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Thiol−Quinone Adduct Formation in Myofibrillar Proteins Detected by LC-MS

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ABSTRACT: Protein oxidation in meat is considered to decrease meat tenderness due to protein disulfide cross-link formation of thiol-containing amino acid residues. An LC-MS method for detection of thiol−quinone adducts (RS−QH_2) in myofibrillar proteins was developed to investigate the interaction between phenols, as protective antioxidants, and proteins from meat under oxidative conditions using aqueous solutions of (i) cysteine (Cys), (ii) glutathione (GSH), (iii) bovine serum albumin (BSA), or (iv) a myofibrillar protein isolate (MPI). The aqueous solutions were incubated at room temperature (30 min) with 4-methyl-1,2-benzoquinone (4MBQ) prepared from oxidation of 4-methylcatechol (4MC) by periodate resin or incubated at room temperature (5 h) with 4MC and Fe(II)/H_2O_2. GSH, BSA, and MPI were hydrolyzed (6 N HCl, 110 °C, 22 h) after incubation, and the cysteine−quinone adduct, Cys−QH_2 (m/z 244.2) was identified according to UV and mass spectra after separation on an RP-C18 column. The thiol−quinone adduct was present in all thiol systems after incubation with 4MBQ or 4MC oxidized by Fe(II)/H_2O_2. Direct reaction with 4MBQ resulted in each case in increased Cys−QH_2 formation compared to simultaneous oxidation of thiol source and 4MC with Fe(II)/H_2O_2. The covalent bonds between quinones and thiol groups may act as a potential antioxidant by inhibiting disulfide protein cross-link formation.

KEYWORDS: protein oxidation, 4-methylcatechol, quinones, thiols, LC-MS

INTRODUCTION

Protein disulfide cross-linking increases the toughness of fresh meat stored in modified-atmosphere packaging (MAP) with high levels of oxygen.[1] Myoglobin becomes catalytic in meat during storage and induces oxidation in meat, especially under high-oxygen conditions.[2,3] Lipid easily becomes oxidized, but protein is also subject to radical attack to yield a variety of products.[4] However, it has now been shown that protein disulfide cross-links formed as a result of oxidation of thiol groups on the cysteine residue of the myosin heavy chain (MHC) in the myofibrillar protein fraction decrease the tenderness and juiciness of meat and, consequently, reduce the meat quality during storage in high-oxygen MAP.[1,5]

The addition of natural antioxidants from plant extracts is commonly used to prevent lipid oxidation in meat products. Recently, studies assessing the antioxidative effect of phenolic extracts toward protein oxidation in meat have been published.[6−9] However, it still seems unclear by which mechanism such phenolic antioxidants protect against protein oxidation.[10,11] A recent study by Jongberg, Skov, Tørngren, Skibsted, and Lund[12] showed that addition of a phenol-rich white grape extract (WGE) interfered with the protein thiol groups of the MHC from the myofibrillar protein fraction of beef patties stored under high-oxygen conditions. The overall concentration of thiol groups decreased with the addition of WGE, but disulfide cross-link formation was inhibited compared to a control without WGE added, indicating that the fate of the thiol groups is not primarily protein disulfide cross-link in the presence of phenols.

Metal-catalyzed oxidation of plant phenols, such as the catechols, to yield peroxyl radicals results in the formation of semiquinones. Semiquinones may dismutate, forming a quinone and the phenol (Scheme 1A), as suggested for phenols in wine.[13] As reviewed by Kroll, Rawel, and Rohn,[14] quinones are reactive electrophilic intermediates and react easily with nucleophilic amino acid residues in a protein chain, such as cysteine, lysine, methionine, or tryptophan residues, and form covalent bonds as shown for a thiol in Scheme 1B.

Protein modifications induced by reaction with phenols become important during food processing and storage as such modifications have been shown to change the solubility and emulsion properties of a protein by changing its hydrophilic/hydrophobic balance.[15] Thiol−quinone modifications have long been applied in the wine and must industries, in which glutathione addition is commonly used to hinder oxidized phenols from forming brown polymers. The reaction product is known as the grape reaction product (GRP).[16] Even though the quinone moiety is regenerated to a hydroxyphenol after reaction with the thiol group of glutathione, the GRP is not a substrate for polyphenol oxidases (PPO); hence, adduct formation limits the polymerization of *o*-quinones.[17,18]

Phenol−protein adduct formation has been studied for various peptides and proteins such as glutathione,[19] α-lactalbumin and lysozyme,[20] bovine serum albumin,[21] whey proteins,[22] and myoglobin.[15] Prigent et al.[20] used the MALDI-TOF-MS technique to characterize the derivatization of lysozyme and α-lactalbumin by quinones of chlorogenic acid, formed either by PPO or in nonenzymatic reactions, and found that the modifications

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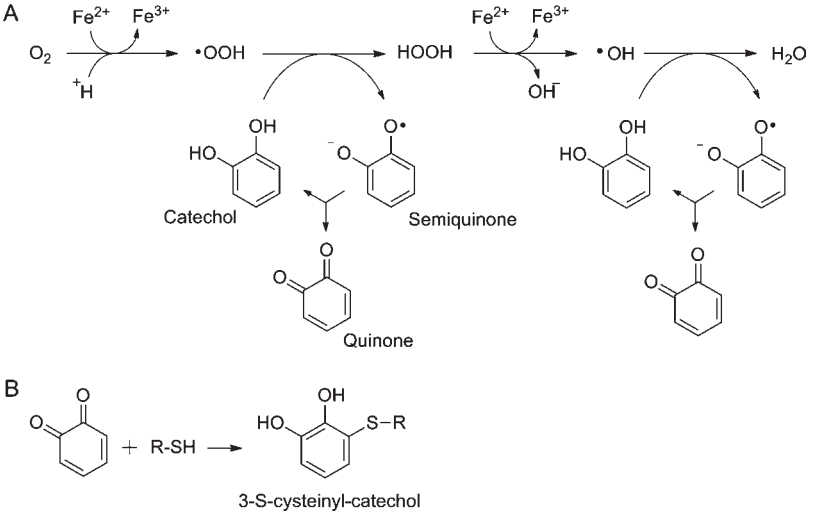
Scheme 1. Proposed Metal-Catalyzed Oxidation Mechanism of Phenols in Meat and Meat Model Systems (Modified from Elias et al.[6]): (A) Oxidation of a Catechol to a Semiquinone and Subsequent Quinone Formation and (B) Formation of the Thiol−Quinone Adduct by Reaction with the Thiol Group of, for Example, Cysteine, Forming 3-*S*-Cysteinylcatechol



often occur via dimerization of the phenolic compounds prior to protein modification. However, earlier studies report monomer addition products from reactions between amino acid nucleophiles and caffeic acid,[23] chlorogenic acid,[18,24] caftaric acid,[16] and epicatechin and catechin.[18]

The aim of the present study was to develop an LC-MS method for the detection of a potential thiol−quinone adduct formed in meat protein and to investigate if such thiol−quinone adducts are formed in a meat model system constituting myofibrillar protein under oxidative conditions generated by hydrogen peroxide and iron(II).

MATERIALS AND METHODS

Chemicals. Maleic acid, Amberlyst A-26(OH) ion-exchange resin, periodic acid, ethylenediaminetetraacetic acid (EDTA), and sodium phosphate were purchased from Sigma-Aldrich, Inc., St. Louis, MO. 4-Methylcatechol (98%), formic acid, and L-cysteine (99%) were purchased from Acros Organics, Morris Plains, NJ. Glutathione (reduced, Biotech grade), hydrochloric acid, sodium hydroxide, acetonitrile, and bovine serum albumin (BSA) were purchased from Fisher Bioreagents, Fisher Scientific, Fair Lawn, NJ. Sodium chloride, tetrahydrofuran (THF), hydrogen peroxide (30%), and ether were from EMD Chemicals Inc., Gibbstown, NJ, and magnesium chloride we purchased from Fisher Scientific Education, Rochester, NY. All chemicals were of analytical grade or of the highest available purity.

Extraction of Myofibrillar Protein Isolate (MPI). Lean beef (minced on the same day as protein extraction was carried out) was obtained from the Meat Laboratory at the Department of Animal Science at the University of California, Davis, CA. A myofibrillar protein isolate (MPI) was prepared according to the method of Park, Xiong, Alderton, and Ooizumi[25] with minor 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 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 described above. This 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 final centrifugation, the suspension was filtered through four layers of cheesecloth. The supernatant was discarded, and the protein pellet was frozen to −80 °C and lyophilized.

Preparation of Quinone by Periodate Resin. Periodate resin was prepared according to the method of Harrison and Hodge[26] with slight modifications. An aliquot of 18.0 g of Amberlyst A-26 resin in 50 mL of Milli-Q water was mixed with 9.9 g of pure periodic acid, and the solution was stirred at room temperature for 2 h. Water was drained off on an inert filter, and the periodate resin was rinsed with 200 mL of water four times in total. Subsequently, the resin was washed with 100 mL of THF two times and 100 mL of ether two times. The resin was transferred to 50 mL Eppendorf tubes and dried in a vacuum centrifuge at 35 °C overnight.

The quinone, 4-methyl-1,2-benzoquinone (4MBQ), was prepared by dissolving 31.0 mg of 4-methylcatechol (4MC) in 2.50 mL of acetonitrile. The solution was mixed using a magnetic stirrer for 5 min under argon. An aliquot of 395 mg of periodate resin was added to the solution and left to react during stirring for 3 min still under argon. The quinone solution was used immediately after preparation. The amount of phenol converted to quinone was estimated to be ∼95% by HPLC-UV−vis, which gives a quinone concentration of ∼0.095 M in the quinone solution. The quinone solution was stable for up to 30 min after preparation.

Preparation of Samples. The thiol sources, L-cysteine (Cys), glutathione (GSH), BSA, and MPI, were diluted in 0.1 M maleic acid buffer adjusted to pH 5.8 with NaOH and incubated either with 4MC, $FeSO_4$, and H_2O_2 at room temperature for 5 h (simultaneous oxidation of thiol source and 4MC) or with 4MBQ, prepared as described above, at room temperature for 30 min (direct reaction of thiol source and 4MBQ). The simultaneous oxidation of thiol source and 4MC with H_2O_2/Fe(II) corresponds to the reaction pathways presented in Scheme 1(A and B), whereas the direct reaction of thiol source and 4MBQ corresponds to the reaction pathway presented in Scheme 1B. The aqueous solutions containing GSH, BSA, or MPI were after incubation hydrolyzed under argon at 110 °C for 22 h by mixing equal

Table 1. Composition and Concentrations of Reagents in Samples Evaluated by LC-MS

		direct reaction of thiol source and 4MBQ	simultaneous oxidation of thiol source and 4MC		
thiol source		4-MBQ (mM)	4-MC (mM)	$FeSO_4$ (mM)	H_2O_2 (mM)
Cys	2.0 mM	2.0	2.0	2.0	2.0
GSH	2.0 mM	2.0	2.0	2.0	2.0
BSA	2.0 mM	1.0	2.0	2.0	2.0
MPI	66 mg/mL	1.0	1.0	1.0	1.0

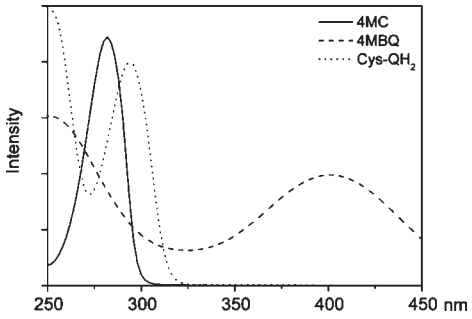


Figure 1. UV spectra of 4MC (—) and 4MC oxidized by periodate resin to yield 4MBQ (– –) and the addition product (Cys−QH_2) in the presence of equimolar concentrations of cysteine (···).

amounts of 12 M HCl and aqueous solution, reaching approximately 4 mL of hydrolyzed protein solution. The final concentrations of samples analyzed by LC-MS are presented in Table 1. All samples were filtered through a 0.22 μm syringe filter before injection in the LC-MS.

Analysis by LC-ESI-MS. The samples (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 diode array detector (DAD). Mass spectrometry analysis was carried out in the positive mode on an 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 (4MC), m/z 244.2 (Cys−QH_2), m/z 366.3 (Cys−QH_2-QH_2), m/z 430.4 (GSH−QH_2), and m/z 552.5 (GSH−QH_2−QH_2). The MS detector parameters were as follows: API-ES, positive polarity; drying gas flow, 12.0 L/min; nebulizer pressure, 35 psig; drying gas temperature, 350 °C; capillary voltage, 3000 V; fragmentor, 30 V.

RESULTS AND DISCUSSION

An LC-MS method was developed to separate the thiol−quinone adducts (RS−QH_2) formed by an addition reaction between 4MBQ and the thiol group of the cysteine residue of myofibrillar protein from meat. The thiol−quinone adducts were generated either by direct reaction with 4MBQ or by simultaneous oxidation with 4MC under oxidative conditions (Fe(II)/H_2O_2).

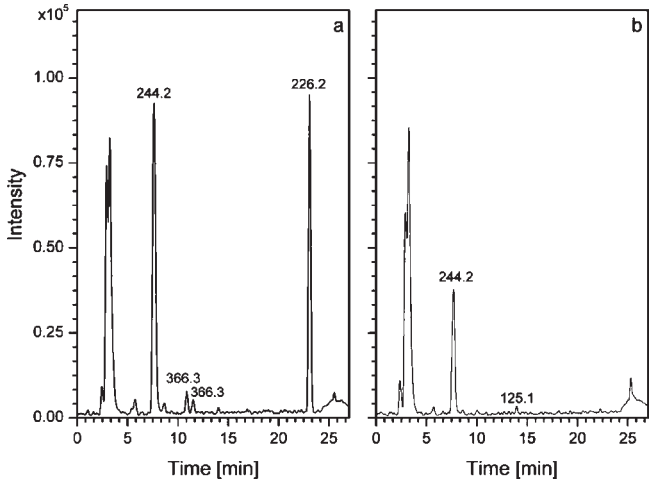


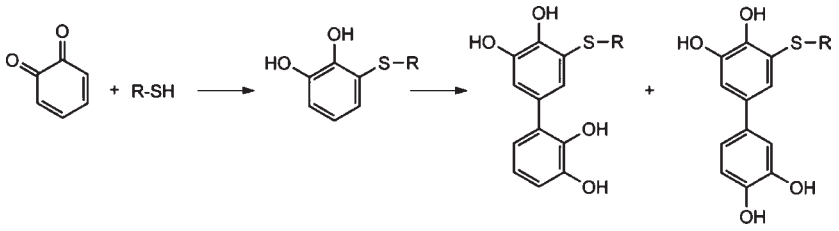
Figure 2. Mass chromatogram (+MS, API-ES, Pos, SCAN) of the thiol−quinone adduct formation in aqueous solutions of (a) 2 mM cysteine and 2 mM 4MBQ or (b) 2 mM cysteine, 2 mM 4MC, 2 mM H_2O_2, and 2 mM $FeSO_4$.

LC-MS Method Development. The LC-MS method development was based on Cys or Cys-derived compounds incubated with 4MBQ. Injection of 4MBQ, prepared from oxidation of 4MC by periodate resin, diluted in acetonitrile with no thiol source, resulted in a single peak at R_T = 7.59 min on the mass chromatogram. The peak had an UV spectrum as shown in Figure 1 (4MBQ) and had, according to the mass spectrum, an m/z of 123.2, which corresponds to the positively charged 4MBQ $[M + H]^+$ or the quinone dianion, which is reported to be formed in nonaqueous solutions by two successive one-electron reduction steps.[27] Injection of 4MBQ diluted in buffer (pH 5.8) with no thiol source resulted in an additional peak at R_T = 13.97 min with a UV spectrum as shown in Figure 1 (4MC) and an m/z of 125.1, which corresponds to the positively charged hydroquinone (QH_2) $[M + H]^+$ formed from 4MBQ in buffered aqueous solutions through two-electron−two-proton reduction steps.[27]

Injection of Cys incubated with 4MBQ resulted in a peak at R_T = 7.63 min. This peak had an UV spectrum different from that of 4MBQ as shown in Figure 1 and had an m/z of 244.2, which corresponds to the $[M + H]^+$ addition product between Cys and 4MBQ (Cys−QH_2) as exemplified by a catechol in Scheme 1B. Similar UV spectra of the 4MC and the Cys−QH_2 were shown by Richard et al.[18] for a system containing 4MC oxidized by PPO in the presence of excess cysteine. The same study identified the structure of the addition compound between cysteine and 4MC by NMR as being 5-*S*-cysteinyl-3,4-dihydroxytoluene (6-*S*-cysteinylcatechol). Cilliers and Singleton[23] found that the addition product of the quinone of caffeic acid and cysteine primarily was 2-*S*-cysteinylcaffeic acid with the concomitant formation of 5-*S*-cysteinylcaffeic acid and 2,5-di-*S*-cysteinylcaffeic acid as secondary products. Furthermore, studies using caftaric acid and GSH found the reaction product to be 2-*S*-cysteinylcaftaric acid.[16] These results show that quinone−thiol reactions are common and that the specific products depend on the character of the phenolic compound.

Oxidation Mechanism of Phenols and Thiol−Quinone Adduct Formation. 4MBQ generated by oxidation of 4MC with Fe(II)/H_2O_2 in buffer (pH 5.8) with no thiol source present was unstable and, therefore, not observed by LC-MS. The

Scheme 2. Formation of Dimeric Addition Isomers of 1-Cysteinyl-5-(catechyl)-2,3-catechol



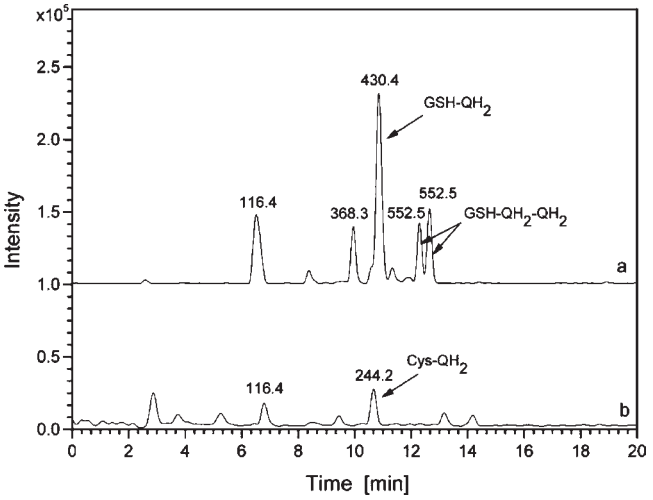


Figure 3. Mass chromatogram (+MS, API-ES, Pos, SCAN) of the thiol−quinone adduct formation in aqueous solutions of 2 mM GSH and 2 mM 4MBQ, which have been (a) injected directly onto the LC-MS or (b) hydrolyzed before injection onto the LC-MS.

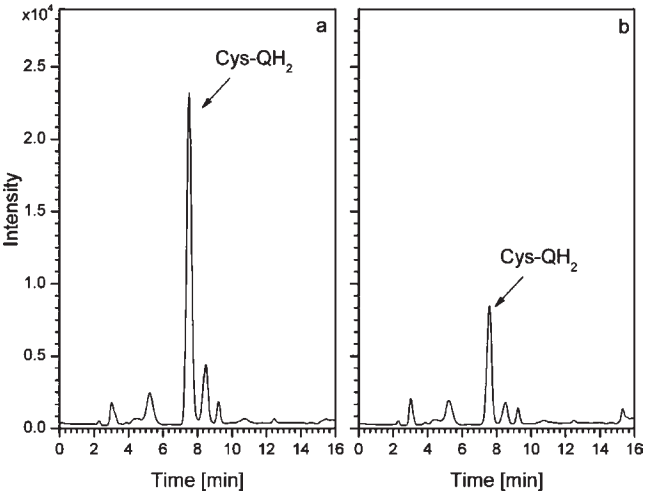


Figure 4. Mass chromatogram (+MS, API-ES, Pos, SIM) of the thiol−quinone adduct formation in hydrolyzed aqueous solutions of (a) 2 mM BSA and 1 mM 4MBQ or (b) 2 mM BSA, 2 mM 4MC, 2 mM H_2O_2, and 2 mM $FeSO_4$.

quinone appeared to be reduced to the catechol (hydroquinone) immediately after oxidation, as also described above for quinones in aqueous buffered solutions.[27] By adding a thiol to the oxidation reaction, in this case, cysteine, the production of 4MBQ was detected indirectly by the detection of Cys−QH_2. By omitting H_2O_2 and Fe(II), only very small amounts of adduct were detected, supporting the proposed reaction mechanism in Scheme 1A. The reaction mechanism suggests that 4MBQ is produced by reaction with peroxyl radicals generated from metal-catalyzed activation of oxygen. However, under these conditions, this reaction is limited by the limited availability of oxygen and Fe(II), and the production of quinones can be accelerated by the addition of hydrogen peroxide and Fe(II), which generate the more reactive hydroxyl radicals (Scheme 1A), although this oxidant also reacts with other components, partly explaining the low conversion. Accelerated formation of 4MBQ increases the formation of Cys−QH_2 as shown in Scheme 1B, and provides sufficient levels of Cys−QH_2 for detection by LC-MS analysis, as shown in Figure 2. Hydrogen peroxide and Fe(II) addition simulate the conditions in meat according to Kanner;[28] however, the relative proportions of the reagents may differ from those of actual meat.

Quinone Reactivity. The formation of Cys−QH_2 adducts was higher for Cys incubated with 4MBQ compared to incubation with 4MC and Fe(II)/H_2O_2 (Figure 2). Moreover, generation of adduct after direct reaction with 4MBQ resulted in the formation of two dimeric addition products between one cysteine and two 4MBQ (Cys−QH_2−QH_2) with an m/z of 366.2 and R_T of 10.88 and 11.52 min (Figure 2a) and an unidentified reaction product with an m/z of 226.2 and R_T = 23.08 min. Elution of the two separate dimeric addition peaks indicates the production of isomers, which, on the basis of previous findings,[18,23] are suggested to be isomeric forms of 1-cysteinyl-5-(catechyl)-2,3-catechol. The two dimeric isomers and the unidentified compound were not generated by simultaneous oxidation of Cys and 4MC with Fe(II)/H_2O_2 (Scheme 2), which suggests that the concentration of the quinone was not as high in the incubation with 4MC and Fe(II)/H_2O_2. Direct addition of the reactive quinones (4MBQ) may result in multiple reaction products, as the high concentration of quinones permits indiscriminate reactions with multiple targets or may polymerize before or after reaction with a nucleophile. This scenario may be different from the simultaneous oxidation of thiol source and phenols by Fe(II)/H_2O_2, when quinones are expected to be formed concurrently with their consumption in the thiol−quinone adduct formation.

Hydrolysis of GSH and Proteins for the Release of the Thiol−Quinone Adduct for LC-MS Detection. Solutions of GSH were employed to verify the use of acid hydrolysis for separating the Cys−QH_2 from the remaining amino acid residues. The thiol−quinone adduct was generated on the cysteine residue of GSH by incubating the GSH solution with 4MBQ prepared by periodate oxidation. The mass chromatogram is shown in Figure 3. A slightly modified LC-MS method was used for the separation of GSH−QH_2 adduct by LC-MS, and as seen in Figure 3 the GSH−QH_2 (m/z 430.4) eluted at the retention

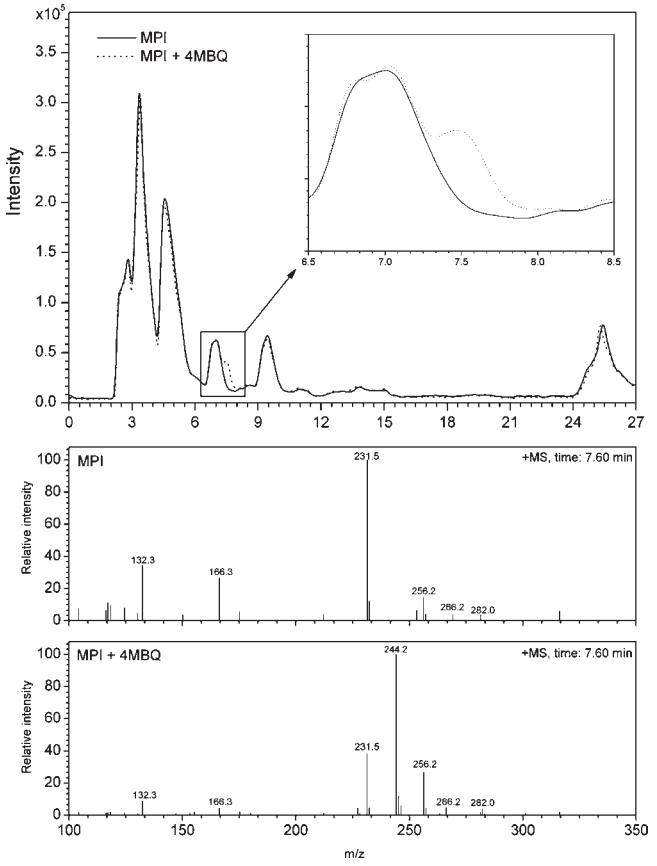


Figure 5. (Top panel) Mass chromatogram (+MS, API-ES, Pos, SCAN) of hydrolyzed MPI (66 mg/mL) incubated without 4MBQ (—) or with 1 mM 4MBQ (···). (Bottom panels) Mass spectra (+MS) of the same samples at R_T = 7.60 min.

time R_T = 10.88 min. Again, dimeric reaction products (m/z 552.5) eluted later with the retention times R_T = 12.32 and 12.67 min. As seen in Figure 3, it was possible to detect Cys−QH_2 after hydrolysis of GSH incubated with 4MBQ. The unlabeled peaks in the mass chromatograms are primarily amino acids or unidentified compounds.

Detection of the Thiol−Quinone Adduct by SIM Mode during LC-MS Analysis. BSA was incubated with 4MBQ prepared by periodate oxidation, and the product solution was hydrolyzed by acid treatment. An UV chromatogram at 254 nm obtained after injection of the hydrolyzed solution showed a peak at R_T = 7.46 min, which had a mass spectrum identical to that of the Cys−QH_2 adduct. However, the peak was asymmetric and partly also contained an unidentified ion of m/z 231.5, which was impossible to separate from the Cys−QH_2 adduct by LC. To detect the Cys−QH_2 adduct in complex hydrolyzed protein samples, SIM mode was used instead of scan mode to detect the ions of interest, and as seen in Figure 4, Cys−QH_2 was found in BSA after hydrolysis. Direct reaction of 2 mM BSA with 1 mM 4MBQ (Figure 4a) resulted in relatively higher adduct formation compared to the simultaneous oxidation of 2 mM BSA and 2 mM 4MC with Fe(II)/H_2O_2 (Figure 4b). As a control, a solution of α-lactalbumin, which contains no free thiol groups, was prepared similarly to the BSA solution. No peak with the characteristics of the Cys−QH_2 adduct was found for α-lactalbumin, which supports the findings of the Cys−QH_2 adduct for proteins containing thiol groups (data not shown).

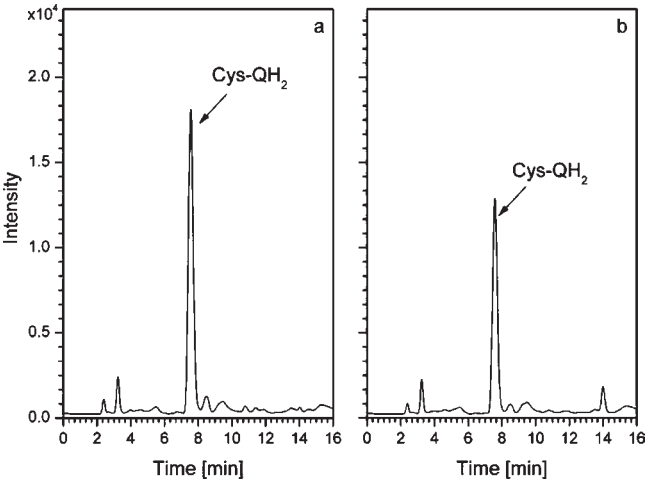


Figure 6. Mass chromatogram (+MS, API-ES, Pos, SIM) of the thiol−quinone adduct formation in hydrolyzed aqueous solutions of (a) 66 mg/mL MPI and 1 mM 4MBQ or (b) 66 mg/mL MPI, 1 mM 4MC, 1 mM H_2O_2, and 1 mM $FeSO_4$.

Thiol−Quinone Adduct Formation in a Myofibrillar Protein Isolate. MPI from beef was incubated with either 4MBQ or 4MC and Fe(II)/H_2O_2. The ratio of phenol to protein was 1.8 mg of phenol/g of protein and corresponds approximately to the ratio used in the study by Jongberg et al.,[12] in which the interactions between phenolic substances of a WGE and the myofibrillar proteins in a storage experiment with beef first were reported. Figure 5 shows the mass chromatograms of the hydrolyzed MPI with or without direct reaction with 4MBQ. The chromatograms show that the hydrolysis products from the two samples are nearly identical apart from the interval from 7.3 to 8.1 min, when the Cys−QH_2 elutes. The only difference in the ion distribution between MPI with or without direct reaction with 4MBQ is the Cys−QH_2 ion with m/z 244.2, as seen in the mass spectra (Figure 5, bottom panels). This indicates that the primary site for quinone−protein interaction in MPI may be the thiol group of the cysteine residue. For comparison, Rawel, Czajka, Rohn, and Kroll[29] found adduct formation with lysine, tryptophan, and cysteine of soy protein, but the much higher pH 9 versus 5.8 used here would have enhanced the availability of the amine nucleophiles as they would not be fully protonated at the higher pH. Similarly, Rawel et al.[22] reported adduct formation with only lysine and tryptophan in whey protein, and this was presumably a result of the limited amount of free thiol groups in β-lactoglobulin and the absence in α-lactalbumin.

Figure 6 shows the mass chromatograms of the MPI incubated with 4MBQ or 4MC and Fe(II)/H_2O_2. Again, an increased adduct formation by direct reaction with 4MBQ compared to the simultaneous oxidation of thiol source and 4MC was observed, as was also observed for the other thiol sources used in this study.

In conclusion, a method for the detection of thiol−quinone (Cys−QH_2) adducts in the myofibrillar fraction of meat protein was developed on the basis of different thiol sources. Furthermore, the present study shows that the Cys−QH_2 adduct was formed in a model system prepared by MPI from beef. Thiol−quinone adducts were formed both by direct reaction with the quinone of 4MC or by simultaneous oxidation of thiol source and 4MC by Fe(II)/H_2O_2. The results imply that such adduct formation may be part of a novel antioxidative mechanism protecting against

disulfide formation in meat by blocking the thiol groups of the cysteine residues. The formation of thiol−quinone adducts as well as the effect on the oxidative stability of protein in beef with 4MC stored under high-oxygen atmospheres are currently being studied. Additional studies are needed to verify if the formation of disulfide cross-link are inhibited by the thiol−quinone interactions in actual meat samples.

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