Phenolic Antioxidant Scavenging of Myosin Radicals Generated by Hypervalent Myoglobin

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ABSTRACT: The scavenging activity of extracts of green tea (GTE), white grape (WGE), and rosemary (RE), all plant material with high phenolic content, and of the phenolic compounds 4-methylcatechol (4-MC), (+)-catechin, and carnosic acid toward long-lived myosin radicals generated by reaction with H_2O_2-activated myoglobin at room temperature (pH 7.5, I = 1.0) was investigated by freeze-quench ESR spectroscopy. Myosin radicals were generated by incubating 16 μM myosin, 800 μM metmyoglobin, and 800 μM H_2O_2 for 10 min, and the phenolic extracts were subsequently added (1% (w/w) phenolic compounds relative to myosin). GTE was able to scavenge myosin radicals and reduce the radical intensity by 65%. Furthermore, a low concentration of 4-MC (33 μM) was found to increase the radical concentration when added to the myosin radicals, whereas a higher concentration of 4-MC and catechin (330 μM) was found to scavenge myosin radicals and reduce the overall radical concentration by ∼65%.

KEYWORDS: myosin radicals, phenolic antioxidants, radical scavenging, hypervalent myoglobin

■ INTRODUCTION

Oxidation of meat during storage affects its quality and nutritional value due to off-flavor formation and oxidative degradation of lipids and proteins. Especially meat packed in the presence of oxygen is labile for oxidative changes. Packaging in high-oxygen modified atmospheres (70−80% O_2/20−30% CO_2) is widely used to prevent microbial growth and to maintain a red meat color during storage and retail display, but has also been shown to accelerate oxidative modifications.[1−4] Lipid oxidation and the resulting formation of off-flavors have been recognized as the primary cause for the deterioration of meat quality.[5,6] Recently, the effects of protein oxidation in meat have been recognized, and the oxidative modifications are found to affect the tenderness and juiciness of fresh meat, as reviewed by Lund et al.,[7] and alter protein functionality in meat and meat products.[8]

Protein oxidation is commonly assessed by the accumulation of oxidation products such as protein carbonyls or loss of protein thiols. Attempts have been made to control the progression of protein oxidation by adding natural phenolic antioxidants to fresh or processed meats, but so far ambiguous results have appeared for the inhibition of protein oxidation by such phenolic components.[1,3,9−11] Phenolic antioxidants are commonly known to inhibit lipid oxidation in meat, and the antioxidative mechanisms are well-characterized.[12,13] Radical scavenging of reactive oxygen species (ROS) and of lipid radicals is the primary antioxidative mechanism, and this prevents the formation of primary and secondary lipid oxidation products.[14] It may be speculated if the same scavenging mechanism is relevant for protection against the formation of protein radicals, which are precursors of oxidative protein modifications.[15]

Myosin is the most abundant protein in muscle tissue (5%, w/w) and constitutes the majority of the myofibrillar protein fraction, which represent the structural proteins of meat.[16] Studies using meat model systems have demonstrated that myosin radicals are formed by reaction of myosin with hypervalent myoglobin species,[17,18] and it is likely that such reactions also take place in meat and meat products. Oxidation of myosin in meat can result in the formation of myosin–myosin cross-links and further aggregation, which have been shown to increase meat toughness and hardness significantly.[19,20]

A recent study showed that protein radical formation increased in beef stored under high-oxygen atmospheres and that the presence of the phenolic compound, 4-methylcatechol (4-MC), limited the formation of protein radicals as detected by ESR spectroscopy.[21] On the other hand, radical signal intensity has been shown to increase in myofibrillar proteins isolated from heat-treated pork sausages with added phenolic-rich plant extracts from green tea (GTE) or from rosemary (RE) compared to control sausages without plant extracts, suggesting a prooxidative effect of plant phenols.[22] However, the character of the spectra from samples with added GTE was different compared to the control samples, indicating that the radicals generated in the presence of plant phenols may be different from those generated in the absence of phenolic compounds.[22]

Mechanistic studies are accordingly needed to verify the radical scavenging mechanisms of phenolic antioxidants toward protein radical species for protection of meat quality during storage. Accordingly, in the present study the radical scavenging effects of phenolic extract and phenolic compounds toward the

formation of myosin radicals induced by hypervalent myoglobin have been investigated. The fate of myosin radicals generated by reaction with H_2O_2-activated myoglobin and following the addition of phenolic antioxidants was analyzed by freeze-quench ESR spectroscopy to understand antioxidant effects of phenols toward protein radical formation in meat.

■ MATERIALS AND METHODS

Reagents. Myoglobin from equine heart (MbFe(III)) of >90% purity, hydrogen peroxide (30% (w/w)), zinc sulfate heptahydrate, and gallic acid were obtained from Sigma-Aldrich (Steinheim, Germany). Mark 12, MES buffer, lithium dodecyl sulfate (LDS) sample buffer, and SYPRO Ruby Protein Gel Stain were obtained from Invitrogen (Carlsbad, CA, USA). Precision Plus Protein Standard All Blue Marker was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Peroxidase from horseradish (HRP) was obtained from Roche (Mannheim, Germany). White grape extract (Grap'Active White H, (WGE)), Guardian Green Tea extract 20 M (GTE), and Guardian Rosemary extract 202 (RE) were obtained from DuPont Nutrition and Biosciences ApS ((formerly Danisco A/S), Brabrand, Denmark). Double-ionized water (Millipore, Copenhagen, Denmark) was used throughout.

Myosin Extraction. Myosin extracts were prepared according to the method described by Frederiksen et al.[18] with slight modifications. An aliquot of 50 g of porcine longissimus dorsi, which had been frozen in liquid nitrogen immediately after slaughter followed by frozen storage (-20 °C), was homogenized in 150 mL of Guba−Straub solution (0.3 M KCl, 0.1 M KH_2PO_4, 0.05 M K_2HPO_4, 1.0 mM EGTA, pH 6.4) using an Ultra-Turrax mixer. The crude myofibrillar extract was diluted to 450 mL with Milli-Q water and filtered (plastic mesh, 400 μm). The filtrate was diluted approximately 6 times by the addition of 2.175 L of Milli-Q water, and the mixture was left to precipitate for 2 h on an ice bath. The clear supernatant was decanted, and the precipitate was centrifuged at 2000g for 15 min (Herolab HiCen 21, Rotor A6.14, Herolab GmbH Laborgeräte, Axeb, Albertslund, Denmark). The pellet was dissolved in 100 mL of extraction buffer (0.5 M KCl, 1 mM EGTA, 10 mM Tris, pH 7.5) containing 2 mM ATP and 5 mM $MgCl_2$ and stirred for 10 min before ultracentrifugation at 70000g for 30 min (Beckman L8-70 M Ultracentrifuge, Rotor SW-28, Ramcon A/S, Birkerød, Denmark) using polyallomer centrifuge tubes, $^5/_8$ × 4 in. (16 × 102 mm) (Beckman, Ramcon A/S). The white myosin layer on top of the actomyosin pellet was resuspended in the supernatant. Precipitated actomyosin (yellow layer at the bottom) was discarded. Myosin was salted out by slow addition of up to 35% saturation with $(NH_4)_2SO_4$ during constant stirring. The sample was centrifuged at 10000g for 15 min, and the supernatant was discarded. The pellet was suspended in extraction buffer and was dialyzed (6−8 kDa molecular cutoff) for 24 h against extraction buffer (100 times sample volume and buffer exchanged twice) followed by one change of extraction buffer without EGTA. The protein concentration was determined spectrophotometrically at 280 nm using the extinction coefficient of 0.496 L/g/cm and M_w of 520 kDa.[17] The myosin extracts were frozen in small aliquots and stored at -80 °C until use.

Preparation of Myoglobin. An aliquot of 500 mg of MbFe(III) was dissolved in 5 mL of 100 mM TRIS buffer with 0.95 mM NaCl (pH 7.5, $I = 1.0$), and low molecular weight constituents were removed using a PD10 Sephadex G-25 column (GE Healthcare, Buckinghamshire, UK). The myoglobin concentration was determined spectrophotometrically at 525 nm using the extinction coefficient of 7.700 M^{-1} cm^{-1}. The myoglobin solution (4.0 mM) was frozen in small aliquots and stored at -80 °C until use.

Analysis by SDS-PAGE. The myosin extract and the myoglobin solution were analyzed by gel electrophoresis using NuPAGE Novex 3−8% TRIS-Acetate gel or 10% Bis-Tris gel, respectively, according to the manufacturer's instructions (Invitrogen). Initially, the myosin extract and the myoglobin solution was diluted 40 and 30 times, respectively, in 100 mM TRIS buffer (pH 7.5, $I = 1.0$). Subsequently, samples were prepared in loading solution by mixing 4 μL of LDS

sample buffer, 10.4 μL of Milli-Q water, and 1.6 μL of sample for preparation of the nonreduced samples and by mixing 4 μL of LDS sample buffer, 1.6 μL of 1.0 M dithiothreitol (DTT), 8.8 μL of Milli-Q water, and 1.6 μL of sample for the reduced samples. Aliquots of 10 μL of loading solution containing sample and 3 μL of marker were loaded to the wells. Precision Plus Protein Standard All Blue Marker was used for the 3−8% TRIS-Acetate gel, which was run at 150 V for 90 min in cassettes containing ice-cold SDS TRIS-Acetate running buffer, and Mark 12 unstained standard was used for the 10% Bis-Tris gel, which was run at 200 V for 35 min in cassettes containing ice-cold MES buffer. Following electrophoresis, the gels were fixed in 50% ethanol/ 7% acetic acid for 30 min, whereafter the fixation solution was exchanged and the gels were left overnight at room temperature. The gels were stained with the fluorescence stain SYPRO Ruby Protein Gel Stain and photographed by a Charge-Coupled Device (CCD) camera (Raytest, Camilla II, Straubenhardt, Germany). The pixel intensity of the protein bands, determined by the peak volume after subtraction of background, was quantified using Phoretix 1D software, version 2003.02.

Quantification of Total Phenolic Content by Folin− Ciocalteu. The phenolic concentration was determined by Folin− Ciocalteu's method as described by Singleton and Rossi.[23] In short, the three extracts (WGE, GTE, and RE) were dissolved in Milli-Q water and left to react with Folin−Ciocalteu phenol reagent for 8 min. Subsequently, 20% sodium carbonate was added and the reaction mixture was left to incubate at room temperature for 2 h. The phenol concentration was determined spectrophotometrically at 765 nm against a standard curve prepared from gallic acid. The concentrations are given in gallic acid equivalents (g/100 g dry extract (% w/w)).

Model System Preparation. Frozen myosin was thawed at room temperature; samples oxidized by MbFe(III)/H_2O_2 were prepared in 100 mM TRIS buffer (pH 7.5, $I = 1.0$), and 100 μL of 50 μM myosin, 60 μL of 4.0 mM MbFe(III), and 4.8 μL of 50 mM H_2O_2 were added to a total volume of 300 μL. Samples oxidized by HRP/H_2O_2 were prepared in 100 mM TRIS buffer (pH 7.5, $I = 1.0$), and 144 μL of 107 μM myosin, 90 μL of 1.0 mM HRP, and 18 μL of 0.5 M H_2O_2 were added to a total volume of 300 μL. Hydrogen peroxide was prepared freshly every day from a stable stock solution of 30% H_2O_2. H_2O_2 concentration was determined spectrophotometrically at 240 nm using the extinction coefficient of 39.4 M^{-1} cm^{-1}. The model system was left to react at room temperature (20 °C), and reaction time refers to the time in minutes after H_2O_2 addition. After exactly 10 min of reaction, phenolic extracts, WGE, GTE, and RE, or phenolic compounds, 4-methylcatechol (4-MC), catechin, or carnosic acid, all diluted in 100 mM TRIS buffer (pH 7.5, $I = 1.0$), were added. The total volume of the complete reaction mixture was always 300 μL. The total concentrations of phenols or specific phenolic compounds are given in the text and refer to the final concentration in the reaction mixture. When relevant, 10 mM $ZnSO_4$ was added together with the addition of phenolic extracts/phenolic compounds. After the addition of phenolic compounds, the model system was incubated at room temperature, transferred to cylindrical ESR tubes (outer diameter, 4 mm; length, 241 mm) (Wilmad, NJ, USA), and frozen in liquid nitrogen at exactly 1 min after the addition of extracts, phenols, or $ZnSO_4$, resulting in a total reaction time of 11 min after H_2O_2 addition.

Freeze-Quench ESR Spectroscopy. Freeze-quenched samples were mounted in the cavity (ER 4103 TM) of an ECS 106 electron spin resonance spectrometer (Bruker, Rheinstetten, Germany) equipped with a Dewar flask filled with liquid nitrogen. The magnetic field was modulated with a microwave frequency of 100 GHz, and ESR spectra were recorded by use of the following settings: gain, 2.5 × 10^5; modulation amplitude, 10 G; time constant, 163.84 ms; sweep time, 335.54 s; center field, 3368 G; field sweep width, 160 G; and microwave power, 2.25 mW, with four acquisitions averaged. The radical signal intensity was determined as the integral of the ESR spectra by use of the software WINEPR.

Data Analysis. All experiments were conducted in triplicates except if otherwise stated, and data are expressed as the mean ± SD. Statistical analysis were performed using R version 2.12.1 (The R

Data were analyzed by analysis of variance using a linear model with "sample" (control, GTE, WGE, and RE) and "replicates" (A, B, and C) as variables. When "replicates" was found to be insignificant for the statistical models, it was excluded as a variable. The significance level used was $P < 0.05$.

■ RESULTS AND DISCUSSION

Formation of Myosin Radicals by H$_2$O$_2$-Activated Myoglobin or Horseradish Peroxidase. Myosin radicals were generated in a model system by hypervalent myoglobin formed by the reaction of metmyoglobin with hydrogen peroxide. Myosin was extracted from pork, and the purity of the extract was evaluated by SDS-PAGE. Protein band intensity showed that the extract contained primarily myosin heavy chain (MHC) (Figure 1, right). The purity of myoglobin (Mb) was
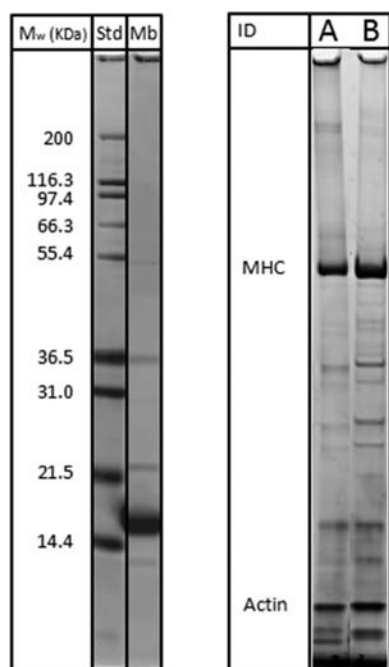

Figure 1. (Left) Myoglobin (Mb) reduced by DTT and separated by SDS-PAGE. Std, marker with proteins of known molecular weight. (Right) Nonreduced myosin extract (A) and reduced myosin extract (B) separated by SDS-PAGE. Myosin heavy chain (MHC) and actin are identified by use of a molecular weight protein marker (not shown).

likewise estimated from protein band intensity on the SDS-PAGE, and the reduced Mb contained ~80% myoglobin and some unspecified impurities (Figure 1, left). Nonreduced Mb showed that the Mb solution contained protein aggregates (data not shown), which were of higher molecular weight than myoglobin and which disappeared by reduction with DTT, indicating that the aggregates originate from reducible protein disulfide interactions.

Figure 2A shows the formation of perferrylmyoglobin radicals (MbFe(IV)=O$^•$) over time generated by reaction between metmyoglobin and hydrogen peroxide (25 °C, pH 7.5, $I = 1.0$). The figure shows the presence of a radical signal for up to 30 min by incubation of only myoglobin and hydrogen peroxide. Application of hydrogen peroxide in the present study facilitates the activation of myoglobin to induce myosin radicals for investigation of radical scavenging activities of phenolics.

Hence, the concentration applied is not typical for any meat conditions, but serves to induce the required concentration of perferrylmyoglobin and, subsequently, myosin radicals for detection. The half-lives of perferrylmyoglobin radicals have been found to be approximately 28 s at 25 °C,[24] and perferrylmyoglobin radicals were expected to be reduced to the less reactive ferrylmyoglobin (MbFe(IV)=O) or metmyoglobin (MbFe(III)) after a short time of reaction. In agreement with this, the small peak in the perferrylmyoglobin radical spectrum at ~3310 G, which is characteristic for the perferrylmyoglobin radical,[25] was found to be absent after 11 min of reaction, indicating that the original perferrylmyoglobin radical was reduced. However, the presence of a radical signal after 30 min of reaction indicates that the protein impurities or the protein aggregates in the Mb solution may have formed stable protein radical species. Hence, the radical signal obtained between 11 and 30 min corresponds to other unspecified long-lived protein radicals, most likely derived from the aggregates observed in the SDS-PAGE gel.

The model system containing myosin and Mb/H$_2$O$_2$ showed a higher radical signal intensity compared to the signal obtained by Mb/H$_2$O$_2$ alone at all time points (Figure 2B). This confirms the generation of myosin radicals by reaction with Mb/H$_2$O$_2$. Subtracting the signal after 11 min of reaction of Mb/H$_2$O$_2$ (Figure 2A) from that of myosin/Mb/H$_2$O$_2$ (Figure 2B) results in a signal in the ESR spectrum, which corresponds to the contribution from myosin radicals (Figure 3A). Integration of the resulting signal obtained after subtraction resulted in a myosin radical signal intensity of $(4.3 \pm 1.2) \times 10^8$ AU and corresponded to the intensity of myosin radicals generated by H$_2$O$_2$-activated horseradish peroxidase (HRP/H$_2$O$_2$), which was found to be $(4.4 \pm 0.9) \times 10^8$ AU (Figure 3B). HRP/H$_2$O$_2$ in the absence of other compounds did not give rise to a signal in the ESR spectrum (data not shown), and the signal shown in Figure 3B was concluded to originate entirely from myosin radicals generated by reaction of myosin with HRP/H$_2$O$_2$. The concentration of myosin was 16 or 51 μM in the system oxidized by Mb/H$_2$O$_2$ or HRP/H$_2$O$_2$, respectively. Furthermore, the concentrations of the radical-generating systems were 800 μM/800 μM or 300 μM/30 mM of Mb/H$_2$O$_2$ or HRP/H$_2$O$_2$, respectively. The concentration of H$_2$O$_2$ is rather high considering the fact that HRP previously has been found to be irreversibly inactivated above 1 mM H$_2$O$_2$.[25] However, this was not true for the HRP-based model system in the present study. Preliminary studies showed that this combination of reactants gave the highest radical signal intensity, and control samples verified that the generated radical signal derives from myosin radicals generated by activated HRP. In agreement with this, previous studies have shown that the generated long-lived protein radicals originate from radical transfer from the heme protein, and not from Fenton-induced oxidation.[17,24]

HRP/H$_2$O$_2$ is a more effective radical generator than Mb/H$_2$O$_2$, and the effect is enforced by the excess amount of H$_2$O$_2$ in the HRP system, which enables the peroxidase to continue its oxidation cycle.[26] Preliminary studies showed that the applied concentrations of reactants in the HRP/H$_2$O$_2$ system resulted in radical signals comparable to those generated by the reactants in the Mb/H$_2$O$_2$ system within the given reaction time (Figure 3). Lund et al.[17] found that generation of myosin (10−13 μM) radicals by 100 μM HRP/10 mM H$_2$O$_2$ or 300 μM Mb/300 μM H$_2$O$_2$ resulted in comparable myosin radical levels determined at room temperature after 2 min of reaction.
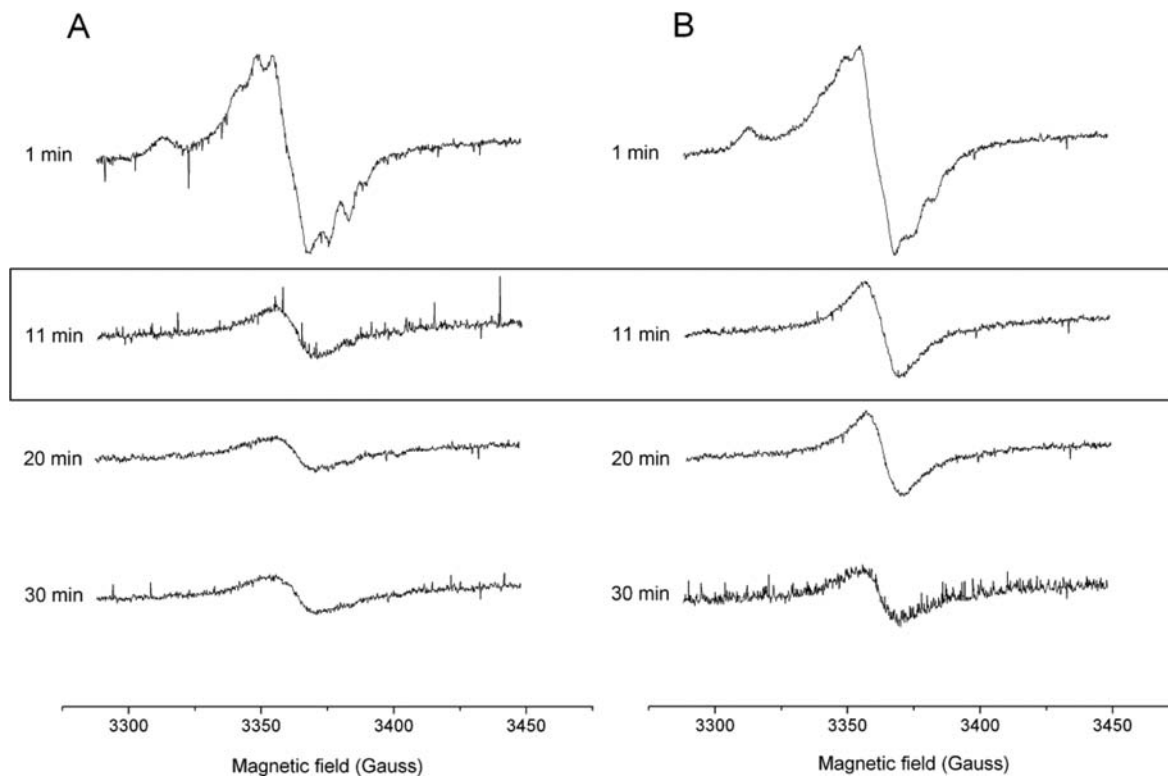
Figure 2. Generation of myosin radicals by H_2O_2-activated myoglobin (Mb/H_2O_2): (A) 800 μM Mb, 800 μM H_2O_2, 100 mM TRIS (pH 7.5, I = 1.0), t = 1, 11, 20, or 30 min; (B) 8.4 mg/mL (16 μM) myosin, 800 μM Mb, 800 μM H_2O_2, 100 mM TRIS (pH 7.5, I = 1.0), t = 1, 11, 20, or 30 min.
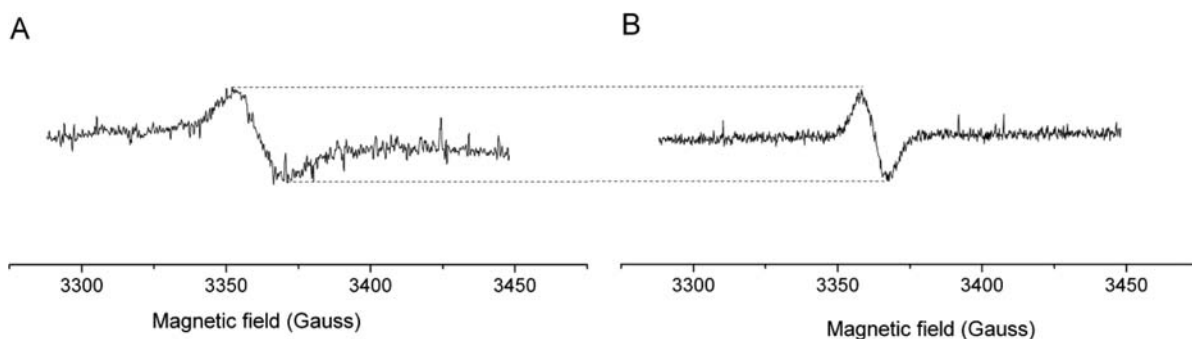

Figure 3. Representative ESR spectra of myosin radicals generated by H_2O_2-activated myoglobin (Mb/H_2O_2) or H_2O_2-activated horseradish peroxidase (HRP/H_2O_2): (A) 8.4 mg/mL (16 μM) myosin, 800 μM Mb, 800 μM H_2O_2, 100 mM TRIS (pH 7.5, I = 1.0), t = 11 min subtracted the spectra of 800 μM Mb, 800 μM H_2O_2, 100 mM TRIS (pH 7.5, I = 1.0), t = 11 min; (B) 26.6 mg/mL (51 μM) myosin, 300 μM HRP, 30 mM H_2O_2, 100 mM TRIS (pH 7.5, I = 1.0), t = 2 min.

However, in the study by Lund et al.,[17] the contribution from perferrylmyoglobin radicals were not subtracted and may, accordingly, constitute part of the myosin radical signal presented. To obtain reproducible spectra with the freeze-quench ESR methodology used for detection of radicals, elevated concentrations of the radical-generating systems were required. Hence, all concentrations of Mb/H_2O_2 or HRP/H_2O_2 were increased 3-fold compared to the study by Lund et al.,[17] but the ratios between Mb and H_2O_2 or between HRP and H_2O_2 were kept the same. Even though the radical signal intensities were comparable between the two oxidizing systems, the spectra seem to vary, giving a narrower spectrum with lower peak to peak width for the HRP system compared to the Mb system (Figure 3). Østdal et al.[27] found that the two oxidizing systems possess different modes of radical transfer, which may

lead to the diverse spectra and may explain also the different myosin levels applied in the present study.

Radical Scavenging by Phenolic-Rich Extracts. The phenolic-rich extracts were added after 10 min of incubation of myosin with Mb/H_2O_2, as the perferrylmyoglobin radicals were expected to be reduced at this point. However, as seen from Figure 2A, unspecified protein radicals originating from the Mb solution may still be present even though the perferrylmyoglobin radical is reduced. The extracts were left to react for an additional 1 min prior to freeze-quench by liquid nitrogen. This procedure enabled evaluation of the scavenging effects directly on the myosin radicals and to a lesser extent on the possible remains of perferrylmyoglobin radicals. The extracts were added on the basis of their total phenolic content to compensate for any variation hereof. As shown in Table 1 the extracts had different total phenolic contents as determined

Table 1. Concentration of Phenolic Compounds in Extracts Presented as the Mean ± SD

extract	phenols[a] (% w/w)
green tea 20 M (GTE)	23.8 ± 1.3
white grape extract (WGE)	67.7 ± 1.8
rosemary extract (RE)	4.78 ± 0.14

[a]Grams of gallic acid equivalents per 100 g of extract.

spectrophotometrically after reaction with the Folin–Ciocalteu reagent. Concentrations of 1% (w/w) phenol (based on gallic acid equivalents) to myosin were added. Preliminary studies showed that this concentration provided differences in radical scavenging activities between the extracts, facilitating evaluation of their antioxidative effect. As shown in Figure 4B, GTE was
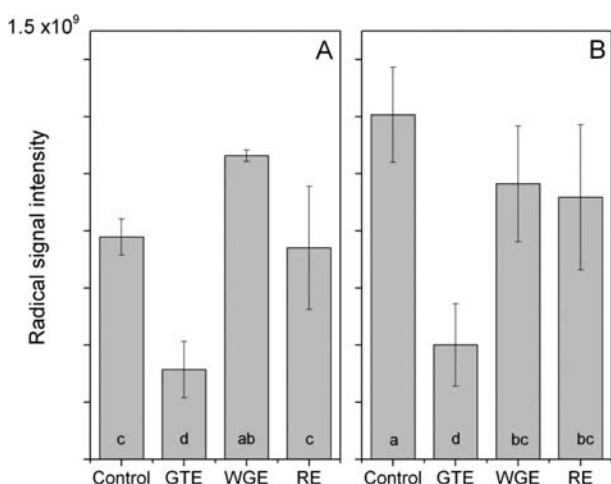

Figure 4. Radical signal intensity after addition of GTE, WGE, or RE to (A) 800 μM Mb and 800 μM H_2O_2 preincubated for 10 min in 100 mM TRIS (pH 7.5, I = 1.0) or (B) 8.4 mg/mL (16 μM) myosin, 800 μM Mb, and 800 μM H_2O_2 preincubated for 10 min. in 100 mM Tris (pH 7.5, I = 1.0). GTE, WGE, or RE was added on the basis of their phenolic content (Table 1) to obtain 0.084 mg/mL of total phenolics in the sample corresponding to 1% (w/w) phenol to myosin. Control refers to a sample with no addition of extract. The reaction was stopped by freeze-quench 1 min after addition of extract (total time, t = 11 min). Levels are expressed as the mean ± SD of a minimum of three replicates. Significant differences, P > 0.05, among samples are denoted with different letters.

able to scavenge the myosin radicals and reduce the radical signal intensity by ~65%. In contrast, WGE and RE showed no significant radical scavenging activity on the myosin radicals at the applied concentration, but preliminary studies had shown that WGE, like GTE, was able to scavenge the myosin radicals at elevated concentrations of 5 and 10% (w/w) phenol to myosin (data not shown).

The radical scavenging activity of the extracts was further determined against the unspecified radical species present in the model system containing Mb/H_2O_2 in the absence of myosin. Figure 4A shows that GTE was able to reduce the unspecified radicals by ~60%, whereas RE showed no radical scavenging effect. Interestingly, WGE increased the radical intensity, indicating that the extract may serve as a prooxidant in the given system by inducing formation of more radical species. Phenolic compounds are coupled to the reduction of oxygen to yield reactive oxygen species, such as hydrogen peroxide and hydroxyl radicals, which may oxidize substrates such as protein or lipid.[28−30] Prooxidative effects have previously been demonstrated for phenolic antioxidants in relation to the formation of protein carbonyls in model systems containing both lipid and proteins[31] and also in myofibrillar model systems oxidized by hypervalent myoglobin species.[32] The results of the present study demonstrate that the extracts exert different radical scavenging potentials depending not only on the phenolic profile of the extract but also on the nature of the protein radical that they are scavenging.

The phenolic profile of WGE was characterized by Jongberg et al.[3] and was found, on the basis of dry matter, to contain 3% (w/w) monomers, 3% (w/w) dimers, 2% (w/w) trimers, 2% (w/w) tetramers, and low levels of polymers of higher degrees of catechin or epicatechin (Scheme 1a,b). Catechin and epicatechin are also found to be the main components in green tea extracts, although primarily in their monomer form.[33] As scavengers of myosin radicals, WGE and GTE exhibited different effects, which may be due to the ability of the phenolic compounds in the extracts to terminate radical species and the ability to regenerate their phenolic structure. Generally, green tea extracts have a high total concentration of phenolic compounds of, primarily, catechins (C), epicatechin (EC), epicatechin gallate (ECG), and epicgallocatechin gallate (EGCG), which appears to account for up to 80% of the antioxidant activity of green tea extracts.[32,34] The radical scavenging effects of ECG, EGCG, and EC against lipid radicals in a lecithin/lipoxygenase system ranged in the mentioned order with ECG having the highest effect.[35] The presence of

Scheme 1. Representative Phenolic Compounds in Green Tea Extract (a), White Grape Extract (b), or Rosemary Extract (c)
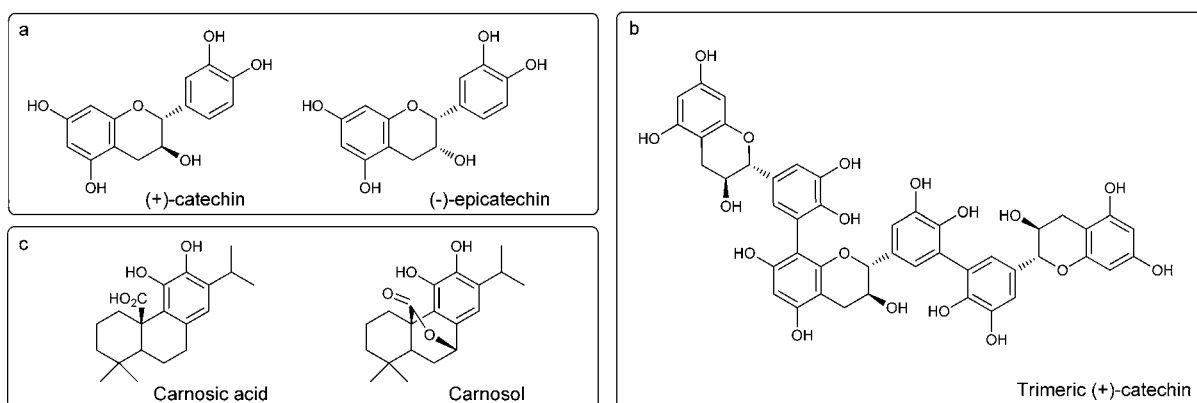
three adjacent hydroxyl groups on the B ring of ECG makes it an effective radical scavenger, and the galloyl group is further found to be responsible for the iron-binding ability of catechins, which makes it an efficient protector against metal-catalyzed oxidation.[36] Donation of hydrogen atoms from the catechol or gallol moiety results in the formation of rather stable phenoxyl radicals. When such species accumulate, polymerization reactions between adjacent phenoxyl radicals may occur by substitution of carbon atoms in their aromatic ring. These polymerization mechanisms regenerate the hydroxyl groups and, hence, their ability to donate hydrogen atom and their antioxidant capacity[37] WGE contains mainly already polymerized catechin and epicatechin and no or only low concentrations of ECG and EGCG, which may explain the differences in radical scavenging activity observed between the two extracts. Furthermore, no radical scavenging activity against the protein radicals was observed for the RE, indicating that the phenolic compounds contained in this extract possess poor radical scavenging activity. This is in agreement with a previous paper on the effect of rosemary extract against protein carbonyl formation in meat[1] and meat products.[22]

Radical Scavenging Mechanisms. Radical−radical interactions resulting in the polymerization of phenolic compounds are commonly referred to as termination reactions as the mechanism scavenges radical species. The ability of the phenoxyl radicals to terminate chain reactions may, to a certain extent, explain the radical scavenging activity of the extracts against the protein radical species. Phenoxyl radicals are stabilized and prevented from dimerizing, and hereby their radical signals are enhanced when coordinated to divalent metal ions, such as Zn^{2+}.[38,39] Accordingly, in the present study, Zn^{2+} was added concomitantly with the extracts to the model systems to evaluate the generation of phenoxyl radicals. Interestingly, WGE and RE generated significantly higher amounts of phenoxyl radicals compared to GTE in both systems without or with myosin (Figure 5). This may indicate that the phenoxyl radicals generated by the addition of GTE terminate by polymerization reactions more efficiently than the phenoxyl radicals generated by the addition of the other extracts and may explain the more efficient radical scavenging activity observed for GTE against the protein radicals (Figure 4). The dihydroxybenzene moieties of catechins contain multiple sites for polymerization reactions (Scheme 1a), which enhance the ability for termination reactions in the GTE. According to the producer, the dominant phenolic compounds in the RE are the diterpenes carnosic acid and carnosol (Scheme 1c), which contain only a single free carbon in the aromatic structure for polymerization, and furthermore, as previously described, WGE comprised primarily already partly polymerized catechins (Scheme 1b). The reduced ability of the phenols to polymerize and thereby terminate radical species may explain the poor radical scavenging activity observed for the WGE and RE. Consistent with the results obtained in the present study, the same GTE was found to be a more efficient antioxidant against both TBARS and protein carbonyl formation than the same RE in Bologna-type pork sausages.[22]

The intensity of the Zn-stabilized phenoxyl radical signal was found to be increased in the absence of myosin (Figure 5). This indicates that myosin acts as an antioxidant in the system by scavenging radical species and thereby diminishing the formation of phenoxyl radicals, in effect protecting the phenolic compounds. In fact, this indicates that myosin acts as a
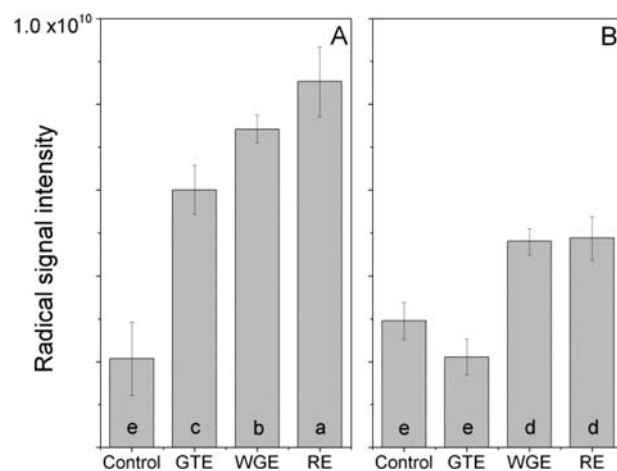

Figure 5. Radical signal intensity after addition of 10 mM Zn^{2+} and GTE, WGE, or RE to (A) 800 μM Mb and 800 μM H_2O_2 preincubated for 10 min in 100 mM TRIS (pH 7.5, I = 1.0) or (B) 8.4 mg/mL (16 μM) myosin, 800 μM Mb, and 800 μM H_2O_2 preincubated for 10 min in 100 mM TRIS (pH 7.5, I = 1.0). GTE, WGE, or RE was added on the basis of their phenolic content (Table 1) to obtain 0.084 mg/mL of total phenolics in the sample. Control refers to a sample with no addition of extract. The reaction was stopped by freeze-quench 1 min after addition of Zn^{2+} and extract (total time, t = 11 min). Levels are expressed as the mean $\pm$ SD of a minimum of three replicates. Significant differences, P > 0.05, among samples are denoted with different letters.

competitor to phenols in the system, in agreement with the increase in radical signal observed in the presence of myosin, but without phenolic compounds. The increased radical signal may be due to the extensive oxidation by ferrylmyoglobin, which oxidizes myosin beyond its antioxidative capacity. Cysteine and methionine residues have been suggested to be involved in intermolecular antioxidant mechanisms in proteins.[40] However, under the extensive oxidation conditions in the given model system, the antioxidant activity of myosin is quickly exhausted, and protein radicals will accumulate as long-lived myosin radicals as seen by the increased radical signal intensity in the system containing myosin compared to the system without myosin (Figure 2). Hence, the antioxidative capacity of myosin may protect against oxidation of the phenols, either by reducing the radical-generating ferrylmyoglobin before phenol addition or merely by reducing ferrylmyoglobin to metmyoglobin at a faster reaction rate than the phenolic compounds. In future studies, further attention should be given the comparison of reaction rates for these compounds to identify the reaction pathways in meat and meat systems. It may be speculated that the unspecified long-lived protein radical generated in the Mb/H_2O_2 without myosin may transfer radicals differently from myosin radicals and, in effect, generate additional phenoxyl radicals than observed in the presence of myosin. Moreover, metal-catalyzed oxidation of phenols may generate hydrogen peroxide,[41] which may contribute to the propagation of pseudo-peroxidase cycle of myoglobin, thus generating more phenoxyl radicals. However, this mechanism has not yet been investigated for heme protein-induced oxidation.

To better understand the scavenging mechanisms of the individual phenolic compounds in the extracts against the myosin radicals, the effect of the three pure phenolic compounds 4-methylcatechol (4-MC), (+)-catechin, and

carnosic acid were evaluated. The pure phenolic compounds represent partly the phenolic profile of the three extracts applied in the present study. Catechin is as previously mentioned the main constituent in green tea extracts, and in WGE, it is found in its polymerized form. 4-MC represents the catechol moiety of catechin, which is suspected to be mainly responsible for the antioxidative activity of catechin,[33] and carnosic acid is one of the main constituents of RE according to the manufacturer. Catechin, 4-MC, and carnosic acid were added to the myosin/Mb/H_2O_2 system in two different molar concentrations, 33 and 330 μM. The higher concentration corresponds to the addition of 0.5% (w/w) 4-MC, 1.0% (w/w) catechin, or 1.3% (w/w) carnosic acid to myosin, and the lower was applied to investigate any possible dose-dependent effect.

The higher concentration (330 μM) of 4-MC or catechin reduced the myosin radical signal intensity by ~65% (Figure 6). In contrast, carnosic acid in both concentrations showed only little or no radical scavenging activity on the myosin radicals, indicating a poor antioxidative activity compared to the higher concentration of 4-MC or catechin. The scavenging effect of 4-MC and catechin was consistent with the degree of inhibition observed for the addition of GTE (Figure 4), which
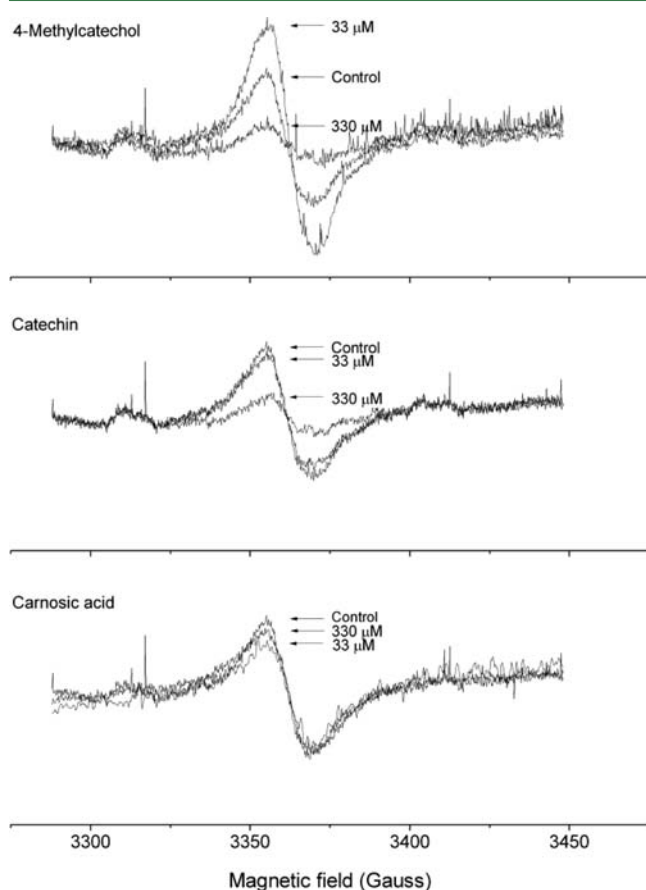

corresponds well with the fact that GTE and catechin were added in equal concentrations. Furthermore, the absent radical scavenging effect of carnosic acid may partly explain why RE was unable to scavenge the protein radicals in the model systems (Figure 4).

Interestingly, the low concentration (33 μM) of 4-MC increased the signal intensity in the ESR spectrum, whereas catechin or carnosic acid had no effect on the signal intensity at the low concentration. As previously mentioned, phenolic compounds may serve as prooxidants, and prooxidative activities have been associated with lower molecular concentrations of especially flavonoids, such as catechins containing catechol moieties.[42] As the form of the spectrum obtained by the addition of 4-MC is comparable to the spectrum of the control sample, it is reasonable to believe that the radical species in the two samples are of similar nature, stressing that the observed elevated radical signal intensity is caused by a prooxidative activity and not the formation and accumulation of phenoxyl radicals. This prooxidative effect may be responsible for the higher radical signal intensity observed by the addition of WGE to the Mb/H_2O_2 (Figure 4A) compared to the control sample, as WGE contains low molecular concentrations of monomer catechins.

The scavenging effect of the extracts and the pure phenolic compounds indicates that the addition of natural extracts rich in phenolic compounds may protect meat proteins against oxidative modifications, which is in agreement with previous findings in frankfurters with added rosemary extract.[9] A previous study showed that the addition of 0.05% (w/w) 4-MC to beef stored in high-oxygen atmospheres significantly inhibited the radical intensity in the myofibrillar protein fraction.[21] The ratio of antioxidants relative to protein used in the present study is higher compared to the previous studies with meat. However, also highly elevated Mb/H_2O_2 concentrations compared to meat were necessary in the present study to generate reproducible myosin radical signals and, accordingly, a stronger antioxidative capacity was needed to obtain clear effects. Nonetheless, in consideration of the high myoglobin and hydrogen peroxide concentrations used in the model system compared to the conditions in meat, the model system may provide the basis for screening and evaluation of phenolic antioxidants to be used in the development of meat products. From this perspective, the pH also needs to be considered, and the development of similar systems reaching meat pH of approximately 5.6−5.8 is consequently being initiated.

In conclusion, the present study demonstrated that the phenolic extract from green tea, GTE, was able to scavenge myosin radicals generated by H_2O_2-activated myoglobin and indicated that the radical scavenging activity of extracts was dependent on the nature of and interactions among the phenolic compounds in the extracts. Furthermore, it was shown that low concentrations of 4-MC had a prooxidative effect on the radical intensity in the myosin system oxidized by Mb/H_2O_2, but that higher concentrations of pure 4-MC or catechin were able to scavenge the myosin radicals. The findings indicate that in consideration of the total phenolic content, green tea extract provides a radical scavenging activity against meat protein oxidation corresponding to the effect of pure catechin, and the scavenging activity is ascribed to the ability of the catechins to terminate and quench radical species. However, further studies are needed to establish a clear guideline for the

Figure 6. Representative ESR spectra of myosin radicals generated by H_2O_2-activated myoglobin with or without addition of the phenolic compounds, 4-methylcatechol (4-MC), (+)-catechin (C), or carnosic acid. Samples contain 8.4 mg/mL (16 μM) myosin, 800 μM Mb, and 800 μM H_2O_2 preincubated for 10 min in 100 mM TRIS (pH 7.5, I = 1.0) and subsequently added 33 or 330 μM concentrations of the phenolic compounds. Control refers to a sample with no addition of phenolic compounds. The reaction was stopped by freeze-quench 1 min after addition of phenolic compound (total time, t = 11 min).

application of phenolic antioxidants against protein oxidation in meat.

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