologna type sausages
Phenolic antioxidants
Thiol oxidation
Thiol-quinone-adducts

ABSTRACT

Bologna type sausages were prepared from oxidatively stressed pork (UV-irradiation, 48 h, 5 °C) using a traditional recipe (control) or the same recipe but added green tea extract (500 ppm total phenolic compounds) or rosemary extract (400 ppm total phenolic compounds). Green tea and rosemary extracts protected against formation of TBARS and protein carbonyls. On the contrary, increased thiol loss and a distinct loss of myosin heavy chain and actin due to polymerization by reducible bonds as determined by SDS-page were found by addition of green tea extract. The enhanced protein polymerization was ascribed to the reaction between quinone compounds from the plant extracts and protein thiol groups to yield phenol-mediated protein polymerization. Analysis by ESR spectroscopy revealed increased radical intensities in sausages added plant extracts, which was ascribed to originate from protein-bound phenoxyl radicals, which may protect against other oxidatively induced protein modifications.

1. Introduction

Raw materials for processed meat products are often stored and distributed as frozen. Depending on handling, storage time and conditions, the meat varies in quality and may be oxidatively stressed before refinement to processed meat products. Refinement is often accompanied by thermal processing, which accelerates oxidation (Frankel, 1998). The radical-mediated chain reaction of lipid oxidation progress faster at elevated temperatures as recently demonstrated by Gatellier, Santé-Lhoutellier, Portanguen, and Kondjoyan (2009) for beef steaks. Further, protein oxidation as evaluated by the accumulation of protein carbonyls has been found to increase in pork sausages subjected to long term mild heat treatment (Sun, Cui, Zhao, Zhao, & Yang, 2011), and in beef steaks subjected to superheating (400 °C for 120–300 s) (Gatellier, Santé-Lhoutellier, Portanguen, & Kondjoyan, 2009).

Phenolic-rich plant extracts from herbs and spices, e.g. rosemary, are effective protectors against oxidative decay in a broad variety of food products (Brewer, 2011). Lipid and protein oxidation may both be inhibited during processing and storage of processed meat products added phenolic-rich extracts (Estévez & Cava, 2006; Estévez, Ventanas, & Cava, 2006; Lara, Gutierrez, Timón, & Andrés, 2011; Vuorela et al., 2005). The ability of the extracts to inhibit oxidation seems to be more pronounced with regards to protection against lipid oxidation than against protein oxidation. Antioxidative mechanisms of phenolic compounds have been extensively studied, and the antioxidative mechanisms against lipid oxidation is primarily assigned to the radical scavenging or metal chelating activity of phenolic compounds as reviewed by Brewer (2011). However, the mechanisms involved in the protection against protein oxidation seem not to be directly comparable to those for lipid oxidation, and prooxidative activities are regularly found by addition of phenolic antioxidants (Estévez & Heinonen, 2010). The complexity of protein oxidation and the large variation in protein oxidation products complicates the characterization of the specific mechanisms and the prediction of the resulting effects.

The amino acid residues, lysine, arginine, proline, and threonine are prone to oxidation in meat through radical-mediated reactions to yield protein carbonyls by reactions also dependent on transition metal ions, such as iron or copper (Estévez, 2011). Studies have shown that addition of phenolic-rich plant extracts prevents protein carbonyl accumulation during storage of fresh meat (Jia, Kong, Liu, Diao, & Xia, 2012; Rababah et al., 2004; Rodríguez-Carpena, Morcuende, Andrade, Kylli, & Estévez, 2011; Rodríguez-Carpena, Morcuende, & Estévez, 2011). However, the protection is inferior to what is commonly observed for lipid oxidation as recently demonstrated in fresh beef patties added a white grape extract (Jongberg, Skov, Tørngren, Skibsted, & Lund, 2011), and in some cases no effect or prooxidative effect of adding plant extracts to fresh meat or meat products have been found (Estévez & Cava, 2006; Lund, Hviid, & Skibsted, 2007).

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0309-1740/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.meatsci.2012.11.005

Protein polymerization has been found to decrease tenderness of fresh meat via disulfide cross-link formation (Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007). Disulfides are generated from oxidation of thiol groups on the amino acid residue cysteine. Lara, Gutierrez, Timón, and Andrés (2011) found that addition of rosemary extract or lemon balm extract inhibited thiol oxidation during cooking of pork patties, and Jia et al. (2012) found preserved thiol levels by addition of black currant extract to fresh pork patties. This is in contradiction to a previous study demonstrating an accelerated loss of thiols during high-oxygen storage of fresh beef added white grape extract rich in catechins (Jongberg, Skov, et al., 2011). The list of contradictory results with regard to protection against protein oxidation by phenolic antioxidants is long, and stresses the complexity of the antioxidative mechanisms towards protein oxidation.

Under oxidative conditions thiols are lost due to nucleophilic interactions with oxidized phenols, the quinones (Pierpoint, 1969). The scavenging of radicals by phenolic compounds results in the formation of semiquinone radicals and quinone compounds. Jongberg, Gislason, Lund, Skibsted, and Waterhouse (2011) found that the quinone of 4-methyl catechol (4-MC) reacted with meat protein thiols in a model system oxidized by Fe(II)/H_2O_2. The covalent bond between protein thiols and the quinone of 4-methyl catechol was later found responsible for a distinct thiol loss during storage of fresh beef under high oxygen atmospheres (Jongberg, Lund, Waterhouse, & Skibsted, 2011). Recently, the mechanism was demonstrated to be responsible for the loss of volatile thiols in musts containing (+)-catechin, (−)-epicatechin, and caftaric acid (Nikolantonaki et al., 2012). The studies underline the importance of an understanding of the reaction mechanisms between proteins and phenolic compounds in the development of meat products with improved oxidative stability by use of natural ingredients, such as phenolic-rich extracts.

The aim of the present study was to characterize the antioxidative mechanisms of phenolic-rich extracts from green tea and rosemary added to Bologna type sausages prepared from oxidatively stressed pork. Protein carbonyl formation, thiol loss, and protein disulfide cross-linking was investigated together with protein radical formation by Electron Spin Resonance (ESR) spectroscopy.

2. Methods and materials

2.1. Chemicals

Reagent-grade chemicals and distilled-deionized (MilliQ) water were used throughout.

2.2. Preparation of Bologna type sausages

Pork shank muscle and jowls were cut to small pieces (20 × 10 mm) and exposed to UV light (105 lx by Philips TUV 36 W UV-C) for 48 h at 5 °C, the meat was subsequently minced by a 3 mm hole plate. Bologna type sausages were prepared by mixing 5 kg shank muscle, 3 kg jowls, 1.88 kg water and 180 g salt (Control). Bologna type sausages containing plant extract were additionally added 25 g green tea extract (Guardian™ Green tea extract 20 M, DuPont Nutrition and Biosciences ApS (formerly Danisco A/S), Brabrand, Denmark) or 102 g rosemary extract (Guardian™ Rosemary extract 202, DuPont Nutrition and Biosciences ApS (formerly Danisco A/S), Brabrand, Denmark), resulting in 500 ppm total phenolic compound from green tea extract or 400 ppm total phenolic compound from rosemary extract. Lower levels of rosemary extract compared to green tea extract were applied to reduce the spicy off-flavor of rosemary. No nitrate was added to the sausages. From each recipe ten sausages of 1 kg (diameter 63 mm) were prepared. Each sausage was dedicated a specific set of analyses; hence, for protein oxidation analyses triplicate myofibrillar protein isolates were prepared from one sausage. The sausages were cooked in a steam cooker at 80 °C until a center temperature of 75 °C was reached. The sausages were rapidly chilled to 2 °C (15 min), sliced in 2 mm thickness, and stored in modified atmosphere containing 30% CO_2/69% N_2/1% O_2 at −80 °C until analysis. Samples used for sensory evaluation after 4 weeks were stored at 5 °C in modified atmosphere containing 30% CO_2/69% N_2/1% O_2 before storage at −80 °C.

2.3. Lipid oxidation by TBARS

Secondary lipid oxidation products were quantified by the TBARS analysis according to Vyncke (1970) and Sørensen and Jørgensen (1996) and expressed as malondialdehyde (MDA) equivalents. Aliquots of 10.0 g Bologna-type sausage were homogenized in 30 ml 7.5% trichloro acetic acid (TCA) with 0.10% propylgallate and 0.10% ethylenediaminetetraacetic acid (EDTA) using an Ultra Turrax for 60 s at 13,500 rpm and filtered. Filtrate (5.00 ml) was mixed with 5.00 ml 20 mM thiobarbituric acid (TBA) and incubated at 100 °C in a water bath for 40 min. Absorbance was measured at 532 and 600 nm at room temperature. Results are mean of two replicates from the same sausage expressed as 2-thiobarbituric reactive substances (TBARS) in μmol MDA/dry matter using a standard curve.

2.4. Extraction of myofibrillar protein isolate (MPI)

Myofibrillar protein isolates (MPI) were prepared from the Bologna type sausages according to the procedure described by Park, Xiong, and Alderton (2007) with slight modifications. Twenty slices of pork sausages (ca. 100 g) were chopped and mixed in a blender, before three aliquots (for triplicate measurements) of 4.0 g pork sausage were homogenized in 20 ml isolation buffer (10 mM NaH_2PO_4, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.0) in 50 ml centrifuge tubes using an Ultra Turrax T-25 Homogenizer (Bie and Berntsen A/S, Denmark). Samples and buffers were kept on ice as much as possible. The homogenate was centrifuged at 2600 *g* at 4 °C for 15 min (Sigma Laborzentrifugen 3k15, Bie and Berntsen A/S, Denmark), the supernatant was discarded and the pellet re-suspended in 10 ml isolation buffer using the homogenization and centrifugation as above. The wash-procedure was repeated three times in total. Subsequently, the supernatant was discarded and the pellet re-suspended in 10 ml 100 mM NaCl, homogenized and centrifuged as above. Lipid residues were removed by a glass spatula after each centrifugation. The supernatant was discarded and the protein pellet was frozen to −80 °C and lyophilized.

2.5. Protein radical intensity by ESR spectroscopy

Lyophilized MPI was transferred to clear fused quartz ESR tubes (inner diameter 4 mm, wall 0.5 mm, Wilmad, Buena, NJ, USA) to reach minimum 5 cm filling of the tube. The tubes were placed in the cavity of a JEOL JES-FR30X ESR spectrometer (JEOL Ltd., Tokyo, Japan) with the following settings: microwave power: 4 mV, center field: 336 mT, sweep width: 7.5 mT, sweep time: 2 min, modulation width: 0.2 mT, amplitude: $6.3 \cdot 10^2$, time constant: 0.03 s, accumulations: 4. The obtained ESR spectra were corrected by an internal standard, Mn(II) (set to 600) and the density of the sample measured as g/cm in the ESR tube, enabling direct comparison between spectra. Spectral manipulation using Savitzky-Golay signal processing was applied for the presentation of selected ESR spectra (Origin 8.6, OriginLab Corporation, Northhampton, MA).

2.6. Protein thiol analysis

The thiol concentration was determined spectrophotometrically after derivatization by Ellman's reagent, 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959). An aliquot of 10.0 mg MPI was dissolved in 5.0% sodium dodecyl sulfate (SDS) in 0.10 M tris(hydroxymethyl)-aminomethane (TRIS) buffer (pH 8.0) by 1 h of incubation in a water bath heated to 80 °C. Samples were centrifuged at 850 g for