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4-Methylcatechol Inhibits Protein Oxidation in Meat but Not Disulfide Formation

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ABSTRACT: The interaction between phenolic compounds and protein thiols was investigated in minced beef with or without 500 ppm 4-methylcatechol (4-MC) that had been stored under oxygen or argon for 7 days (4 °C). Myofibrillar protein isolates were extracted, and the oxidative stability evaluated by the protein radical intensity measured by ESR spectroscopy was found to be improved by 4-MC as well as by storage without oxygen. Significant loss of thiols was found in samples stored under oxygen compared to argon, whereas an additional loss was found in samples with added 4-MC stored under oxygen. In beef with added 4-MC, LC-MS analysis showed formation of thiol−quinone adducts, which may explain the observed additional loss of thiols. Although storage without oxygen inhibited protein cross-link formation as evaluated by SDS-PAGE, both in presence and in the absence of 4-MC, no inhibitory effect of 4-MC was found on the formation of protein disulfide cross-links in beef stored under oxygen.

KEYWORDS: thiol−quinone adduct, protein oxidation, thiols, disulfides, beef, 4-methylcatechol

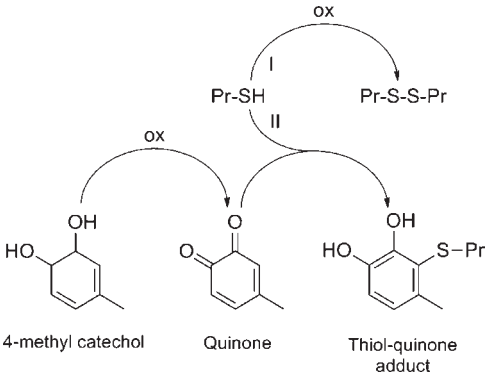
INTRODUCTION

Oxidation processes diminish meat quality because cross-linked proteins increase meat toughness and lipid oxidation produces off-flavors undesirable to consumers.[1,2] Oxidation processes are accelerated by high-oxygen modified atmosphere packaging (MAP), which is commonly applied to protect oxymyglobin and thus the red color of meat.[3] Lipid oxidation reactions in fresh meat and meat products are effectively inhibited by use of phenolic antioxidants from plant sources, such as herbs, spices, and green tea.[4−6] Within recent decades the degrading effects of protein oxidation on meat quality have been recognized, and strategies to prevent this have attracted the attention of both industry and many food scientists.

Oxidative protein cross-link formation diminishes the eating quality of meat and meat products due to decreased tenderness and changed protein functionality.[7] Cross-links are often formed by oxidation of protein side chains to yield protein radicals, which subsequently interact with other side chains forming dityrosine or disulfide bonds. Intra- and intermolecular protein disulfide cross-links are generated in meat when thiol groups (R−SH) in the myofibrillar protein are oxidized to disulfides (R−S−S−R)[8] (Scheme 1, reaction I). Several studies have verified the formation of disulfide cross-links on the myosin heavy chains (MHC) of the myofibrillar protein fraction from meat (see, e.g., Martinaud et al.[9] and Ooizumi and Xiong[10]) and show that protein disulfide cross-link formation has a strong effect on the tenderness and water-holding capacity of meat.[1,11]

Numerous attempts have been made to inhibit the formation of protein oxidation products in general by use of phenolic antioxidants in meat and meat products.[5,12−14] However, carbonyl formation has been evaluated as the principal parameter for protein oxidation, whereas only a few studies have considered the effect of phenolic compounds on thiol oxidation (see, e.g., Eymard et al.[15]). A previous study by Jongberg et al.[16] showed an accelerated decrease in protein thiols of a myofibrillar extract from beef to which a phenol-rich white grape extract had been added prior to storage. However, no signs of increased disulfide cross-link formation were found in the presence of phenolics, indicating that the decrease in thiol groups due to the presence of phenols was not the result of protein disulfide formation but other interactions between the phenols and the protein thiols.

Scheme 1. Proposed Oxidation Mechanisms in Meat with Added 4-Methylcatechol and Chill-stored under High-Oxygen Atmospheres[a]



[a] (I) Oxidation of protein thiol groups (Pr−SH) resulting in cross-linking of proteins by disulfides, and (II) Michael addition between the quinone of 4-methylcatechol and the protein thiol groups forming a thiol−quinone adduct.

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Proteins are modified during processing and storage of meat, and the modifications change the functionality of the proteins, which may or may not be beneficial for the given meat product.[17] Amino acid side chains are a palette of targets for multiple reactions, including glycation, phosphorylation, lactosylation, and oxidation.[18−20] Cysteine, lysine, methionine, and tryptophan all have nucleophilic side chains, which may form covalent bonds with electrophilic groups. In the presence of di- or trihydroxybenzene-containing compounds, such as caffeic acid, chlorogenic acid, quinic acid, or gallic acid, Michael addition reactions have been demonstrated for several proteins, such as α-lactalbumin and lysozyme,[21] bovine serum albumin,[22] whey proteins,[17] and myoglobin.[23] The amino acid side chains form covalent bonds with the quinone formed by oxidation of the phenol, in effect regenerating the hydroxyl groups.[24] Jongberg et al.[25] demonstrated the formation of thiol−quinone adducts by the reaction between the *o*-phenol, 4-methylcatechol (4-MC), and the thiol moiety of the cysteine residues of a myofibrillar protein isolate (MPI) in an aqueous model system under oxidative conditions as established by ferrous iron and hydrogen peroxide.

Formation of thiol−quinone adducts (Scheme 1, reaction II) may serve as an indirect protector against disulfide formation (reaction I) and in that sense act as an antioxidant by blocking the thiol groups in meat protein, thereby potentially inhibiting an increase in toughness due to cross-link formation.

The aim of the present study was to investigate the relative importance of the formation of thiol−quinone adducts in beef with added 4-MC chill-stored under oxygen in comparison to disulfide formation. Experiments were based on an in situ meat model system by use of the phenol 4-MC as a model for a typical plant phenolic moiety.

MATERIALS AND METHODS

Chemicals. Ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), and sodium phosphate were purchased from Sigma-Aldrich, Inc., St. Louis, MO. 4-Methylcatechol (98%) and formic acid were purchased from Acros Organics, Morris Plains, NJ. Hydrochloric acid, sodium hydroxide, and acetonitrile were purchased from Fisher Bioreagents, Fisher Scientific, Fair Lawn, NJ. Sodium chloride was from EMD Chemicals Inc., Gibbstown, NJ, and magnesium chloride was purchased from Fisher Scientific Education, Rochester, NY. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Fluka, Stenheim, Germany. Tris(hydroxymethyl)aminomethane) (TRIS) and L-cysteine were obtained from Merck, Darmstadt, Germany. Sodium borohydride (96%) was obtained from Sigma-Aldrich, Inc., Steinheim, Germany. NuPAGE Novex 3−8% TRIS-acetate gels, LDS sample buffer, SDS TRIS-acetate running buffer, and Molecular Probes SYPRO Ruby Protein Gel Stain were obtained from Invitrogen, Carlsbad, CA. Precision Plus Protein Standard All Blue was obtained from Bio-Rad Laboratories, Inc., Hercules, CA. Dithiothreitol (DTT) was obtained from Applichem GmbH, Darmstadt, Germany. 1-Octanol was purchased from Fluka, Neu Ulm, Germany. All chemicals were of analytical grade or of the highest available purity. Water was prepared using a Millipore Milli-Q purification system (Millipore, Billerica, MA, and Copenhagen, Denmark).

Preparation and Storage of Beef Samples. Freshly ground beef was obtained from the Meat Laboratory at the Department of Animal Science at the University of California, Davis, CA. The meat was divided in two portions of 100 g. One portion was mixed with 8.0 mL of 6.25 mg/mL 4-methylcatechol (4-MC) diluted in water to obtain a final concentration of 500 ppm 4-MC in the beef. The second portion was mixed with 8.0 mL of water containing no phenol (control). Aliquots of 5.0 g of beef sample with or without 4-MC were transferred to 30 mL glass vials, which were purged for 3 min with 100% oxygen or 100% argon and sealed with a cap. The four beef samples, which were prepared in triplicate (A, B, and C) were stored in the dark at 4 °C for 7 days. Each day all samples were purged for 3 min with either oxygen or argon.

Extraction of Myofibrillar Protein Isolates (MPI). MPI were prepared from the beef samples after 7 days of storage according to the procedure described by Park et al.[26] with slight modifications. An aliquot of 4.0 g of beef was homogenized in 20 mL of 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 a General Laboratory Homogenizer (GLH) with a 20 mm probe with a sawtooth at speed 1 (Omni International, Marietta, GA). Samples and buffers were kept on ice as much as possible. The homogenate was centrifuged at 5000 rpm at 4 °C for 15 min (Eppendorf Centrifuge 5403), the supernatant was discarded, and the pellet was resuspended in 10 mL of 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 resuspended in 10 mL of 100 mM NaCl, homogenized, and centrifuged as above. This was repeated three times in total, although, before the last centrifugation, the suspension was filtered through four layers of cheesecloth (grade 20). The supernatant was discarded, and the protein pellet was frozen to −80 °C and lyophilized.

Radical Intensity by ESR Spectroscopy. Lyophilized MPI were transferred to clear fused quartz ESR tubes (inner diameter = 4 mm, wall = 0.5 mm; Wilmad, Buena, NJ) 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, 5 mT; sweep time, 2 min; modulation width, 0.125 mT; amplitude, 200; time constant, 0.3 s; accumulations, 1. The radical intensity was quantified relative to the intensity of the internal standard, Mn(II), and the density of the sample measured as g/cm in the ESR tube (radical intensity = (signal height$_{sample}$/signal height$_{Mn(II)}$)/density (g/cm)).

Acid Hydrolysis of MPI. HCl (6.0 M) was added to MPI, reaching a final concentration of 66 mg MPI/mL, and hydrolyzed under argon in an oil bath at 110 °C for 22 h. The hydrolysates were filtered through a 0.22 μm syringe filter before injection in the LC-MS.

Analysis by LC-ESI-MS. The MPI was analyzed by LC-MS as described by Jongberg et al.[25] Hydrolyzed MPI (30 μL) were injected into an LC (HP 1100 series, Agilent Technologies, Wilmington, DE) equipped with a reversed-phase C18 column (HPLC cartridge 250-4 from Agilent Technologies) and eluted at a flow rate of 0.8 mL/min with a gradient prepared from (A) 1% formic acid and (B) 80% acetonitrile/20% A. The gradient was as follows: 0 min, 15% B; 20 min, 40% B; 22 min, 100% B; 25 min, 15% B; 27 min, 15% B. The column was operated at a constant temperature of 30 °C, and UV detection at 254, 280, and 410 nm was obtained by a diode array detector (DAD). Mass spectrometry analysis was carried out in the positive mode on a HP 1100MSD series (Agilent Technologies) equipped with an ESI interface and LC/MSD Chemstation software (Agilent Technologies, 1990−2002). Conventional ESI-MS data were recorded using a scan range of m/z 100−650 or selected ion monitoring (SIM) mode monitoring ions of m/z 125.1 (4-MC), 244.2 (Cys-QH_2), and 366.3 (Cys-QH_2-QH_2). The MS detector was in API-ES, positive polarity mode, drying gas flow was 12.0 L/min, nebulizer pressure was 35 psig, drying gas temperature was 350 °C, capillary voltage was 3000 V, and the fragmentor was 30 V.

Thiol Groups in Nonreduced MPI. The thiol concentration was determined spectrophotometrically after derivatization by Ellman's reagent, DTNB.[27] An aliquot of 10.0 mg of MPI was dissolved in 5.0% SDS in 0.10 M TRIS buffer (pH 8.0) by 30 min of incubation in an 80 °C water bath. The samples were analyzed according to the method of Liu and Xiong[28] by mixing 500 μL of sample, 2.00 mL of 0.10 M TRIS buffer (pH 8.0), and 500 μL of 10 mM DTNB dissolved in 0.10 M TRIS

buffer (pH 8.0). The absorbance at 412 nm was measured before the addition of DTNB ($ABS_{412\text{-before}}$) and after reaction with DTNB ($ABS_{412\text{-after}}$) against a reference solution of 500 μL of 5.0% SDS and 2.50 mL of 0.10 M TRIS buffer (pH 8.0). The mixture was protected against light and allowed to react for exactly 30 min. A solution containing 2.00 mL of 0.10 M TRIS buffer (pH 8.0), 500 μL of 5.0% SDS, and 500 μL of 10 mM DTNB was used as blank sample. Hence, the absorbance corresponding to the thiol concentration in the sample was $Corr.ABS_{412} = ABS_{412\text{-after}} - ABS_{412\text{-before}} - ABS_{412\text{-blank}}$. The thiol concentration was calculated on the basis of a 5-point standard curve ranging from 0.4 to 83.3 μM prepared from L-cysteine diluted in 5.0% SDS in 0.10 M TRIS buffer (pH 8.0). Thiol content was given in nanomoles of thiol per milligram of protein. Each sample consisted of three independent replicates (A, B, and C).

Thiol Groups in Reduced MPI. The total thiol concentration was determined after reduction with sodium borohydride according to the method of Hansen et al.[29] with a few modifications. An aliquot of 30 mg of MPI was dissolved in 3.00 mL of 5.0% SDS in 0.10 M TRIS buffer (pH 8.0). An aliquot of 100 μL of freshly prepared 30% $NaBH_4$ in 1 M NaOH was added together with 30 μL of 1-octanol, and the mixture was incubated in a sealed glass vial at 50 °C for 1 h with occasional stirring. After 30 min, the vials were carefully opened to release the pressure formed in the vials. After heat treatment, another 30 μL of 1-octanol was added, and 900 μL of 1.0 M HCl was added dropwise to remove excess $NaBH_4$. The samples were placed on a magnetic table and stirred for 1 h. Aliqouts of 1000 μL of sample were transferred to test tubes, and the pH was carefully adjusted to pH 8.0 by the addition of approximately 100 μL of 1.0 M NaOH. The thiol concentration was subsequently determined spectrophotometrically after reaction with DTNB according to the method of Liu and Xiong[28] as described above for the thiol quantification of the nonreduced MPI.

Cross-Link Formation by SDS-PAGE. Samples were analyzed by gel electrophoresis using NuPAGE Novex 3−8% TRIS-acetate gels according to the manufacturer's instructions. The loading solution for the nonreduced samples was prepared with 4 μL of LDS sample buffer, 10.4 μL of Milli-Q water, and 1.6 μL of sample, which was a 15-fold dilution of 10 mg/mL MPI in 5.0% SDS in 100 mM TRIS buffer (pH 8.0). Reduced samples were prepared with 4 μL of LDS sample buffer, 1.6 μL of 1.0 M DTT, 8.8 μL of Milli-Q water, and 1.6 μL of diluted sample. Aliquots of 12 μL of loading solution with sample and 3 μL of Precision Plus Protein Standard All Blue marker were loaded to the wells. Electrophoresis was run at 150 V for 90 min in cassettes containing ice-cold SDS TRIS-acetate running buffer. Following electrophoresis, the gels were fixed in fixing solution (50% ethanol, 7% acetic acid, and 43% Milli-Q water) for 30 min; afterwards, the fixing solution was exchanged and left overnight at room temperature on a rocking table. The gels were stained by the fluorescence SYPRO Ruby Protein Gel Stain and photographed by a charge-coupled device (CCD) camera (Raytest, Camilla II, Straubenhardt, Germany). The intensity of the bands was quantified using Phoretix 1D software, version 2003.02. A minimum profile background was subtracted, and the bands were manually selected for the myosin heavy chain (MHC) and the cross-linked MHC (CL-MHC). The pixel intensity of the bands was determined as the peak height of the selected band in the lane profile.

Identification of Proteins by Mass Spectrometry Analysis. Selected protein bands were cut from the gel and digested with trypsin, and the mass spectrum was recorded using matrix-assisted laser desorption ionization−time-of-flight mass spectrometry (MALDI-TOF-MS). In-gel digestion was performed as described by Jensen et al.[30] Custom-made chromatographic columns were used for desalting and concentration of the peptide mixture prior to mass spectrometric analysis.[31] The proteins were analyzed by use of a MALDI-TOF-TOF instrument (4700 Proteomics analyzer, Applied Biosystems, Foster City, CA) according to the procedure of Jensen et al.[30] Protein identification by peptide mass mapping was performed using the Mascot database search program. The peptide mass maps and protein identification were evaluated as described by Jensen et al.[30]

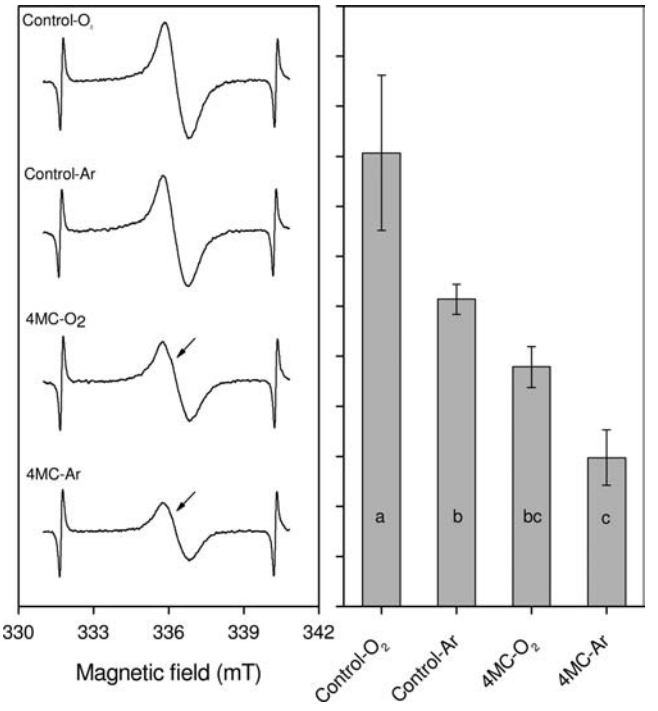


Figure 1. Radical intensity evaluated by ESR spectroscopy of myofibrillar protein isolate (MPI) extracted from beef with or without 500 ppm 4-MC and stored in oxygen or argon for 7 days at 4 °C. (Left panel) ESR spectra obtained from replicate A of the four samples, Control-O_2, Control-Ar, 4-MC-O_2, and 4-MC-Ar. The two outer signals in the spectra are the internal standard Mn(II), and the middle is the radical signal of the MPI. The arrow indicates a weak shoulder on the ESR spectra of MPI from beef with added 4-MC. (Right panel) Radical intensity determined as the mean ± SD of two independent replicates (A and B). Different letters (a−c) denote a statistical difference ($P < 0.05$) between means of independent replicates (A and B).

Statistical Analysis. Statistical analyses were performed using R version 2.12.1 (The R Foundation for Statistical Computing (ISBN 3-900051-07-0)). Data were analyzed by analysis of variance using a linear model with "sample" (control-O_2, control-Ar, 4-MC-O_2, and 4-MC-Ar) and "replicates" (A, B, and C) as variables. The effect of the "replicates" was found to be insignificant for the statistical model and therefore excluded from the model. The significance level was selected as $P < 0.05$.

RESULTS AND DISCUSSION

Oxidative Stability. The overall oxidative stability of MPI extracted from beef with or without 4-MC stored under oxygen or argon for 7 days at 4 °C was evaluated by ESR spectroscopy. The ESR spectra showed a significantly increased oxidative stability of the MPI from beef samples with 4-MC compared to samples without 4-MC as evaluated by the radical intensity as presented in Figure 1. MPI from beef without 4-MC stored under oxygen showed significantly higher radical intensity compared to all other samples, which indicates an increased protein radical formation in meat stored in high-oxygen atmospheres. This observation is consistent with studies showing increased oxidation of proteins in meat stored in high-oxygen atmospheres as

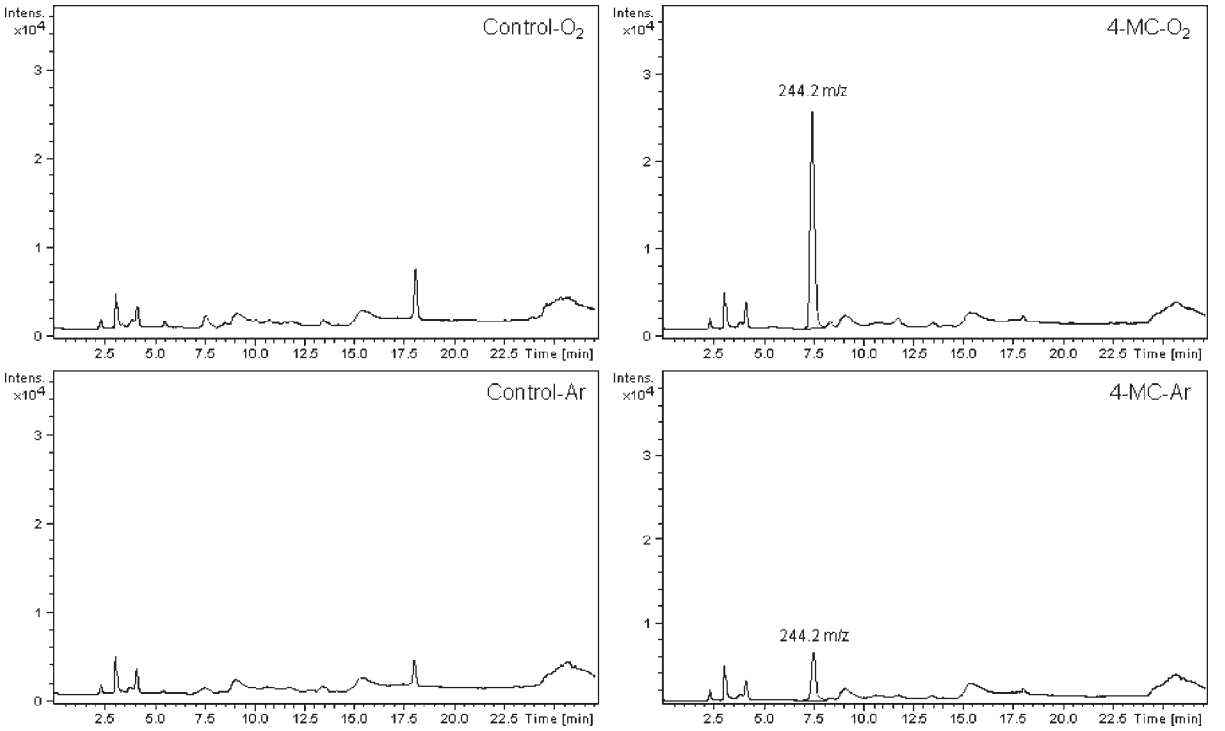


Figure 2. Thiol−quinone adduct formation in myofibrillar protein isolate (MPI) extracted from beef with or without 500 ppm 4-MC and stored in oxygen or argon for 7 days at 4 °C as determined by LC-MS analysis after acid hydrolysis of MPI. The thiol−quinone adduct (Cys-QH_2) of m/z 244.2 eluted with a R_T of 7.5 min.

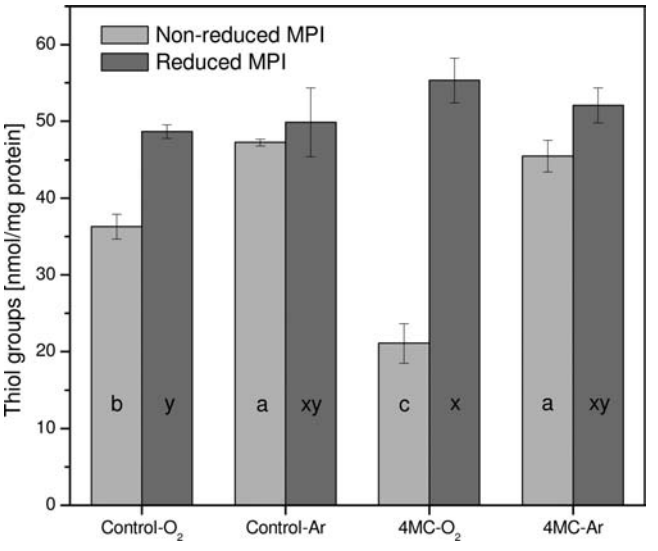


Figure 3. Thiol concentration in nonreduced or reduced myofibrillar protein isolates (MPI) extracted from beef with or without 500 ppm 4-MC stored under oxygen or argon at 4 °C for 7 days. Results are given as the mean ± SD of three independent triplicates (A−C). Different letters on the columns denote a statistical difference ($P < 0.05$) between means of independent triplicates (A−C) within nonreduced MPI (a−c) or reduced MPI (x, y).

reviewed by Lund et al.,[7] but it is to our knowledge the first time that radical formation has been determined directly in the protein fraction of meat stored in high-oxygen atmospheres. However, no significant difference in radical intensity was found between the MPI from beef stored under argon (control-Ar) and beef with 4-MC stored under oxygen (4-MC-O_2), which indicates that 4-MC protects the meat protein from radical formation as efficiently as storage without oxygen. Because lipid residues, water-soluble compounds, and connective tissue were removed during the MPI extraction procedure, the radical signal is considered to originate directly from the salt-soluble myofibrillar proteins. Hence, the results demonstrate a radical scavenging effect of 4-MC on the myofibrillar protein fraction of beef, indicating that phenolic substances have an inhibiting effect on the formation of meat protein radicals. Figure 1 (left panel) shows the ESR spectra of representative samples. The spectra from samples containing 4-MC are slightly different from the control samples as marked by the arrows. The weak shoulder noted in the spectra indicates that the spectra may originate from a modified protein compared to the spectra of the control samples without 4-MC.

Formation of Thiol−Quinone Adducts. A modification of the meat protein may be induced by an electrophilic attack on the nucleophilic protein thiols from quinones generated from oxidation of 4-MC. The presence of a potential thiol−quinone adduct in the MPI was analyzed by LC-MS, and the results showed that thiol−quinone adducts were formed during storage of beef in the presence of 4-MC (Figure 2). The thiol−quinone adduct has previously been detected in a MPI model system,[25] but not previously detected in meat samples added phenolic compounds. As seen in Figure 2, the adduct was found only in the beef sample with added 4-MC stored under oxygen and only to a minor degree in the beef sample with added 4-MC stored under argon. Keeping in mind that formation of thiol−quinone adducts through Michael addition requires a preceding oxidation of the phenol to quinone, formation of the adduct in the sample stored under argon will only occur as a result of residual oxygen from the

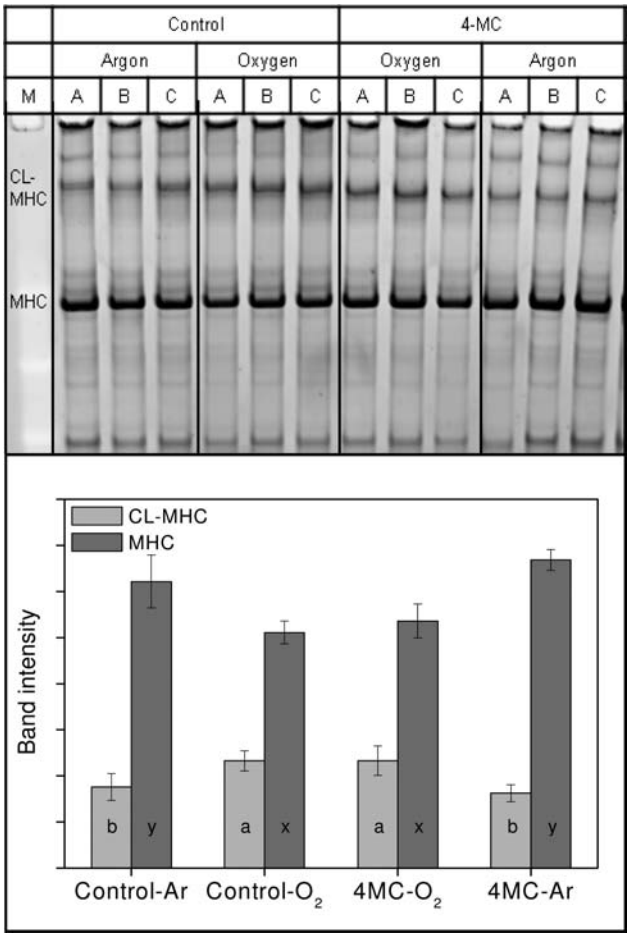


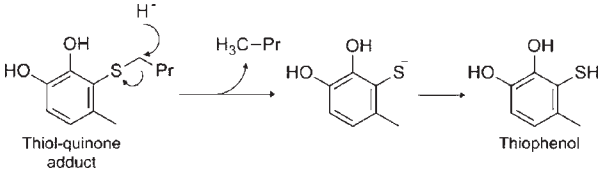
Figure 4. (Top panel) SDS-PAGE gel of nonreduced myofibrillar protein isolates (MPI) extracted from beef with or without 500 ppm 4-MC stored under oxygen or argon at 4 °C for 7 days. The gel shows all samples in triplicate (A−C). CL-MHC, cross-linked myosin heavy chain; MHC, myosin heavy chain. (Bottom panel) Pixel intensity (mean ± SD) based on peak height of MHC and CL-MHC bands. Different letters on the columns denote a statistical difference ($P < 0.05$) between means of independent triplicates (A−C) within CL-MHC (a, b) or MHC (x, y).

atmospheric air dissolved in the beef or from the presence of other oxidative systems naturally present in fresh meat. The results clearly demonstrate formation of the thiol−quinone adduct, but fail to quantify the formed adduct. However, on the basis of a parallel run of a standard prepared from cysteine and the quinone of 4-MC in an aqueous buffered solution as described by Jongberg et al.,[25] an estimated concentration of the thiol−quinone adduct was obtained. By comparison of the intensities of the thiol−quinone adduct from hydrolyzed MPI samples and the thiol−quinone adduct determined from the standard, a thiol−quinone concentration of approximately 6 nmol/mg protein was found. This estimate represents the minimum concentration of formed thiol−quinone adduct in the beef, as the MS signal will be suppressed in a matrix with increased complexity; as well, the total recovery of the adduct is reduced throughout the preparation of the sample (e.g., MPI extraction, hydrolysis).

Thiol Loss and Disulfide Formation. To investigate the impact that formation of thiol−quinone adducts have on the thiol redox status in the meat protein, the thiol concentration was quantified in nonreduced and reduced MPI extracted from the beef samples (Figure 3). The results for the nonreduced MPI showed a significantly lower thiol concentration in samples stored under oxygen compared to the samples stored under argon. This observation is in agreement with the formation of protein disulfides under oxidative conditions from thiol groups as previously observed (see, e.g., Lund et al.[1] or Jongberg et al.[16]). The beef with added 4-MC stored under oxygen showed a distinctly lower thiol content compared to the control beef without 4-MC stored under oxygen, which indicates that 4-MC alters the thiol redox status in meat, for example, by the reactions shown in Scheme 1. The decreased thiol concentration caused by the presence of 4-MC may be explained by a pro-oxidative effect of the quinone of 4-MC generating more disulfides, but may also be explained by the formation of thiol−quinone adducts (Scheme 1, reaction II), as found by the LC-MS analysis presented in Figure 2. Protein modification by thiol−quinone adducts may block the protein thiol group and make it unavailable for detection using DTNB derivatization. More importantly, the modification may block the oxidation site and prevent protein disulfide cross-link formation in the meat. The thiol concentration was also quantified in reduced samples. Figure 3 also shows the thiol concentration in the MPI obtained after reduction with borohydride. The result showed that the thiol groups were regenerated by reduction of the MPI and that significantly higher thiol concentrations were obtained in samples treated with 4-MC stored under oxygen compared to the control stored under oxygen.

To establish if the additional loss in thiol groups in the 4-MC beef stored under oxygen was caused by accelerated disulfide formation or blocking of thiol groups by thiol−quinone adducts, the degree of cross-linked myosin in the MPI was evaluated by SDS-PAGE. The MPI samples were analyzed in both their reduced and nonreduced states to verify that the cross-link formation was caused by reducible disulfide bonds. Relevant bands were subsequently analyzed by MALDI-TOF-MS and identified as MHC and CL-MHC (data not shown). The reduced samples showed no CL-MHC bands on the SDS gel (data not shown) as were found for the nonreduced samples (Figure 4, top panel), which verifies that the cross-link formation is a result of disulfide bonding between MHC molecules. Analysis by SDS-PAGE provides a marker for the degree of protein disulfide cross-link formation, as only the monomer and dimer MHC are visualized on the gel. Other MHC polymers are most likely too big to enter the gel, and the results are consequently only indications of the CL-MHC level in the meat sample. Quantification of the band intensity showed significantly ($P < 0.05$) increased CL-MHC formation and a correspondingly increased MHC degradation in beef stored under oxygen compared to samples stored under argon (Figure 4, bottom panel). However, no significant difference between beef with or without 4-MC stored under oxygen was found; hence, the quinone of 4-MC does not act as a pro-oxidant on the protein thiols. This is in contrast to previous studies proposing a pro-oxidant action of quinones on proteins to yield protein carbonyls.[32,33] The distinct thiol loss observed for the nonreduced MPI from beef with 4-MC stored under oxygen (Figure 3) is therefore most likely caused by a modification of the thiol group, which presumably is generated by Michael addition reaction with the quinone of 4-MC (Scheme 1, reaction II). The difference in thiol concentration between control and 4-MC beef stored under oxygen of approximately

Scheme 2. Proposed Reaction Mechanism for Reduction of the Thiol−Quinone Adduct with Hydride Ions Resulting in Release of a Thiophenol from the Protein Backbone



15 nmol/mg protein corresponds well with the formation of minimum 6 nmol thiol−quinone adduct/mg protein found in the LC-MS analysis. Assuming that the distinct thiol loss in the nonreduced MPI from beef with added 4-MC and stored under oxygen is caused by covalent interactions between a protein thiol group and the quinone of 4-MC, the proposed reaction mechanism in Scheme 2 explains how the hydride ion attacks the carbon adjacent to the sulfur on the cysteine moiety and subsequently releases a thiophenol from the protein backbone. The thiols of the resulting thiophenol will also be derivatized by DTNB in the thiol quantification analysis, and consequently the thiol concentration obtained after reduction of MPI constitutes both protein thiols and thiophenols. As seen in Figure 3, the thiol concentrations obtained after reduction of MPI are significantly ($P = 0.02$) higher for beef with 4-MC compared to beef without 4-MC stored under oxygen. The increased concentration of total thiols can be explained by the release of thiophenols, which are more easily oxidizable and also more easily accessible to DTNB than protein thiols, which may be buried in the myofibrillar protein structure.

Protein-Bound Antioxidant Effects. Modification of myofibrillar proteins through adduct formation between protein thiols and quinones as evaluated in the present study has a considerable effect on the oxidative stability. A covalent interaction between the thiol and the quinone not only blocks the thiol group but also regenerates the phenolic moiety of the dihydroxybenzene (Scheme 1) and may thereby increase the overall antioxidative capacity. It is likely that the regenerated phenol will exert antioxidant action locally on the protein. It was not possible to identify the weak shoulder of the ESR spectra (Figure 1) as a phenoxyl radical due to the freeze-dried nature of the samples, which results in a powder spectrum with indistinct features.

A protein-bound phenolic compound may possess radical scavenging effects within the protein structure and may be a potential intermolecular antioxidant serving as a sink of radicals, thereby protecting against the formation of protein oxidation products, such as carbonyl groups, in other moieties of the protein. However, it should be kept in mind that formation of such protein-bound phenoxyl radicals may increase the cross-link potential of the meat protein and, thus, not contribute to the protection of the eating quality of meat. Further studies are needed to determine changes in protein functionalities resulting from such thiol−quinone protein modification. Moreover, the thiol−quinone adduct or similar adduct formations must be carefully considered in the evaluation of the protein oxidation state based on thiol levels. Protein-bound phenolic compounds may in general be beneficial for the production of other processed food products containing large quantities of proteins (e.g., meat products, infant formulas, and soup powders). The present study demonstrated how protein radical formation in meat can be quantified in a simple manner by purification and lyophilization of the myofibrillar proteins and a subsequent measurement of the radical intensity directly by ESR spectroscopy at room temperature without the addition of spin traps as usually done.

In conclusion, the overall oxidative stability of the myofibrillar proteins as evaluated by ESR spectroscopy was significantly improved by the addition of 4-MC to beef stored with or without oxygen. The observed difference in protein thiols was partly ascribed to the formation of a thiol−quinone adduct; however, the blocking of protein thiols by 4-MC was not found to be sufficient to significantly inhibit protein disulfide cross-link formation in the MPI from beef.

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