ELUCIDATION OF PRO-OXIDANT MECHANISMS OF THE BIOACTIVE POLYPHENOL, (-)-EPIGALLOCATECHIN GALLATE, IN FOOD EMULSIONS

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Abstract

Polyphenols are widely regarded as antioxidants, due in large part to their free radical scavenging activities and their ability to disrupt radical chain propagation. Besides the anticipated antioxidant activity in foods, polyphenols are also attractive ingredients for their potential health benefits after consumption. However, recent studies have demonstrated that the oxidation of some phenolic compounds result in the generation of hydrogen peroxide (H$_2$O$_2$), a precursor for the highly reactive hydroxyl radical (•OH), which can potentially compromise the oxidative stability of foods and beverages.

Due to conflicting results regarding phenolic effects on lipid oxidation in the literature, I surveyed the effects of pH (2-7) and phenolic concentration (0-500 µM) on the generation of lipid oxidation markers in flaxseed emulsions to determine conditions for future oxidation studies. (-)-Epigallocatechin-3-gallate (EGCG), the major polyphenol in green tea, was used as the model polyphenol in all oxidation studies. A low pH (2-4) resulted in pro-oxidant activity, while higher pH values (5-7) resulted in a net antioxidant activity. Higher EGCG concentrations (100, 500 µM) also showed the largest changes in lipid oxidation markers compared to the no EGCG controls after 96 h.

Disrupting redox cycling by metal chelation may be a viable solution for controlling metal-catalyzed phenolic oxidation, since redox cycling of transition metals is essential for the metal to exert a catalytic function. I added the iron chelators, EDTA and 2,2-bipyridine (BPY) to hexadecane emulsions containing EGCG (400 µM) and Fe$^{3+}$ (25 µM) at pH 3 and pH 7 to determine their effects on EGCG oxidation and the resulting ROS.

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produced in the absence of an oxidatively labile lipid. I then repeated the studies in flaxseed emulsions to determine the effect on lipid oxidation. At neutral pH, EDTA accelerated EGCG oxidation and hydroxyl radical (•OH) formation. BPY treatment slightly slowed EGCG oxidation compared to EGCG-only samples, though both treatments showed H₂O₂ accumulation and very slow •OH radical generation. Even with rapid •OH radical formation in EDTA treatments, all EGCG-containing samples showed antioxidant activity at neutral pH. Conversely, at acidic pH, EDTA strongly inhibited EGCG oxidation and •OH radical formation resulting in strong antioxidant activity in preventing flaxseed emulsion oxidation, while EGCG readily oxidized in BPY and EGCG-only treatments, leading to rapid •OH radical generation, and increased lipid oxidation in flaxseed emulsions.

Though EDTA showed promising results by preventing phenolic and lipid oxidation at acidic pH, EDTA may cause problems at neutral pH by accelerating phenolic loss even if no increased lipid oxidation occurs. Proteins may be another viable solution in inhibiting increased lipid oxidation by scavenging the H₂O₂ generated from metal-catalyzed phenolic oxidation, prior to •OH radical formation. We show that casein (CAS) and whey protein isolate (WPI) readily scavenge H₂O₂ in emulsions. However, differences in peroxide scavenging activity were dependent on more than total methionine (Met) and cysteine (Cys) content; Met and Cys being the two amino acids capable of directly reacting with peroxides.
A major factor for the variable peroxide scavenging activity observed in proteins may be due to differences in Met and Cys accessibility. To further examine this, I effectively blocked Met and Cys residues in proteins by reacting them with peroxides and collecting the oxidized proteins. CAS and β-lactoglobulin (BLG) were pre-treated with the peroxides, t-butyl hydroperoxide (TBHP) or H$_2$O$_2$. TBHP is a bulky peroxide whose accessibility to buried Met and Cys residues is sterically hindered; H$_2$O$_2$, on the other hand, has been shown to be able to access buried residues. Using the prepared proteins, I proceeded to examine the proteins’ ability to scavenge exogenously added H$_2$O$_2$ and improve EGCG stability. In Tween-stabilized hexadecane emulsions at neutral pH, CAS treatments showed decreasing peroxide scavenging activity and EGCG stability as peroxide pre-treatments decreased solvent accessible Met concentration. Similar to WPI, BLG displayed much weaker peroxide scavenging activity and lower EGCG stability compared to CAS, since the Met residues were not readily accessible to the ROS present. However, in SDS-stabilized emulsions, BLG readily scavenged H$_2$O$_2$ and showed increased EGCG stability compared to CAS. As SDS denatured BLG, buried Cys residues became accessible to oxidation.

When I repeated experiments in flaxseed emulsions at pH 3, BLG was capable of preventing increased lipid oxidation from the addition of high (400 µM) and low (100 µM) EGCG concentrations in Tween-stabilized flaxseed emulsions. The addition of BLG-CTRL-1% or BLG-HOOH-1% maintained lipid oxidation markers in EGCG treatments well below the control. However, the addition of BLG-HOOH-0.2% with EGCG (400 µM) was unable to keep lipid oxidation markers in EGCG treatments below
the control for the full duration of the 8-day study. This suggests that the presence of solvent accessible Met residues is important to BLG’s protective effect. At the low EGCG concentration (100 µM), both BLG-0.2% treatments showed higher lipid oxidation markers by the end of the study, signifying phenolic concentration also impacts the effects on lipid oxidation.

Overall, I showed that EGCG readily oxidized to generate ROS in emulsions with a low pH being a strong promoter of increased lipid oxidation due to metal-catalyzed phenolic oxidation. Though all my studies were performed using the polyphenol EGCG, many of the mechanistic investigation, such as the effects of metal chelators on oxidation, may be extrapolated to other phenolics that undergo metal-catalyzed oxidation. Further work is necessary to elucidate the impact of the different reactions resulting from metal-catalyzed phenolic oxidation, such as •OH radical formation and Fe reduction, on inducing lipid oxidation. However, controlling metal redox cycling with chelators holds promise as a potential strategy for mitigating the pro-oxidant activity of phenolics in foods. The quenching of EGCG-generated H$_2$O$_2$ by solvent accessible Met and Cys residues in proteins is also a viable strategy for stabilizing lipids in foods. Though uncontrolled metal-catalyzed phenolic oxidation may yield many problems, my research shows that phenolics may be safely incorporated into food emulsions without negative consequences on lipid stability by controlling key phenolic pro-oxidant mechanisms of the polyphenol, such as transition metal reduction and •OH radical formation.
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Finally, I must give thanks to my friends, and family who made sure I was able to enjoy my time here in State College.
1. Introduction and a Review of Relevant Literature

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1.1. Introduction

Phenolic compounds are secondary plant metabolites commonly encountered in fruits and vegetables. These compounds include at least one phenol group, a structure containing an aromatic benzene ring with at least one hydroxyl substituent (Fig. 1.1). Phenolics have long been added to lipid foods to inhibit oxidation reactions. However, in recent years, phenolics have become popular bioactive ingredients in formulated foods as studies have demonstrated that these compounds are beneficial to human health. Unfortunately, many phenolics are labile to oxidation, which complicates their addition to food products. Phenolics are often degraded as a result of nonenzymatic oxidation reactions over the life of food, and these reactions may compromise the oxidative stability of the surrounding food matrix under some conditions. Phenolics may also undergo enzymatic oxidation reactions resulting from polyphenol oxidase (PPO) to yield similar products (eg. H₂O₂ and quinones) (Lee Chang, 1992; Subramanian, Venkatesh, Ganguli & Sinkar, 1999). However, our research will focus on metal-catalyzed phenolic oxidation as it leads to substantial phenolic degradation in food products even in the absence of active PPO.
Fig. 1.1: Chemical structures of various phenolics.

1.1.1. Applications of Phenolics as Antioxidants in Food Lipids

Phenolic compounds, especially di- and tri-hydroxyl substituted phenolics, are known to exhibit strong antioxidant activity in lipid-based foods (Table 1.1). With heightened consumer awareness of the potentially harmful effects of synthetic antioxidants (such as butylated hydroxytoluene and butylated hydroxyanisole) in the diet (Esterbauer, 1993), plant-derived phenolic extracts have become attractive non-synthetic antioxidants in formulated lipid foods. Phenolic extracts from a host of plant-based foods and waste
products (for example, skins, stems, and seeds) have been shown to exhibit equivalent or even higher antioxidant activity than conventional antioxidants, such as ascorbic acid and tocopherols, under many conditions (Kähkönen et al., 1999). Pure phenolic compounds have been shown to inhibit oxidation and discoloration in meat products, bulk oils, and lipid dispersions. The same is true for plant-derived phenolic extracts, which have also been proven to be effective antioxidants in foods.

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Table 1.1: Antioxidant activity from phenolic compounds and extracts observed in lipid systems.
1.1.2. Applications of Phenolics as Bioactive Ingredients in Food Lipids

Adding phenolics to formulated foods is a growing trend as consumers gain awareness of the purported health benefits of dietary phenolics. For example, the consumption of (-)-epigallocatechin-3-gallate (EGCG), the dominant polyphenol in tea, has been associated with the prevention of metabolic syndrome (Bose et al., 2008; Thielecke & Boschmann, 2009) and cancer (Yang et al., 2009). Cocoa polyphenols have been reported to reduce the risk of cardiovascular disease (Shrime et al., 2011). Many of these phenolics are available as dietary supplements; however, high doses may lead to toxicity. Liver toxicity resulting from high EGCG intake has been reported (Lambert et al., 2007), and such high concentrations are easily achieved using dietary supplements. The delivery of biologically significant levels of phenolics in food appears to be a safer approach, with respect to mitigating the toxicity of these compounds.

1.2. Antioxidant Mechanisms of Phenolics in Foods

Phenolic compounds are known to have high antioxidant capacity, as seen in hydrogen abstraction assays, such as the oxygen radical absorbance capacity (ORAC) assay (Alarcón et al., 2008; Dudonné et al., 2009; Hurst et al., 2009; Ma et al., 2011) and in single electron transfer assays, such as the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (Nanjo et al., 1999; Roy et al., 2010) and the ABTS (2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) assay (Cai et al., 2006). Many phenolic compounds are also capable of serving as transition metal chelators (Khokhar & Owusu, 2003), which may augment their antioxidant activity in metal catalyzed oxidation reactions (Guo
et al., 2007). The mechanism by which phenolic compounds scavenge lipid-derived radicals is shown in Fig. 1.2.

![Fig. 1.2: Proposed antioxidant reaction mechanisms in lipid dispersions depicted with a galloyl group.](image)

Formulated foods commonly contain more than one antioxidant; therefore, it is important to understand the synergistic or antagonistic interactions between phenolics and non-phenolic antioxidants (Becker et al., 2007; Dai et al., 2008). Combinations of the phenolics—such as catechin, cyanidin, caffeic acid, quercetin, and ellagic acid—showed additive effects to the inhibition of copper-catalyzed human LDL oxidation, except in combinations with ellagic acid and catechin where antagonistic effects were observed (Meyer et al., 1998). Even among phenolic combinations found naturally in plants, antagonistic, additive, and synergistic interactions between phenolics may occur, as was observed in the ORAC assay with combinations of phenolics at their typical concentrations in navel oranges (examples include chlorogenic acid, hesperidin, luteolin, myricetin, naringenin, p-coumaric acid, and quercetin) (Freeman et al., 2010). Interactions between antioxidants may also result in a reversal in antioxidant activity.
Using a Fe$^{2+}$/histidine-Fe$^{3+}$ system, ascorbate alone (50–200 µM) was demonstrated to have pro-oxidative activity in liposomes, while ascorbate and tocopherols together demonstrated a synergistic antioxidant effect (Thomas et al., 1992). The lipid system also affects interactions. For example, α-tocopherol had a synergistic effect on quercetin in the metmyoglobin-initiated oxidation of methyl linoleate emulsions, but an antagonistic effect in bulk oil (high-oleic sunflower oil) (Becker et al., 2007).

1.2.1. Radical Scavenging Activity

1.2.1.1. Relationship between Phenolic Structure and Radical Scavenging Activity

The antioxidant activity of phenolic compounds is due primarily to resonance stabilization of the phenoxy radical after oxidation. Polyphenols consisting of large, conjugated pi systems are typically more stable radicals, and the introduction of various substituents, such as electronegative carboxyl or hydroxyl groups, may also increase radical stability. The presence of a carboxyl group in monohydroxy benzoic acids causes electrons to withdraw, which significantly increases the antioxidant activity of the meta-substituted monohydroxy acid, compared to the ortho and para positions (Rice-Evans et al., 1996). Di- and tri-hydroxyl substitutions on phenol groups have greater antioxidant activity, especially with adjacent substitutions, because of resonance stabilization. This phenomenon has been seen with the substituted phenol group, presented in Fig. 1.3 (1,2,3-trihydroxybenzene (pyrogallol) > 1,2-dihydroxybenzene (catechol) > 1,3-dihydroxybenzene = 1,4-dihydroxybenzene > phenol) (Kumamoto et al., 2001).
Blocking studies have also supported the importance of the adjacent di- and tri-hydroxyl group substitution on antioxidant activity. O-methylation of the catechol group significantly reduced the antioxidant activity of catechols (Dueñas et al., 2010). Similarly, a loss of resonance occurs in galloyl groups if the middle hydroxyl group is altered, which then causes a significant loss of antioxidant activity (Nanjo et al., 1996). EGCG consists of two galloyl moieties (B- and D-ring) and loses significant antioxidant activity only when both galloyl groups are glycosylated at the middle hydroxyl position (4’ and 4” hydroxyl positions) (EGCG structure and nomenclature shown in Fig 1.4).

Though numerous studies have focused on the non-glycosylated species (that is, the aglycone), it is important to note that phenolic extracts may also contain phenolic glycosides. Phenolic glycosides may result in different antioxidant activities, as seen between quercetin and multiple quercetin glycosides in lipid systems (Huber et al., 2009).
Steric hindrance is another important factor that affects radical scavenging capacity as epimers with greater steric hindrance [(EGCG, epigallocatechin, and epicatechin) versus (gallocatechin gallate, gallocatechin, and catechin the corresponding catechins with less steric hindrance)] tend to show lower antioxidant activity (Zhao et al., 2001). The effect of epimers was more pronounced at low catechin concentrations and larger radicals, as observed with the large radicals generated from 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) versus the smaller, superoxide (O$_2^-$) radical (Guo et al., 1999). These may be important factors for scavenging lipid-derived radicals. Because phenolics represent a diverse class of compounds based on their structure, other features, such as additional double bonds, also impact antioxidant activity. The influence of these other structures will not be further discussed in this chapter; however, extensive studies have examined the effects of various structural motifs on phenolic antioxidant activity (Huvaere et al., 2009; Rice-Evans et al., 1996).
1.2.1.2. Relationship between Matrix pH and Radical Scavenging Activity

Matrix pH also greatly influences the antioxidant activity of phenolics as changes in reduction potentials appear to correlate with the deprotonation of phenolics. Relatively less energy is required to abstract an electron from the dissociated phenolic species. As such, some have argued that the radical scavenging activity of phenolics increases with increasing pH (Amorati et al., 2006).

Reduction or oxidation potentials are often seen as good predictors of antioxidant activity because higher oxidation potentials (that is, lower reduction potentials) correlate to an increased capability to lose an electron. A pH-dependent decrease in oxidation potential was observed in catechin (Janeiro & Oliveira Brett, 2004) as well as in tea catechins, epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and EGCG (Kumamoto et al., 2001). Interestingly, oxidation potentials were pH-independent outside the pH range of 3–9 for catechin (Janeiro et al., 2004) and pH 5–8 for EC and EGCG (Kumamoto et al., 2001), which suggests that radical scavenging activity may not increase above those threshold pH values. The tea catechins showed strong antioxidant activity from pH 5–12; however, under strong alkaline conditions (pH 13), antioxidant activity decreased sharply, which was likely because increased phenolic oxidation leaves little of the unoxidized species intact to act as radical scavengers (Kumamoto et al., 2001).
1.2.1.3. Relationship between Phenolic Partitioning and Radical Scavenging Activity

Food systems are much more complex than the environments employed in the majority of radical scavenging assays. Radical scavenging activity data from assays, such as ORAC, do not always predict the efficacy of antioxidants like phenolics in complex, multiphase food systems (Alamed et al., 2009). The hydrophobicity of phenolic compounds has been suggested to be a major factor influencing a compound’s activity, with respect to inhibiting its lipid oxidation (Aliaga et al., 2009; Iglesias et al., 2010). Polymerization of polyphenolic compounds has been found to increase antioxidant activity with mixed results; radical scavenging activity may increase, but antioxidant activity in emulsions may not change due to preferential partitioning into the lipid core instead of the interface as more units are polymerized (Iglesias et al., 2010; Pazos et al., 2010).

Surface-active phenolic derivatives (such as fatty acid–esterified phenolics) have been the topic of much research in recent years, with the objective of increasing the antioxidant activity of phenolics by directing their partitioning to the sites of lipid oxidation reactions (that is, oil-water interfaces). The conjugation of alkyl groups to phenolics may lead to a decrease in radical scavenging ability. Peroxyl radical scavenging activity was observed as follows (in order of highest to lowest): free p-hydroxyphenylacetic acid (HPA) > dodecyl HPA > butyl HPA (Yuji et al., 2007). Similarly, fatty acid–esterified chlorogenic acid (CGA) exhibited a lower radical scavenging activity than free CGA; CGA showed the highest ORAC value, while dodecyl CGA showed the lowest (Sasaki et al., 2011). To account for the differences in radical scavenging activity, Sasaki et al. (2011) added varying concentrations of the conjugates to emulsions to achieve the same
radical scavenging activity (that is, the dodecyl chlorogenic acid concentration was more than double the concentration of free chlorogenic acid). However, lipid oxidation in fish-oil (menhaden) emulsions showed antioxidant activity on the order of octyl CGA > butyl CGA > CGA > control = dodecyl CGA, suggesting that increasing chain-length and hydrophobicity does not necessarily predict greater antioxidant in emulsions, as would be expected. Also, dodecyl CGA greatly decreased the interfacial tension of the emulsion droplet; because it is located in the interface and not buried in the lipid core where its antioxidant activity may have been lowered, phenolic partitioning is not the sole explanation. Languere et al. (2009, 2010) have also produced multiple studies involving the antioxidant activity of phenolics conjugated to various alkyl chains (C1–C20) in CGA and rosmarinic acid. The authors also witnessed a linear increase in the antioxidant activity of emulsions (that is, medium-chained C8), up until a certain point. The decrease in observed antioxidant activity may be due to many factors, such as partitioning into the lipid droplet core, restricted mobility, steric hindrance, or increased solubilization by excess emulsifier for longer alkyl chains. From 0–17 µM Brij 35, C8–C20 show minimal aqueous phase phenolic concentration. Correspondingly, increasing the emulsifier content increased the partitioning of phenolics into the aqueous phase; C12–C20 conjugates also showed greater partitioning into the aqueous phase as a function of increasing emulsifier concentration, compared to C1–C8 conjugates.

1.2.2. Chelation of Transition Metals

The observed antioxidant activity of some polyphenolic compounds in lipid foods may also be related to their ability to chelate, or form complexes with, transition metal
catalysts. Many phenolics have a strong capacity for binding ferric (Fe$^{3+}$) ions due to the presence of iron-binding motifs, such as $\alpha$-dihydroxyl substituents (Khokhar & Owusu Apenten, 2003). Ligands with catechol-containing moieties can bind Fe$^{3+}$ ions in a ligand:metal ratio from 1:1, 2:1, and 3:1 (Hider et al., 2001) with the ligand:metal complex, whose formation depends on available ligand and metal concentrations. Flavonoids containing 3-hydroxy and 4-oxo, or 5-hydroxy and 4-oxo groups, are capable of binding ferrous (Fe$^{2+}$) ions (Leopoldini et al., 2006). Quercetin contains the 4-oxo, in addition to the 3- and 5-hydroxy, groups, thus displaying a strong affinity for Fe$^{2+}$ ions. As such, quercetin is capable of competing with the strong ferrous chelator Ferrozine, (3-(2-pyridyl)-5,6-bis(phenyl sulfonic acid)-1,2,4-triazine) for Fe$^{2+}$ ions. In addition, it completely suppresses •OH radical generation (via the Fenton reaction), as measured by 2-deoxyribose degradation at pH 7.2 (Guo et al., 2007).

1.3. Pro-Oxidant Mechanisms of Phenolics on Foods

Pro-oxidant activity due to metal-catalyzed phenolic oxidation has also been observed in lipid dispersions in a number of studies (Table 1.2). Green tea extracts (Frankel et al., 1997a) and tea catechins (Huang & Frankel, 1997a) were reported to produce pro-oxidative behavior in oil-in-water emulsions. Gallic acid exhibited higher peroxide values and yielded more oxidation products (such as oxidized triglyceride monomers, triglyceride dimers, and triglyceride oligomers) in sunflower oil–in-water emulsions at pH 7.5–7.8 (Velasco et al., 2004). The effect was also magnified in the presence of transition metals as ECG, EGCG, and myricetin exhibited pro-oxidant effects in the presence of ferric ions in sunflower oil–in-water emulsions at pH 5.5 (Roedig-Penman &
The factors that influence the pro-oxidative effects remain unclear but appear to be related to phenolic concentration (Mei et al., 1999) and pH (Mei et al., 1999), as well as the influence of other matrix constituents. In the presence of phenolic compounds, Mei et al. demonstrated increased lipid oxidation markers in emulsions at pH 3 but antioxidant activity at pH 7 (Mei et al., 1999). The authors reported varying effects depending on phenolic concentration: when the phenolics were present at high concentrations (500 µM), antioxidant activity was observed, while low phenolic concentrations (5 µM) resulted in pro-oxidant activity. The different effects may be due to competition between anti-oxidant (Fig. 1.2) and pro-oxidant reactions (Fig. 1.5). Pro-oxidant activity may result from the generation of reactive oxygen species (ROS) or the regeneration of transition metals to their catalytically active reduced states, which occurs because of the metal-catalyzed oxidation of phenolic compounds.
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Table 1.2: Pro-oxidant activity from phenolic compounds and extracts observed in lipid systems

1.3.1. Metal-Catalyzed Oxidation of Phenolic Compounds

Many dietary phenolic compounds, particularly those bearing catechol or galloyl groups, are readily oxidized in foods, especially at neutral and alkaline pH values. These “autoxidative” reactions, which actually appear to be catalyzed by transition metals, are coupled to the reduction of oxygen to ROS (Lapidot et al., 2002; Li et al., 1994)—namely, superoxide (O₂⁻), or its protonated form, the hydroperoxyl radical •OOH), which eventually leads to the formation of H₂O₂ (Danilewicz, 2003; Elias et al., 2009a;
Waterhouse & Laurie, 2006; Wildenradt & Singleton, 1974) (Fig. 1.5). Though focus is placed on di- and tri-hydroxyl substituted phenolics due to their greater ease of oxidation, the same oxidation pathway may exist in other species capable of reducing transition metals, just as ascorbic acid (Nappi & Vass, 1997) and tocopherols (Fukuzawa et al., 1988) have been shown to undergo the same metal-catalyzed reactions.

Fig. 1.5: Proposed pro-oxidant reaction mechanisms in lipid dispersions depicted with a galloyl group.

The oxidation of phenolic compounds is also affected by their concentration: higher concentrations have greater stability against phenolic oxidation (Sang et al., 2005). In addition, oxidation products, such as quinones resulting from phenolic oxidation, may further propagate oxidation reactions by interacting with unoxidized phenolics to form dimers (Sang et al., 2007). In the presence of multiple catechol moieties, both rings are capable of undergoing oxidation. Both the B- and D-rings of EGCG are labile to
oxidation: the B-ring was shown to be the primary site of metal-catalyzed oxidation for EGCG (Severino et al., 2009; Valcic et al., 2000), while the D-ring was the primary reaction site for (O$_2^-$) (Severino et al., 2009).

1.3.1.1. Effect of Transition Metals on Metal-Catalyzed Phenolic Oxidation

Transition metals appear to be essential catalysts in the oxidation of phenolic compounds as their complete removal was demonstrated to prevent catechol oxidation (Nakagawa et al., 2002). In these reactions, transition metals assist in the transfer of electrons as a direct reaction between ground-state oxygen and catechol or gallate groups (or any organic compound, for that matter) is unfavorable. Oxygen in the ground state is referred to as triplet oxygen, a biradical with two unpaired valence electrons in separate orbitals. Phenolic compounds cannot directly donate an electron to oxygen as the overlapping of these orbitals would violate Pauli’s exclusion principle, which states that no orbital can contain electrons with the same spin. Therefore, phenolic oxidation requires the presence of transition metals to initiate electron transfer between phenolics and triplet oxygen.

It has been suggested that the transfer of electrons is facilitated by the formation of a ternary phenolic-metal-oxygen complex where electrons are transferred to oxygen by a concerted two-electron transfer (Bandy et al., 1990). Jarabak et al provided evidence of ternary complex formation in a study of the redox cycling of polycyclic aromatic hydrocarbon (PAH) o-quinones in the presence of reductases (Jarabak et al., 1998). The investigators removed trace metals from buffers and reaction vessels using a metal chelating resin. Consequently, redox cycling of PAH-o-quinone (9,10-
phenanthrenequinone) was inhibited by 90% in the presence of superoxide dismutase (SOD) (0.18 µM). In the presence of SOD, the initial rate of NADPH oxidation used for quinone reduction was identical to the control as PAH-o-quinone was added to initiate the reaction. However, within 10 min, the rate of NADPH loss was observed to decrease dramatically, indicating that in the presence of SOD, the limiting factor is the formation of substrates for reduction by hydroquinone oxidation. Upon addition of Cu$^{2+}$ (1 µM), redox cycling was fully restored to control rates, while Fe$^{3+}$ (10 µM) partially restored redox cycling (>50%). The addition of metals alone (no SOD) at the concentrations stated did not change the rate of redox cycling. This supports the formation of a ternary complex as SOD no longer significantly inhibited oxidation because no (O$_2^•$) was released in the presence of free transition metals. The formation of a ternary complex is further supported by the introduction of metal chelators that occupy essential coordination sites. Bandy and Davidson (1987) demonstrated that in the presence of SOD, EDTA + SOD completely inhibited quinone formation from the oxidation of 6-hydroxydopamine. By itself, EDTA (1 mM) accelerated 6-hydroxydopamine oxidation, and SOD (20 U/mL) inhibited oxidation by 40%; however, EDTA + SOD together inhibited oxidation by 86%. Because EDTA + SOD showed greater inhibition of phenolic oxidation, O$_2^•$ is expected to play a major role in phenolic oxidation in the presence of EDTA. When EDTA binds transition metals, it likely prevents the formation of a phenolic-metal-oxygen complex, leading to single electron transfers and the release of O$_2^•$. 
Many studies have focused on the role of iron in phenolic oxidation reactions; however, other transition metal catalysts (e.g., copper, manganese) are also present in foods, albeit at relatively lower concentrations. Catechins are known chelators of Cu$^{2+}$ (Kumamoto et al., 2001) and Fe$^{3+}$ (Elhabiri et al., 2007) ions, but the reduction capacity for each of these metals differs, with most catechins exhibiting a higher capacity for reducing Cu$^{2+}$ (Mira et al., 2002). This difference in reduction capacity may result in faster ROS generation and greater pro-oxidant activity, with the addition of Cu$^{2+}$ compared to Fe$^{3+}$ ions (Kumamoto et al., 2001; Zhang et al., 1996). The introduction of both Cu$^{2+}$ and Fe$^{3+}$ has also been shown to exert a synergistic effect with respect to phenolic oxidation rates in wine (Danilewicz, 2007; Danilewicz et al., 2008).

**1.3.1.2. Effect of Matrix pH on Metal-Catalyzed Phenolic Oxidation**

Matrix pH is an important factor influencing polyphenol stability. It has long been thought that the dissociated form of the phenolic is the species that readily undergoes oxidation; therefore, a matrix pH above the pKa of the phenolic compound should produce higher oxidation rates (Mochizuki et al., 2002). Kumamoto et al. (2001) have shown that other components, such as the presence of Fe$^{3+}$ and Cu$^{2+}$, also lower the acid dissociation constant, potentially promoting higher oxidation rates at a lower pH. Higher pH values may stabilize intermediates from phenolic oxidation, such as the semiquinone radical (Mochizuki et al., 2002) and the O$_2^-$ radical (Knowles et al., 1969), thus promoting oxidation.
Matrix pH also leads to changes in transition metal properties and, consequently, reactivity. Metal speciation and solubility depend on pH, with lower pH stabilizing Fe\(^{2+}\) ions that would otherwise readily precipitate at neutral or alkaline pH. Phenolics also influence iron speciation as the ratio of Fe\(^{2+}\) to total Fe is thought to depend on the phenolic compound in solution (epicatechin > catechol > pyrocatechinic acid > gallic acid) (Hider et al., 2001). With increasing pH, the Fe\(^{2+}\) to total Fe ratio decreased such that above pH 6 all iron was in the Fe\(^{3+}\) state, regardless of the different phenolics in solution. The effects of added metals are less pronounced as pH increases due to oxidation rate increases from other factors, such as dissociation and decreased reduction potential, which we discussed above (Bandy et al., 2001).

1.3.2. Generation of Reactive Oxygen Species

The metal-catalyzed oxidation of phenolics yields H\(_2\)O\(_2\), which alone is not a potent oxidant (\(E^{0'} = 320\) mV at pH 7.0 for the H\(_2\)O\(_2\), H\(^+\) / H\(_2\)O, •OH couple). Though H\(_2\)O\(_2\) is relatively stable in the absence of scavengers, its reduction by transition metals via the Fenton reaction results in the production of highly oxidizing •OH radicals (\(E^{0'} = 2310\) mV at pH 7.0 for the •OH, H\(^+\) / H\(_2\)O couple). •OH radicals are capable of reacting with most organic matter (such as lipids, proteins, and DNA) at diffusion-limited rates (Elias et al., 2009b, 2008a; Elias & Waterhouse, 2010; Makrigiorgos et al., 1995). Due to the high reactivity of •OH radicals, an increase in constituents in the lipid system may impede •OH radicals from reaching lipids to initiate oxidation. For instance, •OH radicals are capable of reacting with surfactant micelles; however, Bansal et al. (1971) observed
different reaction rate constants, depending on the aqueous and critical micelle concentrations for the particular surfactant.

1.3.3. Phenolic Oxidation Products and Quality Loss

Quinones are electrophilic phenolic oxidation products that have been shown to exert biological damage by reacting with DNA (Lewis et al., 1996) and proteins, especially thiol groups (that is, cysteine residues). α-Quinones are also capable of isomerizing to p-quinone methides (Boersma et al., 2000), compounds that are relatively less stable and more electrophilic. The reaction between quinone methides and free nucleophilic amino acids, in order of increasing reactivity, is reported as follows: thiol group of cysteine > α-carbon of lysine and α-carbon of histidine > ε-carbon of lysine and π- and τ-carbon of histidine (Bolton et al., 1997). The formation of covalent bonds between quinones and sulfhydryl groups of membrane proteins may lead to the formation of protein aggregates with intermolecular cross-linking in EGCG and ECG (Chen et al., 2011). This, in turn, may have harmful effects on protein solubility or the physical stability of protein-stabilized emulsions.

A common sensory defect arising from phenolic oxidation is color changes caused by polymerization of phenolics. Polymerization resulting from phenolic oxidation, yield high molecular weight tannins that may imbue a yellow, yellow-brown or yellow-red pigmentation. As tannins increase in size, visual faults such as precipitation and haze may result (Poncet-Legrand et al., 2003). Changes in color may also arise from reactions with anthocyanins. Anthocyanins are glucosides of anthocyanidins, a class of compounds that
impart bright colors in plants like blueberries and grapes. However, undesired color loss may result from nucleophilic addition of flavanols to anthocyanins to form colorless adducts (Dueñas et al., 2006; Remy-Tanneau et al., 2003). These colorless adducts further oxidize to yield xanthylium cations (Liao, et al. 1992, Timberlake and Bridle, 1976) as observed in anthocyanins in Roselle that changed from red to colorless and then to yellow after heating (Tsai and Huang, 2004).

1.4. Potential Solutions to Minimize Pro-Oxidant Activity

We can employ various strategies to mitigate the pro-oxidant activity and quality loss in lipid foods that results from the metal-catalyzed oxidation of polyphenols (as shown in Fig. 1.6). First, it is possible to exclude oxygen from—or, at least, minimize its presence in—the system, which would require us to limit headspace oxygen as well as dissolved oxygen in the system. Another strategy is to scavenge radicals and peroxides prior to •OH radical formation. Finally, the removal of metal catalysts, or the inactivation of redox active metals by suitable chelators, may also prove useful in inhibiting oxidation. While steps may be taken to reduce the levels of exogenous transition metals during food processing, it is impractical to completely remove metals (such as iron and copper) given that they are minor, endogenous components of foods. Therefore, changes to the redox activity of transition metals in polyphenol rich foods have the potential to greatly reduce pro-oxidant activity, especially if the chelator is capable of interfering with the redox cycling of metal catalysts.
1.4.1. Protein Interactions: Radical and Peroxide Scavenging Mechanisms

Due to the fact that •OH radicals are a highly reactive species, a better approach to limiting their pro-oxidant activity may lie in scavenging H$_2$O$_2$, the more stable precursor to •OH radicals. Proteins, in particular, are common food constituents that may scavenge peroxides via methionine and cysteine residues. Methionine residues in proteins are known to react with H$_2$O$_2$ and lipid hydroperoxides to form methionine sulfoxide and non-reactive hydroxides by two electron processes (Garner et al., 1998a, 1998b). Sulfhydryl groups may also react with H$_2$O$_2$ (Denu & Tanner, 1998) as L-cysteine and...
glutathione both effectively decreased H$_2$O$_2$ concentrations generated from polyphenol oxidation (Aoshima & Ayabe, 2007). Cysteine reacts with H$_2$O$_2$ via a two step nucleophilic mechanism to generate the intermediate cysteine sulfenic acid. Competition between free thiols and H$_2$O$_2$ for cysteine sulfenic acid dictates the final products which include cystine (disulfide linkages), cysteine sulfinic acid, cysteine sulfonic acid, and thiol sulfinate (Luo, Smith & Anderson, 2005).

Various amino acids in proteins are also capable radical scavengers and metal chelators. Most amino acids may react with •OH radicals though with different reactivities ((Zs.-Nagy & Floyd, 1984)). Besides cysteine and methionine, tryptophan, histidine, tyrosine and phenylalanine are also good radical scavengers, reviewed in (Dávalos, Miguel, Bartolomé & López-Fandiño, 2004; Stadtman, 2003). Antioxidant activity of histidine residues may also be attributed to its strong metal chelation, which may inhibit redox cycling as observed with the His residues in amyloid-β that inhibited ascorbate oxidation and •OH radical formation in the presence of Cu$^{2+}$ or Fe$^{3+}$ (Nakamura et al., 2007).

Antioxidant activity from the different amino acids may account for some of the antioxidant activity of proteins observed in lipid dispersions (Achyuthan et al., 2002; Elias et al., 2008b) and may be a viable route for scavenging radicals and H$_2$O$_2$ in systems that contain polyphenol. It is conceivable that the radical scavenging properties of polyphenols (that is, their ability to disrupt lipid peroxidation by quenching alkoxy radicals) are revealed only when H$_2$O$_2$ is removed. Thus, the observation that proteins scavenge H$_2$O$_2$ may explain why tea catechins show antioxidant activity in protein-rich
multiphase foods, such as meat systems (He & Shahidi, 1997; Tang et al., 2002, 2006b, 2001b; Yilmaz, 2006).

The interaction between phenolic compounds and proteins may also be of interest as varying the protein in the continuous phase leads to different antioxidant activities (Estevez et al., 2008). Berry phenolics and proteins showed a synergistic antioxidant effect in emulsions (Salminen et al., 2010) and adducts resulting from polyphenolic compounds and proteins have also been shown to exert higher antioxidant activity (Almajano et al., 2007). The addition of phenolics that have already formed protein adducts may be an effective strategy, as the phenolic has already undergone oxidation, and ROS would have presumably been generated beforehand.

1.4.2. Transition Metal Chelator Strategies

Disrupting transition metal redox cycling is a potentially effective method for stabilizing phenolics in foods, thereby inhibiting lipid oxidation reactions. The reduction of metals by many phenolics (for example, catechols) occurs relatively quickly. For example, a 1:1 ratio of hydroquinone (HQ) to Cu$^{2+}$ (100 µM) was able to reduce Cu$^{2+}$ to Cu$^{+}$ within 5 s, while conversion to the benzoquinone reached its highest level in 4 min (Li & Trush, 1993). However, it was also demonstrated that O$_2$ consumption and benzoquinone formation in the presence of Cu$^{3+}$ could be inhibited by the copper chelator and bathocuproinedisulfonic acid, with the maximum effect observed at a metal:ligand ratio of 1:4.
As iron is the most ubiquitous transition metal in foods, this section will focus on iron chelator strategies. The effects of chelators on polyphenol oxidation have been mixed and depend on chelator type. Ligands with oxygen-based donor groups tend to bind Fe\(^{3+}\) ions, while nitrogen-based donor ligands often preferentially bind Fe\(^{2+}\) ions. Some ligands are capable of completely inactivating redox cycling. This may occur if the chelator occupies or blocks all of the metal’s coordination sites, in which case oxidants like oxygen will be unable to bind. One such chelator is desferroxamine (DFO), a hexadentate ferric chelator with zero free coordination sites, which has been shown to prevent ascorbate oxidation and •OH radical formation (Mahoney & Graf, 1986). A strong binding affinity to a particular oxidation state, as we see in quercetin and Fe\(^{2+}\) at pH 7.2, also leads to limited redox cycling. Other ligands, such as 2,2-bipyridine and EDTA, stabilize a specific redox state, thus influencing cycling between Fe\(^{3+}\) and Fe\(^{2+}\) states by promoting a preferred oxidation state.

However, it is important to note the effects of metal chelators on the overall oxidation mechanics, if the metal complex is still redox active. For instance, chelation by EDTA, a ferric chelator, results in one free coordination site that is capable of binding oxidants such as water, oxygen, or even H\(_2\)O\(_2\), allowing the complex to readily generate •OH radicals by promoting Fe\(^{2+}\) oxidation. EDTA also stimulates the oxidation of phenolic compounds in the presence of trace metals (Bandy et al., 1987, 2001) by promoting transition metal oxidation. However, EDTA tends to have antioxidative effects on lipid oxidation (Cho et al., 2003; Hu et al., 2004). Thus, the incorporation of metal chelators
that do not fully inactivate transition metal cycling should be carefully studied to ensure that both metal-catalyzed phenolic oxidation and lipid oxidation are inhibited.

1.4.3. Oxygen Exclusion

An obvious yet extremely effective strategy for controlling the oxidation of phenolics is the removal or exclusion of oxygen. This may be accomplished by modified atmosphere packaging as well as vacuum packaging; both are known to greatly enhance lipid stability. The chemical stability of phenolics increases in low oxygen environments; ~95% EGCG (20 µM) was retained after 6 h in buffer flushed with gaseous N₂ for 24 h, while nearly all EGCG was lost within 2 h in the same buffer system under normal atmospheric conditions (Sang et al., 2005). However, the presence of other electron acceptors that may react with reduced transition metals will reduce the effectiveness of oxygen exclusion. Complete oxygen removal may also lead to other issues such as the growth of Clostridium botulinum.
2. Significance and Hypotheses

Plant-derived phenolics are attractive ingredients due to their purported health benefits and antioxidant activity in foods. In order to confer the benefits of phenolic compounds in lipid-based foods, care must be taken to maintain phenolic stability since most phenolics are oxidatively labile. Besides the unwarranted loss of bioactive phenolics, metal-catalyzed phenolic oxidation may also yield increased lipid and protein oxidation in the food matrix due to reactions with generated ROS (e.g., H$_2$O$_2$ and •OH). Thus, I investigated EGCG oxidation in emulsions to gain a better understanding of the factors and conditions that promote phenolic instability and concomitant ROS production. The results from my study will enable food manufacturers to formulate stable lipid foods containing biologically significant levels of phenolics.

The primary aim of my work was to investigate factors that affect metal-catalyzed EGCG oxidation in order to determine interventions that would prevent the loss of bioactive phenolics and the induction of pro-oxidant activity resulting from phenolic oxidation in oil-in-water emulsions. Due to conflicting results in the literature regarding phenolic effects on lipid oxidation, I surveyed different factors to establish model conditions in which EGCG would be expected to promote lipid oxidation. Based on these findings, I employed methods including metal chelation and protein antioxidant strategies to determine metal chelator and protein characteristics that are important to phenolic and lipid stability.
1. **Effect of matrix pH and phenolic concentration**: The effects of matrix pH and phenolic concentration on net anti-/pro-oxidant activity in o/w emulsions were investigated to determine phenolic concentrations and pH that induced perceivable effects on lipid oxidation.

**Hypothesis**: I expect pro-oxidant activity will be observed below a threshold pH, while anti-oxidant activity will be observed above a threshold pH. Pro-oxidant activity at acidic pH is expected as low pH stabilizes Fe$^{2+}$ ions and more easily generates •OH radicals. Higher phenolic concentrations will yield greater antioxidant effects since more EGCG will be available to scavenge radicals.

2. **Effect of iron chelators on EGCG and lipid oxidation**: The role of iron chelators on metal-catalyzed phenolic oxidation and their net effect on lipid oxidation in o/w emulsions were investigated at acidic and neutral pH.

**Hypothesis**: If the metal chelators (ethylenediaminetetraacetic acid; EDTA or 2,2-bipyridine;BPY) completely inhibit redox cycling, I hypothesize that they will show the ideal effect of strongly inhibiting phenolic and lipid oxidation. If the iron-chelator complex is still active, I would expect the Fe$^{3+}$ chelator, EDTA to promote phenolic and lipid oxidation by encouraging Fe$^{2+}$ conversion to Fe$^{3+}$. The Fe$^{2+}$ chelator, BPY, would be expected to inhibit phenolic and lipid oxidation as it will prefer to hold iron in the Fe$^{2+}$ state for iron-chelator complexation. pH will
also play a crucial role in influencing metal-chelator effects due to changes in reduction potentials of metal-chelator complexes with pH.

3. **Effect of dairy proteins on EGCG oxidation.** The effect of the dairy proteins (CAS or WPI) on peroxide scavenging and EGCG oxidation in emulsions was examined.

   **Hypothesis:** I hypothesize that both proteins will show peroxide scavenging activity proteins though differences in peroxide scavenging activity are expected due to Cys and Met availability to H₂O₂. Since, proteins are capable peroxide and radical scavengers radicals, I expect EGCG oxidation to decrease, since propagation of EGCG oxidation by radicals (O₂^- and •OH) will be curbed.

4. **Effect of solvent accessible Met and Cys residues on EGCG and lipid oxidation.** The role of solvent accessible Met and Cys residues in proteins on metal-catalyzed phenolic oxidation and lipid oxidation in o/w emulsions were investigated.

   **Hypothesis:** I hypothesize peroxide scavenging activity by proteins will be dependent on the solvent accessibility of Cys and Met residues. I expect the rate of peroxide scavenging and total scavenging activity to increase as more Cys and Met residues are made available by denaturing agents such as SDS. The reverse will also be true, such that as Cys and Met residues are blocked, total scavenging
activity will decrease. This will impact EGCG oxidation, since the protein stabilizing effect may be related to ROS scavenging as hypothesized above. When EGCG is stabilized in the presence of proteins and any ROS generated rapidly scavenged by proteins, I expect a net anti-oxidant activity in lipid dispersions will prevail. However, if solvent accessible Cys or Met residues are unavailable to scavenge radicals, pro-oxidant activity by metal-catalyzed oxidation will arise.
3. Antioxidant and Pro-oxidant Activity of (-)-Epigallocatechin-3-gallate in Food Emulsions: Influence of pH and Phenolic Concentration

Published as:

3.1. Abstract

Polyphenols have been observed to exert both antioxidant and pro-oxidant activity in lipid foods, and factors that influence that net effect include both polyphenol concentration and matrix pH. In this study, the effects of concentration (1-500 µM) of a model polyphenol, (-)-epigallocatechin-3-gallate (EGCG), and matrix pH (2-7) on the net anti-/pro-oxidant activity of EGCG in flaxseed oil-in-water (o/w) emulsions were systematically evaluated. After 24 h, EGCG (5-100 µM) was observed to exhibit pro-oxidant activity in low pH (pH 2-4) emulsions, as determined by conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) production. At the higher pH values studied (pH 5-7), lower CD and TBARS concentrations were detected in samples with 25-500 µM EGCG at 24 h. Overall, EGCG concentration and pH both played significant roles in determining net antioxidant or pro-oxidant effects, with the largest antioxidant and pro-oxidant effects observed at the higher EGCG concentrations (100-500 µM) tested.

3.2. Introduction

Polyphenols are often regarded as highly effective antioxidants, with many studies reporting antioxidant activity of novel phenolic extracts or phenolic derivatives for use in food applications. Polyphenols are strong antioxidants due to their radical scavenging
activity resulting from resonance stabilization of the resulting radical after oxidation (Kumamoto, Sonda, Nagayama & Tabata, 2001), as well as their ability to chelate transition metals like iron (Khokhar & Owusu Apenten, 2003). However, the addition of polyphenols to lipid dispersions has been shown to result not only in antioxidant effects (Almajano, Delgado & Gordon, 2007a; Maqsood & Benjakul, 2010), but also pro-oxidant activity (Frankel, Huang & Aeschbach, 1997b; Huang & Frankel, 1997; Mei, McClements & Decker, 1999; Roedig-Penman & Gordon, 1997). The conflicting results observed is likely due to competing reactions (Fig. 3.1) between reactive oxygen species generation coupled to metal-catalyzed polyphenol oxidation and its competing radical scavenging activity. Metal-catalyzed oxidation of di- and tri-hydroxylated phenols leads to the formation of reduced transition metals (e.g., Fe$^{2+}$) (Elias & Waterhouse, 2010), which are capable of reducing oxygen to peroxyl radicals (•OO) and, ultimately, hydroxyl radicals (•OH), a highly reactive radical capable of oxidizing most organic compounds at diffusion limiting rates (Makrigiorgos, Bump, Huang, Baranowska-Kortylewicz & Kassis, 1995). Reduced transition metals are also pro-oxidative as they may catalyze lipid oxidation by reduction of lipid hydroperoxides to lipid alkoxyl radicals, thus resulting in the propagation of lipid oxidation reactions. However, polyphenols are capable of scavenging the same free radicals (peroxyl and hydroxyl radicals) they assist in producing, as well as scavenging lipid-derived radicals.

Two factors that appear to play a considerable role with respect to the net anti-/pro-oxidant activity of phenolics in lipid systems are pH and concentration. pH is capable of influencing the antioxidant activity of phenolics, as well as transition metal properties.
Acidic pH increases the solubility and stability of reduced transition metals (*e.g.* Fe\(^{2+}\)) (Hider, Liu, Khodr & Lester, 2001), with reduced transition metals capable of catalyzing pro-oxidative reactions as shown in **Fig. 3.1**. Higher pH increases phenolic antioxidant activity (Amorati, Pedulli, Cabrini, Zambonin & Landi, 2006), but also metal-catalyzed oxidation (Bandy, Walter, Moon & Davison, 2001; Mochizuki, Yamazaki, Kano & Ikeda, 2002) by increasing the amount of dissociated species. Previous studies suggest the combination of acidic conditions and low polyphenol concentration result in pro-oxidant activity. Corn o/w emulsions treated with (+)-catechin, epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG) (5, 20 µM) showed pro-oxidative activity at pH 3.0-3.5 (Huang et al., 1997). Increased lipid oxidation was also observed in a salmon o/w emulsion system with 5 µM galloyl derivatives, gallamide, methyl gallate, and gallic acid at pH 3, but not at pH 7 (Mei et al., 1999). In the same study, varying effects due to concentration differences were also observed: 500 µM galloyl derivatives at pH 3 showed an antioxidant effect even though treatment with 5 µM galloyl derivatives displayed a pro-oxidant effect.
Fig. 3.1: Proposed mixed mechanism of polyphenol-mediated lipid oxidation and polyphenol radical scavenging antioxidant activity. For simplicity, oxidation of a simple galloyl group is depicted.

The objective of the present study is to provide a comprehensive investigation of the effects of pH and polyphenol concentration on the anti- and pro-oxidant activity of a model phenolic, EGCG (Fig. 1.4), in an oxidatively labile flaxseed oil-in-water (o/w) emulsion system. Lipid oxidation was followed over the course of 4 days at 50 °C by measuring the increase in conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) concentrations.
3.3. Materials and Methods

3.3.1. Materials

EGCG (>93% purity) was acquired from Taiyo Green Power Company (Jiangsu, China). 2-Thiobarbituric acid, butylated hydroxytoluene (BHT) and Tween 80 were purchased from Sigma-Aldrich (St. Louis, MO). Ferric chloride hexahydrate, trichloroacetic acid and sodium azide were purchased from Mallinkrodt (Phillipsburg, NJ). 1,1,3,3-tetraethoxypropane was acquired from TCI America (Portland, OR). Flaxseed oil was purchased from a local market and used as received. All other chemicals and solvents were of analytical or HPLC grade. Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA) purification train.

3.3.2. Emulsion Preparation

Oil-in-water emulsions were prepared by adding 10 wt % flaxseed oil into a 2 wt % Tween 80 solution prepared in water. Coarse emulsions were prepared by using an Ultra-Turrax T25 Basic high-speed blender (IKA, Wilmington, NC) on high speed for 0.5 min. Coarse emulsions were then passed through a microfluidizer (Microfluidics M-110Y, Newton, MA) at 40 psi for two passes to achieve a mean particle size $d_{32} = 0.20 \mu m \pm 0.01$, as determined by laser light scattering (Horiba LA 920, Irvine, CA). EGCG (0-500 $\mu$M) was added to emulsions from an EGCG stock solution prepared in 20 mM phosphate buffer (pH 2-7). Emulsions were then diluted to a final concentration of 5 wt % with 20 mM phosphate buffer. Sodium azide (0.02 %) was added to prevent microbial spoilage. Iron from a freshly prepared stock solution in water was added to the stock emulsions to achieve a concentration of 25 $\mu$M Fe$^{3+}$ in the final 5 wt% o/w emulsions.
The pH of emulsions was measured and adjusted with hydrochloric acid (6 N) or sodium hydroxide (10 N) as necessary. Samples were stored in the dark for 4 days at 50 °C and analyzed for CD and TBARS at predetermined time intervals. All emulsions were stable over the duration of the experiment, though browning was observed in EGCG-containing emulsions due to EGCG polymerization. All experiments were performed in triplicate and plotted as log [EGCG] at 24 h (early stage oxidation) and 96 h (late stage oxidation) to account for the asymmetric distribution of EGCG concentrations tested. Kinetic data for all concentrations, time points, and pH values tested is available in Appendix A2.

### 3.3.3. Conjugated Dienes Analysis

Conjugated dienes (CD) were measured using a method adapted from Mei et. al. (Mei, McClements, Wu & Decker, 1998). Emulsion samples (50 µL) were added to 1.3 mL of methanol:butanol (2:1, v/v) and mixed by vortex. The absorbance was then measured at 234 nm using an Agilent 8453 UV-Vis diode array spectrophotometer (Agilent Technologies, Santa Clara, CA). High EGCG concentrations (500 µM) showed higher Abs$_{234}$, thus CD was plotted as change in absorbance over time using the average day 0 value at each EGCG concentration as the respective reference value for the different EGCG treated flaxseed oil emulsions. Negative values were changed to zero.

### 3.3.4. TBARS Analysis

TBARS concentrations were determined according to a method described previously by McDonald and Hultin (McDonald & Hultin, 1987). Briefly, samples (0.5 mL) were mixed with 1.0 mL TBA reagent and subsequently heated (100 °C) for 15 min. Samples
were cooled by holding at room temperature for 10 min and centrifuged at 3200 x g for 15 min. Samples were then stored for 10 min and analyzed at 532 nm. TBARS concentrations were quantified using an external calibration curve prepared with 1,1,3,3-tetraethoxypropane.

3.3.5. Statistical Analysis

One-way ANOVA with Dunnet’s post test was performed between different EGCG concentrations at the same pH against the control with no EGCG using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California, USA). One-way ANOVA with Bonferroni’s post test was also performed between the different EGCG concentrations at the same pH. Treatments were considered significantly different at $p < 0.05$. 
3.4. Results and Discussion

3.4.1. Effect of EGCG Concentration and pH during Initial Stages of Lipid Oxidation

Flaxseed emulsions (5 wt%) were allowed to oxidize for 96 h at 50 °C in the presence of varying concentrations of EGCG under a range of pH conditions (2-7). No significant differences in CD or TBARS levels were observed in the first 0.5 h within treatment groups at the same pH. After 24 h oxidation at 50 °C, the progress of lipid oxidation is shown according to the primary and secondary lipid oxidation markers, CD (Fig. 3.2a) and TBARS (Fig. 3.2b), respectively. The lowest CD levels were observed at the higher pH values (pH 5-7) and higher EGCG concentrations tested (100, 500 µM). The highest CD concentrations were observed under low pH (pH 2-4) conditions and low EGCG concentrations (5 and 10 µM). Trends in TBARS concentrations were consistent with those observed with CD; the lowest TBARS were again observed at the higher pH values and higher EGCG concentrations tested. Though the highest TBARS concentrations were still detected at low pH (pH 2 and 3), high TBARS concentrations also included higher EGCG concentrations (25-100 µM), unlike CD where the highest values were observed at 5 and 10 µM EGCG.
Fig. 3.2: 3D contour plots of (a) CD concentrations measured as change in Abs$_{234}$ and (b) TBARS in 5 wt% flaxseed emulsions with added Fe$^{3+}$ (25 µM) after 24 h incubation with EGCG (0-500 µM) in 10 mM phosphate buffer (pH 2-7). The gray planes in the 3D contour plot represent the control (no EGCG) at each pH.
Fig. 3.2: (continued)

Pro- and antioxidant effects after 24 h oxidation was determined by comparing to CD and TBARS levels to the EGCG-free control as shown in Table 3.1 and Table 3.2, respectively. Pro-oxidant activity was observed in emulsions with aqueous phase pH values from 2-4 and EGCG concentrations from 1-100 µM. No pro-oxidant activity was observed in higher pH (5-7) emulsions, and a net antioxidant effect was observed, if any significant differences were observed at all. In high pH (5-7) emulsions, TBARS levels showed concentration dependent decreases, such that the lowest TBARS concentrations were observed at 500 µM EGCG.
Table 3.1: CD concentrations measured as change in Abs $234$ in 5 wt% flaxseed emulsions with added Fe$^{3+}$ (25 µM) after 24 h incubation with EGCG (0-500 µM) in 10 mM phosphate buffer (pH 2-7).

<table>
<thead>
<tr>
<th>pH</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2</td>
<td>0.5543</td>
<td>0.5947</td>
<td>0.6456</td>
<td>0.7298</td>
<td>0.4783</td>
<td>0.5099</td>
<td>0.478</td>
<td>0.1568$^+$</td>
</tr>
<tr>
<td>pH 3</td>
<td>0.4321</td>
<td>0.4997</td>
<td>0.6203*</td>
<td>0.6536*</td>
<td>0.5228*</td>
<td>0.5027</td>
<td>0.4851</td>
<td>0.0506*</td>
</tr>
<tr>
<td>pH 4</td>
<td>0.4908</td>
<td>0.5242</td>
<td>0.5985*</td>
<td>0.6638*</td>
<td>0.5727</td>
<td>0.4261</td>
<td>0.2301$^+$</td>
<td>0.0005$^+$</td>
</tr>
<tr>
<td>pH 5</td>
<td>0.6962</td>
<td>0.6939</td>
<td>0.7063</td>
<td>0.5785*</td>
<td>0.4494*</td>
<td>0.1223$^+$</td>
<td>0.0470$^+$</td>
<td>0$^+$</td>
</tr>
<tr>
<td>pH 6</td>
<td>0.4738</td>
<td>0.5086</td>
<td>0.4715</td>
<td>0.3794$^+$</td>
<td>0.1824$^+$</td>
<td>0.1319$^+$</td>
<td>0.0725$^+$</td>
<td>0$^+$</td>
</tr>
<tr>
<td>pH 7</td>
<td>0.663</td>
<td>0.6464</td>
<td>0.5548$^*$</td>
<td>0.5124$^*$</td>
<td>0.3297$^*$</td>
<td>0.1877$^+$</td>
<td>0.0227$^+$</td>
<td>0$^+$</td>
</tr>
</tbody>
</table>

+ denotes significantly lower values (p<0.05) compared to the 0 µM EGCG control
* denotes significantly higher values (p<0.05) compared to the 0 µM EGCG control

Table 3.2: TBARS concentration in 5 wt% flaxseed emulsions with added Fe$^{3+}$ (25 µM) after 24 h incubation with EGCG (0-500 µM) in 10 mM phosphate buffer (pH 2-7).

<table>
<thead>
<tr>
<th>pH</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>500</th>
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</thead>
<tbody>
<tr>
<td>pH 2</td>
<td>47</td>
<td>54</td>
<td>72$^*$</td>
<td>88$^*$</td>
<td>75$^*$</td>
<td>91$^*$</td>
<td>93$^*$</td>
<td>30$^+$</td>
</tr>
<tr>
<td>pH 3</td>
<td>44</td>
<td>54$^*$</td>
<td>82$^*$</td>
<td>99$^*$</td>
<td>94$^*$</td>
<td>97$^*$</td>
<td>95$^*$</td>
<td>38</td>
</tr>
<tr>
<td>pH 4</td>
<td>51</td>
<td>58$^*$</td>
<td>67$^*$</td>
<td>69$^*$</td>
<td>71$^*$</td>
<td>69$^*$</td>
<td>56$^*$</td>
<td>44$^+$</td>
</tr>
<tr>
<td>pH 5</td>
<td>61</td>
<td>62</td>
<td>61</td>
<td>62</td>
<td>52$^+$</td>
<td>31$^+$</td>
<td>16$^+$</td>
<td>10$^+$</td>
</tr>
<tr>
<td>pH 6</td>
<td>35</td>
<td>40</td>
<td>40</td>
<td>38</td>
<td>21$^+$</td>
<td>20$^+$</td>
<td>18$^+$</td>
<td>6$^+$</td>
</tr>
<tr>
<td>pH 7</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>36</td>
<td>30$^+$</td>
<td>21$^+$</td>
<td>9$^+$</td>
<td>6$^+$</td>
</tr>
</tbody>
</table>

+ denotes significantly lower values (p<0.05) compared to the 0 µM EGCG control
* denotes significantly higher values (p<0.05) compared to the 0 µM EGCG control
It is interesting and important to note that the primary lipid oxidation marker (CD) did not always correlate with secondary markers (TBARS). For example, though no significant differences were observed with respect to CD at 24 h at pH 2 from 5-100 μM EGCG, each of these emulsions contained significantly higher TBARS concentrations. This suggests that the degradation of primary lipid oxidation markers such as CD and lipid hydroperoxides is accelerated in the presence of polyphenols due to their ability to reduce transition metals to their catalytically active state (i.e., the ferrous state required to reduce lipid hydroperoxides to alkoxy radicals). The effect is most pronounced under acidic conditions and high EGCG concentrations; both conditions expected to promote the speciation of iron to its ferrous state, thus focus will be placed in the secondary lipid oxidation marker for designation of net anti-/pro-oxidant activity.

3.4.2. Effect of EGCG Concentration and pH during Advanced Stages of Lipid Oxidation

The progress of lipid oxidation in emulsions was further examined after 96 h storage at 50 °C by measuring accumulated CD (Fig. 3.3a) and TBARS (Fig. 3.3b). Similar to CD levels observed at 24 h, CD concentrations were at their lowest in emulsions containing higher EGCG concentrations (100 and 500 μM) and higher pH values (pH 6 and 7). With respect to TBARS, the overall trends also coincided well with those observed at 24 h; the highest TBARS concentrations were observed in low pH (2-4) emulsions (Fig. 3.3b), which decrease sharply at pH 5 and above. One notable difference between the early and late stage lipid oxidation measurements occur at the high EGCG concentration (500 μM).
at acidic pH (pH 2-4), which display some of the highest TBARS values at 96 h, but no increase at 24 h oxidation.

Fig. 3.3: 3D contour plots of (a) CD concentrations measured as change in Abs$_{234}$ and (b) TBARS in 5 wt% flaxseed emulsions with added Fe$^{3+}$ (25 µM) after 96 h incubation with EGCG (0-500 µM) in 10 mM phosphate buffer (pH 2-7). The gray planes in the 3D contour plot represent the control (no EGCG) at each pH.
Fig. 3.3: (continued)

A summary of the net anti-/pro-oxidant activity observed with respect to primary and secondary lipid oxidation markers at 96 h is shown in Table 3.3 and Table 3.4, respectively. After 96 h oxidation, pro-oxidant effects became more pronounced, resulting in increased CD values at pH 2 not previously observed at 24 h, as well as a larger divergence in the concentration of the secondary oxidation marker, TBARS, between EGCG-containing emulsions and the control. At pH 2 and 96 h, TBARS concentrations showed concentration-dependent increases as a function of increasing EGCG concentration (5-500 µM EGCG). The largest difference was observed in
emulsions containing 500 μM EGCG: 520 μM TBARS was observed in the EGCG-containing emulsions while its corresponding control showed only 163 μM TBARS, representing a ca. 315% increase in TBARS concentration compared to the control. It is interesting to note the shift to pro-oxidant activity (as viewed by higher observed TBARS concentrations) in emulsions treated with 500 μM EGCG at 96 h. As discussed above, emulsions containing 500 μM EGCG yielded the highest TBARS values at pH 2, and significantly higher TBARS concentrations at pH 3 and 4. This is in contrast to the results observed at 24 h, where 500 μM EGCG exhibited no significant difference at pH 3 and antioxidant activity at pH 2 and 4. An explanation for the different results observed at the early oxidation phase may be due to the high EGCG concentration (500 μM) that increases the likelihood for EGCG to act as an antioxidant, since EGCG oxidation may be limited by the supply of metal catalyst in our system. Further evidence of the competing antioxidant and pro-oxidant reactions resulting from EGCG is observed at pH 3 and 4, where 500 μM EGCG shows significantly lower TBARS concentrations as compared to 100 μM EGCG at the same pH. Without the competing radical scavenging reactions, TBARS concentrations would be expected to continue to increase in a concentration-dependent manner, which does not occur at pH 3 and 4. This suggests EGCG is also exerting a protective effect, even at low pH conditions, resulting in lower TBARS concentrations compared to the 100 μM EGCG treatment. It is possible that if EGCG concentration increased further, pro-oxidant activity may be lost and, eventually, antioxidant mechanisms may dominate with further increases in EGCG concentration.
Table 3.3: CD concentrations measured as change in Abs\textsubscript{234} in 5 wt% flaxseed emulsions with added Fe\textsuperscript{3+} (25 \textmu M) after 96 h incubation with EGCG (0-500 \textmu M) in 10 mM phosphate buffer (pH 2-7).

<table>
<thead>
<tr>
<th>pH</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>100</th>
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<td>2.0317</td>
<td>2.4084*</td>
<td>2.4290*</td>
<td>2.4883*</td>
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<tr>
<td>pH 4</td>
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<td>3.6632</td>
<td>3.9413*</td>
<td>3.9760*</td>
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<td>3.8355*</td>
<td>2.0846*</td>
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<tr>
<td>pH 5</td>
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<td>4.0913</td>
<td>3.3952*</td>
<td>3.5999*</td>
<td>2.4531*</td>
<td>1.5475*</td>
<td>0.6306*</td>
</tr>
<tr>
<td>pH 6</td>
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<td>2.4087</td>
<td>2.3971</td>
<td>2.1790</td>
<td>1.6081*</td>
<td>1.6120*</td>
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<td>pH 7</td>
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<td>2.1833*</td>
<td>1.8914*</td>
<td>0*</td>
</tr>
</tbody>
</table>

+ denotes significantly lower values (p<0.05) compared to the 0 \textmu M EGCG control
* denotes significantly higher values (p<0.05) compared to the 0 \textmu M EGCG control

Table 3.4: TBARS concentration in 5 wt% flaxseed emulsions with added Fe\textsuperscript{3+} (25 \textmu M) after 96 h incubation with EGCG (0-500 \textmu M) in 10 mM phosphate buffer (pH 2-7).

<table>
<thead>
<tr>
<th>pH</th>
<th>0</th>
<th>1</th>
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<th>500</th>
</tr>
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<tbody>
<tr>
<td>pH 2</td>
<td>165</td>
<td>193</td>
<td>232*</td>
<td>288*</td>
<td>276*</td>
<td>341*</td>
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<td>520*</td>
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<td>190*</td>
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<td>pH 4</td>
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<td>346*</td>
<td>372*</td>
<td>336*</td>
<td>259*</td>
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<tr>
<td>pH 5</td>
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<td>90</td>
<td>82</td>
<td>92</td>
<td>89</td>
<td>88</td>
<td>8*</td>
</tr>
</tbody>
</table>

+ denotes significantly lower values (p<0.05) compared to the 0 \textmu M EGCG control
* denotes significantly higher values (p<0.05) compared to the 0 \textmu M EGCG control
Significantly lower TBARS concentrations were observed in emulsions at high pH (pH 5-7) and high EGCG concentrations. Surprisingly, 100 µM EGCG led to significantly higher TBARS concentrations in pH 6 emulsions after 96 h, which was unexpected since 50 and 500 µM EGCG both showed antioxidant activity. The same results occurred upon replication of the study at pH 6. Pro-oxidant activity was also observed in another study where sunflower o/w emulsions at pH 5.5 treated with 50 µM FeCl₃ and 100 µM EGCG showed higher peroxide values and CD (Roedig-Penman et al., 1997). The pro-oxidant activity at pH 6 was unexpected and further studies are needed to examine the potential interactions in the system that promotes the pro-oxidant activity (eg. the removal of added Fe, or the EGCG/Fe ratio by trying EGCG concentrations close to 100 µM EGCG). At pH 7, only 500 µM EGCG yielded lower TBARS concentration, while no other EGCG-treated sample at pH 7 showed any significant difference from the control. The loss in antioxidant capacity seen at pH 7 is likely due to the rapid rate of EGCG oxidation at neutral pH. If EGCG readily oxidizes under these conditions, a relatively low concentration of unoxidized EGCG may remain to scavenge radicals and inhibit lipid oxidation propagation reactions. Additionally, it appears that the presence of higher EGCG concentrations leads to higher antioxidant activity at pH 5-7. At pH 5 and 6, emulsions containing 500 µM EGCG showed only a slight increase in TBARS (10 µM) after 96 h incubation, while the same system at pH 7 showed no increase from initial observed values (7 µM TBARS).

Overall, pH appears to play a significant role in dictating the net antioxidant and pro-oxidant capacity of polyphenols in lipid dispersions. The addition of EGCG to low pH
(pH ≤ 4) matrices was observed to yield pro-oxidant effects. The concentration of the phenolic compound was also observed to play an important role, as higher EGCG concentrations (1-100 µM) resulted in higher TBARS concentrations at pH 2-4. However, further increases in concentration (100 µM vs. 500 µM EGCG) at pH 3 and 4 apparently resulted in a competition between antioxidant and pro-oxidant activities leading to lower TBARS concentrations compared to those observed at 100 µM EGCG. In addition, emulsions containing 500 µM EGCG exhibited a lag phase of at least 24 h before significantly higher lipid oxidation markers were observed. At pH 5-7, high EGCG concentrations appear to exert the best antioxidant effects with 500 µM EGCG resulting in the lowest observed CD and TBARS concentrations in emulsions.

As low pH promotes pro-oxidant activity of polyphenols in lipid dispersions, care should be taken when formulating certain food products. Further examination into other key factors, such as the incorporation of metal chelators, may help to mitigate potential pro-oxidant effects from polyphenols.
4. The Effect of Metal Chelators and pH on the net Anti- and Pro-Oxidant Activity of Polyphenols in Oil-in-Water Emulsions

Published as:

4.1. Abstract

The nonenzymatic oxidation of polyphenols bearing di- and trihydroxyphenol groups results in the generation of hydrogen peroxide (H$_2$O$_2$), a reactive oxygen species that can potentially compromise the oxidative stability of foods and beverages. An investigation of the factors that promote the oxidation of a model polyphenol, (−)-epigallocatechin-3-gallate (EGCG), was undertaken in a model lipid-based food system. Factors affecting oxidative stability, such as exogenous iron chelators (ethylenediaminetetraacetic acid; EDTA and 2,2-bipyridine; BPY) and pH (3 and 7) were evaluated in hexadecane and flaxseed oil-in-water (o/w) emulsions. At neutral pH, H$_2$O$_2$ levels were observed to rise rapidly in hexadecane emulsions except for EDTA-containing treatments. However, EDTA-containing samples showed the highest rate of EGCG oxidation, suggesting that H$_2$O$_2$ was rapidly reduced to hydroxyl radicals (•OH). Conversely, at pH 3, H$_2$O$_2$ concentrations were lower across all treatments. EDTA conferred the highest degree of EGCG stability, with no loss of the catechin over the course of the study. In order to assess whether or not the H$_2$O$_2$ production seen in oxidatively stable hexadecane emulsions translated to pro-oxidant activity in an oxidatively labile food lipid system, the effect of EGCG on the stability of flaxseed o/w emulsions was studied. EGCG displayed antioxidant activity at pH 7 throughout the study; however at pH 3, pro-oxidant activity was seen in EGCG-containing emulsions, with and without BPY. This study attempts to...
provide a mechanistic understanding of the conditions wherein polyphenols simultaneously exert pro-oxidant and antioxidant behavior in lipid dispersions.

4.2. Introduction

Polyphenols represent a class of compounds commonly associated with high antioxidant activity (Cai, Mei, Jie, Luo & Corke, 2006; Chen & Ho, 1995; Clark, 1960; Nanjo, Mori, Goto & Hara, 1999; Roy, Koide, Rao, Okubo, Ogasawara & Juneja, 2010), yet their effect on the oxidative stability of lipid dispersions have been mixed with both antioxidant (Medina, Tombo, Satué-Gracia, German & Frankel, 2002) and pro-oxidant activities reported (Frankel, Huang & Aeschbach, 1997a; Huang et al., 1997; Roedig-Penman et al., 1997). A possible explanation for observed pro-oxidant activity may arise from the non-enzymatic, metal-catalyzed oxidation of polyphenols (Nakagawa et al., 2002), which results in hydrogen peroxide (H$_2$O$_2$) generation (Fig. 1.5). While H$_2$O$_2$ is not a particularly potent oxidant in many food systems, it is capable of undergoing metal-catalyzed reduction to form highly reactive hydroxyl radicals (•OH), a non-specific oxidant capable of reacting with organic matter at diffusion limited rates (Makrigiorgos et al., 1995). One strategy is to limit polyphenol oxidation and its resulting reactive oxygen species (ROS) generation by controlling metal-catalyzed polyphenol oxidation reactions. Iron chelators are commonly added to foods to inhibit metal-catalyzed lipid oxidation reaction. Chelators with oxygen based ligands preferentially bind ferric ions (Fe$^{3+}$), while chelators with nitrogen or sulfur ligands preferentially bind ferrous ions (Fe$^{2+}$). The stabilization of a specific transition metal oxidation state may promote or inhibit certain reactions (Fig. 1.5) when active cycling of iron is necessary for catalysis. For instance,
ethylenediaminetetraacetic acid (EDTA), a ferric ion chelator, has been shown to stimulate the oxidation of ascorbic acid (Mahoney & Graf, 1986) as well as phenolic compounds in the presence of trace metals (Bandy & Davison, 1987; Bandy et al., 2001; Mira, Fernandez, Santos, Rocha, Florêncio & Jennings, 2002) due to the stabilization of the catalytic ferric ion necessary for the oxidation of the above compounds.

As multiphase systems, emulsions contain a number of •OH radical-reactive compounds in the aqueous phase, which may non-selectively scavenge •OH radicals before they are able to reach lipid droplets. The likelihood of •OH radicals initiating lipid oxidation in o/w emulsions is largely unknown, and is most likely dependent on site-specific reactions given the high reactivity of these species. Studies in dispersed lipid systems have shown that the physical location of iron catalysts strongly affects •OH radical-promoted lipid oxidation reactions (i.e., oxidation reactions increased in systems when iron was in close proximity to the interface) (Decker et al., 2001). However, the proximity of iron to interfaces is not the only factor that contributed to oxidative stability, as it has been shown that while ADP and ATP are capable of removing iron from liposome membranes, lipid oxidation reactions increased (Vile & Winterbourn, 1987). Such an effect was not seen in the presence of EDTA and citrate, suggesting that the reactivity of the iron chelate also plays a significant role. Transition metals are also important catalysts in propagating lipid oxidation reactions, as they are able to reduce lipid hydroperoxides to alkoxyl radicals (Huang, Satué-Gracia, Frankel & German, 1999; Nuchi, McClements & Decker, 2001). Though EDTA may result in rapid polyphenol loss and potential •OH radical formation, it appears to exert a net antioxidant effect in lipid dispersions by directing iron
away from interfaces (Mancuso, McClements & Decker, 1999; Mei, Decker & McClements, 1998) where Fe$^{2+}$ has been shown to initiate lipid oxidation by reacting with endogenous lipid hydroperoxides (Tang, Zhang, Qian & Shen, 2000).

The objective of this study was to investigate the role of a ferrous (2,2-bipyridine; BPY) and ferric (EDTA) chelator on epigallocatechin-3-gallate (EGCG) oxidation and H$_2$O$_2$ generation as a function of pH in an oxidatively stable hexadecane o/w emulsion system. As a saturated hydrocarbon, hexadecane will not readily undergo lipid oxidation reactions, thus limiting the confounding effect of polyphenol loss due to reaction with lipid-derived radicals. EGCG (Fig. 1.4) was selected as a model polyphenol in the present study because it is commonly added to foods, both as an antioxidant in lipid systems, and as a bioactive ingredient. The oxidation of a flaxseed o/w emulsion was then studied under identical conditions to determine the net effect of EGCG oxidation on the oxidative stability of lipids more prone to oxidation due to high unsaturated fatty acid content. The elucidation of metal-catalyzed oxidation reactions of polyphenols may aid in preventing the loss of these bioactive polyphenols as well as preventing the potential pro-oxidant activity their oxidation may exert when introduced into lipid foods.
4.3. Materials and Methods

4.3.1. Materials

Butylated hydroxytoluene (BHT), 2,2-bipyridine (BPY) (≥ 99%), ethylenediaminetetraacetic acid (EDTA), Tween 80, xylenol orange tetrasodium salt, and ferrous sulfate heptahydrate were purchased from Sigma-Aldrich (St. Louis, MI). H$_2$O$_2$ (30% w/v) and ammonium thiocyanate were purchased from EMD Chemicals (Gibbstown, NJ). n-Hexadecane (99% purity) was obtained from Acros Organics (Morris Plains, NJ). D-sorbitol (98% purity) and cumene hydroperoxide were purchased from Alfa Aesar (Ward Hill, MA). 1,1,3,3-tetraethoxypropane was acquired from TCI America (Portland, OR). Ferric chloride 6-hydrate and sodium azide were purchased from Mallinkrodt (Phillipsburg, NJ). α-phenyl-N-tert butyl nitrotrone (PBN) was purchased from GeroNova Research (Carson City, NV). α-(4-Pyridyl 1-oxide)-N-tert-butyl nitrotrone (POBN) was purchased from Alexis Biochemicals (Farmingdale, NY). Flaxseed oil was purchased from a local market. EGCG (93% purity) was purchased from Taiyo Green Power Company (Jiangsu, China). All other chemicals and solvents were purchased from Sigma, EMD, Alfa Aesar, and Mallinkrodt, and were of analytical or HPLC grade. Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA) purification rain.

4.3.2. Emulsion Preparation

Oil-in-water emulsions were prepared by dispersing 10 wt % hexadecane or 10 wt % flaxseed oil in water containing 2 wt % Tween 80, a non-ionic surfactant. Coarse emulsions were prepared using an Ultra-Turrax T25 Basic high-speed blender (IKA,
Wilmington, NC) on high speed for 0.5 min. Fine emulsions were prepared by passing coarse emulsions twice through a microfluidizer (Microfluidics M-110Y, Newton, MA) at 40 psi. All emulsions had a mean particle size (d_{32}) of 0.27 ± 0.01 µm, as determined by laser light scattering (Horiba LA 920, Irvine, CA). EGCG (0.4 mM final concentration) was added to 10 wt % hexadecane emulsions from a 10 mM stock solution prepared in 20 mM phosphate buffer (pH 3 or 7). Emulsions were diluted to a final concentration of 5 wt% with 20 mM phosphate buffer (pH 3 or 7). EDTA (50 µM) or BPY (100 µM) was added to 5 wt% emulsions from freshly prepared stock solutions in water. Ferric iron (25 µM) was subsequently added to all samples from a FeCl₃ solution prepared in water. Sodium azide (0.02 wt %) was added to emulsions in order to prevent microbiological growth. Emulsions were stored in the absence of light at 37 °C. Samples were analyzed over time to follow EGCG oxidation and H₂O₂ concentration. For lipid oxidation studies, a separate sample set was prepared for PBN analysis.

4.3.3. Hydrogen Peroxide Analysis

Emulsion samples were chemically destabilized by the addition of methylene chloride:methanol (2:1, v/v) followed by vortex mixing for 30 s and centrifugation at 800 x g for 2 min. The upper aqueous layer was collected and stored at -80° C until analysis. A modified version of the concentrated ferrous oxidation-xylenol orange (FOX) assay (Wolff & Lester, 1994) was used for H₂O₂ analysis. The FOX assay solution consisted of xylenol orange (1 mM), ferrous sulfate (2.5 mM) and sorbitol (1.0 M) in sulfuric acid solution (0.5 N), and was prepared daily from stock solutions. Peroxide analysis was performed by adding the assay solution (20 µL) to the aqueous extract (140 µL). To
remain in the linear portion of the standard curve, samples were diluted as necessary using a 1:2 water:methanol mixture. Samples were mixed by vortex and absorbance values were read at 560 nm following incubation (30 min; ambient temperature) using an Agilent 8453 UV-Vis diode array spectrophotometer (Agilent Technologies, Santa Clara, CA) blanked against aqueous extracts from unoxidized, freshly treated emulsions. Quantitation was performed using an external standard curve prepared using authentic H$_2$O$_2$, the concentration of which was validated using the peroxide’s extinction coefficient $\epsilon_{240} = 40 \text{ M}^{-1} \text{ cm}^{-1}$. A separate standard curve was prepared for H$_2$O$_2$ in the presence of EDTA, as ferric chelators are known to interfere with the FOX assay.

4.3.4. EGCG Analysis

EGCG was extracted from emulsions in the same manner as described above for H$_2$O$_2$ analysis. To prevent further EGCG oxidation during storage and analysis, a preservative solution (10 µL) consisting of ascorbic acid (20 wt%) and ethylenediaminetetraacetic acid (EDTA; 0.1 wt%) in phosphate buffer (pH 3.6; 0.4 M) was added to the aqueous extract (100 µL) prior to storage at -80° C (Lee et al., 2004). Preservative solutions were prepared daily. Chromatographic separation was achieved on a reverse phase Supelcosil LC-18 (4.6 x 150 mm, 5 µm; Supelco Inc., Bellefonte, PA) using a Shimadzu 10ADvp pump (Columbia, MD) with sample introduction by means of a Shimadzu 20ADvp temperature-controlled autosampler (4 °C). Samples were filtered over 0.45 µm PTFE syringe filters. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). EGCG was eluted by gradient according to the following program: 0-8.5 min from 25 to 45% B. The injection volume was 20 µL and the flow
rate was held at 1 mL/min. EGCG was detected at 280 nm using a Shimadzu SPD-M10Avp photodiode array detector, with quantitation based on an external standard curve prepared from EGCG. Apparent first order rate constants were determined by plotting the natural log of EGCG concentrations versus time for the linear portion of the curve (i.e., initial 48 h).

4.3.5. Analysis of PBN-Lipid Radical Spin Adducts by EPR

EPR analysis was measured by the addition of the spin trap, PBN (30 mM), directly to the treated 5 wt % emulsions which were then stored at 37 °C for 8 days. PBN is a lipophilic nitrore spin trap that forms stable radical adducts with lipid-derived radicals. EPR spectra were recorded using a Bruker e-Scan R (Bruker BioSpin, Rheinstetten, Germany) operating in X-band. The instrument settings used were as follows: center field, 3490 G; sweep width, 70 G; microwave power, 6 mW; microwave frequency, 9.78 GHz; modulation frequency, 86 kHz; modulation amplitude, 2.45 G; time constant, 164 ms; conversion time, 20.48 ms for a total of 3 scans per sample. Emulsion samples (50 μL) were transferred to borosilicate capillary tubes before they were introduced to the cavity of the EPR. Analysis was performed at room temperature. Radical concentration was determined by measuring the signal height of the EPR spectrum resulting from PBN-lipid radical adducts.

4.3.6. Lipid Hydroperoxide Analysis

Lipid hydroperoxide concentrations were measured according to the method described by Shantha and Decker (Shantha & Decker, 1994). Flaxseed o/w emulsions (0.15 mL) were
mixed with 0.75 mL of iso-octane/1-butanol (3:1, v/v) and vortexed 3 times for 10 s at 20 s intervals. Samples were subsequently centrifuged for 2 min at 3300 x g. The upper layer of the sample extraction (0.1 mL) was mixed with 1.4 mL of methanol/butanol (2:1, v/v). Sample extracts were diluted with water as needed. The ferrous iron solution was prepared by mixing an equal amount of a solution of 0.144 M FeSO₄ and 0.132 M BaCl₂. The iron solution was centrifuged for 3 min at 3300 x g, and an equal volume of the supernatant and 3.94 M ammonium thiocyanate was mixed to prepare the assay solution. 15 µL of the assay solution was added to the samples and then analyzed at 520 nm after storage for 20 min. Lipid hydroperoxide concentrations were quantified using an external calibration curve prepared with cumene hydroperoxide.

4.3.7. TBARS Analysis

TBARS was determined using a method described by McDonald and Hultin (McDonald et al., 1987). Briefly, samples (0.5 mL) were mixed with 1.0 mL TBA reagent and heated in a boiling water bath for 15 min. Samples were cooled at room temperature for 10 min and centrifuged at 3200 x g for 15 min. Samples were then stored for 10 min and the aqueous layer analyzed at 532 nm. TBARS concentrations were quantified using an external calibration curve prepared with 1,1,3,3-tetraethoxypropane.

4.3.8. Analysis of POBN-1-Hydroxyethyl Radical Spin Adducts

•OH radicals derived from the metal-catalyzed reduction of H₂O₂ were measured indirectly by adding ethanol to emulsions. •OH radicals are known to oxidize ethanol to ethyl radical species (1-hydroxyethyl and 2-hydroxyethyl radicals, 85% and 15%,
respectively), which eliminates the need to add high (i.e., molar) concentrations of spin trapping agents. The EPR spectra of POBN-1-hydroxyethyl (1-HER) spin adducts were obtained after the direct addition of the hydrophilic spin trap, POBN (50 mM), directly to 10 wt % hexadecane o/w emulsions. Emulsions were then diluted with 20 mM phosphate buffer (pH 3 or 7) and 10 mM EGCG prepared in 20 mM phosphate buffer (pH 3 or 7) to achieve a final EGCG concentration of 0.4 mM in a 5 wt% emulsion with 1 vol % ethanol and 25 μM Fe$^{3+}$. EDTA (50 μM) or BPY (100 μM) were quickly added to the emulsions to initiate the reaction. Samples were held at 37°C.

The EPR spectra were recorded using a Bruker e-Scan R (Bruker BioSpin, Rheinstetten, Germany). The instrument settings used were as follows: center field, 3490 G; sweep width, 100 G; microwave power, 6 mW; microwave frequency, 9.78 GHz; modulation frequency, 86 kHz; modulation amplitude, 2.45 G; time constant, 40.96 ms; conversion time, 20.48 ms for a total of 5 scans per sample. Emulsion sample aliquots (50 μL) were transferred to borosilicate capillary tubes and analyzed at room temperature, as described above. Spin adduct intensities were measured according to the method described above.

4.3.9. Statistical Analysis

All experiments were performed in triplicate and results expressed as means and standard deviation. Two-way ANOVA with Bonferroni’s post test was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California, USA). Treatments were considered significantly different at p < 0.05.
4.4. Results and Discussion

4.4.1. Influence of Metal Catalysis and pH on EGCG Stability in Hexadecane Emulsions

The oxidative stability of EGCG in 5 wt% hexadecane emulsions with ferric ions (25 µM) treated with EDTA (50 µM) or BPY (100 µM) was followed over the course of 8 days under both acidic (pH 3) and neutral (pH 7) conditions. At pH 7 (Fig. 4.1a), the rate of EGCG oxidation, in order of highest to lowest, was as follows: EGCG+EDTA > EGCG control > EGCG+BPY with EGCG control not containing any added chelators. The rate of EGCG consumption during the initial phase (i.e., first 48 h) of the study appeared to follow first order kinetics. As such, first order rate constants for EGCG oxidation were calculated as 1.34 s\(^{-1}\), 0.62 s\(^{-1}\), and 0.43 s\(^{-1}\) for EGCG+EDTA, EGCG control, and EGCG+BPY, respectively (R\(^2\) > 0.99 for all lines). At day 2, emulsion samples with EDTA retained only ca. 27 µM EGCG (7% of initial concentration), whereas the control emulsion and emulsions with BPY retained 119 µM (30%) and 171 µM (43%), respectively. By day 8, virtually all EGCG was lost with no observed differences between any of the treatments with respect to EGCG concentration. The presence of the metal chelators EDTA and BPY profoundly affected EGCG oxidation rates, presumably due to their ability to stabilize different iron oxidation states. EDTA is a strong iron chelator, preferentially complexing ferric ions (1:1 ligand:metal ratio), and slightly increasing the reduction potential (E\(_h\)) of the ferric/ferrous couple from 0.110 V (for Fe\(_{3+/2+}\)) to 0.120 V (Fe(EDTA)\(_{3+/2+}\)) (Zoski, 2007). Though EDTA-iron complexes have slightly higher E\(_h\), Fe(EDTA)\(^{2+}\) has still been shown to exhibit higher reducing power as seen by faster Fe\(^{2+}\) auto-oxidation and concomitant reduction of dioxygen to
superoxide radicals (Fig. 1.5) (Welch, Davis & Aust, 2002), as well as faster iron-mediated H$_2$O$_2$ reduction to •OH (Graf, Mahoney, Bryant & Eaton, 1984; Welch et al., 2002) compared to uncomplexed Fe$^{2+}$. Thus, ferric ion stabilization by EDTA promotes the oxidation of Fe$^{2+}$, which likely leads to the increased rate of EGCG oxidation observed due to an increase in redox cycling to the catalytic ferric state required for subsequent EGCG oxidation. Conversely, BPY preferentially chelates ferrous ions (3:1 ligand:metal ratio) (Braterman, Song & Peacock, 1992), thereby increasing the $E_h$ of iron from 0.110 V to 1.074 V for the Fe(BPY)$_3^{3+/2+}$ couple (Zoski, 2007). The higher $E_h$ translates to a decrease in the reducing power of Fe$^{2+}$, and should theoretically retard hydroperoxyl radical formation. An increase in the reduction potential for the BPY-iron complex also facilitates EGCG oxidation, the $E_h$ of which is 0.43 V (Jovanovic, Hara, Steenken & Simic, 1995). However, this increase in $E_h$ also slows the redox cycling of ferrous ions to their catalytically active ferric state, which appears to be necessary for subsequent EGCG oxidation (Fig. 1.5). The $E_h$ of the most effective metal catalysts typically fall in between those reduction potentials of the two species that will ultimately be oxidized or reduced by the metal (i.e., EGCG and molecular oxygen in the system described here) (Bandy et al., 2001). The $E_h$ of the O$_{2(aq)}$/O$_2$•– couple is -0.16 V at pH 7 (Wood, 1988) and 0.43 V for EGCG, suggesting that uncomplexed iron (0.110 V) and Fe(EDTA) complexes (0.120 V) should act as strong catalysts for EGCG oxidation and dioxygen reduction as opposed to the Fe(BPY)$_3^{3+/2+}$ couple (1.074 V). Furthermore, the Fe(EDTA) complex shows better catalytic activity due to the ease of Fe$^{2+}$ oxidation and an increase in iron solubility at neutral pH.
Fig. 4.1: Changes in (a) EGCG and (b) $\text{H}_2\text{O}_2$ concentration resulting from the oxidation of 400 µM EGCG in Tween-stabilized 5 wt % hexadecane emulsions in 10 mM phosphate buffer (pH 7) treated with FeCl$_3$ (25 µM) and EDTA (50 µM) or BPY (100 µM).
The apparent rate of H$_2$O$_2$ production resulting from EGCG oxidation was highest for the EGCG control and BPY treatment yet, surprisingly, virtually no H$_2$O$_2$ was observed in hexadecane emulsions containing EDTA (Fig. 4.1b). In the EGCG control and BPY treatment, H$_2$O$_2$ production rates corresponded well with EGCG oxidation rates, with the EGCG control showing faster EGCG oxidation and subsequent H$_2$O$_2$ production compared to the BPY treatment. By this measure alone, EDTA would appear to be the most effective treatment for preventing H$_2$O$_2$ generation, which would refute our proposed mechanistic interpretation based on EGCG oxidation. However, the low levels of H$_2$O$_2$ observed in the presence of EDTA does not appear to reflect lower peroxide
generation rates, but rather is likely due to the rapid decomposition of H$_2$O$_2$ to •OH radicals *via* the Fenton reaction (Fig. 1.4). As discussed above, the reducing capacity of iron is increased when it is complexed to EDTA, which therefore should accelerate the reduction of peroxides. The ability of EDTA-iron complexes to rapidly generate •OH radicals from H$_2$O$_2$ is, in fact, commonly exploited in •OH radical scavenging studies (Curcio et al., 2009; Halliwell, Gutteridge & Aruoma, 1987; Rivero-Pérez, Muñiz & González-Sanjose, 2008).

![Diagram](image)

**Fig. 4.2:** Indirect •OH radical measurements via POBN-1-HER spin adduct formation resulting from the metal-catalyzed oxidation of polyphenols.
A spin trapping EPR technique was used to confirm that H$_2$O$_2$ was indeed being reduced to •OH radicals in EDTA-containing emulsions. Ethanol (1 vol %, final concentration) was added to emulsions and •OH radicals were measured indirectly as 1-hydroxyethyl radicals (as shown in Fig. 4.2). Under these conditions, •OH radicals oxidize ethanol to 1-hydroxyethyl radicals, which can be quenched by POBN to yield spin adducts with relatively long half-lives (Ramos, Pou, Britigan, Cohen & Rosen, 1992). The time required for the formation of detectable levels of POBN-1-HER adducts (a representative spectrum is shown in Fig. 4.3) was ca. 8 h for both the EGCG control and the BPY treatment (Fig. 4.4); however, POBN-1-HER spin adducts were detected within 1 min in EDTA-containing samples (Fig. 4.4). The rapid formation of •OH radicals in the presence of EDTA accounts for the lack of observed H$_2$O$_2$ accumulation and accelerated
EGCG oxidation at pH 7. Even after 24 h of storage, EGCG control and EGCG+BPY treatments showed intensities of ca. $2 \times 10^5$, while EDTA treatment reached an intensity of $7 \times 10^6$ within just 90 min.

\[ \text{POBN-1-HER Radical Adducts (intensity)} \]

\[ 0 \quad 2 \quad 4 \quad 6 \quad 8 \quad 10 \quad 12 \quad 14 \quad 16 \quad 18 \quad 20 \quad 22 \quad 24 \quad 26 \]

**Fig. 4.4:** POBN-1-HER spin adduct generation resulting from the oxidation of 400 µM EGCG in 5 wt% hexadecane o/w emulsions at pH 7 treated with FeCl$_3$ (25 µM) and the metal chelators EDTA (50 µM) or BPY (100 µM).

The effect of EDTA and BPY on EGCG oxidation and H$_2$O$_2$ production was also investigated at pH 3 (Figs. 4.5a,b, respectively). Nearly 90% of the EGCG was lost in control and BPY-containing emulsions within 8 days, whereas EGCG remained stable in the presence of EDTA over the course of the experiment. First order rate constants for EGCG oxidation were calculated as 0.3147 s$^{-1}$ and 0.3111 s$^{-1}$ for the EGCG control and BPY containing samples ($R^2 > 0.99$ for all lines), respectively, though no significant
difference was observed between these two treatments. In comparison with results obtained at pH 7, no oxidation was observed in EDTA-containing samples at day 2, while both the EGCG control and BPY treatment retained ca. 200 µM EGCG (50%). As expected, EGCG showed greater stability under the acidic conditions employed here compared to the system at pH 7, which is consistent with previous reports (Chen, Zhu, Tsang & Huang, 2000). The control showed greater increased EGCG stability at the acidic pH (0.3147 s⁻¹ vs. 0.6163 s⁻¹) as opposed to the BPY treatment (0.3111 s⁻¹ vs 0.4328 s⁻¹). With decreasing pH, reduction potentials increase, thus increasing the reduction potential of the Fe³⁺/²⁺ couple, which should lower the availability of catalytic Fe³⁺ required for EGCG oxidation. In contrast to what was observed at pH 7, the rate of EGCG oxidation was markedly faster in both the EGCG control and BPY treatment compared to the EDTA treatment. Under these acidic conditions, EDTA no longer accelerated EGCG oxidation as was observed at pH 7, but instead strongly inhibited EGCG oxidation. Consistent with the EGCG oxidation kinetics, the observed levels of H₂O₂ were significantly higher for the EGCG control and BPY treatment compared to the EDTA treatment (Fig. 4.5b); though, little H₂O₂ accumulation was observed in all treatments as compared to H₂O₂ levels at neutral pH.
Fig. 4.5: Changes in (a) EGCG and (b) \( \text{H}_2\text{O}_2 \) concentration resulting from the oxidation of 400 \( \mu \text{M} \) EGCG in Tween-stabilized 5 wt % hexadecane emulsions in 10 mM phosphate buffer (pH 3) treated with \( \text{FeCl}_3 \) (25 \( \mu \text{M} \)) and the metal chelators EDTA (50 \( \mu \text{M} \)) or BPY (100 \( \mu \text{M} \)).
Fig. 4.5: (continued)
EPR analysis of •OH radical formation at pH 3, as measured by POBN-1-HER spin adducts, revealed that H$_2$O$_2$ was quickly reduced to •OH radicals in both the EGCG control and BPY-containing emulsions, but that •OH radical formation was relatively slow in EDTA-containing emulsions (Fig. 4.6). The time required for the formation of detectable levels of POBN-1-HER adducts was ca. 2 h for EDTA-containing emulsions; however POBN-1-HER spin adducts were quickly detected within ca. 10 min in the EGCG control and BPY-containing emulsions. It is interesting to note that while their rate of formation was indeed slow, •OH radicals were observed in emulsions containing EDTA, despite the fact that EGCG was relatively stable and only a small quantity of H$_2$O$_2$ could be measured under the same conditions.
Fig. 4.6: POBN-1-HER spin adduct generation resulting from the oxidation of 400 µM EGCG in 5 wt% hexadecane o/w emulsions at pH 3 treated with FeCl₃ (25 µM) and the metal chelators EDTA (50 µM) or BPY (100 µM).

4.4.2. Effect of EGCG Oxidation and H₂O₂ Production on the Oxidative Stability of Flaxseed Emulsions

The consequences of EGCG oxidation and concomitant H₂O₂ and •OH radical generation on the stability of an oxidatively labile flaxseed emulsion were investigated. The objective of the experiments was to establish if, and under what conditions, EGCG-generated reactive oxygen species (i.e., •OH radicals, H₂O₂) could promote the oxidation of flaxseed o/w emulsions, thereby eclipsing the catechin’s antioxidant capacity.
The rate of formation and final yield of lipid-derived radicals (e.g., lipid alkoxyl and hydroperoxyl radicals) in flaxseed oil emulsions was measured using a PBN spin trapping technique. At pH 7, emulsions containing EGCG+EDTA consistently gave high yields of PBN-lipid radical spin adducts over the 8-day study (Fig. 4.7a). This is consistent with what was observed in hexadecane emulsions, wherein the EDTA treatment resulted in the highest rate of EGCG oxidation (Fig. 4.1a) and •OH radical production (Fig 4.4). Overall, PBN-lipid radical adduct intensities for EGCG-containing samples at day 8 were highest for the EDTA treatment, followed by the EGCG control and finally the BPY treatment (Figure 4.7a). Unlike EGCG+EDTA treatments, at early time points EGCG and EGCG+BPY treatments showed significantly less PBN-lipid radicals compared to their corresponding controls, though by day 6, all EGCG treatments showed higher adduct intensities compared to their respective controls. This is consistent with •OH radical production where EDTA treatment generated •OH radicals immediately while the EGCG-control and BPY treatments showed a lag time prior to •OH radical formation (Fig. 4.4). However, the increased levels of lipid-derived radicals observed in the presence of EGCG, as measured by PBN spin adducts, does not necessarily predict net pro-oxidant activity. In this system, PBN must be present at sufficiently high concentrations to effectively compete with EGCG for lipid-derived radicals. In the same system without PBN, EGCG would be the sole lipid radical scavenger. Therefore, EGCG appears to promote lipid oxidation initiation reactions in emulsions by generating H₂O₂ and, eventually, •OH radicals; however, paradoxically, EGCG would also serve as an antioxidant downstream by quenching the same lipid-derived radicals it helped to promote (Fig. 3.1).
Fig. 4.7: PBN-lipid derived spin adduct generation resulting from the oxidation of 5 wt% flaxseed o/w emulsions treated with FeCl$_3$ (25 μM) and the metal chelators EDTA (50 μM) or BPY (100 μM) in the presence of 400 μM EGCG at (a) pH 7 and (b) pH 3.
At pH 3, the initial rate of PBN-lipid radical formation was higher compared to those at pH 7, with a significantly higher yield of spin adducts observed (Fig. 4.7b). Furthermore, EGCG-containing emulsions showed more PBN-lipid radicals at day 1 compared to their corresponding controls with the order of EGCG-control > EGCG+BPY > EGCG+EDTA. Unlike pH 7 emulsions, EGCG+EDTA samples showed markedly lower radical adducts compared to other EGCG treatments, as was expected based on the EGCG oxidation (Fig. 4.5a) and •OH radical production (Fig. 4.6) results in hexadecane emulsions reported above where POBN-1HER generation followed: EGCG-control > EGCG+BPY > EGCG+EDTA (Fig. 4.6b).
Based on EPR spin trapping analysis alone, it would appear that EGCG is indeed pro-oxidative at pH 3 and 7. This, however, does not necessarily predict the net effect that EGCG will have on overall lipid stability, as was discussed above. Therefore, both lipid hydroperoxides and TBARS concentrations were followed for all treatments for 8 days at both pH 3 and 7 in flaxseed o/w emulsions. At pH 7, lipid hydroperoxide concentrations were markedly lower for all EGCG-containing emulsions, and showed a profound antioxidant effect (Fig. 4.8a). There were no significant differences (p > 0.05) between EGCG treatments and no significant increases (p > 0.05) in lipid hydroperoxides in any of the EGCG treatments over the course of the study. In the absence of EGCG, EDTA inhibited lipid hydroperoxide formation compared to the control and the BPY treatment, which is consistent with previous studies (Alamed, McClements & Decker, 2006; Hu, Julian McClements & Decker, 2004). The observed TBARS concentrations of oxidizing flaxseed oil emulsions followed a similar trend (Fig. 4.8b), with EGCG inhibiting TBARS formation for all treatments. EDTA inhibited TBARS formation for EGCG-free emulsions, which again is consistent with lipid hydroperoxide results (Fig. 4.8a).
Fig. 4.8: Changes in (a) lipid hydroperoxide concentrations and (b) TBARS resulting from the oxidation of 5 wt% flaxseed o/w emulsions in 10 mM phosphate buffer (pH 7) treated with FeCl$_3$ (25 µM) and the metal chelators EDTA (50 µM) or BPY (100 µM) in the presence of 400 µM EGCG.
The above results suggest that, at neutral pH, all EGCG-treated samples display antioxidant activity. At pH 7, EGCG oxidized and H$_2$O$_2$ was observed to accumulate in flaxseed o/w emulsions (data not shown) consistent with what was seen in 5 wt% hexadecane emulsions. The final H$_2$O$_2$ concentration after 8 days in flaxseed emulsions was 415 µM and 310 µM for the EGCG control and the BPY treatment, respectively, which is lower than that seen in 5 wt% hexadecane emulsions, likely due to the fact that EGCG was also consumed by lipid-derived radicals. The lack of pro-oxidant effect in EGCG+EDTA samples may suggest that •OH radicals generated in the aqueous phase are simply too reactive to reach the lipid droplet, or the antioxidant activity of EDTA
resulting from iron chelation mitigated the pro-oxidant effect resulting from EGCG oxidation.

(a)

**Fig. 4.9:** Changes in (a) lipid hydroperoxide concentrations, (b) TBARS, and (c) EGCG concentrations resulting from the oxidation of 5 wt% flaxseed o/w emulsions in 10 mM phosphate buffer (pH 3) treated with FeCl₃ (25 µM) and the metal chelators EDTA (50 µM) or BPY (100 µM) in the presence of 400 µM EGCG.
Fig. 4.9: (continued)
Lipid hydroperoxide and TBARS concentrations were followed in 5 wt % flaxseed oil emulsions at pH 3 (Fig. 4.9a and 4.9b, respectively). Within the first 4 days, all EGCG-containing emulsions showed significantly less lipid hydroperoxides compared to their corresponding EGCG-free treatments. However, after day 4, emulsions containing EGCG only and EGCG+BPY showed marked increases in hydroperoxide production rates, resulting in significantly higher lipid peroxides than the EGCG+EDTA treatment, which showed no significant increase throughout the study. Analysis of TBARS production in flaxseed o/w emulsions at pH 3 were consistent with lipid hydroperoxide analysis for EGCG+EDTA samples and provided further evidence that this treatment inhibited lipid
oxidation reactions (Fig. 4.9b). However, a significant pro-oxidative effect was observed in EGCG only and EGCG+BPY emulsions compared to EGCG-free controls. The fact that lipid hydroperoxides were not observed to accumulate to any significant degree during the early stages of oxidation, yet high TBARS yields were observed, could be due to the fact that EGCG and iron promoted the reduction of hydroperoxides. The presence of BPY under these conditions is also expected to favor the speciation of iron to its reduced oxidation state (i.e., ferrous ions), which are important catalysts for the decomposition of lipid hydroperoxides to alkoxy radicals.

Further examination of EGCG oxidation in flaxseed emulsions at pH 3 revealed a much more rapid loss of EGCG for the EGCG control and EGCG+BPY treatment (Fig. 4.9c), consistent with our proposed scheme of EGCG loss due to lipid-derived radical quenching. Unlike in hexadecane emulsions (Fig. 4.5a), the rate of EGCG oxidation from highest to lowest was in the order of EGCG+BPY > EGCG control > EGCG+EDTA (Fig. 4.9c), with nearly all EGCG oxidized by day 2 in BPY containing samples and only 117 μM EGCG (29%) remaining in the EGCG control compared to the 50% EGCG remaining in hexadecane emulsions. EGCG was, thus, also depleted by reacting with lipid-derived radicals. This is in agreement with our results that show the EGCG+BPY treatment yield the most secondary lipid oxidation markers. Therefore, the pro-oxidant effect resulting from the fast reduction of lipid hydroperoxides and H$_2$O$_2$ may eclipse any antioxidant effect stemming from lipid radical scavenging by EGCG in this system.
Many studies have shown that antioxidant activity in food lipids is achieved when relatively high concentrations of phenolic compounds are used (Maqsood et al., 2010), while some studies showed pro-oxidant activity when the same compounds were present at relatively low concentrations. In a study by Mei et al., low concentrations (5 µM) of galloyl derivatives (methyl gallate, gallamide, gallic acid) resulted in increased lipid hydroperoxide and TBARS concentrations in Brij-stabilized salmon o/w emulsions at pH 3.0, yet this pro-oxidant activity was not seen when high concentrations (500 µM) of the derivatives were used (Mei et al., 1999). With the relatively high concentration of EGCG (400 µM) used in this study, the pro-oxidant effect contributed by \( \text{H}_2\text{O}_2 \) appears to be mitigated by the ability of EGCG to interfere with chain propagation of lipid oxidation reactions. The exception to this appears to be in low pH emulsions, especially when EGCG is added in the presence of a ferrous chelator (e.g., BPY), which results in the rapid generation of TBARS. Other studies have also demonstrated the pro-oxidant activity of EGCG under acidic conditions. Huang and Frankel showed that tea catechins, including (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, and EGCG (5 and 20 µM) were pro-oxidative in low pH (3.0 – 3.5) corn o/w emulsions (Huang et al., 1997). Pro-oxidant activity was also observed in sunflower o/w emulsions treated with 50 µM FeCl\(_3\) and 100 µM tea polyphenols including myricetin, (-)-epicatechin gallate, and EGCG at pH 5.5 (Roedig-Penman et al., 1997). In the same study by Mei et al., no pro-oxidant activity was observed with either 5 or 500 µM galloyl derivatives at pH 7.0 (Mei et al., 1999), suggesting the importance of pH on antioxidant activity. These results are in line with those observed in the present study, where EGCG exhibited strong antioxidant activity in flaxseed o/w emulsions at pH 7 regardless of
chelator treatment while, at pH 3, pro-oxidant activity was observed in EGCG treated samples with the exception of EDTA treatment.

As stated earlier, EGCG may also act as an iron chelator by preferentially binding \( \text{Fe}^{3+} \). Di and tri-hydroxy substituted polyphenols show stability constants (log K) ranging from 7-20 (Perron & Brumaghim, 2009), though a stability constant for EGCG:Fe\(^{3+}\) has not been derived. EDTA has a log K = 25.7 for \( \text{Fe}^{3+} \), suggesting that ligand:metal competition is possible, especially as EGCG concentrations start with at least a 5-fold greater concentration compared to BPY or EDTA. Further work into metal chelator competition and the ligand:metal complexes formed may help clarify the mechanistic effects of the two different iron chelators.

Factors such as pH and the presence and type of transition metal chelators have a profound effect on the stability of trihydroxylated phenolics like EGCG. Under some conditions, these compounds are rapidly oxidized while oxygen is reduced to \( \text{H}_2\text{O}_2 \) in the process. Rapid polyphenol oxidation and subsequent ROS generation did not directly correlate with the net anti-/pro-oxidant effect in lipid dispersions. Instead, factors that increased ferrous iron availability such as ferrous chelators and acidic pH appeared to result in pro-oxidant activity. A more complete understanding of the factors that influence the net anti-/pro-oxidant activity of phenolics will undoubtedly lead to improved technologies for the delivery of biologically significant levels of polyphenols in chemically stable lipid-based foods.

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5.1. Abstract

Polyphenols are widely regarded as antioxidants, due in large part to their free radical scavenging activities and their ability to disrupt radical chain propagation. However, recent studies have demonstrated that the oxidation of some polyphenolic compounds, such as the tea-derived compound (−)-epigallocatechin-3-gallate (EGCG), results in the generation of reactive oxygen species that can potentially compromise the oxidative stability of food lipids under some conditions. In this present study, the rate of hydrogen peroxide (H$_2$O$_2$) generation and its stability, resulting from EGCG oxidation in Tween 80- and sodium caseinate-stabilized oil-in-water (O/W) emulsions in the presence of iron (25 µM Fe$^{3+}$ from FeCl$_3$), were examined. Observed H$_2$O$_2$ levels in protein-stabilized emulsions were significantly lower across all treatments as compared to surfactant-stabilized emulsions. The lower observed H$_2$O$_2$ concentrations seen in the protein system are likely due to the antioxidant effects of the added proteins, which either prevented the generation of or more likely scavenged the peroxide. All protein-stabilized emulsions containing EGCG showed increases in carbonyl concentrations, a marker of protein oxidation, throughout the study. The H$_2$O$_2$ scavenging activity of aqueous phase and interfacial caseinate and whey protein isolate (WPI) was also evaluated. Both proteins showed concentration-dependent scavenging of H$_2$O$_2$ with caseinate displaying significantly higher scavenging abilities at all concentrations. These results suggest that
food proteins may play an important role in mitigating the pro-oxidant effects of polyphenols.

5.2. Introduction

Dietary polyphenols have been associated with a decreased risk of age-related diseases in recent years, making them attractive functional food ingredients. However, the incorporation of these compounds into formulated foods can be challenging due to their susceptibility to oxidative deterioration. Furthermore, nonenzymatic polyphenol oxidation is coupled with oxygen reduction (Clark, 1960), resulting in reactive oxygen species (ROS) formation that can potentially promote the oxidation of other food ingredients (Fig. 1.5). While the pro-oxidant activity of low (i.e., micromolar) concentrations of flavonoids, particularly the catechins, has been observed in lipid foods (Frankel et al., 1997a) and in Chapters 3 and 4, the mechanism underlying this activity is still unclear. Furthermore, the role of $\text{H}_2\text{O}_2$ generation (e.g., factors affecting production, reduction, and fate) in promoting oxidation in these systems has been largely ignored.

(-)-Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in tea (Camellia sinensis) with demonstrated health benefits that include anti-obesigenic and cancer preventative activities (Higdon & Frei, 2003; Katiyar & Mukhtar, 1996; Xu, Ho, Amin, Han & Chung, 1992). Many of these benefits have previously been attributed to EGCG’s antioxidant capacity (Higdon et al., 2003; Yang, Wang, Lu & Picinich, 2009), which stems from its ability to quench radical species (Higuchi, Yonemitsu, Koreeda & Tsunenari, 2003). Catechins, such as EGCG, are thought to behave classically as a chain-
breaking antioxidant by hydrogen atom transfer (HAT) and/or single electron transfer (SET) reactions (Nakanishi et al., 2002), thereby inhibiting peroxidation reactions in lipid foods. Polyphenolic compounds are also known to bind metal ions, which may also account for some of EGCG’s antioxidant activity (Lambert & Elias, 2010). EGCG is known to preferentially bind transition metal ions (e.g., ferric ions; Fe$^{3+}$) with a 1:2 metal:ligand ratio (Qiong, Baolu, Meifen, Shengrong & Wenjuan, 1996), or a 2:1 metal:ligand ratio when examined with a molar excess (pseudo first order conditions) of Fe$^{3+}$, with complexation occurring at both B- and D-rings (Ryan & Hynes, 2007).

Most dietary polyphenols, including EGCG, are readily oxidized in foods, especially at neutral and alkaline pH values. These “auto-oxidative” reactions, which actually appear to be catalyzed by transition metals in most cases (Fig. 1.5), are coupled to the reduction of oxygen to ROS, namely superoxide, or its protonated version (hydroperoxyl radicals) and, eventually, H$_2$O$_2$, as observed in Chapter 4. While H$_2$O$_2$ by itself is not a potent oxidant ($E^0 = 320$ mV at pH 7.0 for the H$_2$O$_2$, H$^+$ / H$_2$O, •OH couple), it is easily reduced to yield highly reactive hydroxyl radicals ($E^0 = 2310$ mV at pH 7.0 for the •OH,H$^+$ / H$_2$O couple) by transition metal catalysts. This reaction is classically referred to as the Fenton reaction, and results in the production of highly oxidizing hydroxyl radicals (•OH) in foods due to the ubiquity of trace levels of iron and copper. The reactivity of •OH radicals is such that they are thought to react with organic matter (e.g., lipids, proteins, DNA) at diffusion-limited rates (Elias, Andersen, Skibsted & Waterhouse, 2009; Elias, Laurie, Ebeler, Wong & Waterhouse, 2008; Elias et al., 2010).
In theory, classical chain-breaking antioxidants are ill-equipped to scavenge \( \cdot \text{OH} \) radicals given their reactivity. A more effective approach would be to prevent the formation of these radicals by scavenging their parent peroxides. Proteins have been shown to quench peroxides under biological conditions and, as such, may be effective preventative antioxidants in polyphenol-containing foods. In particular, methionine residues are known to reduce \( \text{H}_2\text{O}_2 \) and lipid hydroperoxides to non-reactive hydroxides by two electron processes (Garner, Waldeck, Witting, Rye & Stocker, 1998a; Garner, Witting, Waldeck, Christison, Raftery & Stocker, 1998b; Vogt, 1995). This may account for some of the antioxidant activity of proteins observed in lipid dispersions (Achyuthan, Budi, Elias, Cardenas, Cao & McGinnis, 2002; Elias, Kellerby & Decker, 2008), and may be a viable mechanism for scavenging polyphenol-generated \( \text{H}_2\text{O}_2 \). The sulfhydryl groups of cysteine residues may also reduce \( \text{H}_2\text{O}_2 \) by a similar mechanism. Aoshima et al showed that \( \text{H}_2\text{O}_2 \), produced in an aqueous food system by polyphenol oxidation, was effectively reduced in the presence of L-cysteine or glutathione (Aoshima & Ayabe, 2007). Thus, the observation that proteins scavenge \( \text{H}_2\text{O}_2 \) may explain why tea catechins show antioxidant activity in protein-rich multiphasic foods, such as meat systems (He & Shahidi, 1997; Tang, Sheehan, Buckley, Morrissey & Kerry, 2001; Tang, Kerry, Sheehan & Buckley, 2002; Tang, Ou, Huang, Li, Kerry & Buckley, 2006; Yilmaz, 2006). It is conceivable that the radical scavenging properties of polyphenols (e.g., their ability to disrupt lipid peroxidation by quenching lipid radicals) are revealed only once \( \text{H}_2\text{O}_2 \) is removed.

Sodium caseinate and whey protein isolate (WPI) were evaluated for their ability to consume \( \text{H}_2\text{O}_2 \) in this study. Casein and WPI are common functional ingredients in foods
that are often utilized for their ability to stabilize emulsions. However, these proteins also exhibit antioxidant activity in foods (Diaz & Decker, 2004; Diaz, Dunn, McClements & Decker, 2003; Elias, McClements & Decker, 2005), although their mode of protective action appears to be multifaceted and complex. Sodium caseinate is a composite of proteins consisting of $\alpha_{s1}$- and $\alpha_{s2}$-caseins, $\beta$-casein, and $\kappa$-casein, with an average ratio of 11:2.7:10:4 (Walstra, Wouters & Geurts, 2006). Caseinate’s antioxidant activity has been attributed to metal chelation by phosphoseryl groups on $\alpha_{s2}$- and $\beta$-casein, as well as radical scavenging by its constituent amino acids. As is the case with casein, whey protein is not a single protein but rather is comprised principally of $\beta$-lactoglobulin and $\alpha$-lactalbumin at 51% and 20%, respectively, with other minor proteins, such as bovine serum albumin (6%) and immunoglobulins (11%), present as well. Much of WPI’s antioxidant activity has been attributed to the free sulfhydryl groups of $\beta$-lactoglobulin. In one study, sulfhydryl groups were blocked in high molecular weight fractions of WPI, causing a 60% decrease in antioxidant activity in a model food lipid system (Tong, Sasaki, McClements & Decker, 2000). Cysteine and methionine content in casein and major whey proteins are summarized in Table 5.1. The importance of solvent accessibility of these amino acids is also important as $\beta$-Lg hydrolysates and thermally treated $\beta$-Lg inhibited lipid oxidation in oil-in-water emulsions better than native $\beta$-lactoglobulin (Elias, Bridgewater, Vachet, Waraho, McClements & Decker, 2006; Elias, McClements & Decker, 2007).
Table 5.1: Cysteine (Cys) and methionine (Met) content in major dairy proteins.

<table>
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The objective of this study was to follow \( \text{H}_2\text{O}_2 \) generation and consumption rates in an EGCG-containing emulsion system, and to assess the extent that proteins affect both \( \text{H}_2\text{O}_2 \) generation and consumption rates. An emulsion system was used in order to investigate the effects of microenvironments arising from the different emulsion components (lipid; surfactant; proteins) on EGCG oxidation and, thus, \( \text{H}_2\text{O}_2 \) generation.

5.3. Materials and Methods

5.3.1. Materials

Tween-80, xylenol orange tetrasodium salt, and ferrous sulfate heptahydrate were purchased from Sigma (St. Louis, MI). Sodium caseinate (coded Alanate 191) and WPI (coded Alacen 895) were obtained from New Zealand Milk Proteins. \( \text{H}_2\text{O}_2 \) (30% w/v) was purchased from EMD Chemicals (Gibbstown, NJ), and EGCG (93% purity) from Taiyo Green Power Company (Jiangsu, China). 2,4-Dinitrophenylhydrazine (DNPH) was purchased from Matheson Coleman & Bell (Norwood, OH), and n-hexadecane (99%
purity) was obtained from Acros Organics (Morris Plains, NJ). Guanidine hydrochloride and D-sorbitol (98% purity) were purchased from Alfa Aesar (Ward Hill, MA). Trichloroacetic acid (crystalline) was purchased from J.T. Baker (Phillipsburg NJ). All other chemicals and solvents were of analytical or HPLC grade. Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA) purification train.

5.3.2. Emulsion Preparation
Oil-in-water emulsions were prepared by dispersing 10 wt% hexadecane in 10 mM phosphate buffer (pH 7.0) with 2 wt% emulsifier (Tween 80 or caseinate). Hexadecane, a saturated hydrocarbon, was selected as a model for the lipid phase due to the fact that it is chemically stable and is not readily oxidized to lipid hydroperoxides. Sodium azide (0.02 wt%) was added to emulsions in order to prevent microbiological spoilage. Coarse emulsions were made using a high-speed blender (Brinkmann Polytron, Brinkmann Instruments Inc., Westbury, NY) on high speed for 1 min. Fine emulsions were prepared by passing coarse emulsions through a twin-stage valve homogenizer (Niro Soavi Panda, GEA Niro Soavi, Hudson, WI) for at least 3 passes at a pressure of 24 or 40 MPa for Tween- or caseinate-stabilized emulsions, respectively. All emulsions had a mean particle size ($d_{32}$) of 0.59 ± 0.02 µm, as determined by laser light scattering (Horiba LA 920, Irvine, CA). EGCG (0.5 mM or 2.4 mM final concentrations) was added to a 10 wt% hexadecane emulsion from a 10 mM stock solution prepared in 10 mM phosphate buffer (pH 7). The final hexadecane concentration of each emulsion was 5 wt%. Iron (25 µM ferric ions from ferric chloride, FeCl$_3$) was added to the 5 wt% hexadecane emulsion from a freshly prepared stock solution of FeCl$_3$ (10 mM) in water. Emulsions were held
in the absence of light at 37 °C. Samples were analyzed over time to follow EGCG oxidation, H$_2$O$_2$ concentration, and protein carbonyl formation in caseinate-containing samples.

5.3.3. Kinetic Analysis of H$_2$O$_2$ Quenching by Proteins

The reactions between H$_2$O$_2$ and the proteins sodium caseinate and WPI were carried out with the proteins present in either phosphate buffer alone, as non-adsorbed aqueous phase solutes in Tween-stabilized emulsions, or at emulsion interfaces (i.e., the proteins were used as emulsifiers in model emulsions). Surfactant-stabilized 10 wt% hexadecane o/w emulsions were prepared with Tween 80 (2 wt%) in 10 mM phosphate buffer (pH 7.0). Protein stabilized emulsions were prepared with 5 wt% hexadecane and the specified protein concentration (0.5 – 2.0 wt%) in phosphate buffer (pH 7.0; 10 mM). The mean particle sizes ($d_{32}$) of caseinate-, WPI-, and Tween-stabilized emulsions were 0.52 µm ± 0.04, 0.50 µm ± 0.04, and 0.47 µm, respectively. For samples with protein in phosphate buffer alone, the specified protein concentrations were achieved by diluting 10 wt% sodium caseinate or WPI solutions in 10 mM phosphate buffer to the desired final concentrations (0 - 2 wt% protein). For Tween-stabilized systems, emulsions were diluted with the protein stock solutions and buffer to prepare 5.0 wt% hexadecane emulsions with the desired protein concentrations ranging from 0-2 wt% protein. Protein-stabilized emulsions were used as is. Sodium azide (0.02 wt%) was added to all samples. Reactions were initiated by the addition of H$_2$O$_2$ (2.0 mM). Samples were mixed by vortex and stored in the absence of light in a 25 °C shaking water bath with periodic sampling for H$_2$O$_2$ analysis.
5.3.4. Hydrogen Peroxide Analysis

Emulsion samples (162.4 µL) were physically destabilized by the addition of 2:1 methylene chloride:methanol (1 mL) followed by vortex mixing for 30 s and centrifugation at 800 x g for 2 minutes. Protein-containing emulsions were destabilized by the addition of 0.5 N H₂SO₄ (10 µL) in addition to 2:1 methylene chloride:methanol (1 mL) followed by storage for 10 min on ice. Protein-containing samples were mixed for 30 s followed by centrifugation at 11,000 x g for 5 min. The upper aqueous layer was collected and stored at -80° C until analysis. A modified version of the concentrated FOX assay (Wolff et al., 1994) was used for H₂O₂ analysis. The FOX assay solution consisted of xylenol orange (1 mM), ferrous sulfate (2.5 mM) and sorbitol (1.0 M) in sulfuric acid solution (0.5 N), and was prepared daily from stock solutions. Peroxide analysis was performed by adding the assay solution (20 µL) to the aqueous extract (140 µL). Samples were diluted as necessary using a 1:2 water:methanol mixture. Samples were mixed by vortex and absorbance values read at 560 nm following incubation (30 min; ambient temperature) using an Agilent 8453 UV-Vis diode array spectrophotometer (Agilent Technologies, Santa Clara, CA). Quantitation was performed using an external standard curve prepared using authentic H₂O₂, the concentration of which was validated using the peroxide’s extinction coefficient ε₂₄₀ = 40 M⁻¹ cm⁻¹.

5.3.5. EGCG Analysis

EGCG was extracted from emulsions in the same manner as described above for H₂O₂ analysis. To prevent further EGCG oxidation during storage and analysis, a preservative
solution (10 μL) consisting of ascorbic acid (20 wt%) and ethylenediaminetetraacetic acid (EDTA; 0.1 wt%) in phosphate buffer (pH 3.6; 0.4 M) was added to the aqueous extract (100 μL) prior to storage at -80° C (Lee et al., 2004). Preservative solutions were prepared daily. EGCG in extracts was measured by HPLC according to a modified method from Hu et al. (Hu, Zhou & Chen, 2009). Chromatographic separation was achieved on a reverse phase Supelcosil LC-18 (4.6 x 150 mm, 5 μm; Supelco Inc., Bellefonte, PA) using a Shimadzu 10ADvp pump with sample introduction by means of a 20ADvp temperature-controlled autosampler (4 °C). Samples were filtered over 0.45 μm PTFE syringe filters. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). EGCG was eluted by gradient according to the following program: 25 - 45% B in 8.5 min. The injection volume was 20 μL and the flow rate was held at 1 mL/min. EGCG was detected at 280 nm using a Shimadzu SPD-M10Avp photodiode array detector, with quantitation based on an external standard curve prepared from EGCG.

5.3.6. Protein Carbonyl Analysis
Protein carbonyls were measured as their hydrazones, which were prepared by derivatization with 2,4-dinitrophenylhydrazine (DNPH) (Armenteros, Heinonen, Ollilainen, Toldrá & Estévez, 2009; Jongberg, Carlsen & Skibsted, 2009). Briefly, emulsion samples (250 μL) were mixed with DNPH (10 mM; 250 μL) prepared in HCl (2 N). Samples were incubated in a water bath (37 °C; 1 h), with vortex mixing every 10 min. Following incubation, trichloroacetic acid (50% w/v; 162.4 μL) was added. Samples were placed on ice for 10 min and centrifuged for 5 min at 13,000 x g. The supernatant
was decanted and the protein precipitate was washed with 1:1 ethanol:ethyl acetate (v/v; 1 mL). Samples were vortexed and centrifuged for 5 min at 11,000 x g. The supernatant was re-decanted and the wash steps repeated twice more. The resulting protein pellet was dissolved in guanidine hydrochloride (6 M; 1 mL) in sodium phosphate buffer (pH 2.3; 20 mM) and incubated at 37°C for 15 min. Insoluble material was removed following incubation by centrifugation (2000 x g; 2 min). Absorbance values of the hydrazone derivatives were read at 375 nm. The concentration of protein carbonyls (nmol carbonyl/mg protein) was calculated based on the reported extinction coefficient for the hydrazones, $\varepsilon_{375} = 22,000 \text{ M}^{-1}\text{cm}^{-1}$.

5.3.7. Statistical Analysis

All experiments were performed in triplicate and results expressed as means and standard deviation. Two-way ANOVA with Bonferroni’s post test was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California, USA).
5.4 Results and Discussion

5.4.1 Hydrogen Peroxide Generation in Surfactant- and Protein-Stabilized Emulsions

EGCG concentrations were followed by HPLC, with nearly all EGCG lost in both surfactant- and protein-stabilized systems within 96 hours of storage at pH 7 (Fig. 5.1a). EGCG showed significantly lower oxidative stability in the protein-stabilized emulsions, suggesting it also generates H$_2$O$_2$ at a faster rate, as catechol oxidation and oxygen reduction reactions appear to be linked (Lapidot, Walker & Kanner, 2002; Li, Trush & Yager, 1994). While few studies have directly examined the oxidative stability of phenolic compounds in protein-containing foods, some have studied this chemistry under physiological conditions. For example, human serum albumin was observed to increase EGCG stability in buffer at neutral and alkaline pH (Bae et al., 2009; Ishii et al., 2011). EGCG concentration was also shown to be more stable in the presence of N-acetyl cysteine and reduced glutathione (GSH), while the opposite was observed in the presence of oxidized glutathione (GSSG) (Mori, Ishii, Akagawa, Nakamura & Nakayama, 2010). Under wine conditions (pH 3.6), the presence of nucleophiles such as benzenesulfonic acid were seen to markedly increase 4-methylcatechol oxidation, as measured by dissolved oxygen consumption (Danilewicz, Seccombe & Whelan, 2008). The authors argued that the benzenesulfonic acid accelerated oxidation of the catechol by reacting rapidly with its oxidation product, the benzoquinone, by a 1,4-Michael-type addition reaction, thus shifting the equilibrium to the right. Therefore, the fact that EGCG oxidized faster in the presence of caseinate in our system may be attributable to the quenching of EGCG quinones by nucleophilic thiol groups (e.g., cysteine residues) on the
protein. EGCG quinones are known to form covalent adducts with thiol residues (Chen, Wang, Zhang, Ren & Zeng, 2011; Ishii et al., 2008), although lysine and tryptophan adducts have also been reported (Rawel, Kroll & Hohl, 2001).

Fig. 5.1: Changes in (a) EGCG concentration and (b) H$_2$O$_2$ concentration resulting from the oxidation of 0.5 mM EGCG in Tween- and caseinate-stabilized 5 wt% hexadecane emulsion in 10 mM phosphate buffer (pH 7).
An alternative explanation for the observed differences in EGCG oxidation between surfactant- and protein-stabilized systems may be due to the multiphasic nature of the system that allows EGCG to partition into the lipid phase or surfactant micelles. Tea catechins displayed greater oxidative stability in the presence of Tween 20 or lecithin, owing to partitioning into the amphiphilic structures reducing the likelihood of interacting with radicals or transition metals that may otherwise promote its oxidation (Lin, Akesson & Bergenstahl, 2008). In the next set of experiments in Chapters 6 and 7, proteins will be added to Tween-stabilized emulsions to account for differences due to the protective effect of Tween.
The generation of H$_2$O$_2$ due to EGCG (starting concentration, 0.5 mM) oxidation in the presence of iron (25 µM Fe$^{3+}$) in surfactant and protein-stabilized emulsions was examined over 96 hours (Fig. 5.1b). As the FOX assay is not specific to H$_2$O$_2$, catalase was added at the end of each study to determine if other peroxides were formed. No other peroxide species (e.g., hydroperoxides) were observed (data not shown). Samples in Tween-stabilized emulsions showed no further increase after day 4 as levels plateaued at ca. 500 µM H$_2$O$_2$ and persisted throughout the 8-day study (data not shown). At all time points, samples in protein-stabilized emulsions showed significantly lower levels of H$_2$O$_2$ compared to the surfactant-stabilized system. A similar trend for EGCG oxidation and H$_2$O$_2$ generation was observed in the presence of 2.4 mM EGCG. In Tween-stabilized emulsions ca. 52% of the EGCG (corresponding to ~1.25 mM) was oxidized, while in protein-stabilized emulsions ca. 79% of the EGCG (corresponding to ~1.9 mM) was oxidized within the first 24 hours (Fig. 5.2a), suggesting that most of the H$_2$O$_2$ was generated during this time. H$_2$O$_2$ concentration continuously increased in Tween-stabilized emulsions to a final concentration of 1.6 mM at the end of 96 hours (Fig. 5.2b). However, protein-stabilized emulsions displayed H$_2$O$_2$ concentration well below Tween-stabilized emulsions throughout the study.
Fig. 5.2: Changes in (a) EGCG concentration and (b) H$_2$O$_2$ concentration resulting from the oxidation of 2.4 mM EGCG in Tween- and caseinate-stabilized 5 wt% hexadecane emulsion in 10 mM phosphate buffer (pH 7).
The difference in observed H$_2$O$_2$ levels in surfactant- and protein-stabilized emulsions is likely due to the peroxide scavenging activity of the protein. It is unlikely that H$_2$O$_2$ generation rates were slower in the protein-containing system given the relative instability of EGCG in that same system, as oxygen reduction rates, and thus H$_2$O$_2$ formation rates, should mirror EGCG oxidation rates. Further evidence of H$_2$O$_2$ scavenging by protein can be seen by measuring protein oxidation. Therefore, protein carbonyl concentration, a general marker of protein oxidation, was measured in protein-stabilized emulsions in the presence of 0.5 and 2.4 mM EGCG (Fig. 5.3). Protein carbonyl concentrations were observed to increase with increasing EGCG concentrations,

![Fig. 5.2: (continued)](image)
most likely as a result of the protein reacting with H₂O₂. In protein-stabilized emulsions containing 0.5 mM EGCG, protein carbonyl concentrations increased markedly within 24 hours, coinciding with a large reduction in H₂O₂ concentration during that time (as seen in Fig. 5.1b), even as ca. 89% of the catechin (corresponding to ~ 0.44 mM EGCG) had been oxidized. When 2.4 mM EGCG was added, protein carbonyl content once again showed a sharp increase in the first 24 hours, as 79% of the catechin oxidized to produce H₂O₂, with H₂O₂ concentrations significantly (~600 µM) below concentrations observed in Tween-stabilized emulsions. EGCG may also covalently bind to proteins to form adducts that may retain their redox activity (Akagawa et al., 2006) and further induce oxidative stress on the protein by generating more H₂O₂ near the adduct site. Thus H₂O₂ was added exogenously to proteins in the latter quenching experiments.
Fig. 5.3: Protein carbonyl formation resulting from EGCG oxidation in caseinate-stabilized 5 wt% hexadecane emulsion.

5.4.2 Hydrogen Peroxide Quenching by Aqueous Phase and Interfacial Proteins in Emulsions

The H$_2$O$_2$ scavenging activity of sodium caseinate and WPI as aqueous solutes and as components of oil-in-water emulsions was examined in the following experiment. The concentration dependent quenching of exogenous H$_2$O$_2$ (2 mM starting concentration) by caseinate solutions in 10 mM phosphate buffer pH 7 was measured over 96 hours (Fig. 5.4). The quenching of H$_2$O$_2$ by caseinate appears to follow first order reaction kinetics at high protein concentrations (1.0 and 2.0 wt%). The highest concentration of caseinate (2.0 wt%) tested quenched nearly all of the added peroxide (2 mM H$_2$O$_2$). This suggests that the concentration of caseinate (1 wt%) used in the previous protein-stabilized
emulsion was incapable of quenching all the H$_2$O$_2$ generated from the oxidation of 2.0 mM EGCG.

**Fig. 5.4:** Quenching of H$_2$O$_2$ by caseinate solutions (0-2.0 wt%) in 10 mM phosphate buffer pH 7.0.

The peroxide scavenging activity of WPI in 10 mM phosphate buffer pH 7 was also measured for 96 hours (**Fig. 5.5**). Unlike caseinate solutions, WPI solutions did not show first order reaction kinetics, even at higher protein concentrations. WPI solutions displayed a significantly lower capacity for scavenging peroxides, as 2.0 wt% caseinate was capable of scavenging virtually all of the added H$_2$O$_2$, while the equivalent concentration of WPI was only capable of scavenging ca. 61% of the peroxide (1.2 mM H$_2$O$_2$). This difference in protein scavenging activity may be a function of the protein’s primary amino acid sequence as well as the accessibility of certain amino acids to the
aqueous solvent phase. Amino acids that are known to react with peroxides include cysteine (Requejo, Hurd, Costa & Murphy, 2010; Rosas-Rodríguez & Valenzuela-Soto, 2011) and methionine (Snijder, Rose, Raijmakers & Heck, 2010). αs2-Casein and κ-casein both contain two cysteine residues, whereas αs1-casein and β-casein lack free cysteines. The predominant whey proteins, β-lactoglobulin and α-lactalbumin contain five and eight cysteine residues, respectively. Though it appears that the proteins in WPI contain more cysteines, many of these residues are not solvent accessible (Nagano, Ota & Nishikawa, 1999), or participate in disulfide linkages (Boye & Alli, 2000; Hoffmann & van Mil, 1997). β-Lactoglobulin contains two disulfide linkages (Hattori et al., 2005), while α-lactalbumin may be stabilized by four disulfide bonds (Ewbank & Creighton, 1993; Hendrix, Griko & Privalov, 1996), thus limiting the cysteine’s reactivity. With respect to solvent accessibility, it has been shown that enzymatic hydrolysates have increased antioxidant activity in lipid systems (Peng, Xiong & Kong, 2009), presumably due to increased solvent accessibility of radical scavenging amino acids (Elias et al., 2006). Methionine residues have also been shown to reduce peroxide species via a 2-electron (non-radical) reaction (Garner et al., 1998a; Garner et al., 1998b). Levine et al showed that the H2O2 induced oxidation of bacterial glutamine synthetase caused an increase in methionine sulfoxide formation. It was observed that the most oxidatively labile methionine residues were those that were solvent exposed, while those residues buried within the protein’s hydrophobic core remained unaffected (Levine, Mosoni, Berlett & Stadtman, 1996). Methionine residues have been studied previously for their antioxidant activity, and many studies have also shown that this activity is a function of the solvent accessibility to key amino acid residues (Duenas, Keck, DeVos, Jones &
Based on solvent accessibility calculations, three of β-lactoglobulin’s methionine residues were determined to be buried within the protein’s hydrophobic core, and thus, not solvent accessible. Methionine residues in β-lactoglobulin in that study were shown to be oxidatively stable in a surfactant-stabilized emulsion containing the protein as an aqueous phase solute. Furthermore, based on GETAREA (Fraczkiewicz & Braun, 1998), an algorithm designed to calculate solvent accessible surface areas using atomic coordinates from protein data bank files, α-lactalbumin’s sole methionine residue does not appear to be solvent accessible either. Crystallography data for the casein fractions...
comprising sodium caseinate were not available at the time of writing, therefore solvent accessibility data for its methionine residues could not be calculated. However, it has been shown that ca. 75% of bovine casein’s methionine residues were capable of undergoing oxidation to their sulfoxides under similar conditions (6 mM H₂O₂) to those employed in the above described experiment (Cuq, Provansal, Guilleux & Cheftel, 1973).

The peroxide scavenging activity of caseinate and WPI were also investigated in various systems, which included the proteins as solutes in a simple buffer system, as aqueous phase components of surfactant-stabilized emulsions, and as emulsifiers themselves. The peroxide scavenging activity in the various protein (1.0 wt%) containing systems were examined for 72 hours (Fig. 5.6). No significant differences (p < 0.05) between WPI treatments (WPI solution vs. WPI in Tween emulsion vs. WPI emulsion) were observed over the course of the study; however, a marked difference between caseinate treatments was observed. The rate of H₂O₂ consumption by caseinate solutions exceeded that of caseinate in both surfactant- and protein-stabilized systems. The rate of H₂O₂ was slowest in the caseinate-stabilized emulsion system. We hypothesize that this effect may be due to the adsorption behavior of casein. In one study, it was shown that β-casein preferentially adsorbed to the droplet interfaces of 30% soybean oil-in-water emulsions stabilized with <2.0 wt% casein, while at higher protein concentrations α-casein was preferentially adsorbed (Srinivasan, Singh & A. Munro, 1999). Our study employed lower total oil levels, so it may be possible that in the case of a 5 wt% hexadecane emulsion, α-casein may be preferred to stabilize the emulsion droplet. The predominance of α-casein at the emulsion interface may reduce the ability of the protein to scavenge
H$_2$O$_2$, as $\alpha$-casein will no longer be free to interact with the peroxide in the aqueous phase. This is consistent with the observation that aqueous phase $\alpha$-casein has higher radical scavenging activity (Almajano, Delgado & Gordon, 2007b) and antioxidant properties by delaying lipid peroxidation induction time in liposomal suspensions (Cervato, Cazzola & Cestaro, 1999) compared to the other casein proteins.

**Fig. 5.6:** Quenching of H$_2$O$_2$ by 1.0 wt% caseinate or WPI in buffer only, Tween-stabilized emulsion, and protein-stabilized emulsion.

Proteins appear to play a significant role in the production and fate of H$_2$O$_2$ in polyphenol-containing foods. While EGCG oxidized faster in protein-stabilized emulsions, and, in turn, would generate ROS (O$_2^*$ and H$_2$O$_2$) faster, the protein was observed to rapidly quench the peroxide in a concentration-dependent manner, preventing
its accumulation. \( \text{H}_2\text{O}_2 \) quenching rates were seen to vary between caseinate and WPI, with higher peroxide scavenging observed with the aqueous phase caseinate. Further research is needed to elucidate which amino acid residues are responsible for \( \text{H}_2\text{O}_2 \) quenching under these conditions, and how this quenching translates to lipid oxidation inhibition in a real food or beverage system.
6. Influence of Cysteine and Methionine Availability on Protein Peroxide Scavenging Activity and Phenolic Stability in Emulsions

6.1. Abstract

Plant phenolics are micronutrients that have been shown to confer beneficial health effects. However, many of these compounds undergo metal-catalyzed oxidation reactions, leading to the generation of hydrogen peroxide ($\text{H}_2\text{O}_2$) and other reactive oxygen species (ROS) that may negatively impact product stability. Proteins are known peroxide scavengers; specifically, methionine (Met) and cysteine (Cys) residues are capable of reacting directly with peroxides. To examine the effect of solvent accessibility on peroxide scavenging capacity, casein (CAS) and $\beta$-lactoglobulin (BLG) were pretreated with tert-butyl hydroperoxide (TBHP), a bulky peroxide, to oxidize only solvent accessible Met residues or $\text{H}_2\text{O}_2$, the smallest peroxide, to oxidize more buried Met residues. Proteins were examined for their ability to scavenge $\text{H}_2\text{O}_2$ (400 $\mu$M) and influence EGCG oxidation (400 $\mu$M) in Tween- or SDS-stabilized hexadecane emulsions. In Tween-stabilized emulsions, all CAS treatments showed significantly higher radical scavenging activity compared to BLG treatments. The lack of Met or Cys loss supports the inaccessibility of these residues in BLG. In SDS-stabilized emulsions, BLG peroxide scavenging activity was greatly enhanced due to protein denaturation and subsequent exposure of previously buried Cys residues. EGCG stability mirrored the protein’s peroxide scavenging activity in Tween-stabilized emulsions with the slowest oxidation in CAS-CTRL, while BLG-containing samples showed the slowest EGCG oxidation in SDS-stabilized emulsions. CAS treatments showed that a reduction in accessible Met content led to decreased peroxide scavenging activity and EGCG stability.
6.2. Introduction

Polyphenols are attractive functional ingredients in foods; they serve as natural antioxidants in foods (Ganhão, Estévez & Morcuende, 2011; Sørensen, Nielsen & Jacobsen, 2010) and their consumption is associated with numerous health benefits (Soto-Vaca, Gutierrez, Losso, Xu & Finley, 2012). Polyphenols act as antioxidants by scavenging free radicals (Iacopini, Baldi, Storchi & Sebastiani, 2008; Rice-Evans, Miller & Paganga, 1996) and by chelating transition metals under some conditions (Elhabiri, Carrère, Marmolle & Traboulsi, 2007; Hider et al., 2001). Phenolics containing di- or tri-hydroxy substituted phenol rings, such as (-)-epigallocatechin-3-gallate (EGCG) (Fig. 1.4) are especially effective radical scavengers due to resonance stabilization of their one electron oxidation product (semiquinone radical) (Kumamoto et al., 2001). However, di- and tri-hydroxy substituted phenolics are also capable of reducing transition metals even at neutral pH (Fe$^{2+}$, Cu$^{1+}$) (Chvátalová, Slaninová, Brezinová & Slanina, 2008; Mira et al., 2002), resulting in the generation of reactive oxygen species (ROS) such as superoxide radicals (O$_2^-$) which are subsequently reduced to H$_2$O$_2$ and potentially hydroxyl radicals (•OH) (Fig. 1.5). The metal-catalyzed oxidation of phenolics not only results in the undesirable loss of a potentially bioactive compound, it also has the potential to decrease product shelf-life due to sensory defects such as undesired color changes from phenolic polymerization or metallic and fishy notes from increased lipid oxidation. Pro-oxidant activity in oil-in-water emulsions due to the addition of phenolics has previously been observed (Huang et al., 1997), therefore preventing phenolic oxidation and scavenging the oxidants produced may be helpful in expanding phenolic use in food formulations.
In chapter 4, we examined the use of metal chelators as a strategy for controlling phenolic oxidation. The ferric chelator, EDTA, was shown to increase EGCG stability in emulsions under acidic conditions, but induced rapid EGCG oxidation at neutral pH, suggesting that different approaches to preventing phenolic oxidation will be necessary depending on food matrix conditions. Proteins may be an alternate solution due to their ability to scavenge H$_2$O$_2$, mitigating •OH radical formation.

H$_2$O$_2$ is a known precursor for the •OH radical, a potent and non-selective oxidant (Makrigiorgos et al., 1995) for which chain-breaking antioxidants are ineffective. It therefore stands to reason that the most effective way to prevent •OH radical-mediated oxidation is to quench its parent compound, H$_2$O$_2$, before it is reduced by the Fenton reaction. In proteins, cysteine (Cys) (Luo et al., 2005) and methionine (Met) (Garner et al., 1998a; Garner et al., 1998b) residues have been shown to react directly with peroxides via two electron (i.e., non-radical) reduction. We recently showed that the dairy proteins, casein (CAS) and whey protein scavenge H$_2$O$_2$ in emulsions (Chapter 5). Though CAS and whey protein both contain sufficient Cys and Met to scavenge all exogenous H$_2$O$_2$, only casein completely scavenged the H$_2$O$_2$. Differences in the relative solvent accessibility of Cys and Met on the two proteins may offer an explanation, as both Cys and Met residues are relatively hydrophobic. Studies have shown preferential oxidation of solvent accessible Met residues in proteins (Requena et al., 2004; Snijder et al., 2010). After oxidation with H$_2$O$_2$, LC-MS analysis of prion proteins after digestion demonstrated that Met residues in the unstructured, solvent accessible region were the
most readily oxidized, while oxidation of buried Met residues were undetectable (Requena et al., 2004). Similarly, Met oxidation of specific residues in calmodulin were well correlated with their calculated solvent accessibility (Snijder et al., 2010).

The objective of this experiment was to examine the $\text{H}_2\text{O}_2$ scavenging activity of CAS and $\beta$-lactoglobulin (BLG). $\text{H}_2\text{O}_2$ was either added as an exogenous component, or $\text{H}_2\text{O}_2$ was generated from EGCG oxidation. The effect of emulsifier type (Tween vs. SDS) on the peroxide scavenging capacity of the proteins was also investigated. A specific focus was placed on the role of Met’s and Cys’s solvent accessibility on peroxide scavenging activity.
6.3. Materials and Methods

6.3.1. Materials

EGCG (93% purity) was acquired from Taiyo Green Power Company (Jiangsu, China). n-Hexadecane (99% purity) was obtained from Acros Organics (Morris Plains, NJ). Phthaldialdehyde (OPA) reagent, methionine, methionine sulfoxide, tryptophan, tyrosine, sodium dodecyl sulfate (SDS), Tween-80, ethylenediaminetetraacetic acid (EDTA), xylencol orange tetrasodium salt, ferrous sulfate heptahydrate and the enzymes leucine aminopeptidase M (L5006), protease (P5147), and prolidase (P6675) were purchased from Sigma (St. Louis, MI). Sodium caseinate (Alanate 191) was obtained from New Zealand Milk Proteins. β-Lactoglobulin was donated by Davisco Foods International (Eden Prairie, MN). H2O2 (30% w/v) was purchased from EMD Chemicals (Gibbstown, NJ). 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), guanidine hydrochloride, TBHP (70%), phenylalanine and D-sorbitol (98% purity) were purchased from Alfa Aesar (Ward Hill, MA). Ferric chloride 6-hydrate lumps and sodium azide were purchased from Mallinkrodt (Phillipsburg, NJ). Trichloroacetic acid (crystalline) was purchased from J.T. Baker (Phillipsburg NJ). Leucine was acquired from Amresco (Solon, OH). BD Falcon™ Clear 96-well Microtest™ Plates, 0.45 μm PTFE syringe filters and Spectra/Por® regenerated cellulose dialysis tubing with a 12-14 kDa MWCO were purchased from VWR (Radnor, PA). BCA Protein Assay Kit was purchased from Thermo Fisher (Rockford, IL). All electrophoresis materials listed were acquired from Biorad (Hercules, CA):4–20% Mini-PROTEAN® TGX™ Precast Gel, glycine (aminoacetic acid) powder, Tris base (Tris[hydroxymethyl]aminomethane) and Precision Plus Protein™ Kaleidoscope Standards. All other chemicals and solvents were of analytical or HPLC
grade. Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA) purification train.

6.3.2. Preparation of Methionine-Oxidized Protein

Protein solutions (1% w/v CAS or BLG) were prepared in 10 mM phosphate buffer pH 7 and oxidized with H$_2$O$_2$ (2 mM) or TBHP (7.77 mM) for 5 days at room temperature. NaN$_3$ (0.02%) was added to inhibit microbiological activity. The concentrations selected were previously shown to oxidize Met residues while minimizing the oxidation of other oxidatively labile amino acids (Keck, 1996). Controls without added peroxides were also incubated under identical conditions. CAS was precipitated by adjusting the pH to its isoelectric point (pH 4.6). BLG was precipitated by adjusting the pH to 2.6 followed by the addition of NaCl (30% w/v). Protein was collected after centrifugation at 3300 x g for 15 min at 4 °C. Proteins were then resuspended in water and dialyzed (MWCO = 10 kDa) against water for 3 days at 4 °C with 3 water changes per day. The water was analyzed for residual peroxides using the FOX method described below to ensure their complete removal. Following dialysis, protein samples were frozen at -80 °C and lyophilized using a FreeZone 2.5 Liter Benchtop Freeze Dry System (Labconco, Kansas City, MO). Dry protein samples were stored at 4 °C until use. Native-PAGE was run on all protein samples, as well as freshly prepared casein and BLG solutions. No differences in relative mobility were observed (data not shown).
6.3.3. Emulsion Preparation

Oil-in-water emulsions were prepared by dispersing 10 wt% hexadecane in water with 2 wt% emulsifier (Tween 80 or SDS). Hexadecane was selected as the organic dispersed phase for the model system because it is a saturated hydrocarbon that is oxidatively inert. Coarse emulsions were made using a high-speed blender (Brinkmann Polytron, Brinkmann Instruments Inc., Westbury, NY) on high speed for 0.5 min. Fine emulsions were prepared by passing coarse emulsions twice through a microfluidizer with the interaction chambers, H307 - 200 μm porosity and F20Y - 75 μm porosity placed in series (Microfluidics M-110Y, Newton, MA) at a pressure of 40 psi. All emulsions had a mean particle size of (d_{32}) of 0.23 ± 0.02 μm, as determined by laser light scattering (Horiba LA 920, Irvine, CA).

6.3.4. Hydrogen Peroxide Scavenging and EGCG Oxidation Experiments

Protein solutions (2% w/v) were prepared in 20 mM phosphate buffer (pH 7). Sodium azide (0.04% w/v) was added to solutions to prevent microbiological spoilage. Protein solutions were then diluted 1:1 by volume with the 10 wt% hexadecane emulsion to achieve a 1% w/v protein concentration. Ferric iron (25 μM) was added from a freshly prepared stock solution of FeCl₃ (10 mM) in water. The pH was then adjusted to pH 7.0 using 6 N HCl or 10 N NaOH, as required. Peroxide scavenging experiments were initiated upon the addition of H₂O₂ (400 μM). The pH was measured again and readjusted when necessary. Emulsions were then held in the absence of light at 37 °C for 48 h.
For EGCG oxidation experiments, an appropriate mass of EGCG powder was added directly to 10 wt% hexadecane emulsions to achieve a final concentration of 800 µM. EGCG was observed to readily dissolve in emulsions at pH 7 after magnetic stirring for 5 min. Similar to the peroxide scavenging experiment, emulsions were diluted with the protein solutions 1:1 by volume and ferric iron (25 µM) added. The pH was adjusted as necessary and emulsions were then held in the absence of light at 37 °C for 96 h.

6.3.5. Hydrogen Peroxide Analysis

Emulsion samples (162.4 µL) were destabilized by the addition of 0.5 N H₂SO₄ (10 µL) and 2:1 methylene chloride:methanol (1 mL). Samples were mixed for 30 s followed by 10 min incubation on ice. Samples were then centrifuged at 10,000 x g for 2 min. The upper aqueous layer was collected and stored at -80° C until analysis. Peroxide analysis was performed by adding the FOX assay solution (20 µL) to the aqueous extract (140 µL). The FOX assay solution contained xylenol orange (1 mM), ferrous sulfate (2.5 mM) and sorbitol (1.0 M) in sulfuric acid solution (0.5 N). Samples were diluted as necessary using a 1:2 water:methanol mixture. After vortex mixing, samples were stored for 30 m at ambient temperature. Absorbance values were then read at 560 nm using a Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Rockford, IL). Quantitation was performed using an external standard curve prepared using H₂O₂ after validation with the peroxide’s extinction coefficient ε₂₄₀ = 40 M⁻¹ cm⁻¹.
6.3.6. EGCG Analysis

EGCG was extracted from emulsions in the same manner as described above for H$_2$O$_2$ analysis. Prior to storage at -80°C, a preservative solution (10 µL) consisting of ascorbic acid (20% w/v) and ethylenediaminetetraacetic acid (EDTA; 0.1% w/v) in phosphate buffer (pH 3.6; 0.4 M) was added to the aqueous extract (100 µL) (Lee et al., 2004). Preservative solutions were prepared daily. Chromatographic separation was achieved on a reverse phase Supelcosil LC-18 (4.6 x 150 mm, 5 µm; Supelco Inc., Bellefonte, PA) using a Shimadzu 10ADvp pump with sample introduction by means of a 20ADvp temperature-controlled autosampler (4 ºC). Samples were filtered over 0.45 µm PTFE syringe filters. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). EGCG was eluted by gradient according to the following program: 25 - 45% B in 8.5 min. The injection volume was 20 µL and the flow rate was held at 1 mL/min. EGCG was detected at 280 nm using a Shimadzu SPD-10Avp UV-Vis detector, with quantitation based on an external standard curve prepared from EGCG.

6.3.7. Protein Precipitation

Emulsion samples (200 µL) were precipitated after the addition of 40% w/v trichloroacetic acid for Tween-stabilized emulsions or 2 N HCl for SDS-stabilized emulsions. Samples were mixed by vortex and incubated on ice for 10 min. The protein was then centrifuged at 10,000 x g for 3 min. The supernatant was discarded and the protein precipitate was washed with 400 µL of ethanol:ethyl acetate (1:1). Samples were again vortexed and centrifuged at 10,000 x g for 3 min. The supernatant was discarded.
and the wash step repeated twice more. After the final wash, protein samples were dried under N\textsubscript{2} to remove residual wash solution and stored at -80 °C until analysis.

### 6.3.8. Enzymatic Hydrolysis

Protein pellets were dissolved in 6 N guanidine hydrochloride (100 µL) to prepare an ~2% w/v solution. Protein solutions (30 µL) were then diluted with 100 mM phosphate buffer pH 7.5 containing 0.1% w/v sodium azide (370 µL). Enzymatic hydrolysis was performed as described by Baxter et. al (Baxter et al., 2007). Protease (920 U/mL in water) and prolidase (180 U/mL in water) solutions were prepared and stored at -20 °C. Protease solutions were further diluted to a final concentration of 9.2 U/mL in water prior to use. Leucine aminopeptidase M was stored at 4 °C and the slurry diluted in water to 24 U/mL prior to use. For digestion, 24 U/mL leucine aminopeptidase M (6 µL), 9.2 U/mL protease (15 µL), and 180 U/mL prolidase (3 µL) was added to the diluted protein sample (300 µL). The sample was gently mixed and incubated at 37 °C for 20 h. Digested samples were then analyzed by HPLC.

### 6.3.9. Amino Acid Analysis by HPLC

Amino acids were separated using the same HPLC system described above after pre-column OPA derivatization. The OPA reagent (150µL) was added to enzymatically digested protein samples (15 µL). Samples were derivatized immediately prior to analysis, since OPA-derivatives were not stable for extended periods. After mixing, samples were filtered over 0.45 µm PTFE syringe filters. Samples (20 µL) were then eluted using the following flow and gradient program: 0 min – 0.4 mL/min, 19% B; 15
min – 0.4 mL/min, 25% B; 15.1 min – 1.0 mL/min; 30 min – 65% B. The mobile phase consisted of (A) 4% tetrahydrofuran (THF) in 35 mM sodium acetate buffer pH 5.7 and (B) methanol. The OPA derivatized amino acids were detected at 210 nm and quantified based on external amino acid standard curves.

6.3.10. Protein Quantification

Total protein was quantified using the BCA methods, as described previously (Smith et al., 1985). Working reagent was prepared by mixing 50 parts BCA reagent A with 1 part BCA reagent B. Diluted protein solutions prepared for enzymatic hydrolysis above was used for protein quantification, since samples fell within the standard curve. Working reagent (400 µL) was added to protein samples (20 µL) and incubated at 37 °C for 30 min. Mixtures were then cooled at ambient temperature for 10 m. Samples (200 µL) were transferred to 96-well plates for analysis at Abs 562 using a Thermo Scientific* Multiskan GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Rockford, IL). Quantification was performed using external standard curves prepared for each protein in the same guanidine hydrochloride and buffer mixture found in the diluted protein samples.

6.3.11. Cysteine Determination

Free cysteine was quantified by Ellman’s method, as described previously (Ellman, 1959). The reagent solution was prepared by dissolving 4 mg DTNB in 0.1 M phosphate buffer pH 8.3 containing 1 mM EDTA. 6 N Guanidine hydrochloride (200 µL) adjusted to pH 8.3, was mixed with undiluted protein samples (20 µL) prepared in the enzymatic
hydrolysis step above. Samples were mixed after adding the reagent solution (4 µL) and then incubated at room temperature for 15 m. Sample (200 µL) was transferred to 96 well plates and analyzed at Abs 412 using a Thermo Scientific® Multiskan GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Rockford, IL).

6.3.12. Statistical Analysis

All experiments were performed in triplicate and results expressed as means plus or minus their standard deviation. Two-way ANOVA with Bonferroni’s post test was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California, USA).
6.4. Results and Discussion

6.4.1. Hydrogen Peroxide Scavenging Activity in Tween-stabilized Emulsions Containing Methionine-Blocked Protein

H$_2$O$_2$ scavenging activity was measured in Tween 80-stabilized hexadecane emulsions in the presence of 1% protein for 48 h (Fig. 6.1). After 6 h incubation with exogenous H$_2$O$_2$ (400 µM), CAS treatments showed increasing peroxide scavenging activity in the following order: CAS-CTRL > CAS-TBHP > CAS-HOOH. Within 48 h, CAS-CTRL scavenged 381 µM H$_2$O$_2$ (95%), while CAS-TBHP and CAS-HOOH scavenged 260 µM and 175 µM H$_2$O$_2$, respectively. For BLG treatments, BLG-CTRL showed significantly higher peroxide scavenging after 30 h incubation compared to BLG-TBHP and BLG-HOOH; however, overall, BLG samples showed significantly lower peroxide scavenging ability compared to CAS samples. BLG-CTRL, BLG-TBHP, and BLG-HOOH scavenged only 175, 132, and 129 µM H$_2$O$_2$, respectively after 48 h, with only BLG-CTRL showing comparable scavenging to CAS-HOOH, the most extensively oxidized CAS treatment. The low peroxide scavenging activity observed in BLG samples corroborates our previous results in chapter 5, where CAS was shown to have greater H$_2$O$_2$ scavenging activity compared to whey protein, which is comprised of ~51% BLG. Casein is a heterogenous composite of proteins consisting of $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$ and $\kappa$ casein (~11:2.7:10:4). $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$ and $\kappa$ casein contain no free Cys residues, yet have five, four, six, and two Met residues, respectively. BLG contains four Met residues and one free Cys residue. Based on the total Met and Cys content in 1% w/v protein solutions, both protein controls (CAS-CTRL and BLG-CTRL) contained sufficient Cys and Met to theoretically
scavenge 400 µM H₂O₂. To gain a better understanding for this difference in scavenging activity, the accessibility of Cys and Met residues after oxidation by H₂O₂ was examined.

**Fig. 6.1:** H₂O₂ loss in Tween-stabilized 5% hexadecane emulsions containing 1% w/v protein (CAS or BLG) in the presence of 400 µM H₂O₂ and 25 µM Fe³⁺ at pH 7.

The effect of the two peroxide pretreatments (TBHP or H₂O₂) on Met content in CAS and BLG is shown in **Fig. 6.2a** and **Fig. 6.2b**. CAS samples had significantly different Met concentrations following peroxide pre-treatment: 1930, 913, and 669 µM Met in CAS-CTRL, CAS-TBHP, and CAS-HOOH, respectively. Likewise, MetO content increased in peroxide pre-treated samples from 0 µM MetO in the control to 566 and 697 µM MetO in CAS-TBHP and CAS-HOOH, respectively. Unexpectedly, a stoichiometric conversion of Met to MetO was not observed, leading to a lower total Met content (Met+ MetO) in
CAS-TBHP and CAS-HOOH. However, all other measured amino acids (Val, Trp, Phe, Leu) did not show significant differences between CAS treatments suggesting no compositional changes in the heterogenous CAS resulting from protein precipitation. Peroxide pretreatment of BLG samples also resulted in decreased Met concentration, although no significant differences between TBHP and H₂O₂ treatment were observed. Met concentrations decreased from 1910 µM to 1600 and 1670 µM following TBHP and H₂O₂ treatments, respectively. MetO content increased from 185 µM in the control to 551 µM in BLG-TBHP and 537 µM in BLG-HOOH. Compared to the free Met content in the controls, a loss of 1017 µM (53% loss) and 1261 µM (65% loss) Met was measured in CAS-TBHP and CAS-HOOH, respectively, while BLG samples showed only an average 14% reduction (loss of 275 µM Met) in Met after pre-treatment with TBHP and H₂O₂.
Fig. 6.2: Changes in Met and MetO content after 48 h incubation with 400 μM H$_2$O$_2$ in Tween-stabilized hexadecane emulsions at pH 7 with 25 μM Fe$^{3+}$ and 1% w/v protein: (a) CAS and (b) BLG.
Changes in Met residue concentrations are shown following 48 h incubation with 400 µM H₂O₂ (Fig. 6.2a, b). Excluding CAS-CTRL, no significant changes in Met, MetO, or Cys content were observed in CAS or BLG samples incubated in hexadecane emulsions alone at 37 °C for 96 h (data not shown). CAS-CTRL showed 40 µM and 160 µM Met loss after 48 and 96 h, respectively. CAS treatments resulted in increases in MetO content of 330, 309, and 341 µM MetO to a final concentration of 330, 875, and 1040 µM MetO in CAS-CTRL, CAS-TBHP, and CAS-HOOH, respectively. In terms of free Met content, CAS-CTRL retained 1420 µM Met, CAS-TBHP retained 440 µM Met and CAS-HOOH retained 269 µM Met. In BLG treatments, only BLG-CTRL showed a significant
decrease in Met content with a 130 μM Met decrease and a 150 μM MetO increase. The observable Met conversion to MetO in BLG-CTRL may account for the higher H₂O₂ scavenging activity compared to BLG-TBHP and BLG-HOOH.

Fig. 6.3: Free cysteine content in BLG after 48 h incubation with 400 μM H₂O₂ in Tween-stabilized or SDS-stabilized hexadecane emulsions at pH 7 with 25 μM Fe³⁺ and 1% w/v protein.

No significant loss with respect to free thiols in any BLG samples was measured in Tween-stabilized emulsions after 48 h oxidation with 400 μM H₂O₂ (Fig. 6.3). Similar to Met residues, Cys was also likely inaccessible in BLG, explaining the lack of peroxide scavenging activity observed with BLG even though BLG has a higher total Cys and Met content.
6.4.2. Hydrogen Peroxide Scavenging Activity in SDS-stabilized Emulsions Containing Methionine-blocked Protein

H$_2$O$_2$ scavenging studies were repeated in SDS-stabilized hexadecane emulsions in order to increase the solvent accessibility of Met residues through protein denaturation (Fig. 6.4). In the presence of SDS, proteins showed increasing peroxide scavenging activity in the following order: CAS-HOOH < CAS-TBHP < BLG-CTRL = BLG-TBHP = BLG-HOOH < CAS-CTRL. After 6 h, all CAS and BLG containing samples showed significantly greater H$_2$O$_2$ scavenging activity in SDS-stabilized emulsions compared to their Tween-stabilized counterparts. BLG samples showed a larger increase in H$_2$O$_2$ scavenging activity in SDS-stabilized emulsions. By 48 h, all BLG treatments were capable of scavenging 400 µM H$_2$O$_2$ in SDS-stabilized emulsions, while 225 µM (56%), 268 µM (67%), and 271 µM (68%) remained in Tween stabilized emulsions containing BLG-CTRL and BLG-TBHP, and BLG-HOOH, respectively.
**Fig. 6.4:** H$_2$O$_2$ loss in SDS-stabilized 5% hexadecane emulsions containing 1% w/v protein (CAS or BLG) in the presence of 400 µM H$_2$O$_2$ and 25 µM Fe$^{3+}$ at pH 7.

Under the denaturing conditions of SDS, a greater proportion of Cys and Met residues would be expected to become solvent accessible to H$_2$O$_2$. However, all protein samples in SDS-stabilized emulsions showed similar Met loss and MetO formation compared to their respective treatments in Tween-stabilized emulsions (**Fig. 6.5a, b**). This is expected for CAS samples where Met residues were already shown to be solvent accessible in Tween-stabilized emulsions. An increase in accessibility may account for the faster H$_2$O$_2$ scavenging; however, a kinetic analysis of Met changes or measurement at earlier time points would be required. BLG samples also did not show any large difference in Met content between Tween and SDS-stabilized emulsions even though BLG samples showed substantial peroxide scavenging activity in SDS-stabilized emulsions. This results from
increased Cys accessibility, as observed in the significant decreases in Cys in SDS-stabilized emulsions (Fig. 6.3). No differences between treatments were observed with a measured Cys loss of 348, 342, and 373 µM Cys in BLG-CTRL, BLG-TBHP, and BLG-HOOH, respectively. As 1% w/v BLG contained 543 µM Cys, Cys residues were capable of preferentially reacting with the 400 µM H₂O₂. Increasing the H₂O₂ concentration may help determine whether Met residue accessibility was also greatly increased in the presence of SDS.

(a)

Fig. 6.5: Changes in Met and MetO content after 48 h incubation with 400 µM H₂O₂ in SDS-stabilized hexadecane emulsions at pH 7 with 25 µM Fe³⁺ and 1% w/v protein: (a) CAS and (b) BLG.
In general, CAS-CTRL showed the highest H$_2$O$_2$ scavenging activity in both Tween- and SDS-stabilized emulsions. Despite the fact that all BLG samples contained a larger total Met content, many of those residues appear to be inaccessible to the aqueous phase, especially in BLG-TBHP and BLG-HOOH where most of the solvent accessible Met residues were already oxidized during pre-treatment. The same is true for CAS samples, where Cas-TBHP and CAS-HOOH showed progressively lower rates of H$_2$O$_2$ scavenging. This suggests that it takes longer for the peroxide to react with the remaining Met due to loss of the more solvent exposed Met residues after peroxide pretreatment. SDS likely denatured both proteins to make Cys and Met more accessible to H$_2$O$_2$. This
was especially important for BLG samples, since it dramatically increased Cys availability. Increased Met accessibility due to SDS denaturation in BLG require verification as free Cys alone was sufficient to scavenge the exogenously added H₂O₂ (400 µM).

6.4.3. Effect of Methionine-blocked Proteins on EGCG Stability in Tween-stabilized Hexadecane Emulsions

We have recently shown that some phenolics (e.g., EGCG) are capable of generating H₂O₂ in emulsions (Chapter 4 and 5). In Chapter 5, H₂O₂ generated from EGCG oxidation was readily scavenged in CAS-stabilized emulsions though EGCG loss was also accelerated. Thus, experiments were repeated in the presence of EGCG (400 µM) to determine the effect of Met-blocked CAS and BLG on EGCG stability and H₂O₂ levels resulting from differences in O₂•⁻ and H₂O₂ scavenging after blocking Met residues. After 48 h in Tween-stabilized emulsions (Fig. 6.6a), the rate of EGCG loss was as follows (from highest to lowest): control > BLG-CTRL = BLG-TBHP = BLG-HOOH > CAS-HOOH > CAS-TBHP > CAS-CTRL. After 96 h, ~100 µM EGCG (25%) remained in BLG samples, while 205 µM (51%), 184 µM (46%), and 120 µM (30%) EGCG remained in CAS-CTRL, CAS-TBHP, and CAS-HOOH samples, respectively. CAS samples showed greater EGCG stability in proteins containing greater Met content. This may be due to the ability of Met residues to scavenge radicals (O₂•⁻, •OH) that would otherwise propagate EGCG oxidation. CAS is also known to effectively chelate iron (Diaz et al., 2003), which may serve to retard the metal-catalyzed oxidation of EGCG.
Fig. 6.6: (a) EGCG loss and (b) H$_2$O$_2$ concentration in Tween-stabilized hexadecane emulsions with 25 µM Fe$^{3+}$ in 10 mM phosphate buffer pH 7.0 containing 1% protein (CAS or BLG).
H$_2$O$_2$ resulting from EGCG oxidation in Tween-stabilized emulsions is shown in Fig. 6.6b. At 24 h, differences in H$_2$O$_2$ concentrations follow the same trend as EGCG stability with H$_2$O$_2$ concentrations from highest to lowest as follows: control > BLG-CTRL = BLG-TBHP = BLG-HOOH > CAS-HOOH > CAS-TBHP > CAS-CTRL. Even after 96 h, no significant differences were observed in BLG samples with 254 µM H$_2$O$_2$ in BLG-CTRL, 288 µM H$_2$O$_2$ in BLG-TBHP, and 252 µM H$_2$O$_2$ in BLG-HOOH. CAS treatments showed no further loss in H$_2$O$_2$ concentration after 48 h, when 22, 51, and 91 µM H$_2$O$_2$ was measured in CAS-CTRL, CAS-TBHP, and CAS-HOOH, respectively. The differences in H$_2$O$_2$ concentrations were expected as emulsions with CAS showed greater
EGCG stability (Fig. 6.6a) and greater H$_2$O$_2$ scavenging activity (Fig. 6.2) compared to their respective BLG treatments.

To examine the role that Met plays as an antioxidant in scavenging radicals and peroxides from EGCG oxidation, Met loss and MetO formation were measured after incubation with EGCG for 96 h. In CAS samples (Fig. 6.7a), each treatment showed comparable Met loss with a decrease of 537, 505, and 489 µM Met to yield a final concentrations of 1390, 408, and 180 µM Met in CAS-CTRL, CAS-TBHP, and CAS-HOOH, respectively. MetO concentrations showed increases of 303 µM in CAS-CTRL, 158 µM in CAS-TBHP, but no significant increase in CAS-HOOH. BLG samples also demonstrated similar Met changes with 311 µM loss in BLG-CTRL, 202 µM loss in BLG-TBHP, and 184 µM loss in BLG-HOOH. However, BLG-CTRL was the only BLG-containing sample to show a significant increase in MetO concentration (increase of 186 µM MetO) (Fig. 6.7b). The fact that observed MetO concentration increases could not fully account for the magnitude of Met loss during EGCG oxidation require further examination.
**Fig. 6.7**: Changes in Met and MetO content after 96 h incubation with 400 µM EGCG in Tween-stabilized hexadecane emulsions at pH 7 with 25 µM Fe^{3+} and 1% w/v protein: (a) CAS and (b) BLG.
All BLG treatments in Tween-stabilized emulsion showed a drop in free Cys after incubation with EGCG (Fig. 6.8). BLG samples showed no treatment differences with Cys loss of 161, 232, and 190 µM in BLG-CTRL, BLG-TBHP, and BLG-HOOH, respectively. Similar to Met analysis, Cys residues were accessible to ROS generated by EGCG in Tween-stabilized emulsions that were not accessible in the presence of H₂O₂ alone. Some Cys loss may also be the result of reactions with quinone via the 1,4-Michael addition, which would explain the higher than expected Met and Cys loss.
Fig. 6.8: Free Cys content after 96 h incubation with 400 µM EGCG in Tween-stabilized or SDS-stabilized hexadecane emulsions at pH 7 with 25 µM Fe^{3+} and 1% w/v BLG.

6.3.4 Effect of Methionine-blocked Proteins on EGCG Stability in SDS-stabilized Hexadecane Emulsions

EGCG oxidation studies in the presence of proteins were repeated in SDS-stabilized emulsions for 96 h (Fig. 6.9a). In all treatments, EGCG showed reduced stability in SDS-stabilized emulsions compared to Tween-stabilized emulsions. After 24 h, all samples retained less than 100 µM EGCG in SDS-stabilized emulsions, while all Tween-containing samples retained higher concentrations of EGCG (> 250 µM). This difference arises from Tween’s greater protective effect on EGCG oxidation. A separate EGCG oxidation study confirmed that emulsifiers showed different capabilities to protect EGCG
from oxidation. 1% Tween 80 showed significantly slower EGCG oxidation compared to 1% SDS (Fig. 6.10), suggesting the importance of emulsifier type when examining EGCG oxidation.

Fig. 6.9: (a) EGCG loss and (b) \( \text{H}_2\text{O}_2 \) concentration in SDS-stabilized hexadecane emulsions with 25 \( \mu \text{M} \) \( \text{Fe}^{3+} \) in 10 mM phosphate buffer pH 7.0 containing 1% protein (CAS or BLG).
In SDS-stabilized emulsions, EGCG stability after 6 h incubation showed concentrations from highest to lowest as follows: BLG-CTRL > BLG-HOOH = BLG-TBHP > control = CAS-TBHP = CAS-CTRL = CAS-HOOH (Fig. 6.9a). Interestingly, the influence of BLG and CAS on EGCG stability was reversed in SDS-stabilized emulsions. This change in protein effect suggests that peroxide scavenging activity is not the sole factor influencing EGCG stability. When H$_2$O$_2$ was added exogenously to SDS-stabilized hexadecane emulsions, CAS-CTRL still showed the greatest scavenging activity, followed by BLG-containing samples (Fig. 6.4). However, peroxide scavenging
capabilities did not predict EGCG stability, suggesting that other factors, such as stabilization by protein-phenolic binding may also be important.

H₂O₂ concentrations in SDS-stabilized emulsions showed H₂O₂ levels increase rapidly in the first 6 h (Fig. 6.9b), corresponding to the equally rapid EGCG oxidation. At 6h, H₂O₂ concentrations were at one of their highest levels in all protein treatments, while the absence of proteins yielded a final concentration of 513 µM H₂O₂ after 96 h. Similar to SDS-stabilized hexadecane emulsions, H₂O₂ detected in protein treatments after 96 h showed concentration differences from highest to lowest as follows: control > CAS-HOOH > CAS-TBHP > CAS-CTRL > BLG-CTRL = BLG-TBHP = BLG-HOOH. Similar to the peroxide scavenging study, CAS samples once again showed decreasing H₂O₂ scavenging activity with increased Met-blocking, since CAS treatments shared comparable EGCG oxidation profiles and resulting ROS generation. As H₂O₂ was observed to accumulate in the protein-free control after 24 h, it is likely that CAS-TBHP and CAS-HOOH still scavenged H₂O₂, although no net H₂O₂ loss was observed after 24 h in these two treatments. CAS-CTRL and BLG treatments, on the other hand, showed a continuous decrease in H₂O₂ over 96 h, with an average of 46 µM H₂O₂ remaining in BLG treatments and 79 µM H₂O₂ in CAS-CTRL.
Fig. 6.10: EGCG loss in 1 % w/v Tween 80, SDS, or CAS solutions prepared in 10 mM phosphate buffer pH 7 with 25 µM Fe\(^{3+}\) at 37 °C.

The loss of oxidatively labile amino acids was also measured in SDS-stabilized emulsions. All CAS treatments showed significant decreases in Met content, but only CAS-CTRL showed a significant change in MetO with an increase of 330 µM (Fig. 6.11a). Similarly, BLG-containing samples all showed a decrease in Met content (Fig. 6.11b), but only BLG-CTRL showed an increase in MetO levels with a change of 308 µM. The total observed Met content was lower in all treatments, once again suggesting that products other than MetO were produced.
Fig. 6.11: Changes in Met and MetO content after 96 h incubation with 400 µM EGCG in SDS-stabilized hexadecane emulsions at pH 7 with 25 µM Fe^{3+} and 1% w/v protein: (a) CAS and (b) BLG.
Fig. 6.11: (continued)

Similar to the \( \text{H}_2\text{O}_2 \) scavenging study, free Cys content dropped dramatically in SDS-stabilized emulsions (Fig. 6.8). No significant differences in Cys were observed between BLG treatments with Cys loss of 445, 469, and 446 \( \mu \text{M} \) observed in BLG-CTRL, BLG-TBHP, and BLG-HOOH, respectively. SDS improved Cys availability, which may improve EGCG stability by readily scavenging radicals and peroxides.

Contrary to our previous study with CAS-stabilized emulsions in chapter 5, in this study 1% w/v of any protein treatment led to increased EGCG stability in Tween-stabilized emulsions. This difference may be explained due to the protective effect Tween exerts on
EGCG stability as previously shown in Fig. 6.10. Tween’s protective effect on EGCG stability also exceeded CAS, as observed in a study where EGCG (400 µM) with 25 µM Fe$^{3+}$ was allowed to oxidize for 6 h at 37 °C in 1% Tween or 1% CAS solutions (Fig. 6.10). CAS-stabilized emulsions prepared in chapter 5 would also have a lower aqueous protein content, which may factor towards a weaker effect on preventing EGCG oxidation.

A comparison between EGCG stability in Tween and SDS-stabilized emulsions is hard to interpret as EGCG oxidation varied greatly between the controls in Tween- and SDS-stabilized emulsions and the surfactants may also have effects on protein-EGCG binding if EGCG preferentially partitions into surfactant micelles. This confounds any observed differences that may result from increasing solvent accessibility by protein denaturation, since the two surfactants may exert different effects on EGCG oxidation and protein-EGCG binding.

It appears that proteins are capable peroxide scavengers with the added benefit of stabilizing EGCG. The importance of solvent accessible Met residues is readily apparent when comparing CAS and BLG samples; 1930 µM Met was present in CAS-CTRL while 1910 µM Met and 543 µM Cys was present in BLG-CTRL. However, CAS-CTRL greatly exceeded BLG-CTRL in peroxide scavenging in Tween-stabilized emulsions. CAS samples pre-treated with peroxides also demonstrated that reduced availability of readily accessible Met slows peroxide scavenging activity, as well as decreases EGCG stability. The introduction of SDS caused significant increases in peroxide scavenging
activity in both proteins, presumably due to protein denaturation. An increase in antioxidant activity accompanying denaturation has been previously observed in emulsions (Elias et al., 2007). However, care must be taken to ensure other properties, such as metal chelating abilities dependent on structure, are not negatively affected.

Met and Cys availability appears to play a significant role in regulating protein peroxide scavenging activity and in promoting phenolic stability in emulsions. Further work is needed to determine which other factors influence phenolic and protein interactions, since increasing peroxide scavenging activity did not always correlate with increased EGCG stability. However, the results are promising that proteins may be capable of mitigating the pro-oxidant activity of phenolics by increasing phenolic stability and scavenging ROS.
7. \(\beta\)-lactoglobulin Mitigates the Pro-oxidant Activity of Polyphenols in Emulsions.

7.1. Abstract

Phenolics are susceptible to rapid oxidation, which may promote additional oxidation of other food constituents like lipids. Proteins may scavenge oxidants arising from phenolic oxidation (namely peroxyl radicals and hydrogen peroxide), thus preventing further oxidation. In this study, \(\beta\)-lactoglobulin’s (BLG) potential to mitigate pro-oxidant activity induced by (-)-epigallocatechin-3-gallate (EGCG) (400 \(\mu\)M), on the lipid oxidation in flaxseed emulsions at pH 3 was examined. As methionine (Met) solvent accessibility in proteins was previously demonstrated to slow EGCG oxidation, a pre-oxidized BLG treatment (BLG-HOOH) was also tested. EGCG loss was greatly reduced in the presence of BLG, such that 1.0% w/v BLG samples retained 50% EGCG, 0.2% w/v BLG retained 36% EGCG, while EGCG alone retained only 22% EGCG after 8 days in oxidatively stable hexadecane emulsions. In flaxseed emulsions, EGCG-only treatment showed significant pro-oxidant activity, but the addition of protein and EGCG together yielded antioxidant activity compared to the no protein, no EGCG control. After 6 days, only BLG-HOOH at 0.2% w/v matched the TBARS levels in the control, likely due to the loss of accessible amino acids. Met content analysis revealed decreases in Met content with corresponding increases in the oxidation product, methionine sulfoxide (MetO), suggesting that Met oxidation may be important to the observed antioxidant activity. Overall, the addition of proteins may be an effective strategy to stabilize phenolics and to mitigate the pro-oxidative effects of polyphenols.
7.2. Introduction

Phenolics are commonly incorporated into food products naturally through fruits and vegetables inherent in the product or increasingly as an additive. Plant phenolics are attractive to consumers for increasing their antioxidant exposure, as well as for specific effects like cardiovascular health (Vita, 2005) and cancer prevention (Yang et al., 2009). Phenolics in foods are expected to exert antioxidant activity similar to those observed in chemical based assays such as ORAC (Freeman, Eggett & Parker, 2010) and DPPH (Roy et al., 2010). Di- and tri-hydroxy substituted phenols are especially good radical scavengers; they readily donate electrons to radicals due to resonance stabilization in the semiquinone formed after oxidation. However, this ease of oxidation also promotes pro-oxidant activity by undergoing oxidation reactions with transition metals. Metal-catalyzed oxidation of phenolics yield reduced transition metals and reactive oxygen species (superoxide/hydroperoxyl radicals, hydrogen peroxide, hydroxyl radicals), as shown in Fig. 1.5, both of which may promote oxidation in lipids, proteins, and other food components. Phenolics have shown increased lipid oxidation markers in oil-in-water emulsions (Frankel et al., 1997b; Huang et al., 1997), with acidic pH being a major promoter of pro-oxidant activity as shown in chapter 3. This is worrisome as many beverages, such as fruit juices and sodas, are high-acid foods.

The use of the metal chelator, EDTA, has been shown to strongly inhibit phenolic oxidation and lipid oxidation at acidic pH (Chapter 3). However, EDTA is an artificial additive many manufacturers are abandoning for more “clean” labels, especially in foods vying for an all natural label. Proteins may be a good alternative for controlling oxidation
Dairy proteins are readily available with numerous studies showing inhibition of lipid oxidation (Elias et al., 2005). The presence of proteins has also been shown to inhibit phenolic oxidation at physiological pH (Ishii et al., 2011), likely due to the antioxidant activity of cysteine (Cys) and methionine (Met) residues that are capable radical and peroxide scavengers.

Thus, an examination into the potential stabilizing effect of β-lactoglobulin (BLG) on EGCG and their net effect on lipid oxidation at an acidic pH was examined. BLG was selected as it is a readily available homogenous and food-grade protein, while EGCG (Fig. 1.4) is the main polyphenol in green tea with numerous reported health benefits (Bose, Lambert, Ju, Reuhl, Shapses & Yang, 2008; Pan, Chiou, Wang, Ho & Lin, 2011). To assess the importance of solvent accessible amino acids, an oxidized BLG treatment was also tested for comparison.
7.3. Materials and Methods

7.3.1. Materials

EGCG (93% purity) was acquired from Taiyo Green Power Company (Jiangsu, China). n-Hexadecane (99% purity) was obtained from Acros Organics (Morris Plains, NJ). Phthaldialdehyde (OPA) reagent, butylated hydroxytoluene (BHT), methionine, methionine sulfoxide, tryptophan, tyrosine, Tween-80, ethylenediaminetetraacetic acid (EDTA), xylanol orange tetrasodium salt, ferrous sulfate heptahydrate and the enzymes leucine aminopeptidase M (L5006), protease (P5147), and prolidase (P6675) were purchased from Sigma (St. Louis, MI). β-Lactoglobulin was donated by Davisco Foods International (Eden Prairie, MN). H₂O₂ (30% w/v) and ammonium thiocyanate were purchased from EMD Chemicals (Gibbstown, NJ). 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), guanidine hydrochloride, tert-butyl hydroperoxide (70%), phenylalanine, D-sorbitol (98% purity) and cumene hydroperoxide were purchased from Alfa Aesar (Ward Hill, MA). 1,1,3,3-tetraethoxypropane was acquired from TCI America (Portland, OR). Ferric chloride 6-hydrate lumps and sodium azide were purchased from Mallinkrodt (Phillipsburg, NJ). Trichloroacetic acid (crystalline) was purchased from J.T. Baker (Phillipsburg NJ). Leucine was acquired from Amresco (Solon, OH). BD Falcon™ Clear 96-well Microtest™ Plates, 0.45 µm PTFE syringe filters and Spectra/Por® regenerated cellulose dialysis tubing with a 12-14 kDa MWCO were purchased from VWR (Radnor, PA). BCA Protein Assay Kit was purchased from Thermo Fisher (Rockford, IL). Flaxseed oil was purchased from a local market and used as received. All other chemicals and solvents were of analytical or HPLC grade. Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA) purification train.
7.3.2. Preparation of Met-Blocked BLG

Protein solutions (1% w/v BLG) were prepared in 10 mM phosphate buffer pH 7 and oxidized with H₂O₂ (2 mM) for 5 days at room temperature. The concentrations selected were previously shown to oxidize Met residues while minimizing the oxidation of other oxidatively labile amino acids (Keck, 1996). Controls without oxidants added were also prepared. BLG was precipitated by adjusting the pH to 2.6 followed by the addition of NaCl (30% w/v). Protein was collected after centrifugation at 3300 x g for 15 m at 4 °C. Proteins were then resuspended in water and dialyzed in dialysis bags (MWCO = 10 kDa) against water for 3 days at 4 °C with 3 water changes per day. The water was analyzed for residual peroxides using the FOX method described below to ensure complete removal of oxidants. After dialysis, protein slurries were frozen at -80 °C and lyophilized using a FreeZone 2.5 Liter Benchtop Freeze Dry System (Labconco, Kansas City, MO). Dry protein samples were stored at 4º C until use. Native-PAGE was run between all prepared proteins and fresh casein or BLG solutions. No differences in relative mobility were observed.

7.3.3. Emulsion Preparation

Oil-in-water emulsions were prepared by dispersing 20 wt% hexadecane or flaxseed oil with 4 wt% Tween 80 in water. Hexadecane, a saturated hydrocarbon, was used to create an emulsion system that will not readily undergo oxidation. Flaxseed oil was selected as an oxidizeable oil high in omega-3s. Coarse emulsions were made using a high-speed blender (Brinkmann Polytron, Brinkmann Instruments Inc., Westbury, NY) on high speed
for 0.5 m. Fine emulsions were prepared by passing coarse emulsions twice through a microfluidizer with the interaction chambers, H307 - 200 µm porosity and F20Y - 75 µm porosity placed in series (Microfluidics M-110Y, Newton, MA) at a pressure of 40 psi. All emulsions had a mean particle size of \(d_{32}\) of 0.22 ± 0.02 µm, as determined by laser light scattering (Horiba LA 920, Irvine, CA).

7.3.4. EGCG Oxidation Experiments

Protein solutions (2% w/v) were prepared in 20 mM phosphate buffer pH 3.0. Sodium azide (0.04% w/v) was added to solutions to prevent microbiological spoilage. pH was then adjusted to pH 3.0 using 6 N HCl or 10 N NaOH as necessary. EGCG from a stock solution (10 mM in water) was added to emulsions (20 wt%) and diluted to achieve a 10 wt% oil emulsion with 800 µM EGCG. Protein solutions were then diluted 1:1 by volume with the 10 wt% oil emulsion to achieve a final emulsion system with: 1% w/v protein, 400 µM EGCG in a 5 wt% oil-in-water emulsion. Ferric iron (25 µM) was added from a freshly prepared stock solution of FeCl₃ (10 mM) in water. pH was checked and adjusted again if necessary. Emulsions were then held in the absence of light at 37 °C for 8 days.

7.3.5. Hydrogen Peroxide Analysis

Emulsion samples (162.4 µL) were destabilized by the addition of 0.5 N H₂SO₄ (10 µL) and 2:1 methylene chloride:methanol (1 mL). Samples were mixed for 30 s followed by a 10 m incubation on ice. Samples were then centrifuged at 10,000 x g for 2 minutes. The upper aqueous layer was collected and stored at -80°C until analysis. Peroxide analysis was performed by adding the FOX assay solution (20 µL) to the aqueous extract (140
µL). The FOX assay solution contained xylenol orange (1 mM), ferrous sulfate (2.5 mM) and sorbitol (1.0 M) in sulfuric acid solution (0.5 N). Samples were diluted as necessary using a 1:2 water:methanol mixture. After vortex mixing, samples were stored for 30 min at ambient temperature. Absorbance values were then read at 560 nm using a Thermo Scientific* Multiskan GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Rockford, IL). Quantitation was performed using an external standard curve prepared using H₂O₂ after validation with the peroxide’s extinction coefficient ε₂₄₀ = 40 M⁻¹ cm⁻¹.

7.3.6. EGCG Analysis
EGCG was extracted from emulsions in the same manner as described above for H₂O₂ analysis. Prior to storage at -80°C, a preservative solution (10 µL) consisting of ascorbic acid (20% w/v) and ethylenediaminetetraacetic acid (EDTA; 0.1% w/v) in phosphate buffer (pH 3.6; 0.4 M) was added to the aqueous extract (100 µL) (Lee et al., 2004). Preservative solutions were prepared daily. Chromatographic separation was achieved on a reverse phase Supelcosil LC-18 (4.6 x 150 mm, 5 µm; Supelco Inc., Bellefonte, PA) using a Shimadzu 10ADvp pump with sample introduction by means of a 20ADvp temperature-controlled autosampler (4 ºC). Samples were filtered over 0.45 µm PTFE syringe filters. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). EGCG was eluted by gradient according to the following program: 25 - 45% B in 8.5 min. The injection volume was 20 µL and the flow rate was held at 1 mL/min. EGCG was detected at 280 nm using a Shimadzu SPD-10Avp UV-Vis detector, with quantitation based on an external standard curve prepared from EGCG.
7.3.7. **Protein Precipitation**

Emulsion samples (200 µL) were precipitated after the addition of 40% w/v trichloroacetic acid for Tween-stabilized emulsions or 2 N HCl for SDS-stabilized emulsions. Samples were mixed by vortex and incubated on ice for 10 min. The protein was then centrifuged at 10,000 x g for 3 min. The supernatant was discarded and the protein precipitate was washed with 400 µL of ethanol:ethyl acetate (1:1). Samples were again vortexed and centrifuged at 10,000 x g for 3 min. The supernatant was discarded and the wash step repeated twice more. After the final wash, protein samples were dried under N₂ to remove residual wash solution and stored at -80 °C until analysis.

7.3.8. **Enzymatic Hydrolysis**

Protein pellets were dissolved in 6 N guanidine hydrochloride (100 µL) to prepare an ~2% w/v solution. Protein solutions (30 µL) were then diluted with 100 mM phosphate buffer pH 7.5 containing 0.1% w/v sodium azide (370 µL). Enzymatic hydrolysis was performed as described by Baxter et. al (Baxter et al., 2007). Protease (920 U/mL in water) and prolidase (180 U/mL in water) solutions were prepared and stored at -20 °C. Protease solutions were further diluted to a final concentration of 9.2 U/mL in water prior to use. Leucine aminopeptidase M was stored at 4 °C and the slurry diluted in water to 24 U/mL prior to use. For digestion, 24 U/mL leucine aminopeptidase M (6 µL), 9.2 U/mL protease (15 µL), and 180 U/mL prolidase (3 µL) was added to the diluted protein sample (300 µL). The sample was gently mixed and incubated at 37 °C for 20 h. Digested samples were then analyzed by HPLC.
7.3.9. Amino Acid Analysis by HPLC

Amino acids were separated using the same HPLC system described above after pre-column OPA derivatization. The OPA reagent (150 µL) was added to enzymatically digested protein samples (15 µL). Samples were derivatized immediately prior to analysis, since OPA-derivatives were not stable for extended periods. After mixing, samples were filtered over 0.45 µm PTFE syringe filters. Samples (20 µL) were then eluted using the following flow and gradient program: 0 min – 0.4 mL/min, 19% B; 15 min – 0.4 mL/min, 25% B; 15.1 min – 1.0 mL/min; 30 min – 65% B. The mobile phase consisted of (A) 4% tetrahydrofuran (THF) in 35 mM sodium acetate buffer pH 5.7 and (B) methanol. The OPA derivatized amino acids were detected at 210 nm and quantified based on external amino acid standard curves.

7.3.10. Protein Quantification

Total protein was quantified using the BCA methods, as described previously (Smith et al., 1985). Working reagent was prepared by mixing 50 parts BCA reagent A with 1 part BCA reagent B. Diluted protein solutions prepared for enzymatic hydrolysis above was used for protein quantification, since samples fell within the standard curve. Working reagent (400 µL) was added to protein samples (20 µL) and incubated at 37 °C for 30 min. Mixtures were then cooled at ambient temperature for 10 min. Samples (200 µL) were transferred to 96-well plates for analysis at Abs 562 using a Thermo Scientific* Multiskan GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Rockford, IL). Quantification was performed using external standard curves prepared for
each protein in the same guanidine hydrochloride and buffer mixture found in the diluted protein samples.

7.3.11. Cysteine Determination

Free cysteine was quantified by Ellman’s method, as described previously (Ellman, 1959). The reagent solution was prepared by dissolving 4 mg DTNB in 0.1 M phosphate buffer pH 8.3 containing 1 mM EDTA. 6 N Guanidine hydrochloride (200 µL) adjusted to pH 8.3, was mixed with undiluted protein samples (20 µL) prepared in the enzymatic hydrolysis step above. Samples were mixed after adding the reagent solution (4 µL) and then incubated at room temperature for 15 min. Sample (200 µL) was transferred to 96 well plates and analyzed at Abs 412 using a Thermo Scientific Multiskan GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Rockford, IL).

7.3.12. Lipid Hydroperoxide Analysis

Lipid hydroperoxide concentrations were measured according to the method described by Shantha and Decker (Shantha et al., 1994). Flaxseed o/w emulsions (0.15 mL) were mixed with 0.75 mL of isooctane/1-butanol (3:1, v/v) and vortexed 3 times for 10 s at 20 s intervals. Samples were subsequently centrifuged for 2 min at 3300 x g. The upper layer of the sample extraction (0.1 mL) was mixed with 1.4 mL of methanol/butanol (2:1, v/v). Sample extracts were diluted with water as needed. The ferrous iron solution was prepared by mixing an equal amount of a solution of 0.144 M FeSO₄ and 0.132 M BaCl₂. The iron solution was centrifuged for 3 min at 3300 x g, and an equal volume of the supernatant and 3.94 M ammonium thiocyanate was mixed to prepare the assay solution.
15 μL of the assay solution was added to the samples and then analyzed at 520 nm after storage for 20 min. Lipid hydroperoxide concentrations were quantified using an external calibration curve prepared with cumene hydroperoxide.

### 7.3.13. TBARS Analysis

TBARS was determined using a method described by McDonald and Hultin (McDonald et al., 1987). Briefly, samples (0.5 mL) were mixed with 1.0 mL TBA reagent and heated in a boiling water bath for 15 min. Samples were cooled at room temperature for 10 min and centrifuged at 3200 x g for 15 min. Samples were then stored for 10 min and the aqueous layer analyzed at 532 nm. TBARS concentrations were quantified using an external calibration curve prepared with 1,1,3,3-tetraethoxypropane.

### 7.3.14. Statistical Analysis

All experiments were performed in triplicate and results expressed as means and standard deviation. Two-way ANOVA with Bonferroni’s post test was performed on EGCG, H$_2$O$_2$, PV, and TBARS results, while amino acid results were examined for significant with t-test using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California, USA). Treatments were considered significantly different if p < 0.05.
7.4. Results and Discussion

7.4.1. Effects of BLG on EGCG Oxidation in Hexadecane Emulsions

EGCG oxidation was followed at pH 3 in Tween-stabilized 5% hexadecane emulsions containing BLG for 8 days (Fig. 7.1a). BLG was observed to stabilize EGCG in a concentration dependent manner; at day 2, EGCG concentrations from highest to lowest were: BLG-1% > BLG-0.2% > control. Throughout the study, BLG-CTRL and BLG-HOOH at the same protein concentrations showed no significant differences in EGCG stability. The protective effect of BLG appeared to be dependent on concentration, with 1% and 0.2% BLG showing an average loss of 50% and 64% EGCG, respectively, at day 8; no protein control showed a 77% loss. The protective effect is likely due to non-covalent protein-EGCG interactions combined with protein ROS scavenging activity. For example, EGCG was shown to be stabilized in human serum at physiological pH (Bae et al., 2009). The authors showed that EGCG formed complexes with human serum albumin (HSA) in serum, such that the addition of HSA but not human serum γ-globulin increased EGCG stability in phosphate buffer (pH 7.4). Free sulfhydryl content in HSA decreased as a result of EGCG incubation, suggesting that phenolic-protein association and protein ROS scavenging activity are both important to EGCG stabilization. In the present study, non-covalent interactions between EGCG and BLG at pH 3 was demonstrated by filtrating aqueous solutions of EGCG (0.4 mM) and BLG-CTRL-1% or BLG-HOOH-1% through 10 kDa MWCO filters following 15 min equilibration at room temperature. No EGCG was detected in the filtrate, while complete recovery was measured in unfiltered samples. EGCG-protein association may also prevent EGCG-metal complexation, as the phenolic’s hydroxyl groups are involved in protein interactions.
H$_2$O$_2$ concentrations generated via EGCG oxidation were also followed (Fig. 7.1b); little H$_2$O$_2$ was observed and with no consistent trends in concentration changes. This corresponds with previous studies where H$_2$O$_2$ accumulated at pH 7 but not at pH 3, due to rapid metal-catalyzed peroxide decomposition under those acidic conditions (Chapter 4).

![Graph](image)

**Fig. 7.1:** (a) EGCG loss and (b) H$_2$O$_2$ concentration in hexadecane emulsions containing 400 µM EGCG, 25 µM Fe$^{3+}$ and BLG (0.2% or 1.0% w/v) in 10 mM phosphate buffer pH 3.
An analysis of Met residues revealed significant decreases in Met concentration in BLG-CTRL-1%, BLG-HOOH-0.2%, and BLG-HOOH-1% compared to their controls at day 0 (Fig. 7.2a); however, no statistically significant change was observed in BLG-CTRL-0.2%. In terms of absolute Met content in the emulsion sample, BLG-CTRL-0.2% and BLG-HOOH-0.2% lost 48 and 58 µM Met, respectively. BLG-CTRL-1% showed a decrease of 269 µM Met, while BLG-HOOH-1% showed a decrease of 251 µM Met. The greater Met loss observed in 1% BLG solutions may explain the increased EGCG stability, as it is conceivable that BLG scavenged ROS that would otherwise propagate EGCG loss. In terms of MetO formation, BLG-CTRL-0.2%, BLG-CTRL-1%, and BLG-
HOOH-1% showed significant increases in MetO compared to their respective controls (Fig. 7.2b). BLG-CTRL samples showed increases of 60 and 270 µM MetO in 0.2% and 1% BLG-CTRL solutions, respectively which corresponds well to the Met decrease observed. However, BLG-HOOH-1% showed only an increase of 70 µM MetO, lower than the observed Met loss. The formation of other Met oxidation products besides MetO during EGCG oxidation may account for the difference in Met loss as observed previously in CAS and BLG samples at pH 7 (Chapter 6). No significant changes in Met, MetO, or Cys content was observed after incubation in hexadecane emulsions in the absence of EGCG.

(a)

**Fig. 7.2:** Changes in (a) Met and (b) MetO content in BLG-CTRL and BLG-HOOH after incubation with 400 µM EGCG and 25 µM Fe$^{3+}$ in hexadecane emulsions at pH 3.
Cys content revealed significant loss in the presence of EGCG (Fig. 7.3); decreases of 75, 79, 223, and 224 µM Cys was observed in BLG-CTRL-0.2%, BLG-HOOH-0.2%, BLG-CTRL-1%, and BLG-HOOH-1%, respectively. Starting Cys content between BLG-CTRL and BLG-HOOH were not significantly different with 51 µmol/g protein in BLG-CTRL and 53 µmol/g protein in BLG-HOOH; in absolute terms, 103, 107, 515, and 534 µM Cys were recorded for BLG-CTRL-0.2%, BLG-HOOH-0.2%, BLG-CTRL-1%, and BLG-HOOH-1%. As expected, lower BLG concentration (0.2% BLG) showed more Cys loss per unit protein. 0.2% BLG retained 27% Cys, while 1% BLG retained 58% Cys.
Fig. 7.3: Free Cys content in BLG samples after 8-days incubation with 400 µM EGCG in Tween-stabilized hexadecane emulsions at pH 3 with 25 µM Fe$^{3+}$.

7.4.2. Effects of proteins on EGCG (400 µM EGCG) pro-oxidant activity in flaxseed emulsions

The experiments described above were repeated in the presence of an oxidatively labile lipid (flaxseed oil), to determine if BLG is capable of reversing EGCG’s pro-oxidant activity in emulsions. Lipid hydroperoxides (Fig. 7.4a) and TBARS (Fig. 7.4b) were measured as primary and secondary lipid oxidation markers, respectively. Control emulsions with no EGCG or proteins showed a steady increase in peroxides over the 8-day study. Emulsions containing only EGCG (400 µM) showed significantly lower PV at day 2 (134 µM vs 236 µM), no significant difference at day 4, followed by pro-oxidant activity at day 6 (939 µM vs 545 µM) and day 8 (2098 µM vs 723 µM). All EGCG plus
protein containing treatments showed significantly lower peroxides compared to the control over the first 6 days. At day 8, BLG-CTRL-1%, BLG-HOOH-1%, and BLG-CTRL-0.2% still showed antioxidant activity with PV of 293, 183, and 259 µM cumene hydroperoxide equivalents, respectively. However, BLG-HOOH-0.2% showed higher PV in comparison to the other protein treatments with a PV of 584 µM cumene hydroperoxide equivalents, a value comparable to that observed in the control.

(a)

Fig. 7.4: Lipid oxidation markers (a) lipid hydroperoxide concentration and (b) TBARS in flaxseed emulsions containing 400 µM EGCG, 25 µM Fe³⁺ and BLG at pH 3.

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Fig. 7.4: (continued)

In general, TBARS values (Fig. 7.4b) corresponded well to lipid hydroperoxide values (Fig. 7.4a). The EGCG-only treatment showed no antioxidant activity at early time points, with pro-oxidant activity starting at day 4. All samples containing EGCG plus protein showed significantly lower TBARS at day 2 and day 4. Protein concentration effects were observed in BLG samples at early time points (1h and day 2), such that significantly lower TBARS was observed in emulsions with 1% BLG compared to 0.2% BLG. Similar to lipid hydroperoxide values, BLG-HOOH-0.2% treatment showed a lag time of less than six days after which rapid TBARS was observed. By the end of the study, control emulsions contained 131 µM TEP eq. and BLG-HOOH-0.2% contained
180 µM TEP equivalents. TBARS levels in EGCG-only emulsions were 10-fold higher with 1280 µM TEP equivalents. All other BLG with EGCG samples continued to display antioxidant activity at day 8 showing 62, 50, and 74 µM TBARS in BLG-CTRL-1%, BLG-HOOH-1%, and BLG-CTRL-0.2%, respectively.

Protein treatments without EGCG were also monitored for lipid hydroperoxides (**Fig. 7.5a**) and TBARS (**Fig. 7.5b**), but no differences were observed compared to the control with no EGCG or protein added. This was unexpected, as studies have shown proteins to inhibit lipid oxidation reactions in emulsions; however, this may be due to the emulsion conditions used. In protein-stabilized emulsions, positively charged BLG repels catalytic transition metals to inhibit lipid oxidation at acidic pH (Hu, McClements & Decker, 2003). The antioxidant activity on lipid oxidation due to interfacial protein at pH 3 was also supported when interfacial WPI was displaced by Tween 20 which resulted in increased lipid oxidation (Donnelly, Decker & McClements, 1998). However, in this present study emulsions were Tween-stabilized, so the benefits of positively charged interfacial BLG were not observed. Though, other studies have shown antioxidant activity in surfactant stabilized emulsions with continuous phase protein, those studies were performed at neutral pH (Elias et al., 2006; Elias et al., 2005).
Fig. 7.5: Lipid oxidation markers (a) lipid hydroperoxide concentration and (b) TBARS in all flaxseed emulsion controls without EGCG.
EGCG and H$_2$O$_2$ concentrations were also followed throughout the 8-day lipid oxidation study. EGCG loss followed the same order from fastest to slowest as observed in hexadecane emulsions: EGCG-only > BLG-0.2% > BLG-1% (Fig. 7.6a) with no differences between BLG-CTRL or BLG-HOOH treatments. Compared to their corresponding treatments in hexadecane emulsions, the rate of EGCG loss in flaxseed emulsions was faster, purportedly due to the scavenging of lipid-derived radicals by the phenolic. Unexpectedly, the BLG-HOOH-0.2% treatment did not show faster EGCG oxidation compared to BLG-CTRL-0.2% as might be expected from the faster lipid oxidation that would deplete phenolics as they reacted with lipid-derived radicals. This
suggests that up until day 6, BLG-HOOH-0.2% was able to protect EGCG and the oxidatively labile lipid to the same extent as the other protein treatments, which coincides with the sharp increase in lipid oxidation markers after day 6. With respect to \( \text{H}_2\text{O}_2 \), concentrations remained low in flaxseed emulsions (Fig. 7.6b). EGCG-only samples were the only treatments that showed a gradual increase in \( \text{H}_2\text{O}_2 \) from day 4 to day 8.

![Fig. 7.6: (a) EGCG loss and (b) \( \text{H}_2\text{O}_2 \) concentration in flaxseed emulsions containing 400 \( \mu \text{M} \) EGCG, 25 \( \mu \text{M} \) \( \text{Fe}^{3+} \) and BLG (0.2% or 1.0% w/v) in 10 mM phosphate buffer pH 3.](image-url)
A loss in Met content was observed in all BLG-containing samples after 8 days in flaxseed emulsions (Fig. 7.7a), with the 0.2% BLG samples showing the greatest loss per unit protein. This was expected, as 0.2% BLG contains fewer total Met residues, causing more Met residues to be oxidized in an individual protein molecule. In terms of absolute Met loss, BLG-CTRL-0.2% showed a 123 μM Met loss in flaxseed emulsions and a 251 μM Met loss in the presence of EGCG. BLG-CTRL-1% samples showed a decrease of 353 μM Met without EGCG and 288 μM Met in the presence of EGCG, with no significant difference observed with the addition of EGCG. Similar changes were observed in BLG-HOOH samples: BLG-HOOH-0.2% showed a decrease of 243 μM Met...
in the presence of EGCG, while only a 114 µM loss was measured in the absence of EGCG. A kinetic analysis of Met loss would help explain the weaker antioxidant activity observed in the EGCG plus BLG-HOOH-0.2% samples if it was revealed that BLG-HOOH-0.2% lost all its solvent accessible Met residue at an earlier time point (e.g., no further Met loss after day 4 thus no protective effect by BLG after day 4).

(a)

**Fig. 7.7:** Changes in (a) Met and (b) MetO content in BLG-CTRL and BLG-HOOH resulting from oxidation in flaxseed emulsions containing EGCG (100 or 400 µM) and 25 µM Fe³⁺ at pH 3.
The same differences were observed in MetO formation where all BLG samples demonstrated increased MetO after 8 days (Fig. 7.7b). In absolute concentrations, BLG-CTRL-0.2% showed an increase in MetO content of 136 µM, while BLG-CTRL-0.2% with EGCG showed an increase of 266 µM MetO, which correspond well with Met loss. Similar to Met loss, BLG-CTRL-1% showed no MetO concentration differences between samples in the presence or absence of EGCG with an increases 286 µM and 288 µM MetO. 0.2% BLG-HOOH also showed corresponding increases in MetO content with an increase of 190 µM and 100 µM MetO in sample with or without EGCG, respectively.
Similar to BLG and EGCG oxidation experiments in hexadecane above, 1% BLG-HOOH treatments showed no significant increases in MetO content suggesting that the formation of MetO does not account for all Met lost.

Significant Cys loss was also detected in all BLG samples in flaxseed emulsions (Fig. 7.8), but no significant difference was observed between BLG samples with or without EGCG. BLG-CTRL-1% showed a loss of 225 µM Cys with EGCG and 190 µM Cys in the absence of EGCG, while BLG-HOOH-1% showed a loss of 265 µM Cys with EGCG and 224 µM Cys in the absence of EGCG. For 0.2% BLG, BLG-CTRL samples with or without EGCG showed an average loss of 42 µM Cys, while BLG-HOOH samples showed an average loss of 45 µM Cys.
7.3.3 Effects of proteins on EGCG (100 µM EGCG) pro-oxidant activity in flaxseed emulsions

To determine the potential effects of EGCG concentration on the lipid protective effects of EGCG plus BLG, a lower EGCG concentration (100 µM EGCG) in flaxseed emulsions was also examined. Similar to the study above using 400 µM EGCG, EGCG-only samples showed increased lipid hydroperoxides starting at day 6 (Fig. 7.9a). EGCG plus both 1% BLG treatments showed antioxidant activity throughout the study, while PV in both 0.2% BLG treatments matched the control after 6 days oxidation. By day 8, lipid hydroperoxide concentrations were: 723 µM in the control, 2001 µM in EGCG-only
samples, and averaging 483 µM and 846 µM in 0.2% and 1% BLG samples, respectively. No significant differences between BLG-CTRL and BLG-HOOH were observed. Different from the study above with 400 µM EGCG, 0.2% BLG-CTRL with 100 µM EGCG was unable to keep lipid hydperoxide levels below that of the control throughout the full 8-day accelerated oxidation study.

Following the secondary lipid oxidation markers, TBARS (Fig. 7.9b), EGCG-only treatment showed significantly higher TBARS compared to the control after 4 days oxidation. At day 8, TBARS markers followed the trend from highest to lowest as: EGCG only (743 µM) > 0.2% BLG (avg. 228 µM) > control (131 µM) = 1% BLG (avg. 120 µM). 0.2% BLG samples were unable to prevent pro-oxidant activity from EGCG for the full duration of the study, since at day 8 higher TBARS levels compared to the control was observed. Interestingly, both 1% BLG samples with 100 µM EGCG were also not able to maintain lower TBARS values compared to the control till the end of the study.
Fig. 7.9: Lipid oxidation markers (a) lipid hydroperoxide concentration and (b) TBARS in flaxseed emulsions containing 100 µM EGCG, 25 µM Fe$^{3+}$ and BLG at pH 3.
EGCG (100 µM) oxidation in flaxseed emulsions followed the same trends as the higher EGCG concentration such that EGCG stability increased with increasing BLG concentration but with no differences between BLG-CTRL and BLG-HOOH (Fig. 7.10). 0.2% BLG samples averaged a 90% EGCG loss, 1% BLG samples averaged a 78% loss, and the no protein control showed complete EGCG loss after 4 days. No detectable H$_2$O$_2$ was observed in EGCG-treated flaxseed emulsions.
Fig. 7.10: EGCG loss in flaxseed emulsions containing 100 µM EGCG, 25 µM Fe$^{3+}$ and BLG (0.2% or 1.0% w/v) in 10 mM phosphate buffer pH 3.

After 8 days oxidation in flaxseed emulsions, all BLG containing emulsions with EGCG (100 µM) showed a significant decrease in Met content compared to unoxidized BLG at day 0 (Fig. 7.7a). However, no significant differences were observed between BLG samples with or without EGCG (100 µM) after 8 days of oxidation. Similar results were observed with MetO content (Fig. 7.7b), where BLG with EGCG (100 µM) showed significantly higher MetO compared to the unoxidized control but no difference compared to BLG samples alone in flaxseed emulsions. As expected, EGCG (400 µM) showed greater Met oxidation and MetO formation in flaxseed emulsions, since the
higher EGCG concentration would increase the presence of radicals that may oxidize Met residues not accessible to more bulky lipid-derived radicals or lipid hydroperoxides.

Cys content also decreased in BLG with EGCG (100 µM) with no differences between BLG-CTRL and BLG-HOOH (Fig. 7.8). An average loss of 47 µM and 266 µM Cys was observed in 0.2% BLG and 1% BLG, respectively. The only significant difference in Cys content in BLG after 8 days oxidation was observed between BLG-HOOH-1% and BLG-HOOH-1% with EGCG (100 µM).

Higher EGCG concentration appears to improve BLG-EGCG antioxidant activity. In the presence of 400 µM EGCG, both BLG-1% treatments with EGCG showed no increase in TBARS after 8 days oxidation. However with the lower EGCG concentration (100 µM), samples treated with 1% BLG showed TBARS levels comparable to the control after 8 days oxidation. This difference was even more noticeable at low protein concentrations (0.2% BLG) where EGCG (100 µM) and BLG showed TBARS values that exceeded the control after 8 days. However, at the higher EGCG concentration (400 µM), only BLG-HOOH-0.2%, the BLG treatment with fewer accessible Met residues, showed an increase in TBARS levels comparable to the control.

The addition of BLG was capable of preventing the pro-oxidant activity initiated by EGCG at low pH. This may be due to the radical and peroxide scavenging activity of BLG that is capable of decreasing EGCG oxidation and reacting with lipid hydroperoxides and lipid-derived radicals, as proposed in Scheme 2. Proteins may
directly scavenge lipid-derived radicals and lipid hydroperoxides, but increased EGCG stability will also increase the phenolic concentration available to react with radicals. Proteins alone showed no effects on lipid oxidation in flaxseed emulsions, suggesting that the interaction between BLG and EGCG results in the observed effects. Additionally, BLG-HOOH-0.2% showed identical EGCG oxidation compared to BLG-CTRL-0.2% in both hexadecane and flaxseed emulsions, but BLG-HOOH-0.2% showed higher lipid oxidation by the end of the study. Therefore, the availability of solvent accessible Met residues is important in maintaining the antioxidant effects observed in emulsions. No differences between BLG-CTRL-1% and BLG-HOOH-1% were observed, potentially due to incomplete blocking of all solvent accessible Met residues with H$_2$O$_2$ pre-treatment. EGCG binding to proteins may also yield ROS generation in close proximity to Met residues increasing the accessibility of H$_2$O$_2$ and radicals to Met residues previously unavailable during pre-treatment. This is similar to metal catalyzed oxidation studies in proteins where amino acids near the transition metal chelation site are preferentially oxidized (Stadtman, 1990).

Overall, BLG addition prevented the pro-oxidant activity resulting from metal-catalyzed phenolic oxidation with higher BLG and EGCG concentrations yielding greater antioxidant effects on lipid oxidation. The availability of solvent accessible Met and Cys residues was a contributing factor lipid oxidation inhibition, suggesting that the substitution of proteins with higher concentrations of solvent accessible Met and Cys since the previous chapter demonstrates that the majority of the Met and Cys residues are not readily available unless BLG is denatured. Further work focusing on the antioxidant
synergy between phenolics and proteins is needed to assess the importance of protein and phenolic binding on phenolic stability as well as the effects of other proteins like casein that have more solvent accessible Met and Cys residues.
8. Conclusions and Recommendations for the Future Work

The incorporation of phenolics in lipid-based foods presents different challenges depending on matrix conditions. At acidic pH, EGCG tended to be more oxidatively stable, yet EGCG exerted its maximum pro-oxidant activity in lipid dispersions under such conditions (pH 2-4). EGCG in low acid foods, like milk, will be subject to faster phenolic loss, but with a low likelihood for pro-oxidant activity despite the fact that H$_2$O$_2$ is rapidly generated. Higher EGCG concentrations increased overall antioxidant activity with respect to lipid oxidation. At low pH, higher EGCG concentrations may push the competing reactions towards antioxidant activity by increasing the pool of phenolics capable of acting as scavengers of lipid-derived radicals. The same is true at higher pH values, since more EGCG will be available and the H$_2$O$_2$ formed due to metal-catalyzed phenolic oxidation is not readily reduced to •OH radicals at neutral pH. Though EGCG was used in all experiments, results may be applied to other di- and tri-hydroxy substituted phenolics, since they are likely to share similar properties in terms of redox activity and metal chelation.

I observed that the addition of iron chelators to EGCG-containing emulsions resulted in mixed effects with respect to phenolic and lipid stability that are impossible to attribute solely to changes in Fe reduction potentials. At acidic pH, BPY increased lipid oxidation possibly due to increased Fe$^{2+}$ stabilization, while EDTA prevented EGCG and lipid oxidation. However, the reverse was true at neutral pH where EDTA led to increased phenolic oxidation and •OH radical formation, though no increased lipid oxidation was observed in any EGCG-containing treatments. The lack of pro-oxidant activity even with
rapid \( \cdot \text{OH} \) radical generation, implies further work is needed to determine the likelihood of \( \cdot \text{OH} \) radicals reaching the lipid droplet surface to initiate lipid oxidation. Studies may be performed with EPR spin probes that preferentially partition to the interface. If the spin probe is localized solely at the lipid droplet interface, the formation of a \( \cdot \text{OH} \) radical spin adduct will signify \( \cdot \text{OH} \) radicals may reach the lipid droplet to initiate oxidation. The effect of excess aqueous constituents may also be examined with the spin probe, since the \( \cdot \text{OH} \) radicals generated may have reacted with excess surfactant or buffer components in my current system. Care should be taken in choosing metal chelators, since pH greatly affected the reduction potentials and potentially the binding characteristics of the chelators. Further work on selecting metal chelators for characteristics other than their influence on reduction potential may be valuable (e.g. free coordination sites in metal complexes).

I also investigated an alternative strategy for scavenging \( \text{H}_2\text{O}_2 \) prior to \( \cdot \text{OH} \) radical formation using proteins. CAS and WPI both decreased \( \text{H}_2\text{O}_2 \) levels after \( \text{H}_2\text{O}_2 \) was added exogenously or generated \textit{in situ} from EGCG oxidation in hexadecane emulsions. Unexpectedly, EGCG oxidation rates were higher in CAS-stabilized emulsions compared to Tween-stabilized emulsions at pH 7; however, when the experiments were repeated with aqueous phase proteins (1% CAS or BLG) in Tween-stabilized hexadecane emulsions at neutral pH, the presence of CAS and BLG both resulted in increased EGCG stability. The differing effects of EGCG oxidation in the presence of proteins occurred due to the presence of Tween micelles when emulsions were Tween-stabilized, which
acted to protect EGCG from oxidation, though a lower aqueous CAS content in CAS-stabilized emulsions may also play a role.

In studies with the same emulsion conditions, I demonstrated that proteins are capable of slowing EGCG oxidation under neutral and acidic conditions, which translated to a reduction in lipid oxidation even in the presence of EGCG at acidic pH. The effective concentration of solvent accessible Met and Cys residues was found to be an important factor, as higher concentrations of accessible Met and Cys residues yielded better peroxide scavenging activity and EGCG stability. Oxidized proteins showed considerably slower peroxide scavenging activity due to reduced accessible Met concentration, and potentially increased difficulty to access the remaining Met residues. Solvent accessible Met content also played a role in preventing phenolic promoted lipid oxidation, since a lack of accessible Met and Cys residues at low BLG concentrations may have led to the loss in BLG plus EGCG antioxidant activity on lipid oxidation. This suggests that the substitution of a protein with more solvent accessible Cys and Met may garner better antioxidant activity on lipid oxidation in emulsions. CAS would be a good candidate, though the pre-treated CAS samples were not readily soluble at acidic pH.

Detangling the effects of phenolic-protein binding and solvent accessible Cys and Met residues may also be useful, since protein modifications such as denaturing may impact both factors. Short chained peptides or proteins that do not bind phenolics may be used to investigate whether phenolic stability is still improved in the absence of binding. Similarly, the ability of protein-phenolics to preferentially bind and inhibit the formation
of EGCG-metal-O₂ ternary complexes may be interesting. A quick experiment may be performed at neutral pH where EGCG and Fe form a purple complex. If the addition of protein decreases the concentration of the purple complex, the protein interferes with the EGCG-Fe complex, which may inhibit phenolic oxidation. Finally, the analysis of other phenolics may also be performed. As stated earlier, one study showed that EGC did not demonstrate increased stability with HSA at neutral pH, even though EGCG did. This may also be used to determine if there are any major structural characteristics of proteins or phenolics that help protein-phenolic binding.

Overall, proteins and metal chelators are both viable solutions to control the metal-catalyzed oxidation of phenolics which results in phenolic loss and reduced lipid stability at acidic pH. EDTA showed the best EGCG and lipid stability at acidic pH, while CAS showed significantly greater EGCG stability in emulsions at neutral pH compared to any of the metal chelators. A protein’s ability to inhibit phenolic and lipid oxidation also appears dependent on the accessible oxidatively labile amino acid content allowing room for improvement by selectively choosing proteins or denaturing proteins. Integrating the different solutions may also produce better results if multiple hurdles are placed to prevent phenolic oxidation.
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Appendix A: Evaluating novel high-methionine proteins as antioxidants

Methionine residues are important peroxide and radical scavengers, as I have demonstrated in previous chapters (Chapters 6 and 7). Compared to my method of pre-oxidizing Met residues, oxidation studies where additional Met residues are expressed in proteins have yet to be reported. However, researchers have performed the reverse where Met residues were removed and the new protein subjected to oxidative stress to analyze Met oxidation and enzyme activity (Ju, Lin, Chien & Hsu, 2000; Roger Chien, Hsu, Hu, Wang & Hsu, 2002; Weng, Zheng, Bao, Cai, Yin & Zou, 2009).

I embarked to synthesize proteins with step-wise increases in Met content as a novel approach to exploring protein antioxidant activity. Soluble and correctly folded BLG has been successfully expressed and purified from yeast (Kuwata, Era, Hoshino, Forge, Goto & Batt, 1999) and Escherichia coli (Ariyaratne et al., 2002; Ponniah, Loo, Edwards, Pascal, Jameson & Norris, 2010). Genes encoding for zero and two additional Met residues were synthesized by replacing solvent accessible alanine residues assessed according to an algorithm for determining solvent accessible surface area (Fraczkiewicz et al., 1998). Start codon (ATG) codes for Met and adds an additional Met residue to both proteins.
**BLG-0M:**

**MLIVTQTM KGLDIQKVAG TWYSAMAAS DISLLDMQSA PLRVYVEELK**
PTPEGDLEIL LQKWENGECAM QKIIAEKTK IPAVFKIDML NENKVLVLDT
DYKKYLLFCM ENSAEPQSL ACQCLVRTPDEVDDEALEKFD KALKALPMHI
RLSFNPTQLE EQCHI**

**BLG-2M:**

**MLIVTQTM KGLDIQKVAG TWYSAMAAS DISLLDMQSA PLRVYVEELK**
PTPEGDLEIL LQKWENGECAM QKIIAEKTK IPAVFKIDML NENKVLVLDT
DYKKYLLFCM ENSAEPQSL ACQCLVRTPDEVDDEALEKFD KALKALPMHI
RLSFNPTQLE EQCHI**

**Fig. A.1:** DNA synthesis for BLG B variant. Underlined alanine residues are solvent accessible assessed according to an algorithm for determining solvent accessible surface area (Fraczkiewicz et al., 1998). Start codon (ATG) codes for Met and adds an additional Met residue to proteins.

**Methodology**

**Gene Synthesis**

Two genes, BLG-0M and BLG-2M (primary sequence shown in **Fig. A1.1**), were synthesized by GenScript (GenScript USA Inc., Piscataway, NJ). The genes were synthesized between the Hind III restriction sites of pUC 57 plasmids. Codons were optimized by GenScript for *E. coli* expression. Plasmid number was increased by introducing plasmids into DH5-α competent cells by electroporation. Cells were plated onto LB-Amp agar, and single colonies were isolated and stored in a glycerol stock solution at -80 °C.
**Subcloning**

Genes were subcloned from pUC 57 vectors into the T7 promoter expression vector, pET 21(+) at the Hind III restriction site. Plasmids were used to transform DH5-α competent cells by electroporation. Cells were plated on LB-Amp agar and single colonies isolated for DNA sequencing. DNA sequencing was performed to verify correct gene sequence and correct insertion direction in pET 21(+). Primers used for PCR amplification of the gene in pUC 57 plasmid were GTAAAACGACGGCCAGTG and GGAAACAGCTATGACCATG. Primers used for PCR amplification of the gene in pET 21(+) plasmid were GCTAGTTATTGCTCAGCGG and TAATACGACTCACTATAGGG. Colonies with the correct gene insertion direction in pET 21(+) were stored in glycerol stock solutions at -80 °C.

**BLG Induction in *E. Coli***

Extracted pET 21(+) plasmids were used to transform SHuffle® T7 Express Competent *E. coli* cells (New England Biolabs, Ipswich, MA) according to NEB High Efficiency Transformation Protocol (C3029). Transformed cells were plated on LB-Amp agar and single colonies isolated and stored in glycerol solutions. A single colony was resuspended in LB-Amp media and incubated at 37°C until OD$_{600}$ reached 0.4 - 0.6. Cells were then induced with IPTG to achieve a final concentration of 0.4 mM IPTG in the media. Cultures were allowed to incubate overnight (~16 h) at ambient temperature.
BLG Confirmation by Western Blot

Samples were diluted 1:1 with Laemmli loading buffer (Bio-Rad Laboratories, Hercules, CA) and heated at 90 °C for 5 min. Samples (10 µL) was loaded onto BioRad Ready Gel Tris-HCl Precast Gels (12 well; 4-20% polyacrylamide gels). Running buffer was prepared from 10x Tris/Glycine/SDS buffer (BioRad). Gels were run for 2 h at 110 V. Proteins were transferred onto PVDF membranes at 80 V for 1 h in transfer buffer prepared with 2 parts methanol, 1 part 10x Tris/Glycine/SDS buffer, and 7 parts DDW. Membranes were blocked for 1 h with Odyssey blocking buffer (15 mL) under agitation and washed three times with PBS buffer (15 mL) for 5 min. Membranes were incubated in the dark overnight at 4 °C with the primary antibody (15 mL after 1:2500 dilution in blocking buffer). The primary antibody solution was removed and the membrane washed with 0.1% Tween 80 in PBS buffer three times for 5 min. Membranes were incubated for 0.5 h at ambient temperature under gentle agitation with the secondary antibody (15 mL after 1:15,000 dilution in blocking buffer). The secondary antibody was removed and the membrane washed three times with 0.1% Tween 80 in PBS buffer. Membranes were imaged using Odyssey instrument.
Fig. A.2: Western blot confirming BLG expression by SHuffle® cells. Wells 1 and 2 are BLG-0M after precipitation by 30% w/v NaCl, followed by running concentrated samples through a desalting column. Well 5 contained BLG-0M cell lysate without any purification.

Once sufficient protein has been purified from each treatment group, oxidation studies will be performed similar to the lipid oxidation studies above to determine the ability of the recombinant protein in preventing lipid oxidation. Recombinant protein-stabilized emulsions will be compared against a control with zero substitutions expressed in *E. coli*, BLG purified from milk, and a non-ionic surfactant stabilized o/w emulsion.
Appendix B: Influence of pH and Phenolic Concentration on Lipid oxidation in EGCG-containing Food Emulsions at All Time Points (Chapter 3 results)

(a)

Fig. B.1: Lipid oxidation markers (a) CD concentrations measured as change in Abs 234 and (b) TBARS in 5 wt% flaxseed emulsions with added EGCG (0-500 µM) and Fe^{3+} (25 µM) in 10 mM phosphate buffer pH 2.
Fig. B.1: (continued)
Fig. B.2: Lipid oxidation markers (a) CD concentrations measured as change in Abs$_{234}$ and (b) TBARS in 5 wt% flaxseed emulsions with added EGCG (0-500 µM) and Fe$^{3+}$ (25 µM) in 10 mM phosphate buffer pH 3.
Fig. B.2: (continued)
Fig. B.3: Lipid oxidation markers (a) CD concentrations measured as change in Abs$_{234}$ and (b) TBARS in 5 wt% flaxseed emulsions with added EGCG (0-500 µM) and Fe$^{3+}$ (25 µM) in 10 mM phosphate buffer pH 4.
Fig. B.3: (continued)
Fig. B.4: Lipid oxidation markers (a) CD concentrations measured as change in Abs$_{234}$ and (b) TBARS in 5 wt% flaxseed emulsions with added EGCG (0-500 µM) and Fe$^{3+}$ (25 µM) in 10 mM phosphate buffer pH 5.
Fig. B.4: (continued)
Fig. B.5: Lipid oxidation markers (a) CD concentrations measured as change in Abs$_{234}$ and (b) TBARS in 5 wt% flaxseed emulsions with added EGCG (0-500 µM) and Fe$^{3+}$ (25 µM) in 10 mM phosphate buffer pH 6.
Fig. B.5: (continued)
Fig. B.6: Lipid oxidation markers (a) CD concentrations measured as change in Abs$_{234}$ and (b) TBARS in 5 wt% flaxseed emulsions with added EGCG (0-500 µM) and Fe$^{3+}$ (25 µM) in 10 mM phosphate buffer pH 7.
Fig. B.6: (continued)
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