Influence of the Oxidation States of 4-Methylcatechol and Catechin on the Oxidative Stability of β-Lactoglobulin

Sisse Jongberg,*† Mariana Utrera,‡ David Morcuende,‡ Marianne N. Lund,‡ Leif H. Skibsted,‡ and Mario Estévez‡†

†Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg, Denmark
‡IPROCAR Research Institute, TECAL Research Group, University of Extremadura, 10003 Cáceres, Spain

ABSTRACT: Chemical interactions between proteins and phenols affect the overall oxidative stability of a given biological system. To investigate the effect of protein–phenol adduct formation on the oxidative stability of β-lactoglobulin (β-LG), the protein was left to react with an equimolar concentration of 4-methylcatechol (4MC), catechin (Cat), or their respective quinone forms, 4-methylbenzoquinone (4MBQ) and catechin–quinone (CatQ), and subsequently subjected to metal-catalyzed oxidation by Fe(II)/H<sub>2</sub>O<sub>2</sub> for 20 days at 37 °C. The reaction with 4MBQ resulted in 60% thiol loss and 22% loss of amino groups, whereas the addition of 4MC resulted in 12% thiol loss. The reaction with Cat or CatQ resulted in no apparent modification of β-LG. The oxidative stability of β-LG after reaction with each of 4MC, 4MBQ, Cat, or CatQ was impaired. Especially 4MC and 4MBQ were found to be pro-oxidative toward α-aminoacidic semialdehyde and γ-glutamic semialdehyde formation as well as the generation of fluorescent Schiff base products. The changes observed were ascribed to the redirection of oxidation as a result of the blocking of thiol groups but also to the oxidative deamination pathway, accelerating the production of semialdehydes and subsequently Schiff base structures.

KEYWORDS: β-lactoglobulin, 4-methylcatechol, catechin, quinone, protein oxidation, metal-catalyzed oxidation

1. INTRODUCTION

Plant phenolics are used in food products and pharmaceuticals for their bioactivity with regard to protection against primarily lipid oxidation. Lately, phenolic antioxidants have also been applied to various products for the protection against protein oxidation. However, in combination with proteins, phenolic compounds result in different consequences to the redox status of the protein, including an increase in protein oxidation, where the phenols act as pro-oxidants but also with synergistic antioxidative effects that protect lipids. The ambiguous effects are likely associated with the binding affinity of the phenols toward proteins, which includes both covalent and non-covalent bonds, and in differences in antioxidant mechanisms between different polyphenols. The binding between proteins and phenols is controlled by a number of factors, including the oxidative status of the biological system, the structure of the phenol, and the amino acid composition and conformation of the protein. Hence, it is difficult to estimate the effect of a given antioxidant in a specific system.

As recently reviewed by Le Bourvellec and Renard, the covalent interactions between proteins and phenols include nucleophilic addition, which yields chemical bonds that may result in polymerized reaction products composed of both components. Oxidation of the phenol to its oxidized quinone is a prerequisite for the nucleophilic addition and requires a one-electron transfer to generate a semiquinone radical followed by a second electron transfer to yield the quinone. The oxidation occurs spontaneously in the presence of molecular oxygen under alkaline conditions (pH > 9.0) or can be catalyzed by polyphenol oxidases (PPO), or metallic cations. Scheme 1 shows the two-step metal-catalyzed oxidation of phenols to quinones.

Quinones are electrophilic compounds and react rapidly with nucleophilic side chains, especially amino or thiol groups. Scheme 2 shows the reaction between a quinone and a protein thiol group to yield a thiol–quinone (TQ) adduct (reaction A) and between a quinone and a protein amino group to yield amino–quinone (AQ) adducts (reactions B1 and B2). The thiol and amino groups may react with the quinone via a Michael-type addition, where a carbon atom in the aromatic ring is substituted with the protein residue to yield a carbon-substituted protein–phenol adduct (reactions A and B1). Additionally, amino groups may also react through a nucleophilic reaction with the ring group of the quinone to generate iminoquinones (Schiff base-like structures) (reaction B2).

Many previous reports have focused on the structure–affinity relationship of binding between phenols and proteins and the effect of such covalent interactions on the physicochemical properties and digestibility of diverse food proteins. However, only few works have studied the oxidative stability of the protein–phenol adducts and rarely any considered the antioxidative capacity of the modified protein and its own oxidation status.

A better understanding of the nature and redox consequences of the protein–phenol interactions is required to comprehend the chemistry behind their biological effects and apply these phytochemicals in food systems so that reliable and predictable antioxidant effects are gained. The aim of the
To Yield Amino Adduct or between a Quinone and a Protein Amino Group (cuproine, Amberlyst A26, and benzoic acid) (DTNB), N-Sentmenat, Spain. Cat, 4MC, Trizma base, 5,5-dithiobis(2-nitro-}

Scheme 1. Two-Step Metal-Catalyzed Oxidation of a Phenol To Yield the Intermediate Semiquinone Radical and Subsequently the Quinone and Hydrogen Peroxide

Scheme 2. Nucleophilic Addition between a Quinone and a Protein Thiol Group (A) To Yield a Thiol—Quinone (TQ) Adduct or between a Quinone and a Protein Amino Group To Yield Amino—Quinone (AQ) Adducts by Either Michael Addition (B1) or Formation of Schiff Base-Like Structures (B2)

present study was to determine how protein—phenol adduct formation between the quinones of 4-methylcatechol (4MC) or catechin (Cat) affects the metal-catalyzed oxidation of β-lactoglobulin (β-LG). The two phenolic compounds were added in either their reduced phenolic form or in their oxidized quinoic form to identify possible changes in the oxidation pattern of β-LG depending upon the redox status of the phenolic compounds. The oxidation was followed for prolonged incubation times to investigate both early and later stages of oxidation.

2. MATERIALS AND METHODS

2.1. Reagents. β-LG (Mₙ ~ 18 300) consisting of a mixture of the variants A and B was isolated from bovine milk as described by Kristiansen et al. 13 Malic acid was obtained from Scharlau SL, Sentmenat, Spain. Cat, 4MC, Trizma base, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), Trolox, neocuproine, Amberlyst A26, and fluorescamine was obtained from Sigma-Aldrich, St. Louis, MO. Reagent-grade chemicals and double-deionized (Milli-Q) water were used throughout.

2.2. Peroxidation of Phenols by Periodate Resin. Periodate resin was prepared according to Harrison and Hodge, 14 with slight modifications as recently described by Jongberg et al. 15 4MC and Cat was oxidized by mixing 158 mg of periodate resin with 12.4 mg of 4MC or 14.5 mg of Cat dissolved in acetone for 3 min during stirring. The amount of 4MC converted to 4-methylbenzoquinone (4MBQ) was estimated to be ~95% by high-performance liquid chromatography—ultraviolet/visible spectroscopy (HPLC—UV/vis). 16 giving an estimated concentration of ~95 mM 4MBQ or 47.5 mM catechin—quinone (CatQ). The quinone solutions were used immediately for the preparation of samples.

2.3. Preparation of Samples. Samples were prepared in 0.1 malic acid buffer (pH 5.8) to contain 5.0 mg/mL (273 μM) β-LG alone or with addition of 273 μM 4MC or Cat or their corresponding quinones, 4MBQ or CatQ, and left to react for 10 min at room temperature (20 °C). In parallel, blank phenol and quinone samples were prepared in the same concentrations without the addition of β-LG, NEM-derivatized β-LG was prepared by letting 273 μM β-LG react with 7.2 mM NEM for 90 min at room temperature (20 °C). A corresponding blank NEM sample was prepared in parallel without the addition of protein. In total, 11 samples were prepared in triplicate: 6 samples with protein, β-LG, β-LG—4MC, β-LG—4MBQ, β-LG—Cat, β-LG—CatQ, and β-LG—NEM, and 5 samples without protein, 4MC, 4MBQ, Cat, CatQ, and NEM.

2.4. Metal-Catalyzed Oxidation. Oxidation of all 11 samples was initiated by the addition of 50 μM Fe₃(OH)₂⁺ and 5 mM H₂O₂ both dissolved in Milli-Q water. The samples were oxidized in the dark at 37 °C for up to 20 days with constant stirring. Prior to initiation, day 0 samples were collected for thiol and Schiff base analyses, which were carried out on the same day. Samples for α-amino adipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) quantification were frozen at ~80 °C until analysis. Onward, samples were collected at days 1, 3, 5, 8, 12, and 20, and the same procedure as for the day 0 samples was followed.

2.5. Antioxidative Activity by the Cupric Reducing Antioxidant Capacity (CUPRAC) Assay. Samples were prepared as described above (section 2.3), although including additional concentrations of 4MC, 4MBQ, Cat, and CatQ. For each, three concentration levels were added to 273 μM β-LG in 0.1 M malic acid buffer (pH 5.8), namely, 137, 273, or 546 μM, corresponding to the molar ratio between β-LG and the phenol or quinone of 1:0.5, 1:1, or 1:2, respectively. In parallel, blank samples without the addition of β-LG were prepared, containing only 137, 273, or 546 μM 4MC, Cat, 4MBQ, or CatQ, respectively. Samples containing β-LG—NEM and its corresponding blank NEM was prepared as described above. An aliquot of 0.1 mL sample was added to a test tube containing 1.0 mL of 10 mM CuCl₂ dissolved in water, 1.0 mL of 1 M ammonium acetate buffer (pH 7.0), 1.0 mL of 7.5 mM neocuproine in ethanol, and 1.0 mL of water. The mixture was mixed and left to react at room temperature (20 °C) for 30 min before the absorbance at 450 nm was read on a HITACHI U-2000 spectrophotometer. Trolox dissolved in 80% ethanol was used as the standard, and data are given as Trolox equivalent antioxidant capacity (TEAC) and presented as millimolar...
Trolox equivalents (mean ± standard deviation) of three independent replicates (n = 3). Three different TEAC values are presented: one of the model system containing β-LG, one with of the blank model system without β-LG, and finally, a summarized TEAC, which represents the sum of the TEAC of the blank model system plus the TEAC of β-LG alone.

2.6. Quantification of Thiol Groups. Protein thiol groups in samples prior to oxidation by FeSO₄/H₂O₂ were determined by derivatization with DTNB17 and recently described by Jongberg et al.15 The thiol concentrations given as nanomoles of thiol per milligram of protein and are calculated on the basis of a standard curve prepared from cysteine. The contribution measured in the corresponding blank sample without protein.

2.7. Quantification of Free Amino Groups. Free amino groups in samples prior to oxidation by FeSO₄/H₂O₂ were determined as described by Weigele et al.16 and Strauss and Gibson.19 An aliquot of 850 μL of sample containing ~0.01 mg/mL β-LG was added to 2.0 mL of 0.05 M sodium tetraborate (pH 8.5) in a 4 mL quartz spectrophotometer cell, and 150 μL of 0.7 mM fluorescamine solution in acetonitrile was added. The cell was inverted 4 times, and the resulting fluorescence was measured using 390/485 nm for excitation and emission, respectively, on a PerkinElmer LS45 fluorescence spectrometer (Llantrisant, U.K.). The contribution from the malic acid buffer (pH 5.8) was recorded under the same conditions and subtracted all samples. The free amino group concentration was calculated on the basis of a standard curve prepared from lysine diluted in malic acid buffer (pH 5.8). The concentration is given as micromoles of amino groups per milligram of protein.

2.8. Quantification of the Semialdehydes AAS and GGS. The protein oxidation products AAS and GGS were quantified in samples from the samples containing protein according to the method described by Utrea et al.31 An aliquot of 250 μL of sample was precipitated with 1.3 mL of ice-cold 10% trichloroacetic acid (TCA) and centrifuged at 5000 rpm for 30 min and the supernatant was discarded. The pellet was precipitated again with 1.5 mL of ice-cold 5% TCA and centrifuged for 5 min and the supernatant was discarded. The pellet was derivatized by the addition of 0.5 mL of 1% sodium dodecyl sulfate and 1 mM diethylenetriaminepentaacetic acid in 0.25 M MES buffer at pH 6.0 (SDS/DTPA), 0.5 mL of 50 mM sodium tetraborate (pH 8.5) was recorded under the same conditions and subtracted all sample spectra. The concentration of the Semialdehydes AAS and GGS was quantified in samples prior to oxidation by FeSO₄/H₂O₂ were determined as nanomoles of thiol per milligram of protein and are calculated on the basis of a standard curve prepared from cysteine. The contribution measured in the corresponding blank sample without protein.

2.9. Quantification of Schiff Base Structures. The emission of fluorescence by protein oxidation products (Schiff base structures) was quantified in samples (pH 5.8, no oxidants added) containing 273 μM β-LG alone or after reaction with 273 μM 4MC, 4MBQ, Cat, or CatQ for 10 min or after treatment with excess NEM for 90 min at room temperature. The asterisks (*) indicate the sample is significantly different (p < 0.05) from the model system containing only β-LG.

3. RESULTS

3.1. Phenol- or Quinone-Derived Modifications of β-LG. To evaluate the extent of protein—phenol adduct formation, the concentration of amino and thiol groups of β-LG were determined by derivatization with DTNB17 and recently described by Jongberg et al.15 The samples without β-LG were used as blank samples in the analyses to compensate for any interference from the phenols or quinones in the assays. The results showed a significant decrease of 22% amino groups by the addition of 4MBQ to β-LG (upper panel of Figure 1). Higher reactivity of the phenols and quinones was observed for the reaction with thiol groups, where the addition of 4MBQ and 4MC significantly reduced the thiol group concentration by 60 and 12%, respectively.

![Figure 1. Free amino and thiol groups (mean ± standard deviation; n = 3) in samples (pH 5.8, no oxidants added) containing 273 μM β-LG alone or after reaction with 273 μM 4MC, 4MBQ, Cat, or CatQ for 10 min or after treatment with excess NEM for 90 min at room temperature. The asterisks (*) indicate the sample is significantly different (p < 0.05) from the model system containing only β-LG.](image-url)
As a control, the thiol concentration was also determined in NEM-treated β-LG, and NEM was found to block 98% of the thiol groups.

### 3.2. Antioxidative Capacity of the β-LG Samples

The antioxidative capacity of the samples prior to oxidation by Fe(II)/H2O2 was evaluated by the CUPRAC assay (Figure 2). The overall antioxidative capacity was determined for all samples with or without β-LG, including not only the 1:1 molar ratio between the protein and phenol or quinone, but also the ratios 1:0.5 and 1:2. The TEAC obtained for all samples increased proportionally with the concentration of phenol or quinone (panels B–E of Figure 2). Comparing the measured TEAC of the sample with the summarized TEAC indicates whether the combination of protein and phenol or quinone interact to provide an additive, synergistic, or antagonistic antioxidative capacity. A statistically significant difference (marked by * in Figure 2) between the measured TEAC and the summarized TEAC indicates a synergistic or antagonistic effect, whereas the effect is additive for the samples with no significant difference between the measured and summarized TEAC. In this way, additive effects were observed in the β-LG samples containing 4MC in the ratios 1:1 and 1:2 and in the β-LG sample containing Cat in the ratio 1:2 (panels B and D of Figure 2). All β-LG samples containing quinones (panels C and E of Figure 2) as well as the low ratios of 4MC (1:0.5) and Cat (1:0.5 and 1:1) resulted in antagonistic antioxidative capacities, which means that the combination of β-LG and phenols or quinones in most cases reduces the overall antioxidative capacity. Also, derivatization of β-LG with NEM (Figure 2A) led to a significant decrease of TEAC, as compared to β-LG alone.

### 3.3. Thiol Loss during in Vitro Oxidation

The samples were oxidized by Fe(II)/H2O2 at 37 °C for up to 20 days. The results showed that thiols were rapidly lost and reached steady state already at day 1 after initiation for most samples (Figure 3). The samples containing only β-LG reached ~15 nmol of thiols/mg protein at day 1, and this level was not significantly different from the level at day 20 (Figure 3). The same pattern was observed in the β-LG samples containing 4MBQ, Cat, and CatQ (Figure 3), although with a lower starting point for the 4MBQ as a result of the modifications induced to the protein thiols, as shown in Figure 1. In contrast, a significant increase in...
the thiol level from days 1 to 8 was found in the β-LG sample containing 4MC, which unfortunately cannot be definitely explained on the basis of the experimental design of the present study.

3.4. AAS and GGS Formation during in Vitro Oxidation. Both AAS and GGS were quantified in the β-LG samples, and results showed that limited but significant formation took place up to day 12 in the samples containing only β-LG (Figure 4). In β-LG treated with NEM, where all thiol groups were blocked, a rapid and significant increase in AAS was observed until day 5, where the concentration of carbonyl peaked to decrease afterward. For β-LG containing 4MC, 4MB, Cat, or CatQ, the concentration of AAS increased significantly until day 12 and then subsequently decreased (upper panel of Figure 4). The presence of 4MC or 4MBQ significantly increased the concentration of GGS already at day 1, which continued to be significantly higher until day 12, as compared to β-LG alone, whereas Cat and CatQ had no significant effect on GGS formation in β-LG (lower panel of Figure 4). For β-LG treated with NEM, no GGS was found.

3.5. Formation of Schiff Base Structures during in Vitro Oxidation. The reaction between various carbonyl and amino groups leads to the formation of unspecific Schiff base structures that can be quantified by fluorescence spectroscopy. In the sample containing only β-LG, the Schiff base structures increased significantly after 12 days of storage, whereas in β-LG treated with NEM, the structures increased significantly already from day 1 (Figure 5). In the present study, the emission spectra for the sample containing only β-LG or β-LG and NEM showed a maximum at 420 nm. However, the maximum shifted toward a higher wavelength (440 nm) by the addition of phenols or quinones. Both emission wavelengths were monitored, but because no differences in the overall results were observed, only the data from 440 nm are presented. In the β-LG samples containing 4MC or 4MBQ, significantly elevated fluorescence was observed at day 3, as compared to β-LG alone. In contrast, in β-LG samples containing Cat or CatQ, the Schiff base structures increased significantly after day 8 (Figure 5).
apparent differences were observed between Cat and CatQ with regard to Schiff base structures.

4. DISCUSSION

4.1. β-LG Oxidation and Effect of NEM-Blocking of Thiol Groups. The overall antioxidative capacity of β-LG as determined by CUPRAC was significantly reduced after treatment with NEM, indicating that β-LG was less resistant toward oxidation when the thiols were blocked. The CUPRAC assay was selected as a marker of overall antioxidative activity because it also includes the reducing effect of protein thiols, and the result is in accordance with a recent report showing that thiol groups in a myofibrillar protein isolate (MPI) are important for the scavenging of perferrylmyoglobin radicals. In the present study, the thiols were rapidly lost during the first day of storage in the presence of Fe(II) and hydrogen peroxide generating hydroxyl radicals (Figure 3). NEM effectively blocked the thiol groups of β-LG, and consequently, no thiols were available for oxidation in β-LG treated with NEM, which changed the target of the oxidants. This is evident from the rapid formation of AAS during the first days of storage in β-LG treated with NEM as opposed to a slower formation of AAS in untreated β-LG. Similarly, the formation of Schiff base structures also increased more rapidly in NEM-treated β-LG compared to untreated β-LG. These data show that thiols are oxidized more rapidly in our system than alkaline amino acids and are, therefore, better markers of early oxidation events in systems without polyphenols, which is consistent with the higher rate constants reported for the reaction of hydroxyl radicals with thiols than with alkaline amino acids. Sulfur-containing amino acid residues, such as Cys and Met, may act as intramolecular antioxidants in proteins by scavenging radical species. Elias et al. found no loss of Met during oxidation of β-LG but a rather rapid initial loss of thiols, followed by a plateau during continuing oxidation. This sacrificial protection by the protein thiols was not possible in β-LG treated with NEM, and therefore, other susceptible amino acid residues, such as alkaline amino acids, were subsequently attacked by the oxidants. Concomitantly, in β-LG treated with NEM, AAS increased rapidly during the first days of storage, whereas GGS increased slowly until day 12 together with the formation of Schiff base structures, which continued to increase throughout the reaction period. This finding emphasizes the role of metal-catalyzed oxidation in the carboxylation of β-LG when thiols are not available for oxidation. AAS, GGS, and other protein oxidation products may engage in reactions involving a carbonyl group and an amino group, leading to advanced protein modifications similar to Maillard reactions with the generation of Schiff base structures. The overall higher concentration of AAS compared to that of GGS in the β-LG systems is in agreement with previous reports and may be derived from the higher concentration and susceptibility of lysine in β-LG toward metal-catalyzed oxidation compared to other alkaline amino acids.

4.2. Effect of Protein–Phenol Adducts on β-LG Oxidation. In the present study, quantification of amino and thiol groups prior to initiation of oxidation showed that the addition of 4MBQ decreased the concentration of protein amino and thiol groups, suggesting the formation of covalent TQ and AQ adducts for β-LG in the presence of 4MBQ (Scheme 2). In a previous study, it was shown by liquid chromatography–mass spectrometry (LC–MS) analysis that >20% of β-LG was found as protein–phenol adducts when 4MBQ was added in an equimolar ratio at pH 8.0, while 40% of the thiols were lost. In the present study, 60% of the thiols were lost by the addition of an equimolar concentration of 4MBQ at pH 5.8. However, the concentration in the present study was 5 times higher, and pH was lower than in the aforementioned study, which may have led to an increased reaction rate and changes in the protein structure that may have altered the reactivity of specific amino acid side chains as a result of changed exposure to the environment. The smaller loss of amino groups (ca. 20%) suggests that the reaction with quinones was slower than that with thiols. This may be explained by the difference in pKi of the nucleophilic sites (e.g., pKi for ε-amino group of Lys is 10.79, and pKi for thiol group of Cys is 8.37) because a larger proportion of the thiols will be deprotonated and, thereby, more reactive at pH 5.8 compared

Scheme 3. Formation of AAS and the Schiff Base Product Azomethine via (A) Metal-Catalyzed Oxidation of a Protein Lysine Residue or (B) Oxidative Deamination Reaction of the AQ Adduct Iminoquinone

**R** represents the methyl of 4-MC or the A and C ring of Cat.
to the amines. On the basis of these data, it is not possible to clarify whether the thiol loss is caused by pro-oxidative behavior oxidation or adduct formation. During the in vitro oxidation, the thiol level at day 1 was found to be similar in β-LG independent of the presence of 4MC or 4MBQ.

The addition of both 4MC and 4MBQ also caused an increase in the formation of AAS, GGS, and Schiff bases. This apparent pro-oxidative effect was similar to the observation of the NEM-blocked β-LG and may be explained by three different mechanisms. First, it may likely be caused by the elimination of the thiols as oxidation targets or radical scavengers in β-LG, exposing other amino acids to oxidation. Second, phenols may also act as pro-oxidants, forming hydrogen peroxide in the presence of transition metals (Scheme 1). Zhou and Elias showed that 400 μM (−)-epigallocatechin-3-gallate (EGCG) produced 350 μM hydrogen peroxide during 4 days of storage of emulsions in the presence of 25 μM Fe(II) (pH 7.0). In the present study, the phenols or quinones (273 μM) and Fe(II) (50 μM) may in a similar manner generate hydrogen peroxide during oxidation as part of a pro-oxidant mechanism. However, 5 mM hydrogen peroxide was added initially as the oxidation initiator, and this concentration indeed exceeds any possible hydrogen peroxide formation by oxidation of the phenols. Previous studies have shown that a similar concentration of whey protein was able to quench 0.45 mM hydrogen peroxide during 3 days of storage. This indicates that any hydrogen peroxide generated by phenol oxidation may be considered insignificant compared to the excess hydrogen peroxide added as an initiator. Finally, besides additions to Cys, quinones may also react, as already mentioned with other nucleophiles, and both Lys and Trp have previously been found as targets. The reaction with amino groups from Lys to yield AQ adducts can occur by two mechanisms: (i) substitution of an aromatic carbon by the 1,4-Michael addition or (ii) formation of a Schiff base, where the oxo group of the quinone reacts with the amino group to yield iminoquinones and the release of water (Scheme 2). As depicted in Scheme 3, the iminoquinone may transform to the iminophenol, which through an oxidative deamination mechanism releases an aminophenol and the corresponding semialdehyde, AAS. This carbonyl, in turn, may engage further in advanced reaction pathways to yield Schiff base structures with other amino groups. Extracts from green tea, black tea, and complex muscle foods also in in vitro demonstrated the drawback of antioxidant assays and demonstrates how they rarely provide exact information on the antioxidant capacity of a given system and that the reducing capacity or antioxidant activity is highly dependent upon the oxidation substrate and the actual conditions. However, in the present study, the antioxidant assay may provide information on the reaction between the protein and quinones and the effect that their interactions may have on the given system. For the equimolar concentrations applied during the oxidation of β-LG, the presence of 4MBQ, Cat, and CatQ resulted in antagonistic antioxidative effects in β-LG, whereas 4MC showed an additive affect. Antagonistic effects has previously been observed for quercetin-derivatized bovine serum albumin (BSA), where cofaval attachment between the phenol to the protein reduced TEAC, as compared to free quercetin. The formation of covalent TQ or AQ adducts may explain the antagonistic effects because the reaction between the quinone and a nucleophile results in the substitution at a carbon in the aromatic ring of the quinone. The substitution
reduces the quinone to the hydroquinone, and this substitution is commonly considered as the mechanism by which quinones regenerate in cycles of polymerization reactions with other phenols to yield polymeric compounds. In aqueous media, if no nucleophiles or other phenols are available, quinones are reduced spontaneously in a single-step two-electron two-proton process to yield the phenol, and this may explain why a reducing activity was observed for the quinonic compounds, 4MBQ and CatQ, in the CUPRAC assay in the present study. For each polymerization cycle or nucleophile addition reaction, the ability of the quinone to regenerate is reduced, which leads, in turn, to a less antioxidative activity of the compound. The larger difference in reducing capacity observed between Cat and CatQ (4-fold) than that between 4MC and 4MBQ (1.7-fold) may result from different reduction or polymerization mechanisms. It may be hypothesized that Cat preferably is reduced through polymerization and 4MBQ is reduced through spontaneous reduction in the aqueous media. Spontaneous reduction will not compromise the antioxidative capacity in contrast to polymerization, and hence, less difference between the antioxidant capacity of the phenol and quinone may be observed for 4MBQ.

In the present study, the nucleophile reaction between β-LG and the quinones were simulated by the NEM reaction with the reduction will not compromise the antioxidative capacity in mechanisms. It may be hypothesized that Cat preferably is protected and in dietary supplements.

evaluation prior to use of phenolic compounds for food chemistry of the interaction mechanisms between phenols and aldehydes and subsequently Schi deamination pathway accelerating the production of semi-
result of the blocking of thiol groups but also to the oxidative
observed were ascribed to the redirection of oxidation as a
4MBQ served as apparent pro-oxidants, and the changes
impaired during 20 days of storage in the presence of Fe(II)
of
a 22% decrease in amino groups was found by the addition of
had a decrease of thiol groups by 12 and 60%, respectively, and
β-LG was reduced by blocking the thiol groups with NEM. The
β-LG by promoting AAS and Schi deamination pathway accelerating the production of semi-
formed in this sample. However, this does not explain the
γ-LG after reaction with 4MC, 4MBQ, Cat, or CatQ was
β-LG after reaction with 4MC, 4MBQ, Cat, or CatQ was
combined with Cat or CatQ because neither amino groups nor thiols were lost in those samples prior to oxidation.

In conclusion, after reaction with 4MC and 4MBQ, β-LG had a decrease of thiol groups by 12 and 60%, respectively, and a 22% decrease in amino groups was found by the addition of 4MBQ. The addition of Cat or CatQ did not lead to any instant decrease in either thiol or amino groups. The oxidative stability of β-LG after reaction with 4MC, 4MBQ, Cat, or CatQ was impaired during 20 days of storage in the presence of Fe(II) and hydrogen peroxide. Especially, the presence of 4MC and 4MBQ served as apparent pro-oxidants, and the changes observed were ascribed to the redirection of oxidation as a result of the blocking of thiol groups but also to the oxidative deamination pathway accelerating the production of semialdehydes and subsequently Schiff base structures. The complex chemistry of the interaction mechanisms between phenols and different protein residues urges the necessity of more mechanistic studies in this field. The pro-oxidant effect and, hence, potential harmful effect of certain phenolic compounds on proteins provide advice toward a more detailed toxicological evaluation prior to use of phenolic compounds for food protection and in dietary supplements.

**AUTHOR INFORMATION**

**Corresponding Author**

*Telephone: +45-3533-2181. Fax: +45-3533-3344. E-mail: jongberg@food.ku.dk.*

**Funding**

The authors acknowledge The Danish Council for Independent Research/Technology and Production within The Danish Agency for Science Technology and Innovation for granting the project entitled “Antioxidant Mechanisms of Natural Phenolic Compounds against Protein Cross-Link Formation in Meat and Meat Systems” (11-117033). The study was partially supported by the project “IB13132” funded by Gobierno de Extremadura and co-funded by FEDER.

**Notes**

The authors declare no competing financial interest.

**REFERENCES**


