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PHYSICOCHEMICAL AND FUNCTIONAL PROPERTY MODIFICATION OF MYOFIBRILLAR PROTEIN BY PHENOLIC COMPOUNDS UNDER OXIDATIVE STRESS

Anqi Guo
University of Kentucky, guo.anqi@outlook.com
Author ORCID Identifier:
https://orcid.org/0000-0001-7571-7207
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Anqi Guo, Student

Dr. Youling L. Xiong, Major Professor

Dr. David Harmon, Director of Graduate Studies

PHYSICOCHEMICAL AND FUNCTIONAL PROPERTY MODIFICATION OF MYOFIBRILLAR PROTEIN BY PHENOLIC COMPOUNDS UNDER OXIDATIVE STRESS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

Anqi Guo

Lexington, Kentucky

Director: Dr. Youling L. Xiong, Professor of Animal and Food Sciences

Lexington, Kentucky

2021

https://orcid.org/0000-0001-7571-7207

ABSTRACT OF DISSERTATION

PHYSICOCHEMICAL AND FUNCTIONAL PROPERTY MODIFICATION OF MYOFIBRILLAR PROTEIN BY PHENOLIC COMPOUNDS UNDER OXIDATIVE STRESS

Polyphenol-rich spices and extracts of phenolic compounds are widely utilized in meat processing to modify product flavors. Chemically, polyphenols are reactive with myofibrillar protein (MP), the most functional fraction of all muscle proteins responsible for texture development in comminuted meat products. Such protein–polyphenol interaction is prevalent under oxidative conditions that are common in meat processing. As a large group of phytochemicals with diverse structures, phenolic compounds are known to interact with MP with varying efficacies. Yet, the structure-function relationship of polyphenols in eliciting modification of MP is poorly understood. The overall objective of this dissertation research was to elucidate the effect of structurally related phytophenols on the physicochemical properties of MP and resultant changes in protein functionalities, i.e., gelation and emulsification.

To establish appropriate testing conditions, a mild oxidative environment was introduced using glucose oxidase (GOx), and the simplest phenolic compound, gallic acid (GA), was used to investigate the effect on the physicochemical and gelling behavior of MP. Compared with non-oxidized (control) MP, GOx-mediated oxidation facilitated both covalent and noncovalent interactions between GA (6, 30, and 60 μmol/g protein) and protein through promoting protein structural unfolding. Such modifications significantly enhanced the gelling capacity of MP, which was evidenced by up to 86% and 53% increases ($P < 0.05$) in gel elasticity (G′) and breaking strength, respectively.

Based on the above observations, six structurally related monophenolic acids varying in hydroxyl substitution and sidechain groups, i.e., GA, syringic acid (SA), coumaric acid (CMA), caffeic acid (CFA), ferulic acid (FA), and chlorogenic acid (CA), were examined for their effects on MP conformation and gelation under GOx oxidative stress. The elasticity and breaking strength of MP gels were markedly enhanced by all phenolic acids, of which GA and CA induced the highest final G′ values of 291 and 281 Pa ($P < 0.05$), respectively, as compared with 214 Pa of the control MP sample without phenolic addition. Different reaction modes were evident for these two most effective phenolic acids in improving protein gelation. With the least structural hinderance, the

smallest GA facilitated protein cross-linking through covalent adduction to amino acid sidechains. On the other hand, having a bulky sidechain group, CA was the most effective in promoting protein unfolding due to the multiple functional groups, including 5 hydroxyl groups and 1 extra hydrocarbon ring (quinic acid). The findings of structure-dependency of phenolic activity prompted the following experiment where phenolic compounds with more than one phenol structures were included to investigate their influence on MP functionalities.

Here, in addition to three monophenols, i.e., GA, CA, and propyl gallate (PG), two diphenols, i.e., quercetin (QT) and catechin (CC), and one triphenol, i.e., (–)-epigallocatechin-3-gallate (EGCG), were selected to further explore the structure-activity relationship of phenolic compounds on MP functionalities under GOx oxidation. MP-stabilized oil-in-water emulsions were prepared to assess protein emulsifying properties, and an emulsion-filled composite gel system was adopted as a model to mimic comminuted meat products in which MP acted as both an emulsifier and a building block for the protein matrix within the gel. In the emulsion system, phenolic compounds with less polarity, i.e., PG, QT, and CC, significantly improved the emulsifying capacity of MP by increasing protein partition at the oil-water interface by 15, 17, and 23%, respectively ($P < 0.05$). In the MP–emulsion composite gel system, all three monophenols (GA, CA, and PG) and the diphenol QT increased the MP gel strength to a greater extent than CC (diphenol) and EGCG (triphenol). The flavanol structure in CC appeared to interfere with gel structure development. The multiple phenol structures in EGCG caused protein aggregation so severe that both emulsifying and gelling properties of MP were weakened. Lipid oxidation was retarded by all phenols in MP–emulsion composite gels during storage at 4 °C for 7 days with PG and QT being the most effective.

The above findings established that the type and size of the sidechain groups, the number of hydroxyl attached to the benzene ring, as well as the number of the phenol moiety have an important role in affecting phytophenol–MP interaction and the protein functionality under oxidative condition. Small-sized phenolic compounds tend to promote MP gelation and emulsification, and larger sized (such as EGCG) exhibited negative effects due to the propensity to facilitate extensive protein aggregation.

KEYWORDS: Myofibrillar Protein, Phenolic compound, Gelation, Texture, Lipid Oxidation

Anqi Guo

(Name of Student)

06/15/2021

Date

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By
Anqi Guo

Dr. Youling L. Xiong
Director of Thesis

Dr. David Harmon
Director of Graduate Studies

06/15/2021
Date

DEDICATION

To my parents for their unconditional love and support

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CHAPTER 1. INTRODUCTION

Fresh and processed meats are an important part of the human diet in modern society, providing high-quality protein with a well-balanced amino acid profile as well as abundant micronutrients, including vitamin B_{12} and iron (Wood, 2017). The characteristic texture, mouthfeel, and flavor, which are unique to muscle tissue, are other essential contributing factors to its appeal (Romans, Costello, Carlson, Greaser, & Jones, 2000). However, muscle foods are prone to quality deterioration due to chemical and biological reactions, of which lipid oxidation is a primary mechanism when microbial spoilage is prevented. When the membrane of muscle cells is disrupted during processing, oxidation is initiated where prooxidants attack the double bonds in unsaturated fatty acids, which produces lipid peroxyl radicals and other oxygen species through free-radical chain reactions (Campo et al., 2006). Radical-initiated lipid oxidation generates secondary products, such as aldehydes, ketones, and esters, which can have a negative impact on the sensory and nutritional properties of meat products (Domínguez et al., 2019). Oxidative rancidity, warmed-over flavors, discoloration, destruction of vitamins, and formation of atherogenic toxins are common consequences of lipid oxidation (Kanner, 1994; Suman & Joseph, 2013).

Antioxidants are a primary means to counteract undesirable changes induced by lipid oxidation in muscle foods (Ribeiro et al., 2019). Of different types of antioxidants available to meat processors, plant-derived phenolic compounds have become an increasingly attractive choice due to their wide abundance, efficacy, and perceived health benefits (Fernandes, Trindade, & Melo, 2018; Shah, Bosco, & Mir, 2014). Phenolic compounds have the basic structure of an aromatic ring attached by one or more hydroxyl

groups with diverse variations ranging from simple phenols to polymerized complex molecules. In addition to inhibiting lipid oxidation, polyphenols may modulate the oxidation of another major component in muscle foods: proteins.

Phenolic compounds can interact with proteins directly through either non-covalent interactions (reversible) or covalent bonds (irreversible). The latter usually occurs in an alkaline or oxidizing environment where the phenol ring is converted to a quinone form (Ozdal, Capanoglu, & Altay, 2013). The interaction between myofibrillar protein (MP) and phenolic compounds and its effect on protein functionality has been the subject of extensive investigations in recent years. Cao and Xiong (2015) applied chlorogenic acid and a Fenton-mediated oxidation system to modify MP, reporting that the gelling capacity of MP was significantly enhanced by 6–30 μmol/g of the phenolic compound. Li, Liu, Liu, Kong, and Diao (2019) claimed that emulsifying activity of MP was improved by the addition of sage extract. Jongberg, Terkelsen, Miklos, and Lund (2015) discovered that high dosages of green tea extract diminished the textural stability of meat emulsions. It is plausible that the diverse structure attributes of phenolic compounds are responsible for their various effects on the physicochemical and functional properties of MP. However, the role of phenolic structures in the protein–phenol interactions remain unclear.

The aim of this research was to evaluate MP modification by phenolic compounds and elucidate the interaction mechanisms as affected by phenolic structures and oxidation. To simulate the commonly occurring oxidation stress in muscle food processing and convert phenolic species to their more reactive quinone derivatives, glucose oxidase (GOx)-mediated oxidation was used to provide a mild oxidative environment where free

radicals were produced in a progressive and controllable manner. The specific objectives of my dissertation research were:

1. To investigate the impact of gallic acid (GA) on the gelling properties of MP under GOx-induced oxidation.
2. To elucidate the effect of structure-activity relationship of phenolic acids on their roles in modifying physicochemical and gelling properties of MP.
3. To evaluate and understand the effect of six structurally related mono-, di-, and triphenols on the structural properties of MP–emulsion composite gels.
4. To study the effect of six structurally related phenolic compounds on the emulsifying properties of MP.

CHAPTER 2. LITERATURE REVIEW

2.1 Myofibrillar protein (MP) and its functionalities

In muscle foods, proteins have a primary role in the structure-forming process of cooked products. In particularly, gelation and emulsification are responsible for the fine texture, sliceability, water-binding, and adhesion properties of a variety of processed meats, such as frankfurters, bologna, and restructured ready-to-eat deli-style meat (Acton, Ziegler, Burge, & Froning, 1983). Of the three groups of muscle proteins (i.e., myofibrillar, sarcoplasmic, and stromal proteins), myofibrillar protein (MP), especially the myosin component, has been subjected to extensive studies due to its abundance in muscle tissue and structural role in processed meat (Asghar, Morita, Samejima, & Yasui, 1984).

As shown in Figure 2.1, myosin is a fibrous and hexametric molecule composed of four light chains (15–27 kDa) and two identical heavy chains (200 kDa each) (Harrington & Rodgers, 1984). The rich content of sulfhydryl groups in myosin, over 40 in total (Reisler, 1982), gives rise to its excellent functionalities, including gelation and emulsification (Xiong, 1994). When thermally processed, as in the case of commercial production of comminuted meat products, individual MP molecules will undergo a series of structural changes leading to denaturation, aggregation, cross-linking, and ultimately, the formation of a three-dimensional gel network (Xiong, 1997). This process is critical to the textural properties of resultant muscle foods (Sun & Holley, 2011). Being the principal functional component in MP, thermal aggregation of myosin through head-head or tail-tail association has been widely studied. MP are mostly amphiphilic macromolecules comprised of hydrophilic and hydrophobic amino acid residues suitable for the formation of the interfacial membrane in emulsions. In an oil-in-water (O/W) meat emulsion, fibrous

myosin molecules are adsorbed as a monolayer at the interface where their nonpolar head anchors in the oil phase and the hydrophilic tail remains in the aqueous phase to thermodynamically stabilize the system (Jones & Mandigo, 1982). Such structural orientations are applicable to other myofibrillar components. The membrane formed in a typical meat emulsion is unique in that the monolayer of myosin is overlaid with a relatively thick layer of additional MP (Gordon, Barbut, & Schmidt, 1992).

2.2 Natural phenolic compounds applied in meat processing

Substances from plant materials, such as dry herbs and smoke, have been used to preserve meat since prehistoric times although the underlying mechanisms were not understood (Berdahl, Nahas, & Barren, 2010). It is now recognized that among the active compounds responsible for such preservative effects are phenolic substances. As ubiquitous secondary metabolites in plants, phenolic derivatives can promote plant growth and health by imparting a self-defense mechanism, which includes chemical reactions to protect against pathogens, parasites, and predators (Nayak, Liu, & Tang, 2015). Naturally occurring phenolic antioxidants are extremely diverse in chemical forms; representative categories include phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Shahidi & Ambigaipalan, 2015). Even within the same category, there are numerous derivatives that vary in size and substituent groups in the aromatic rings. Indeed, over 8000 phenolic species have been discovered and the list is growing (Fernandes et al., 2018).

In meat processing, phenol-rich herbs, seeds, and spices are commonly incorporated into the product formulation to enhance and complement flavor. This practice imparts an added benefit: the inhibition of oxidation. A myriad of phytophenols have been

identified in various plant materials commonly used in muscle food processing, e.g., catechin acid present in cinnamon, gingerol in ginger, gallic acid and eugenol in clove, and rosmarinic acid (RosA) in rosemary, basil, sage, oregano, and thyme (Milevskaya, Temerdashev, Butyl′skaya, & Kiseleva, 2017; Shan, Cai, Sun, & Corke, 2005). However, in these plant materials, the concentration of phenolic compounds is generally low, affected by farming conditions, post-harvest processing, handling, and storage (Fernandes et al., 2018). Therefore, the use of pure phytochemicals is extremely expensive. Consequently, plant extracts, i.e., mixtures of phytophenols with different structures and activities, are commonly used instead (Wu et al., 2013). Active antioxidants can be extracted from different parts of a plant, including leaves, roots, stems, fruits, seeds, and bark. It is both environmentally friendly and economically viable to extract phenolic materials in this way to produce value-added ingredients from the waste and by-products of plant materials generated during processing (Galanakis, 2018; Lorenzo et al., 2018). Because more than one active antioxidative compound is extracted, plant phenolic extracts are typically complex mixtures of phytophenols consisting of individuals with different structures and activities. The antioxidant activities of plant extracts have undergone extensive studies in recent decades. Several have been commercially available as natural antioxidants from sources such as grape seed, tea, and rosemary (Shah et al., 2014). The antioxidative efficacy of phytophenols has been found to be affected by their chemical structures. For example, the hydrogen atoms from the adjacent hydroxyl groups (ortho-diphenol) promote, whereas glycosylation inhibits, the antioxidant activity of phenolic compounds (Minatel et al., 2017; Shahidi & Ambigaipalan, 2015).

2.3 Interaction of phytophenols with MP

The interactions between MP and plant phenols are governed by multiple mechanisms. Both reversible, non-covalent and irreversible, covalent bonds are involved, providing the driving force of such interactions in meat products and delivering measurable impact on muscle protein functionality and texture-related meat quality. Analytical measurement of these interactions can be focused on changes in protein amino acid side chains (e.g., -SH and -NH groups), conformation (e.g., surface hydrophobicity and intrinsic fluorescence), and formation of protein–phenol adducts (e.g., electrophoresis and mass spectrometry) (Table 2.1).

2.3.1 Non-covalent interactions

Four different types of non-covalent bonds can be formed between MP and phenolic compounds: hydrogen bonding, hydrophobic association, electrostatic attraction, and van der Waals forces. The relative contributions of these forces are related to the molecular structure of phenolic compounds, amino acid side chains of MP, polarity of the phenols, and processing conditions such as pH and redox status (Ozdal et al., 2013). Several proposed non-covalent interactions and the bonds involved are shown in Figure 2.2 (Rohn, 2019; Strauss & Gibson, 2004). Hydrogen bonding is a dipole-dipole attraction occurring between an electronegative atom and a hydrogen atom bonded to another electronegative element such as N, O, and S. Thus, hydroxyl groups in a phenolic compound can form hydrogen bonds with protein sidechain carboxylic groups, amine groups, and sulfhydryls. Where steric hindrances are absent, phenolic hydroxyl groups may also form hydrogen bonds with peptide bonds (C=O or -NH).

Hydrophobic association is the tendency of nonpolar molecules in a polar solvent (usually water) to interact with one another to achieve thermodynamic stability. In the case of MP–phenol interaction, this could be formed through π-bonded complexes by overlapping hydrophobic amino acid side chains in MP, such as phenylalanine, and the aromatic ring structure in phenols. This is thought to be especially relevant for polyphenols with three or more phenol rings, such as tannins (Prigent et al., 2003). Electrostatic forces are the interaction between charged groups, which could occur between acidic or basic amino acid side chain groups in MP and negatively charged phenoxide ions after the oxidation of phenols. van der Waals interactions are attractive or repulsive forces formed through dipoles or induced dipoles via polarization of the electron cloud when atoms from phenol rings or phenolic side chains are in the proximity of protein backbones or polar amino acid side chains (Buitimea-Cantúa, Gutiérrez-Uribe, & Serna-Saldivar, 2018).

2.3.2 Covalent interactions

Irreversible interactions occurring between phytophenols and muscle proteins can be characterized by the formation of covalent linkages which usually take place in an oxidative environment. At alkaline pH, phenols can be readily oxidized to quinone derivatives that are capable of forming covalent bonds with MP. The mechanisms involved in such irreversible interactions are depicted in Figure 2.3. The orthoquinone structure with unsaturated diketone groups are deficient in electrons, thus, can oxidize cysteines to promote disulfide formation. In this process, it is reduced back to the phenolic form (eq. 1). Alternatively, the electrophilic quinone, being a reactive intermediate, is easily attacked by nucleophilic amino acid, such as cysteine, in a protein chain (Kroll, Rawel, & Rohn, 2003; Rohn, 2019). After covalent binding with a cysteine molecule (eq. 1'), the phenol

moiety could be regenerated, re-oxidized, and subsequently bound to another cysteine side chain, forming a protein–phenol–protein cross-link as shown in eq. 2 (Rysman, Van Hecke, De Smet, & Van Royen, 2016). In another possible pathway (eq. 3), two quinone–myosin adductions can dimerize through covalent linkages between quinones and produce a cross-link through diphenols, as proposed by Strauss and Gibson (2004). Therefore, a reduction in the quantity of the nucleophilic amino acid side chain, cysteine, can be viewed as indirect evidence for the presence of protein−quinone covalent adduction. Significant reductions in sulfhydryl (-SH) content with increasing amounts of treatment phytophenols have been reported in several studies (Cao, True, Chen, & Xiong, 2016; Jia, Wang, Shao, Liu, & Kong, 2017; Wang et al., 2018). In those assays, non-covalent interactions are disrupted by sodium dodecyl sulfate (SDS) or urea, so that changes in the quantity of these groups can be assigned to covalent bonds between quinone and reactive amino acid groups in proteins.

Covalent interactions between MP and phytophenols are likely to form cross-linked protein polymers through mechanisms shown in Figure 2.3. Myosin, the most abundant and functional constituent in MP, is known to exhibit modified cross-linking properties upon the reaction with phytophenols (Choi & Kim, 2009). Experimentally, the cross-linking can be detected by electrophoresis, which is a simple yet powerful tool to separate proteins based on their molecular weights. Quinone-induced protein polymers have higher molecular weights so their presence can be evidenced by the reduction in myosin heavy chain band and the concomitant appearance of bands in the stacking gel (Guo & Xiong, 2019; Prodpran, Benjakul, & Phatcharat, 2012). Furthermore, to differentiate between disulfide protein–protein and quinone-mediated cross-links, reducing reagents are used to

cleave disulfide bonds so polymers formed through covalent MP–phenol adduction can be revealed. To semi-quantitatively determine the number of thiol groups covalently attached to MP, a thiol-specific staining, 5-iodoacetamidofluorescein (IAF), has been used to stain and visualize unreacted free sulfhydryl groups (Jongberg et al., 2015).

2.4 Effect of phytophenol binding on MP gelation

The interaction with phytophenols could modify the above physicochemical properties, thus, affecting the association and cross-linking of protein molecules in the gelation process. Several factors affecting protein–phytophenol interaction and the impact on protein gelation have been investigated, which include phenol concentration and type, oxidation, salt content, and the co-presence of other additives.

2.4.1 Impact of oxidation

The conditions under which muscle proteins and phytophenols interact are important for the outcome of protein gelation, and oxidation is one of recognized factors. Oxidation not only facilitates the formation of quinone derivatives and protein–quinone adducts but also modifies protein–protein interactions *per se*. Oxidized amino acid side chains in MP contribute to protein cross-linkages through disulfide bonds, dityrosine, the Schiff's base, and other intermolecular bridges that affect protein aggregation and polymerization (Zhang, Xiao, & Ahn, 2013). Phenolic compounds can mediate these interactions by acting as anti- or pro-oxidants, depending on their chemical structure and concentration (Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008). A specific myosin-phenol binding mechanism is proposed in Figure 2.3. Non-covalent interactions are dominant in non-oxidizing environments. However, under oxidative stress, covalent

interactions occur when phenol molecules are converted to their more reactive quinone derivatives.

Several methods have been employed to introduce oxidative stress in the study of MP–phenol interactions and consequential structure-related functionality. They include Fenton oxidation (Cao & Xiong, 2015; Chen, Diao, Li, Chen, & Kong, 2016; Feng et al., 2017; Jia et al., 2017; Tang et al., 2017), enzymatic oxidation (Guo & Xiong, 2019; Vate & Benjakul, 2016a), malondialdehyde (MDA)-mediated oxidation (Lv et al., 2019; Lv et al., 2020), and processing-induced oxidation (Jiang et al., 2020; Pan et al., 2020). A general conclusion based on these investigations is that mild oxidation promotes gelation while strong oxidation jeopardizes the process (Figure 2.5). Fenton reaction has been the most commonly used oxidation method because •OH radicals are readily generated through the reaction between H_2O_2 and Fe^{2+}. In a study conducted by Chen et al. (2016), MP were mixed with phenol-rich clove extract in a Fenton reaction system, and the gelling properties were compared at different incubation times. The oxidation was so strong that MP insolubilization and aggregation occurred, leading to a poor gel texture. Although clove extract provided some protective antioxidant effect, it was inadequate to counteract the overall negative impact induced by oxidation, especially during a long incubation time of 5 h.

Compared to chemical oxidation, enzyme-mediated oxidation is moderate, controllable, and has the propensity to enhance the gel-forming of MP. This is largely due to the progressive nature of H_2O_2 production and its conversion to •OH radicals to allow gradual protein structure unfolding and exposure of reactive groups in the process of building an ordered protein gel network (Wang, Xiong, Sato, & Kumazawa, 2016). The

rate at which protein matrices in a gel are formed is critical to the gel properties (Ziegler & Foegeding, 1990). As reported recently, in the presence of 60 μmol gallic acid, the gelling capacity of MP was markedly promoted by glucose oxidase for up to 86% in terms of storage modulus (G′) and 53% in gel strength, when compared with non-oxidized samples where the phenolic acid showed no remarkable effect (Guo & Xiong, 2019). Similarly, squid ink tyrosinase, a polyphenol oxidase, successfully improved gelling properties of fish surimi incubated with tannic acid (Vate & Benjakul, 2016b).

Protein and lipid are usually coexistent in muscle food systems, therefore, MP are inevitably susceptible to modification by lipid oxidation products, such as MDA. Lv et al. (2019) compared textural properties of MP emulsion gels treated with varying concentrations of EGCG and MDA. They found that low and intermediate levels of MDA (3 and 6 mM) facilitated the gelation, but this effect was diminished by the presence of 0.5, 1.0, and 2.0 mM EGCG. Interestingly, at a high concentration of MDA (12 mM), where the quality of the MP emulsion gels was otherwise decreased, the addition of 0.5, 1.0, and 2.0 mM EGCG provided a protective effect on MP gelation under the oxidative condition. In a subsequent study, MP gels treated with an even higher MDA concentration (24 mM) showed a poor structure and the addition of EGCG at the same concentrations was found to exacerbate the textural deterioration caused by MDA (Lv et al., 2020).

As processing techniques, ultraviolet irradiation (UVA) and ultrasonication have been applied to initiate free radical formation for the study of gelation of modified MP in the presence of phytophenols. Jiang et al. (2020) reported that under the treatment with the same amount (625 μmol/g protein) of phlorotannin extracts (PTE), the gel strength of UVA-irradiated MP was 74.97% higher than the control sample without irradiation. In the

same study, free radicals were only detected in UVA irradiated and PTE-treated MP and not in the control samples with only UVA or PTE, indicating that both UVA irradiation and PTE are required to generate and maintain free radicals. Similarly, ultrasonication, which produced ˙H, drastically increased gel storage modulus (G′) from 410 Pa to 14137 Pa in MP treated with 5 μmol/g of gallic acid (Pan et al., 2020). Mass spectrometry analysis confirmed the formation of covalent adduction of oxidized GA to cysteine and lysine residues in proteins, which likely contributed to the improved gelling properties.

In model systems without an artificially simulated oxidative condition, phenolic compounds are still able to modify MP gelling properties through non-covalent and possibly covalent interactions due to their anti-/pro-oxidative activities and auto-oxidation *in situ*. Jia et al. (2017) investigated the effect of catechin on MP gelling properties and reported that it caused an approximate 42% reduction in water-holding capacity and 80% loss in MP gel strength. Similar results were obtained by Cao, Ai, True, & Xiong (2018) who showed that EGCG drastically decreased gel strength, increased cooking loss, and reduced the viscoelasticity of emulsified MP gels in the absence of artificial oxidants. Moreover, Balange and Benjakul (2011) tested the effect of tannins on the gel strength of fish surimi gel without added oxidants but at various pH (3, 7, 8, and 9). They showed that alkaline pH significantly enhanced gel breaking force due to the interaction with quinones generated by the deprotonation of phenolic hydroxyl groups. At acidic or neutral pH, few quinone molecules are produced because the hydroxyl groups remained mostly in the reduced forms, hence, no improvement for surimi gelation was observed.

Therefore, in research aimed at elucidating the impact of oxidation on polyphenol-mediated MP gelation, it is important to not only include proper control(s), e.g., systems

free of oxidation, but also consider both the type and the potency of artificially produced oxidative conditions when simulating or mimicking the indigenous oxidation stress normally encountered in muscle food production. Moreover, it is necessary to evaluate the extent of quinone self-polymerization prior to, as well as during, the interactions with MP, because modification of the phenolic structures will conceivably affect their association with proteins.

2.4.2 Influence of concentration and type of phytophenols

During thermal gelation, MP begins with unfolding first to expose binding sites then aggregation follows through protein–protein interactions leading to gel matrix formation and development. To form an isotropic protein gel network, it is important that protein unfolding proceeds slowly enough to allow denatured protein molecules to orient and interact before the initiation of aggregation (Xiong, 1994). Therefore, moderate protein unfolding is beneficial for gelation while abrupt or severe unfolding is conducive to random aggregation thus hindering gel network formation. The extent of MP modification and the properties of resultant gels are dependent on the application level of phenolic compounds. In general, unfolding and cross-linking of MP at moderate phenolic treatment concentrations may aid in protein gelation due to the facilitation of orderly protein aggregation. However, if a large amount of polyphenols is added, the superfluous phenols would not only disrupt the structure of MP but also shield the reactive amino acid side chains (such as thiol and free amine), thereby interfering with gelation. Such concentration-dependent "dual effects" have been observed in several studies. As an example, Cao et al. (2016) reported that 6 and 30 μmol/g GA significantly improved the elasticity and hardness of MP gels while 150 μmol/g impaired gelation due to excessive aggregation and

insolubility of proteins promoted by the high concentration of GA. Similar dose-dependence phenomena have been noted for chlorogenic acid and tea polyphenols (Cao & Xiong, 2015; Li et al., 2020a). The effects of different concentrations (0.05, 0.25, and 1.25 mM) of RosA on the gelling behavior of MP as well as the characteristics of covalent MP–phenol adducts were evaluated by Tang et al. (2017). At low-to-medium concentrations (0.05 and 0.25 mM RosA), RosA–Cys and Arg/His–RosA–Cys adducts were formed, exerting a positive effect on the rheological attributes of the MP gels. At high RosA concentration (1.25 mM), however, RosA–Cys became the dominant type of cross-link, and the extensive blockage of the thiol groups (an important force in MP gels) impeded gel formation and produced a poor gel texture.

In some reported studies, the effect of phytophenols on MP gelation only followed a single trend (promoting or suppressing) rather than both because either the particular phenols are inherently detrimental (or beneficial) to protein gelation or the optimal dosage level had not been attained in the concentration range tested. The specific structures of the phenolic compounds have a major influence on their functionality, hence, their unique concentration-dependence pattern. Epicatechin and EGCG are flavonoids with different numbers of phenol and hydroxyl groups. Li et al. (2019) reported that the elasticity (G′) of MP gels was positively correlated with epicatechin at concentrations of 0.1, 0.2, 0.5, 1, and 2 mM. However, EGCG was found to impair MP gelation in the concentration range 10, 50, 100, and 200 μmol/g protein from a prior study (Jia et al., 2017) and at 50, 100, 200, 500, and 1000 mg/L in another study (Cao et al., 2018). Variations among polyphenols in their efficacy are conspicuously related to the structural diversity as manifested by differences in molecular weight, hydrophobicity, molecular flexibility, methylation,

hydroxylation, glycosylation, and other attributes (Yildirim-Elikoglu & Erdem, 2018). The strong dose-dependence effect entails the establishment of an appropriate working concentration for an individual phenolic compound intended as a functional additive to produce a desired level of protein modification. A condition that favors MP gelation would allow an interactive internal structure and well-textured meat product. Continuing research is required to elucidate the underlying mechanisms and the structure–activity relationship of phenolic compounds applied to muscle food processing.

Although the presence of certain high amounts of phytophenols can be detrimental to MP gelation, their widely recognized antioxidant potential provides the incentive for developing strategies to minimize the undesirable effects on protein gelation and structure-forming potential. Cyclodextrins (CDs), a family of natural cyclic oligosaccharides with a high affinity for polyphenols, have been added to bind with excessive polyphenols and mitigate physicochemical deteriorations associated with extensive MP–phenol interaction. Zhang et al. (2018a) found that methyl-β-cyclodextrin (M-β-CD) was effective in increasing the polyphenol loading capacity by interfering with protein–phenol interactions. The increased cooking loss of the MP emulsion gel caused by high concentrations of EGCG was offset by the addition of M-β-CD in a dose-dependent manner. At the highest load of M-β-CD (160 μmol/g), the cooking loss was reduced to a similar level as the control. In a subsequent study (Zhang et al., 2018b), three chemically related cyclodextrins, namely, M-β-CD, β-cyclodextrin (β-CD), and (2-Hydroxypropy l)-β-cyclodextrin (2HP-β-CD), were compared for their ability to mitigate the negative impact of EGCG on MP gelation. It was found that at the application level of 80 μM/g protein, the CDs reduced cooking loss caused by EGCG and improved the structure of MP emulsion gel in the order of M-β-

CD>2HP-β-CD>β-CD. The amphiphilicity of CDs coupled with the entrapping cyclic structure enabled the formation of CD–EGCG complexes through non-covalent association, thus, disrupting interactions between EGCG and proteins. Methyl and hydroxypropyl in the substituent groups increased hydrophobicity of CD so its binding affinity for EGCG was enhanced through hydrophobic forces.

2.5 Effect of phytophenol binding on MP emulsification

Polyphenol-induced physicochemical changes of MP could affect their ability to form an interfacial membrane, hence, emulsifying properties that play an important role in the textural properties of emulsified meat products. Indeed, both physicochemical and anti- or pro-oxidative changes induced by phenolic compounds were found to modify the emulsifying properties of MP (Estévez et al., 2008). Compared to control protein, a MP–phenol complex tends to be more easily adsorbed onto the oil/water interface and increases the surface charge of the protein-membrane (Figure 2.4). This often leads to stronger electrostatic repulsions between particles hence a more stable emulsion with smaller oil droplets dispersed in the aqueous phase (Cheng, Zhu, & Liu, 2020; Li et al., 2019). Sage extract (100 μg/mL) increased the emulsion zeta potential (negative) from –18.7 mV to –37.7 mV, and a thicker and more compact interfacial protein membrane which stabilizes oil droplets was revealed under the microscope (Li et al., 2019). On the other hand, the presence of certain large polyphenols, such as EGCG, especially in large quantities, can be detrimental to the emulsifying properties of MP. This is because extensive protein unfolding and aggregation incurred would result in less protein being adsorbed to the interfacial membrane (Cao et al., 2018; Feng et al., 2017). Nonetheless, in a moderate-to-

strong oxidizing environment, EGCG can act as an antioxidant to inhibit protein aggregation and polymerization caused by oxidation, producing more stable emulsions (Lv et al., 2019). Therefore, the role of EGCG seems to be complex subjecting to the specific meat processing conditions.

To mimic the composition of emulsified meat products, including frankfurters, bologna, and a wide variety of comminuted luncheon meats, emulsion composite gel model systems are frequently used in the investigation of the effect of phytophenols on those products (Feng et al., 2017; Lv et al., 2019; Wang et al., 2018). In these complex, heterogeneous systems, the physicochemical and structure-forming properties of phytophenol-modified MP are found to be affected by and can be assigned to the individual behavior of emulsified fat droplets and the continuous protein gel matrix. For example, the presence of 12 μM and 60 μM RosA modified functional amino acid side chains (free amine and sulfhydryl) and enhanced cross-linking of MP. Consequently, a firmer and more elastic MP composite gel was produced, in which smaller oil droplets were dispersed in a strengthened protein network (Wang et al., 2018). On the other hand, excessive protein aggregation and reduced surface hydrophobicity caused by the addition of 1000 ppm EGCG impaired the textural properties of MP composite gels with 40.5% cooking loss, versus 4.3% for the control (Feng et al., 2017). The microstructure of these composite gels exhibited oil coalesced into larger droplets and a high-degree shrinkage of the gel matrix in the continuous protein phase.

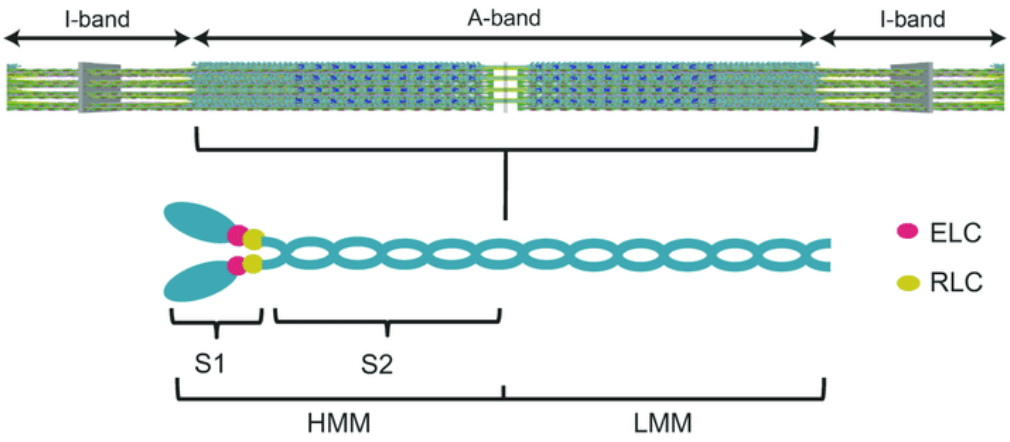


Figure 2.1. Diagram of the muscle sarcomere and the myosin molecule. HHM: heavy meromyosin, LLM: light meromyosin, ELC: essential light chain, RLC: regulatory light chain (Tang et al., 2017).

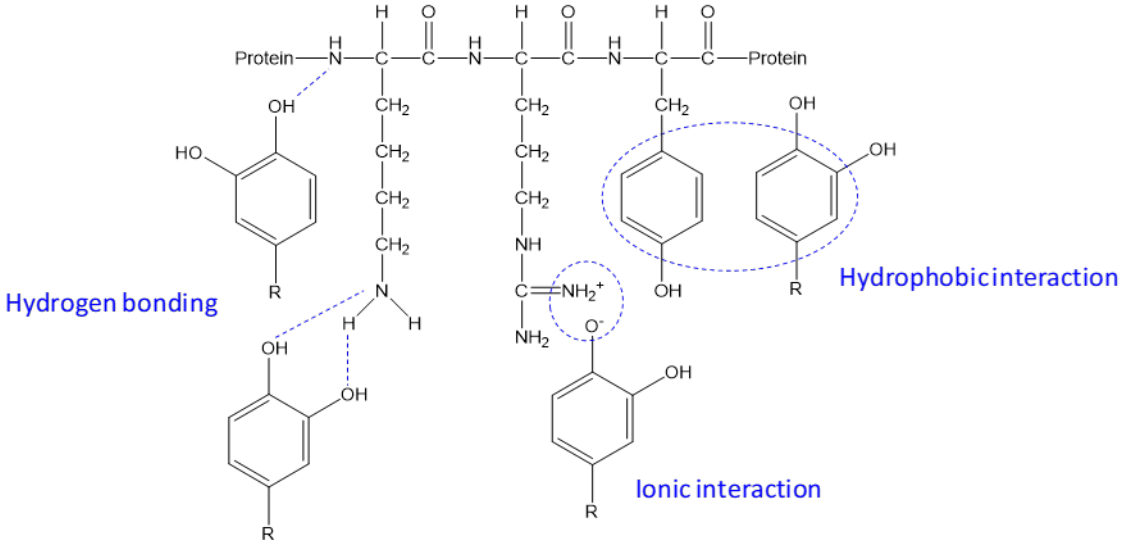


Figure 2.2. Proposed mechanism of non-covalent interactions between proteins and phenolic compounds (Le Bourvellec & Renard, 2012).

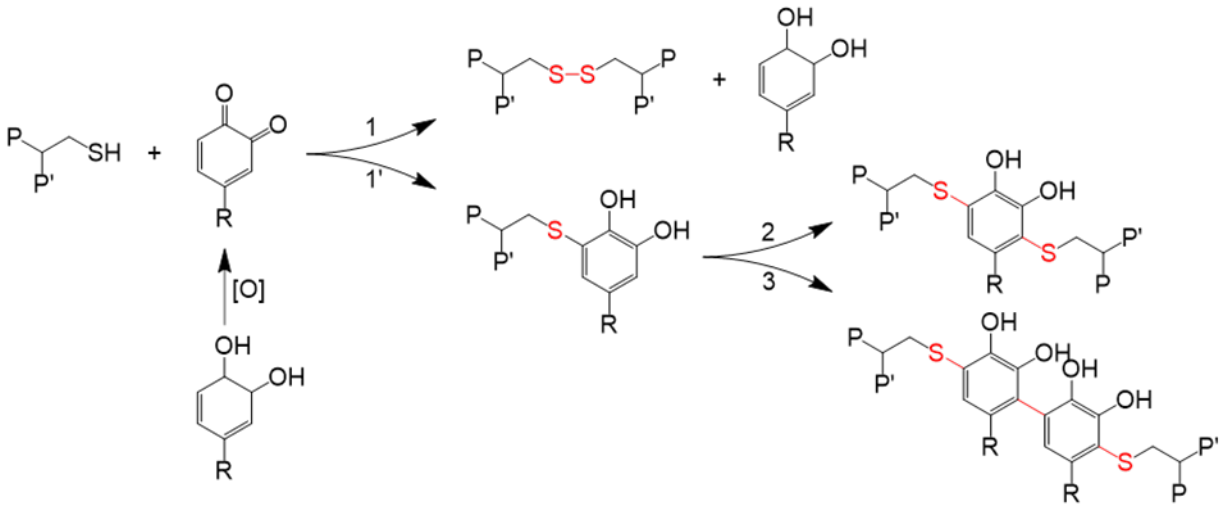


Figure 2.3. Proposed mechanism of covalent interactions between muscle proteins and phenolic compounds focusing on the role of protein thiol groups. 1: production of disulfide bond in redox reaction; 1': adduction of quinone to -SH group in proteins; 2: formation of cross-linking by another protein binding to the phenol derivative; 3: formation of cross-linking through quinone–myofibrillar protein adduct dimerization.

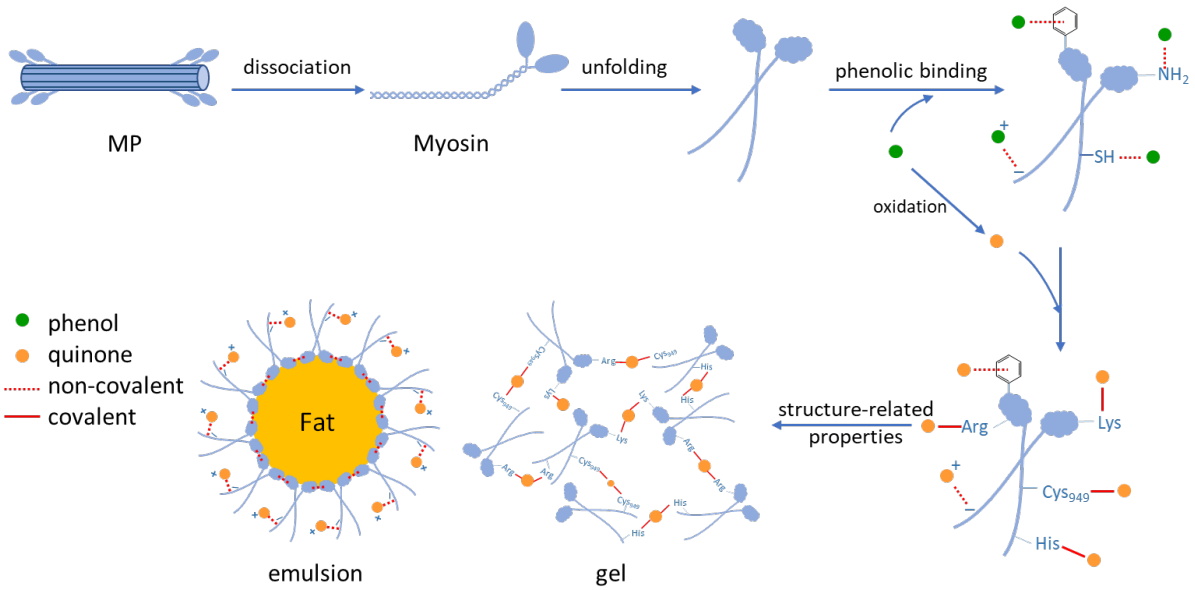


Figure 2.4. Schematic presentation of myosin-phenol binding and the effect on structure-forming functionality in processed muscle food systems.

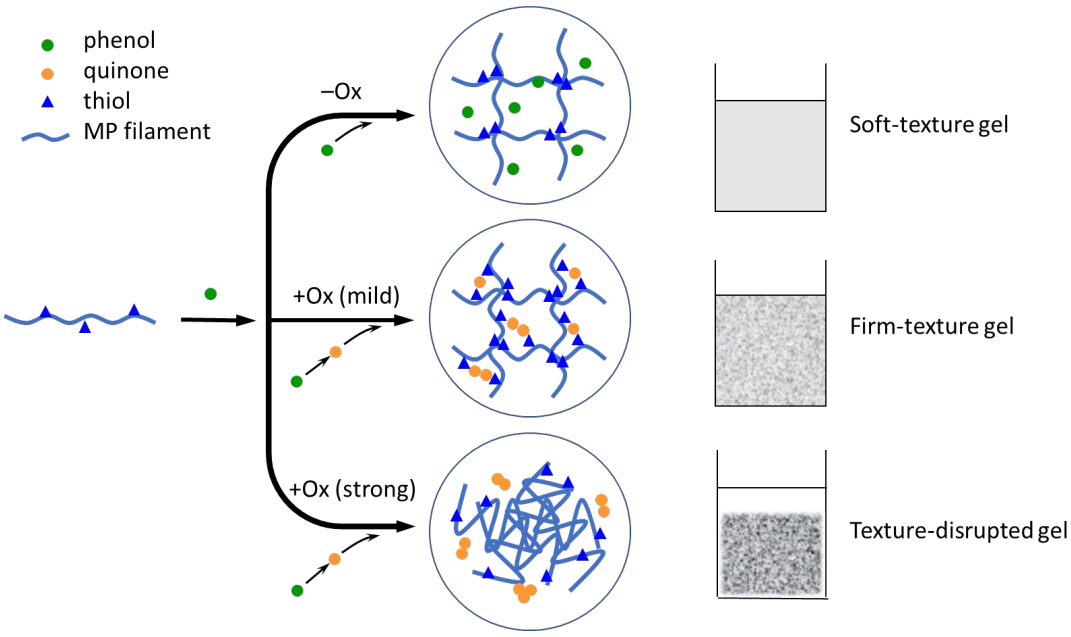


Figure 2.5. Proposed effects of quinones on thermal gelling properties of myofibrillar protein (MP) under different oxidative (Ox) conditions. Mild oxidation promotes gelation while strong oxidation disrupts the gel texture.

Table 2.1. Common analytical methods for the characterization of interactions between myofibrillar protein (MP) and phenolic compounds.

Techniques	Principles
Amino acid side chains	
Sulfhydryl and amine groups	Nucleophilic side chain groups in MP, including sulfhydryl and amine, are attacked by electrophilic quinones
Conformation	
Intrinsic fluorescence	Protein structure unfolding caused by phenol interaction exposes tryptophan, tyrosine, and phenylalanine to a more hydrophilic (aqueous) environment, hence, fluorescence quenching
Surface hydrophobicity	Protein structure unfolding and hydrophobic interaction between MP and phenols modify the surface hydrophobicity
Differential scanning calorimetry	Interaction with phenols alters MP conformational stability, which is reflected by the shift in thermal transitions (temperature and enthalpy)
Circular dichroism	Protein–phenol interaction alters secondary structures of MP, which affects protein backbone absorption of polarized light
Fourier transform infrared	Protein–phenol interaction alters secondary structures of MP, which changes chemical bond vibration measured by absorption spectra
Raman spectrometry	Protein–phenol interaction alters secondary structures of MP, which changes molecular vibration measured by scattering spectra
Phenol adduction and protein cross-linking	
Mass spectrometry	Formation of protein–phenol adducts changes the mass-to-charge (m/z) ratio of protein fragments
Electrophoresis	Formation of protein polymers is promoted by phenol-mediated conversion of free sulfhydryl to disulfide bonds; quinones act as bridges to dimerize or polymerize proteins

CHAPTER 3. GLUCOSE OXIDASE PROMOTES GALLIC ACID–MYOFIBILLAR PROTEIN INTERACTION AND THERMAL GELATION

3.1 Summary

The effect of glucose oxidase (GOx) catalytic oxidation on the efficacy of gallic acid (GA) to modify the chemical structure and gelling behavior of myofibrillar protein (MP) was investigated. In contrast to non-oxidized MP samples where GA induced very little changes, GA (0, 6, 30, and 60 μmol/g MP) under GOx treatment promoted sulfhydryl and amine loss (up to 58% and 49%, respectively). The attenuation of intrinsic tryptophan fluorescence in the GA/GOx-treated MP corroborated the finding. The gelling capacity of MP, corresponding to disulfide and non-disulfide bond formation in protein aggregates, was markedly enhanced by 60 μmol GA under GOx, up to 86% in gel storage modulus G′ and 53% in gel strength. The GOx-aided GA modification of MP could be a potential ingredient strategy in meat processing to promote textural attributes of cooked products.

3.2 Introduction

Myofibrillar protein (MP), constituting up to 60% of muscle protein, is well-known for its vital role in texture characteristics of both fresh and processed meats. In particular, the ability of MP to form an adhesive gel in cooked muscle foods is responsible for meat and fat particle binding, water immobilization, and flavor entrapment, which contribute to palatability particularly mouthfeel, juiciness, and flavor (Sun & Holley, 2011). Of various processing factors that affect the gel properties of MP, oxidation is recognized as an important one (Xiong, Blanchard, Ooizumi, & Ma, 2010). Hence, modulating protein–protein interactions and cross-linking via oxidative modification was considered as a possible strategy to produce processed meat products with modified texture (Xiong, 1996; Zhang et al., 2013).

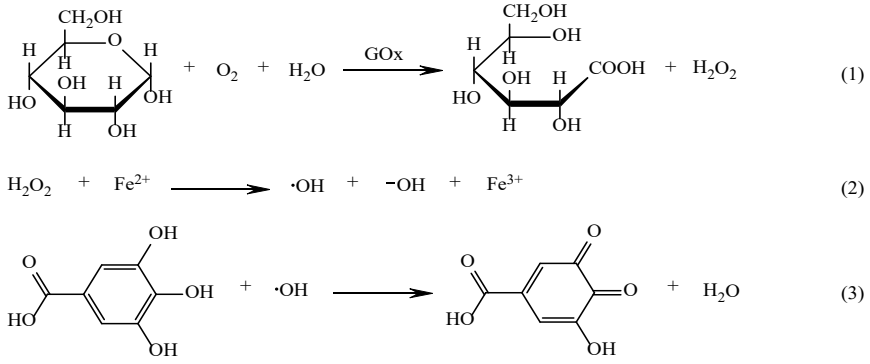
Phenolic compounds are one of the most widely occurring diverse groups of phytochemicals that have been incorporated to meat and meat products for preservation and flavor development (Balasundram, Sundram, & Samman, 2006; Falowo, Fayemi, & Muchenje, 2014). Moreover, the potential of synthetic food additives causing toxicological effects has led to a strong demand for natural substitutes in the consumer market as well as the meat industry. Thus, there has been a growing research interest in the interaction between phenolic compounds and proteins and its implication for protein functionality. Accordingly, studies have been conducted to investigate the effect of phytophenolics on the gelling potential of MP, and oxidation has been introduced as a processing factor. As found in a recent study (Cao et al., 2016), structural modification of MP by gallic acid (GA) under chemically induced oxidation altered inter- and intramolecular cross-linking of myosin in a GA concentration-dependent manner. Such protein–protein interactions as

well as the attachment of GA to MP had either a synergistic or antagonistic effect on MP gelation depending on the GA concentration.

Previously, Balange and Benjakul (2009) reported that treatment of fish surimi (a protein concentrate) with oxidized phenolic acids could enhance the surimi gel strength while non-oxidized phenolics showed no such effect. However, Jia, Wang, Shao, Liu, and Kong (2017) noted that non-oxidized catechin was still able to modify protein conformation and impair the microstructure of pork myofibrillar protein gels. The latter observation supported the findings of Jongberg et al. (2015) who showed green tea extract disrupted meat emulsion gels by the suppression of protein S–S cross-linking. Nonetheless, covalent binding between phenolics and reactive amino acid sidechain groups, including amine and sulfhydryl, is facilitated under oxidizing conditions when the phenols are converted to their corresponding quinone derivatives (Bittner, 2006; Jongberg, Gislason, Lund, Skibsted, & Waterhouse, 2011; Kroll et al., 2003).

Several methods have been developed to introduce an oxidative environment in model systems to explore MP–phenolic interactions, of which chemically induced Fe–H_2O_2 Fenton reaction is the most commonly used. In these studies, hydrogen peroxide and ferrous ion are added directly to the systems to introduce oxidation by generating hydroxyl radicals (Cao et al., 2016; Tang et al., 2017; Wang et al., 2018). However, chemical production of H_2O_2 has the disadvantage that the sudden burst of $^{\bullet}OH$ will cause severe conformational changes and aggregation of proteins, resulting in the insolubility and the tendency to lose functionality (Wang et al., 2016). Hence, oxidative conditions without direct use of hydrogen peroxide would be preferable. Glucose oxidase (GOx), a generally regarded as safe (GRAS) food additive, has been widely used as an oxidizing agent to

remove glucose by the conversion to gluconic acid or to produce H_2O_2 (Eq. (1)). The latter is the precursor of ˙OH (Eq. (2)) that promotes the oxidative transformation of sulfhydryls to disulfide bonds, which has found its use in strengthening the gluten network for bread texture improvement (Rasiah, Sutton, Low, Lin, & Gerrard, 2005; Wong, Wong, & Chen, 2008). In our recent research, we have found that GOx was capable of aiding in the production of firmer and more elastic MP gels than the Fenton oxidation system, and this effect was attributed to the progressive production of H2O2 which was subsequently converted to ˙OH in a controllable manner (Wang et al., 2016; Wang, Xiong, & Sato, 2017).



In the present study, we aimed to investigate the impact of gallic acid (GA), a simple yet common phenolic acid present in spices and herbs, on the gelling properties of MP under GOx-induced oxidation. Hydroxyl radicals generated from the enzymatic process were expected to oxidize gallic acid phenol into the more reactive quinone species (Eq. (3)). Our hypothesis was that by means of slow release of H_2O_2, GOx could promote the interaction between MP and GA thereby enhancing the viscoelastic properties of MP gels. Chemical, structural, and morphological analyses were performed to illustrate the underlying mechanisms.

3.3 Materials and methods

3.3.1 Materials

Longissimus lumborum muscle samples were collected from pork carcasses (24 h post-mortem) harvested at the University of Kentucky Meat Laboratory, a USDA-approved facility. The loin muscle samples were cut into 1 cm chops before being individually vacuum-packaged and stored in a −30 °C freezer until use. Gallic acid (GA, purity ≥ 98.5%) was purchased from Sinopharm Chemical Reagent (Shanghai, China). Glucose oxidase was donated by Ajinomoto Co., Inc. (Kawasaki, Japan). All other chemicals were of at least analytical grade from Sigma–Aldrich (St. Louis, MO, USA) or Thermo–Fisher Scientific (Waltham, MA, USA). Double-deionized water (NANO pure Diamond, Barnstead, IA, USA) was used in all experiments.

3.3.2 Sample preparation

Extraction of MP. Frozen muscle samples were thawed at 4 °C overnight and then chopped into small pieces. MP was isolated from the minced muscle using an extraction buffer consisting of 10 mM sodium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EGTA at pH 7.0 (Park, Xiong, & Alderton, 2007). In the last washing step, the pH of MP suspended in 0.1 M NaCl was adjusted to 6.25. The MP pallet was kept on ice and utilized within 48 h. The preparation was conducted in a 4 °C walk-in cooler. Protein concentration was determined by the Biuret method using bovine serum albumin as a standard (Gornall, Bardawill, & David, 1949).

Oxidation. The MP pellet was suspended in 50 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid) (PIPES) buffer containing 0.6 M NaCl (pH 6.25) to a final concentration of 20 mg/mL. This salt concentration–pH condition was selected because it

was a typical condition for meat processing. GA at four final concentrations (0, 6, 30, and 60 μmol/g MP) were thoroughly mixed with the protein suspensions. Samples were then oxidatively modified with an enzymatic system (50 μg glucose and 8 μg GOx per mg of MP in the presence of 10 μM $FeSO_4$, all final concentrations) by incubation at 4 °C for 8 h. As a set of controls, MP samples with respective amounts of GA but no GOx were prepared for comparison. After the incubation, samples were immediately analyzed.

3.3.3 Measurement of MP chemical changes

Carbonyls. The carbonyl content was determined using the 2,4-dinitrophenylhydrazine (DNPH) colorimetric method as described by Levine et al. (1990). MP samples were mixed with DNPH solution and precipitated with 20% TCA. Recovered protein was washed with ethanol/ethyl acetate (1:1, v/v) solution to exhaustively remove unreacted DNPH, and then dissolved in 6 M guanidine hydrochloride (pH 2.3) for re-solubilization. The absorbance (at 370 nm) was read and a molar extinction coefficient of 22,000 M^{-1} cm^{-1} was used for carbonyl content calculation.

Sulfhydryls. The total sulfhydryl content was determined using the Ellman's reagent (Jongberg et al., 2015). After reaction with the urea-SDS solution (8 M urea with 3% SDS in 0.1 M phosphate buffer, pH 7.4), MP samples were incubated with 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent at 25 °C for 15 min. The absorbance at 412 nm was read and a molar extinction coefficient of 13,600 M^{-1} cm^{-1} was used for calculation. Both reagent and sample blanks were run to correct for the background color.

Free amines. The free amine content was measured following the method described by Adler-Nissen (1979). Protein samples were diluted to 4 mg/mL and mixed thoroughly with SDS solution (1% SDS in 0.2 M phosphate buffer, pH 8.2) and then reacted with 0.1%

2,4,6-trinitrobenzenesulfonic acid (TNBS) reagent in a water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 0.1 M sodium sulfite. The absorbance (at 420 nm) was read and a standard curve of L–leucine was used for calculation. All samples were washed three times with prechilled deionized water and then re-dissolved in 50 mM PIPES buffer containing 0.6 M NaCl (pH 6.25) to avoid interference from GA.

3.3.4 Determination of MP structural changes

The intensity of intrinsic tryptophan fluorescence was measure using a FluoroMax-3 fluorometer (Horiba Jobin Yvon Inc., Edison, NJ USA) with a dilute suspension of MP (0.4 mg/mL in 50 mM PIPE buffer, 0.6 M NaCl, pH 6.25). The emission spectra were recorded from 300 to 450 nm at an excitation wavelength of 283 nm. Background spectra under the same conditions were collected and subtracted from the respective spectra of the MP samples. The data of fluorescence quenching under either nonoxidizing or oxidizing conditions were analyzed using the Stern–Volmer equation:

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensities before and after the addition of GA; K_q is the biomolecular quenching-rate constant; τ_0 is the lifetime of the fluorophore in the absence of a quencher; [Q] is the concentration of the quencher; and K_{sv} is the Stern–Volmer quenching constant. According to Lakowicz and Weber (1973), τ_0 for biopolymer is 10^{-8} S^{-1}. K_{sv} was determined by plotting linear regression F_0/F against [Q].

3.3.5 Identification of protein cross-linking

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted to determine protein patterns in unheated (sol) and heated (gel) MP samples according to the method of Laemmli (1970) with a 3% polyacrylamide stacking gel and a

10% polyacrylamide resolving gel. To each diluted MP sample (2 mg/mL), 1 mM N-ethylmaleimide (NEM, a thiol-blocking agent) or 10% β-mercaptoethanol (βME) was added before boiling for 3 min for the detection of protein aggregates via S–S bonds. Detailed SDS–PAGE sample preparation conditions were described by Balange and Benjakul (2009). For both oxidized and unoxidized MP samples, the degree of loss of myosin heavy chain (MHC), relative to MP control with neither GA nor GOx, was established using the UN–SCAN–IT software (Silk Scientific, Orem, Utah, U.S.A.) with the following formula:

$$\text{Relative loss (\%)} = \frac{\text{pixel intensity in control} - \text{pixel intensity in sample}}{\text{pixel intensity in control}} \times 100$$

3.3.6 Gelling properties of MP

Dynamic rheological properties. MP sols (20 mg/mL protein in 0.6 M NaCl, pH 6.25) were de-aerated by centrifuging at 1000g for 1 min then subjected to oscillatory shear analysis using a Model CVO rheometer (Malvern Instruments, Westborough, MA, USA). Thermal gelation was achieved by heating sols loaded between the parallel plates (30 mm upper plate diameter, 1 mm gap) from 20 to 72 °C at a 1 °C/min heating rate. The exposed sample rim was covered with a thin layer of silicon oil to prevent dehydration. During heating, the storage modulus (G′) was recorded every 30 s at a fixed frequency of 0.1 Hz and a maximum strain of 0.02.

Gel strength. MP sols (20 mg/mL protein in 0.6 M NaCl, pH 6.25) were de-aerated and then transferred to glass vials (18.0 mm inner diameter) and then heated in a water bath from 20 to 72 °C at a 1 °C/min heating rate. After reaching the final temperature, gels were immediately chilled in an ice slurry for 30 min and kept at 4 °C overnight. The set gels were then equilibrated at room temperature for 1 h and extruded with a stainlesssteel, flat-

ended probe (10 mm diameter) attached to a Model 4301 Instron universal testing machine (Canton, MA, USA) at a crosshead speed of 20 mm/min until structural failure. The initial puncture force required to disrupt the gels was recorded as gel strength (N).

Scanning electron microscopy (SEM). MP gels prepared with 40 mg/ml protein in 0.6 M NaCl at pH 6.25 were used for SEM examination after fixation as described by Wang et al. (2017) with slight modifications. Gel cubes (approximately 3 mm^3) were cut and fixed in 0.1 M phosphate buffer (pH 7.2) containing 3% glutaraldehyde for 24 h at 4 °C. Fixed samples were washed with 0.1 M phosphate buffer (pH 7.2) three times and then postfixed for 20 h in 1% osmium tetroxide. The postfixed samples were washed three times, each time with 0.1 M phosphate buffer (pH 7.2) and dehydrated in a series of ethanol (50, 75, 90, 95, and twice 100%) for 30 min per solution. Samples were further dehydrated by critical point drying. Dried samples were sputter-coated with gold and examined under a Model S4300 Hitachi scanning electron microscope (Hitachi, Ltd., Tokyo, Japan) with 10 kV accelerating voltage.

3.3.7 Statistical analysis

Experiments were conducted with three independent trials (n = 3) on different days, each with a new batch of isolated MP. Data were subjected to the analysis of variance using a general linear model's procedure in Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA). Significant ($P < 0.05$) differences between means were identified by LSD all-pairwise multiple comparisons.

3.4 Results and discussion

3.4.1 Changes involving amino acid side chains

3.4.1.1 Carbonyls

Carbonylation is considered to be one of the most remarkable chemical modifications in oxidized proteins which has been widely used for the quantification of oxidation. Non-oxidized muscle proteins are free of carbonyl groups, but in oxidatively stressed proteins, carbonyls are always generated (Estévez & Heinonen, 2010; Xiong, 1996). The most common precursors of carbonyl derivatives are sidechains of amino acid residues such as histidine, lysine, arginine, proline, and threonine (Stadtman, 2006; Zhang et al., 2013). As presented in Table 3.1, the carbonyl content (1.95 nmol/mg) in samples subjected to GOx-mediated oxidation doubled that of the non-oxidized control ($P < 0.05$). Hydrogen peroxide (H_2O_2) was simultaneously produced when GOx catalyzed the oxidative conversion of glucose to gluconic acid by utilizing molecular oxygen as an electron acceptor (Bankar, Bule, Singhal, & Ananthanarayan, 2009). Extremely reactive hydroxyl radicals ($^{\bullet}OH$) were then continuously generated from H_2O_2 in the presence of ferrous iron (Fe^{2+}), resulting in MP oxidation and an increased carbonyl content (Wang et al., 2016).

The addition of GA did not affect the carbonyl formation in either oxidized or non-oxidized MP samples (Table 3.1). Plant phenolics could inhibit carbonyl-forming protein oxidation through two major pathways. One is donation of electrons to disrupt the oxidation cycle through scavenging free radicals; the other one is chelation of metal ions and removal of free radical initiators by nature of stabilizing them (Falowo et al., 2014). On the other hand, phenolic compounds could contribute to carbonyl formation when they

are oxidized to quinone species and catalyze the oxidative deamination of susceptible amino acids to form the corresponding semialdehydes (Estévez & Heinonen, 2010). The overall anti- or pro-oxidative effect of phenolic compounds is complicated depending on several factors, including chemical structure and concentration of the specific phenolic compounds, the pH of the food matrix, and the oxidizing agents (Utrera & Estévez, 2012; Zhou & Elias, 2013). In the present study, GA exerted no antioxidative effect on protein either with or without GOx, indicating that protein radicals were not scavenged by GA.

3.4.1.2 Total sulfhydryls

MP is rich in sulfhydryl (SH) groups (up to 42 per mole of myosin), which are susceptible to oxidation and can be readily converted to various thiol derivatives such as sulfenic acid, sulfinic acid, sulfonic acid, and disulfides. The disulfide bond (S–S) is among the most dominant products in oxidatively stressed MP (Liu, Xiong, & Butterfield, 2000; Lund, Heinonen, Baron, & Estévez, 2011). As shown in Table 3.1, the total SH content of MP decreased slightly from 55.1 nmol/mg to 43.4 nmol/mg after GOx oxidation ($P < 0.05$). The addition of GA further reduced the sulfhydryl content in a dose-dependent manner with a total loss of 58% at 60 μmol/g of GA treatment ($P < 0.05$). The further sulfhydryl loss could be caused by the adduction of the electrophilic quinone carbonyls produced by GA oxidation to the nucleophilic cysteine SH groups (Jongberg et al., 2011). Similar results were obtained from several other studies (Jia et al., 2017; Wang et al., 2018). On the other hand, low concentrations (6 and 30 μmol/g MP) of GA had negligible effect on total SH content in non-oxidized MP samples, apparently due to limited SH–quinone adduction (Prigent et al., 2003). Nevertheless, a slight but significant loss of SH (14%, $P < 0.05$) was

observed at a high GA concentration (60 μmol/g MP), which can be attributed to the auto-oxidation occurring during the preparation of the mixed MP/GA samples.

3.4.1.3 Free amines

In both non-oxidized and oxidized MP samples, a continuous reduction ($P < 0.05$) in free amine content was observed when MP was incubated with increasing concentrations of GA (Table 3.1). However, the loss of amines was more pronounced in GOx-oxidized MP samples: from 69.5 to 44.2 nmol/mg when compared with 82.5 to 65.1 nmol/mg for non-oxidized MP ($P < 0.05$). It has been proposed that in an oxidative environment, readily accessible lysine residues can be deaminated and form the Schiff's base adduct with carbonyl derivatives (Levine et al., 1990). The increased availability of protein carbonyls in GOx-stressed MP samples, as described above, would contribute to the loss of amines. Since 1% SDS was used in the assay of free amine content (which dissociates non-covalent aggregation), covalent interactions were responsible for amine loss in the present study. Furthermore, as aforementioned, in GOx-treated MP/GA mixture, quinone derivatives of GA would react with α- or ε-NH_2 groups of amino acid sidechains forming quinone–protein adducts, further contributing to the decreased amine content (Kroll et al., 2003). The GA-dose dependent loss of amines in GOx-oxidized MP samples supported this premise.

3.4.2 Protein structures

The photophysical properties of intrinsic tryptophan are highly sensitive to the polarity of its surrounding environment. For native folded proteins, tryptophan residues are embedded deep into a hydrophobic pocket, so they have a high quantum yield and high fluorescence intensity. When proteins unfold and tryptophan residues are exposed to a hydrophilic environment, the fluorescence will attenuate (Ghisaidoobe & Chung, 2014).

Therefore, the characteristics of intrinsic tryptophan spectra are commonly used as indicators of the tertiary structure of proteins. In the present study, the intrinsic tryptophan fluorescence in both non-oxidized and oxidized MP samples was suppressed by GA in a dose-dependent manner (Figure 3.1A), suggesting progressive MP unfolding with increasing GA content. However, the quenching of fluorescence by GA in GOx-oxidized samples was stronger than in non-oxidized samples. As shown in Figure 3.1B, the quenching constant K_{sv} rose from 1.6×10^3 M^{-1} to 2.7×10^3 M^{-1} by GOx-mediated oxidation. Without oxidation, the GA-induced structure change would mainly be attributed to non-covalent forces such as hydrophobic interactions between the aromatic ring of GA and aromatic amino acid residues, hydrogen bonds formed between hydroxyl groups in GA and hydrogen acceptors in MP, and van der Waals interactions (Buamard & Benjakul, 2015; Wu et al., 2011). Under GOx-mediated oxidation, additional covalent binding between MP and quinone derivatives of GA would facilitate protein structure change. It is also possible that the interactions between GA and MP were further promoted by exposed reactive groups preceded by protein unfolding under oxidative conditions. The quenching constant (K_{sv}, 2.7×10^3 M^{-1}) of GA was at the same order but slightly lower than that found in another study (K_{sv}, 6.9×10^3 M^{-1}) where the model system was oxidized by Fenton reaction (Cao et al., 2016). Compared to the strong and rapid release of $^{\bullet}OH$ produced by chemically induced oxidation, progressive $^{\bullet}OH$ production from GOx would enable less severe protein structure modification. Variations in quenching constants have been found to stem from other factors, including proteins sources, molecular weight and structure of phenolics, and temperature (Soares, Mateus, & De Freitas, 2007).

3.4.3 Cross-linking of MP

MP cross-linking before and after thermal gelation was evaluated by SDS–PAGE (Figure 3.2). In non-oxidized and non-reducing MP sol (Figure 3.2A), no detectable difference in the intensity of protein bands was observed between GA-treated samples and the control ($P > 0.05$). However, when MP was subjected to GOx oxidation, the loss of myosin heavy chain (MHC) increased slightly from 36% to 46% with increasing concentrations of GA (6–60 μmol/g, $P < 0.05$). Under reducing conditions (+βME) where disulfide bonds were cleaved, the differences caused by GOx were largely diminished and MHC was mostly recovered in all samples (Figure 3.2B). Hence, S–S produced in myosin was the major force in the enhanced cross-linking of MP sol.

After the heating process, the MHC band in GOx-treated MP gel samples became rather faint (over 90% reduction). Concomitantly, a significant amount of polymers/oligomers that were too large to enter the resolving gel appeared on the top of the porous stacking gel (Figure 3.2C). When treated with βME, some of the lost MHC was recovered. Yet, the amount of MHC that remained unrecoverable by the reducing compound increased from 20% (control) to 61% if the MP sample was previously treated with 60 μmol/g of GA ($P < 0.05$). The extra loss (41%) was evidence of covalent bonds other than S–S linkages (Figure 3.2D). The oxidation-induced polymers in the stacking gel were quantitatively related to the GA concentration. This could be explained because non-disulfide covalent bonds such as di-tyrosine and Schiff's base were formed under oxidative conditions, leading to enhanced MP cross-linking (Zhang et al., 2013). Another possible mechanism is that quinones derived from GA oxidation acted as bridges to produce protein polymers through thiol–quinone or amine–quinone adduction. These interactions were

intensified with thermal gelation where phenolic compounds or their quinone derivatives could bind more effectively (Balange & Benjakul, 2009).

3.4.4 Gelling properties

3.4.4.1 Dynamic rheological behavior of MP

GA-induced changes in dynamic viscoelastic behavior of the MP during thermal gelation was assessed by measuring the storage modulus (G′) evolution (Figure 3.3). During heating, all MP samples exhibited a typical thermogram with two major G′ peaks (around 45 °C and 50 °C), corresponding to the association of denatured myosin head and tail groups, respectively (Xiong et al., 2010). Compared to the non-oxidized control sample, the addition of GA to GOx-oxidized MP drastically increased G′ of the second transition peak (up to 85% with 60 μmol/g GA), evincing stronger myosin tail–tail interaction. Upon heating to above 55 °C, the G′ increased steadily and especially in samples treated with GA, indicating networking of previously formed protein aggregates. The G′ reached a plateau toward the final heating temperatures, and the final G′ (72 °C) of the gel was enhanced by 86% when MP was treated with 60 μmol/g GA under GOx oxidation. In contrast, despite the tendency of increasing in the intermediate temperature range (45–50 °C) by the presence of GA, the G′ development in non-oxidized samples was much less affected by GA treatments, which could be due to limited quinone production without GOx. Quinone derivatives of GA could promote the MP gelation by facilitating the SH/S–S interchange and conversion, itself acting as cross-linker through binding two polypeptides, or dimerizing after adduction with nucleophilic groups in proteins (Strauss & Gibson, 2004; Vate & Benjakul, 2016a). These results, in corroboration with the sulfhydryl, amine, and carbonyl measurements (Table 3.1), suggest that the production of quinone under GOx-

mediated oxidation was necessary for the efficacy of GA in the development of a more elastic MP gel.

3.4.4.2 Gel strength

The gelling properties of MP were further analyzed by measuring the strength (the initial force required to rupture the structure) of set protein gels. The enhancement of gel strength by GA and GOx was in agreement with the rise in the final G′ value at 72 °C (Figure 3.3). Without the GOx oxidation, the gels prepared from GA-treated MP exhibited no obvious difference in gel strength ($P > 0.05$, Figure 3.4). However, upon oxidation, the gel strength tended to increase gradually with the GA concentration. The maximum gain was observed at the highest GA concentration level (60 μmol/g) where the gel strength showed a 53% increase ($P < 0.05$). The result supported Balange and Benjakul (2009) who found the breaking force of heated fish surimi only increased with the addition of oxidized ferulic acid while no change was noted when ferulic acid was not oxidized in advance.

3.4.4.3 Microstructure of gels

SEM was performed to visualize the detailed structure of MP gels. Compared with the rugged and uneven surfaces observed on the control MP gel, the GOx-treated gels displayed a denser and more uniform matrix with less pores (Figure 3.5). This compact structure could resist disrupting forces, thus, explaining the enhanced gel strength in Figure 3.4. More obvious structural differences were revealed when GA (6 and 30 μmol/g) were added in combination with GOx: a filamentous gel network structure with fine strands (pointed by arrow) was formed while their non-oxidized counterparts had continuous and aggregated matrices (Figure 3.5). Oxidized GA enhanced myosin tail–tail association

(Figure 3.3) and cross-linking (Figure 3.2) during thermal gelation, which could contribute to the formation of such filaments formed within the protein gel.

3.5 Conclusion

In conclusion, the presence of GA under the GOx-mediated oxidation condition promoted the gelation of MP by modifying the chemical, structural, and cross-linking patterns of MP. Such effects were attributed to the covalent adduction between GA quinone derivatives and nucleophilic amino acid side chains, formation of disulfide and nondisulfide linkages, and progressive protein unfolding. Further studies are needed to identify and quantify the specific quinone–protein adducts and the site on myosin where cross-links occur. Moreover, phytochemicals from spices and plant extracts, which are commonly added to meat products, are a large family of natural compounds with various structures and activities. Therefore, the effect of these phenolics as individuals or a mixture on MP gelation under normal meat processing conditions should be investigated.

Table 3.1. Carbonyl, total sulfhydryl, and free amine contents in myofibrillar protein (MP) treated with various amounts of gallic acid (GA) under nonoxidizing (–Ox) or oxidizing (+Ox) conditions.[1]

GA treatment (μmol/g MP)	Carbonyl (nmol/mg MP)	Sulfhydryl (nmol/mg MP)	Free amine (nmol/mg MP)
–Ox			
0	0.97±0.05[a]	55.1±3.2[a]	87.5±2.0[a]
6	0.98±0.08[a]	59.6±0.8[a]	82.5±3.7[ab]
30	0.93±0.06[a]	56.1±1.6[a]	71.8±1.8[c]
60	0.98±0.13[a]	47.5±2.4[b]	65.1±2.1[c]
+Ox			
0	1.95±0.07[b]	43.4±1.4[b]	72.6±4.8[bc]
6	1.89±0.13[b]	42.9±1.1[b]	69.5±3.3[c]
30	2.01±0.08[b]	33.6±1.5[c]	54.4±0.7[d]
60	2.11±0.13[b]	23.3±2.0[d]	44.2±3.0[e]

[1] Oxidation was carried out with 8 μg GOx and 50 μg glucose (per mg of MP) and 10 μM $FeSO_4$.

[a–e] Means within the same column without a common letter differ significantly ($P < 0.05$).

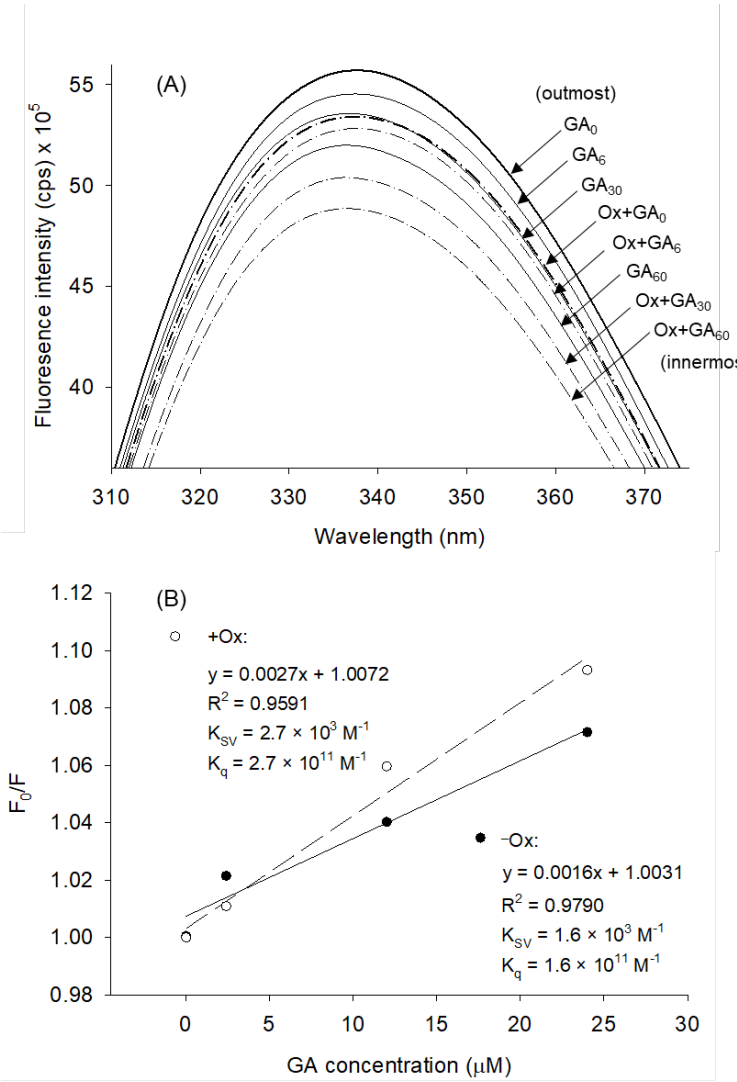
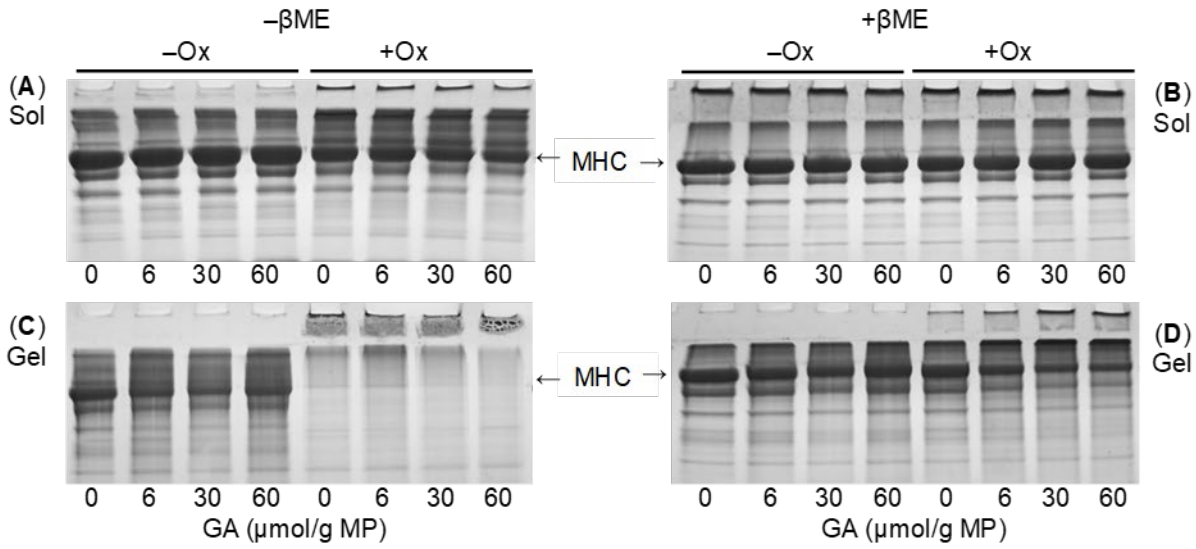


Figure 3.1. Tryptophan fluorescence (A) and Stern–Volmer plots (B) of myofibrillar protein (MP) treated with various amounts of gallic acid under nonoxidizing (–Ox) or oxidizing (+Ox) conditions. The GA concentrations used for the Stern–Volmer plots (0, 2.4, 12, and 24 µM GA) were derived from the respective treatment concentrations (0, 6, 30, and 60 µmol GA/g MP) after a 50 times dilution. Oxidation was carried out with 8 µg GOx and 50 µg glucose (per mg of MP) and 10 µM $FeSO_4$.



Relative reduction of MHC induced by GA and GOx oxidation

Gel	GA concentration (μmol/g MP)							
	–Ox				+Ox			
	0	6	30	60	0	6	30	60
(**A**)	0.0	8.0a	9.3a	11a	36b	36b	38b	46c
(**B**)	0.0	5.5a	11b	13b	17bc	21c	11b	13b
(**C**)	0.0	27a	46b	43b	94c	90c	95c	96c
(**D**)	0.0	20a	28a	24a	20a	41b	55c	61c

Figure 3.2. Representative SDS–PAGE patterns of myofibrillar protein (MP) focusing on changes in myosin heavy chain (MHC). The MP samples were treated with various amounts of gallic acid (GA) under nonoxidizing (–Ox) or oxidizing (+Ox) conditions where oxidation was carried out with 8 μg GOx and 50 μg glucose (per mg of MP) and 10 μM $FeSO_4$. Protein samples were prepared in the presence (+βME) or absence (−βME) of 10% β-mercaptoethanol. Relative reduction of MHC = (pixel intensity in control − pixel intensity in sample) / pixel intensity in control × 100. Means in the same column without a common letter (a−c) differ significantly ($P < 0.05$).

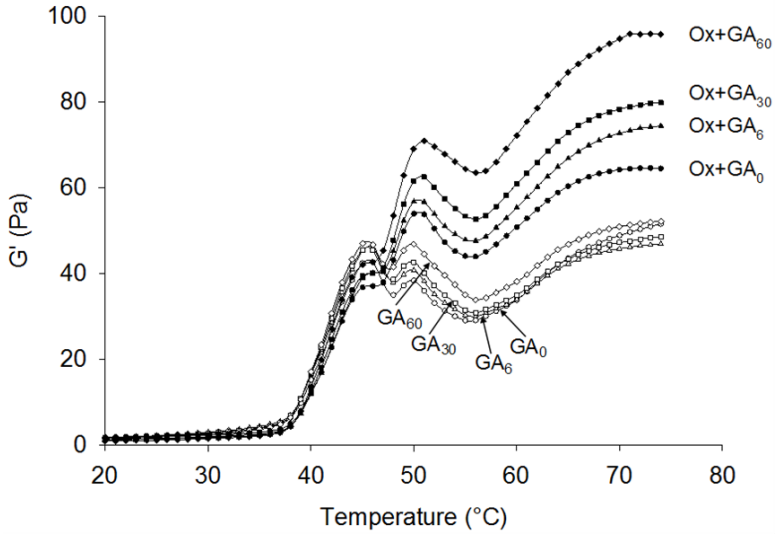


Figure 3.3. Storage modulus (G′) development during thermal gelation of MP with various amounts of gallic acid (GA: 0, 6, 30, and 60 µmol/g MP) under nonoxidizing (–Ox) or oxidizing (+Ox) conditions. Oxidation was carried out with 8 µg GOx and 50 µg glucose (per mg of MP) and 10 µM $FeSO_4$.

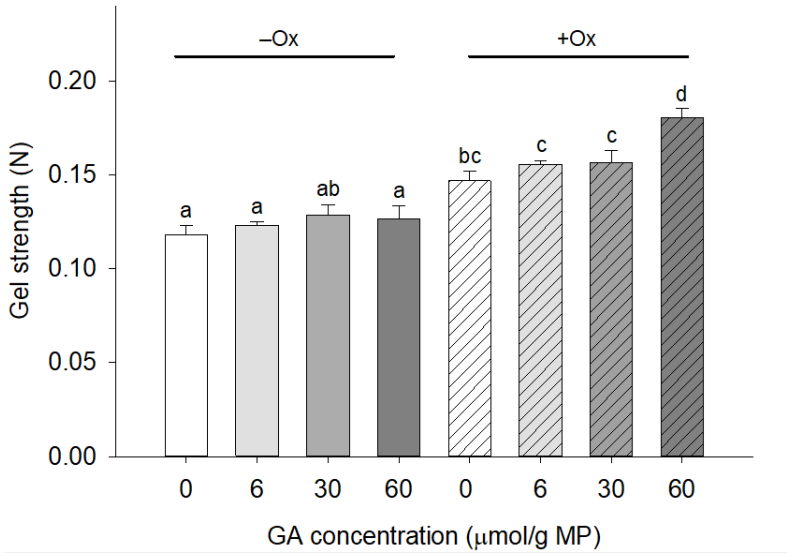


Figure 3.4. Gel strength of MP treated with various amounts of gallic acid (GA) under nonoxidizing (–Ox) or oxidizing (+Ox) conditions. Oxidation was carried out with 8 µg GOx and 50 µg glucose (per mg of MP) and 10 µM $FeSO_4$. Means without a common letter (a−d) differ significantly ($P < 0.05$).

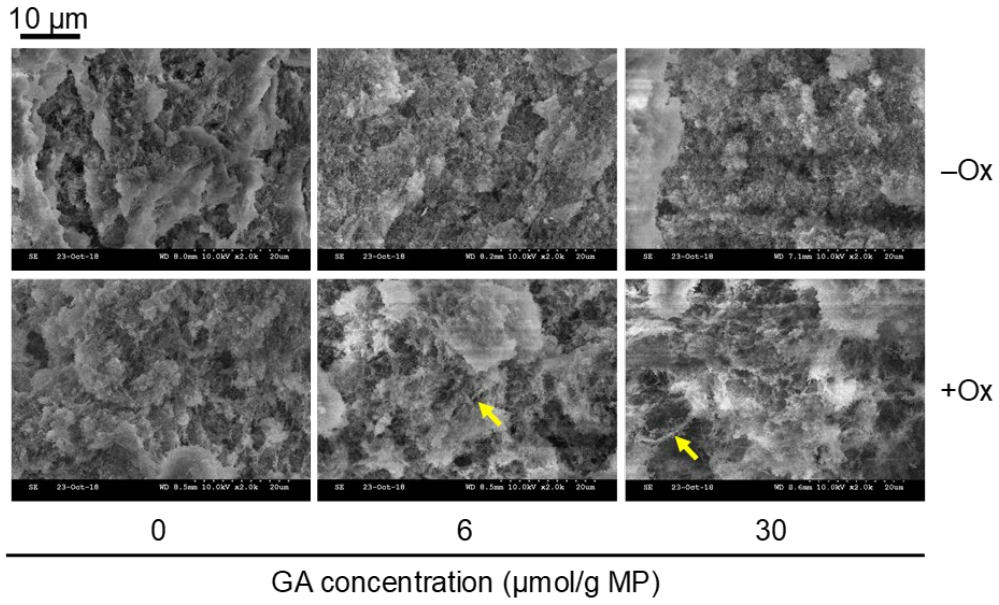


Figure 3.5. SEM images of gels made from MP (40 mg/mL protein) after treatment with various amounts of gallic acid (GA) under nonoxidizing (–Ox) or oxidizing (+Ox) conditions. Oxidation was carried out with 8 µg GOx and 50 µg glucose (per mg of MP) and 10 µM $FeSO_4$. A filamentous network structure with fine strands is pointed by the arrow.

CHAPTER 4. EFFECT OF STRUCTURALLY RELATED PHENOLIC ACIDS ON THE GELLING PROPERTIES OF OXIDATIVELY STRESSED MYOFIBRILLAR PROTEIN

4.1 Summary

Six phenolic acids (PA) with different structures, i.e., gallic acid (GA), syringic acid (SA), coumaric acid (CMA), caffeic acid (CFA), ferulic acid (FA), and chlorogenic acid (CA), were compared for their effects on the gelling properties of myofibrillar protein (MP). Of the six PA, the smallest GA caused significant reductions of free amine and sulfhydryl content (by 26% and 7%, respectively) but introduced low fluorescence quenching. In contrast, the other PA showed no effect on amino acid side chains but more strongly enhanced fluorescence quenching. The largest molecule, CA, was the most effective which decreased the fluorescence intensity 38% and increased λ_{max} from 338 nm to 350 nm. During thermal gelation, the GA-modified MP displayed the strongest cross-linking. The elasticity (G′) and breaking strength of MP gels were markedly enhanced by the addition of PA and the final G′ value followed the order GA>CA>FA>CMA>SA>CFA.

4.2 Introduction

Phenolic acids (PA) are a group of phytophenols ubiquitous in herbs and spices with gallic acid in cloves, caffeic acid in thyme, and coumaric acid in oregano being some of the common compounds (Embuscado, 2015). In meat processing, phenolic extracts are commonly incorporated into muscle foods to improve sensory attributes and oxidative stability (Kausar, Hanan, Ayob, Praween, & Azad, 2019). The additional nutrition and health benefits of PA as well as customers' growing demands for naturally sourced ingredients rendered them a preferrable and promising replacement for synthetic antioxidants used in the meat industry (Ribeiro et al., 2019).

PA are hydroxybenzoic or hydroxycinnamic acids with the basic structure of one or more hydroxyl groups and a carboxylic acid derivative attached to the benzene ring (Heleno, Martins, Queiroz, & Ferreira, 2015). The functional groups in PA are known to be readily interactive with the muscle proteins through both non-covalent (irreversible) and covalent (reversible) mechanisms (Guo & Xiong, 2021). Non-covalent forces, including hydrogen bond, hydrophobic association, and electrostatic attraction, can be introduced by hydroxyl groups, phenoxide ions, and the aromatic ring in PA (Ozdal et al., 2013). Under oxidative conditions, covalent adduction would occur between PA-derived electrophilic quinone species and nucleophilic amino acid side chains in proteins (Rohn, 2019). One of the major consequences of the protein–PA interactions is the modifications of the physicochemical and functional properties of myofibrillar protein (MP), which is the most important meat protein component responsible for textural properties of processed muscle foods (Xiong & Guo, 2021; Zhao, Xu, & Zhou, 2021).

The effects of PA on the physicochemical properties of MP have been subjected to extensive studies and different efficacies have been reported. As oxidation is generally introduced in meat processing due to mincing, chopping, and blending, many of the studies were conducted under oxidizing conditions. At low-to-medium concentrations, chlorogenic acid was found to promote the gel formation of oxidative stressed MP by enhancing protein unfolding and non-covalent association (Cao & Xiong, 2015). In comparison, gallic acid at the same addition levels not only facilitated MP unfolding but also induced protein cross-linking through the formation of intermolecular disulfide bonds (Cao et al., 2016). In another study, enhanced cross-linking was reported in caffeic acid-modified MP while ferulic acid showed no such effect (Prodpran et al., 2012). The discrepancies observed between PA are likely related to structural differences, and the large number of structurally diverse natural PA compounds with different molecular arrangements warrant structure-activity relationship exploration. Some phenolic structures affecting the efficacy of protein modifications have been recognized, for example, the number and position of hydroxyl groups, size of substituent groups, and derivatization (methoxylation, hydroxylation, or glycosylation) (Guo, Jiang, True, & Xiong, 2021; Xiao et al., 2011). However, little is known about how these attributes affect their interactions with MP.

The objective of the present study was to elucidate the effect of structure-activity relationship of selective PA on the roles in modifying physicochemical and gelling properties of MP. Six structurally related PA were compared, including two hydroxybenzoic acids and four hydroxycinnamic acid derivatives (Figure 4.1). Glucose oxidase (GOx), a food grade enzyme widely used in breadmaking for texture improvement,

was applied to imitate oxidative stress that would likely be encountered in meat processing (Wang et al., 2016). Their potential to modulate PA–MP interactions was specifically examined.

4.3 Materials and methods

4.3.1 Materials and reagents

Protein *Longissimus lumborum* muscle samples were collected from pork carcasses (24 h post-mortem) harvested at the University of Kentucky Meat Laboratory, a USDA-approved facility. The loins were cut into 1 cm chops which were individually vacuum-packaged and stored in a −30 °C freezer until use. Glucose oxidase was donated by Ajinomoto Co., Inc. (Kawasaki, Japan). Gallic acid (GA, purity ≥ 99.0%), coumaric acid (CMA, purity ≥ 98.0%), and ferulic acid (FA, purity ≥ 99.0%) were purchased from J&K Scientific (Beijing, China). Syringic acid (SA, purity ≥ 97.0%) and caffeic acid (CFA, purity ≥ 98.0%) were purchased from TGI (Shanghai, China). Chlorogenic acid (CA, purity ≥ 98.0%) was purchased from Spring & Autumn Co. (Nanjing, China). All other chemicals, all a minimum of analytical grade, were acquired from MilliporeSigma (St. Louis, MO, USA), Thermo Fisher Scientific (Waltham, MA, USA), or VWR (Radnor, PA, USA). Double-deionized water was used in all experiments.

4.3.2 Sample preparation and treatment

Extraction of MP. Frozen muscle samples were thawed at 4 °C overnight and then minced. MP was isolated using an extraction buffer consisting of 10 mM sodium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EGTA at pH 7.0 (Park et al., 2007). In the last washing step, the protein pellet was suspended in 0.1 M NaCl and the pH was

adjusted to 6.25. Purified MP pellet was kept on ice and utilized within 48 h. The entire MP sample preparation was conducted in a 4 °C walk-in cooler except for centrifugation (4 °C). Protein concentration was determined by the Biuret method using bovine serum albumin as a standard.

Oxidation. The MP pellet was suspended in 50 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid) (PIPES) buffer containing 0.6 M NaCl (pH 6.25) to a final concentration of 20 mg/mL. PA at 60 μmol/g MP were thoroughly mixed with the protein suspensions and then oxidatively modified by incubation at 4 °C for 8 h with an enzyme system comprised of 50 μg glucose and 8 μg GOx per mg MP in the presence of 10 μM $FeSO_4$ (Guo et al., 2021). Non-oxidized and oxidized MP samples without PA were prepared for comparison. Samples were immediately analyzed after the incubation.

4.3.3 Protein chemical and structural changes

Total sulfhydryls, free amines, and carbonyls. These selective amino acid sidechain groups and carbonyl derivatives were analyzed using colorimetric methods as described in detail in a previous study (Guo & Xiong, 2019). For *sulfhydryls*, the absorbance of the chromophore developed by the reaction of unfolded MP (in 8 M urea with 3% SDS) with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was measured at 412 nm, and a molar extinction coefficient of 13,600 M^{-1} cm^{-1} was applied to calculate total sulfhydryls. For *free amines*, MP samples (in 1% SDS) were reacted with 0.1% 2,4,6-trinitrobenzenesulfonic acid (TNBS) at 50 °C for 30 min in the dark to form a chromophore, and the absorbance at 420 nm was fit in a standard curve of *L*–leucine to determine the amine concentration. For *carbonyls*, the absorbance of the chromophore formed between MP (washed with 1:1, v/v ethanol/ethyl acetate and resolubilized in 6 M

guanidine hydrochloride) and 2,4-dinitrophenylhydrazine (DNPH) was measured at 370 nm. A molar extinction coefficient of 22,000 M^{-1} cm^{-1} was used for carbonyl content calculation. In all the analyses, reagent blanks were run to correct for the background color.

Surface hydrophobicity. The protocol developed by Li-Chan, Nakai, and Wood (1985) was followed to measure MP hydrophobicity using an 8-anilinonaphthalene-1-sulfonic acid (ANS) fluorescence probe and a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA). The excitation and emission wavelengths were set at 390 and 470 nm, respectively.

4.3.4 Detection of protein aggregation

Turbidity scan. MP samples were diluted to 2 mg/mL in 50 mM PIPES buffer containing 0.6 M NaCl (pH 6.25). Thermally induced protein–protein association was determined by monitoring turbidity change of the protein solution during heating from 20 to 72 °C at 1 °C/min, expressed as the absorbance at 500 nm recorded every 30 s using an 8-Abs UV-VIS spectrophotometer (Shimadzu UV-2700, Kyoto, Japan) connected to a temperature controller (Quantum northwest, Liberty Lake, WA, USA). The differential change of the absorbance as a function of heating temperature (dAbs/dT) was calculated to determine the rate of protein aggregation.

Particle size. Dilute MP samples (2 mg/mL in 50 mM PIPES buffer containing 0.6 M NaCl, pH 6.25) were subjected to particle size measurement using a Nano-ZS ZetaSizer (Malvern Instruments, Worcestershire, UK) at room temperature under the following settings: measurement angle, 90°; equilibration time, 300 s; number of runs, 2; and number of measurements, 3. Since heating produced large particles that exceed the size limit for the measuring device, only unheated MP samples were analyzed.

4.3.5 Detection of protein cross-linking

To identify covalent protein cross-linking, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS–PAGE) was conducted for unheated (sol) and heated (gel) MP samples with a 5% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel. For the detection of non-disulfide linkages, 10% β-mercaptoethanol (βME) was added to cleave disulfide bonds. Detailed SDS–PAGE sample preparation has been previously described (Cao et al., 2016).

4.3.6 Gelling properties of MP

Two different tests were conducted to evaluate the gelling properties of MP samples treated with PA. In the first test, 20 mg/mL MP sols (in 50 mM PIPES, 0.6 M NaCl, pH 6.25) were de-aerated and then subjected to *dynamic oscillatory shear* measurement using a Model CVO rheometer (Malvern Instruments, Westborough, MA, USA). Samples were gradually gelled by heating the sols from 20 to 72 °C at a 1 °C/min heating rate (Guo et al., 2021). Storage modulus (G′) was recorded every 30 s during the sol-to-gel transformation. In the second test, the *gel breaking strength* test, de-aerated 20 mg/mL MP sols were transferred to glass vials (18.0 mm inner diameter) and then heated in a water bath from 20 to 72 °C (1 °C/min rate) to form set gels. The gels were extruded with a stainless-steel, flat-ended probe (10 mm diameter) attached to a Model 4301 Instron universal testing machine (Canton, MA, USA) at a puncture speed of 20 mm/min. The initial force required to disrupt the gel structure was recorded as gel breaking strength (N).

4.3.7 Statistical analysis

Experiments were conducted with three independent trials (n = 3) on different days, each with a new batch of isolated MP. Results were subjected to the analysis of variance

using the procedure of the general linear model in the Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA). LSD (least significant difference) all-pairwise multiple comparisons was used to identify significant ($P < 0.05$) differences between means.

4.4 Results and discussion

4.4.1 Amino acid sidechain groups

Free radicals generated in an oxidative system can typically attack the sulfhydryl and amine groups in proteins and convert these reactive side chains into various oxidized products such as sulfenic acid, sulfinic acid and disulfides derived from -SH, and Schiff's base adduct derived from $-NH_2$ (Lund et al., 2011; Xiong & Guo, 2021). In the present study, GOx-mediated oxidation decreased total sulfhydryl and free amine content by 27% (Figure 4.2A) and 42% (Figure 4.2B), respectively ($P < 0.05$). A further reduction of 7% and 26% ($P < 0.05$) induced by GA was observed. The additional loss of the functional groups may be attributed to the adduction of electrophilic quinone derivative from GA with nucleophilic thiol and amine groups through covalent bonds (Rohn, 2019; Tang et al., 2017). Compared with other PA, the small size of GA has the advantage of easier penetration to the inter-myofibrillar regions of MP to gain a close proximity of the reactive protein sidechain groups (Zhang, Cheng, Wang, & Fu, 2020). It is plausible that the least structural hinderance in GA could also facilitate covalent binding of GA with MP.

4.4.2 Carbonyls

Carbonylation, one of the most common consequences of oxidative stress, is a sensitive marker of protein oxidaiton (Suzuki, Carini, & Butterfield, 2010). As compared with the control sample (non-oxidized), GOx-mediated oxidation markedly increased the

MP carbonyl content, i.e., from 0.26 to 1.39 nmol/mg ($P < 0.05$, Figure 4.2C). With the exception of SA, at the treatment concentration tested (60 μmol/g protein), PA did not inhibit oxidation-induced carbonylation. Even though phenolic compounds are generally effective antioxidants against lipid oxidation, their role to mitigate protein oxidation is variable. Both anti- and pro-oxidation and the lack thereof can occur depending on the chemical structure and concentration of PA as well as the oxidative conditions (Cao et al., 2016; Lund et al., 2011). The significant SA-induced carbonyl reduction (~20%) may be attributed to its strong scavenging activity of $^{\bullet}$OH radicals in aqueous media (Vo et al., 2020).

4.4.3 Surface hydrophobicity

Surface hydrophobicity is an important predictor of protein gelling behavior because it measures the extent of exposures of protein hydrophobic domains (unfolding) and hence, possible hydrophobic association during the protein gel network formation (Li-Chan et al., 1985). CA, the largest PA with most hydroxyl groups (5) and a bulky quinic acid group, was the only molecule that significantly decreased S_0, from 150 to 131 (Figure 4.2D, $P < 0.05$). This effect may result from the blockage of exposed hydrophobic groups in MP by the quinic moiety thereby decreasing the accessibility by ANS.

4.4.4 Intrinsic fluorescence

The intrinsic fluorescence is extremely sensitive to the microenvironment of fluorophores (aromatic amino acids), so it is commonly adopted to the evaluation of tertiary structural changes of proteins. Three fluorescencing amino acids, i.e., tryptophan, tyrosine, and phenylalanine, are buried in the hydrophobic core of native (folded) proteins so they yield a high fluorescence intensity after being excited (Royer, 2006). Protein unfolding

exposes the fluorophores to a hydrophilic (aqueous) environment, resulting in an attenuated fluorescence intensity and a longer maximum emission wavelength (λ_{max}) (red shift) (Ghisaidoobe & Chung, 2014). The interactions between PA and MP promoted protein unfolding, which was indicated by the suppressed fluorescence and red shift (Figure 4.3). Covalent binding between electrophilic quinones and the nucleophilic indole group of tryptophan has bene suggested as another contributing factor for fluorescence quenching (Rohn, 2019). Compared with the two hydroxybenzoic acids (GA and SA), the four hydroxycinnamic acids (CMA, FA, CFA, and CA) were more effective in modifying the fluorescence spectra of MP. The strong reactivity potential of hydroxycinnamic acids with proteins might result from their ability to get easily oxidized and bound to MP (Rawel & Rohn, 2010). Consistent with our results, Li et al. (2020b) observed that the binding affinity of hydroxycinnamic acids for β-lactoglobulin was significantly higher than that of hydroxybenzoic acids. Compared with the simplest hydroxycinnamic acid, CMA, the additional methoxyl group in FA and hydroxyl group in CFA facilitated MP unfolding, suggested by their stronger suppressing effect on fluorescence intensity. It is noteworthy what the largest hydroxycinnamic acids with the most functional groups, CA, induced the greatest reduction of fluorescence intensity (38%) and the largest λ_{max} shift (from 338 nm to 350 nm), in agreement with a previous finding (Cao & Xiong, 2015).

4.4.5 Cross-linking of MP

Heat-induced cross-linking of MP molecules is known to be affected by the interaction with phenoli compounds (Tang et al., 2017; Zhao et al., 2021). Covalently bound PA could promote protein cross-linking through acting as a bridge to connect two peptides or dimerizing through covalent linkages between two quinones (Strauss & Gibson,

2004). As shown in Figure 4.4D, GA caused noticeable loss of myosin heavy chain, and concomitantly, protein polymers were formed which was evidenced in the stacking gel where exceedingly large polymers were unable to enter the resolving gel. Since disulfide bonds were cleaved by β-mecaptoethanol during SDS–PAGE sample preparation, the presence of these polymers verified the formation of non-disulfide linkages in GA-treated MP, which could include quinone–MP adduction through NH_2 or SH groups. The result is consistent with the most effective modifications that GA exerted on amine and sulfhydryl groups (Figure 4.2). Compared with other PA compounds, the stronger reducing potential of GA could facilitate its re-oxidation after one site adduction and subsequent formation of cross-linking (Cheng, Ren, Li, Chang, & Chen, 2002). Because phenol-induced protein cross-linking was promoted at high temperatures (> 60 °C) as shown previously (Guo et al., 2021), little differences were observed in the unheated MP sol samples under non-reducing (Figure 4.4A) or reducing conditions (Figure 4.4B). For thermally gelled MP samples, however, covalently linked protein aggregates were produced under oxidative conditions, and they were too large to enter the stacking gel. Therefore, no protein patterns appeared for the oxidized and PA-treated samples prepared under non-reducing conditions (Figure 4.4C).

4.4.6 Protein aggregation and particle size

Turbidity is a simple method recommended for the detection of heat-induced protein–protein association (Hall et al., 2016). As shown in Figure 4.5A, the turbidity of all the MP samples, measured as optical density at 500 nm, was relatively stable before 40 °C. The value increased gradually till 50 °C, rose rapidly to around 60 °C, and ascended slowly to the final heating temperature 72 °C. Note that before heating (at 20 °C), oxidized

MP samples, either with or without PA, were slightly more turbid and the particle size (Figure 4.5A inset) was larger than non-oxidized control (by 6.8–17%, $P < 0.05$). Oxidation induced unfolding and ensuing hydrophobic aggregation were responsible for the observed turbidity increase during heating. At the end of the heating process (72 °C), most PA treated samples exhibited higher turbidity than the control samples due to enhanced cross-linking. This was most remarkable for the GA treatment, which was the most effective in promoting protein aggregation in which non-disulfide covalent bonds were clearly involved (Figure 4.4D).

The rate of protein aggregation was compared by calculating the first derivative of the turbidity change. All samples showed two transition peaks around 45 °C and 58 °C (Figure 4.5B). They are attributed to, respectively, the association of heavy meromyosin (head) and light meromyosin (tail) of myosin (Wang & Smith, 1994). Based on the peak height of the major peak (~58 °C), the treatment with GA resulted in a slower protein aggregation rate than the other PA treatments, which showed no remarkable differences between them. A slower aggregation rate might be conducive to better gelling potential, because it allows sufficient time for protein aggregates to cross-link in an ordered manner leading to a viscoelastic gel matrix (Ziegler & Foegeding, 1990). The GA-mediated MP cross-linking by the galloyl moiety with three hydroxyls, presumably in the form of either protein–GA–GA–protein or protein–GA–protein (Guo & Xiong, 2019), may require relatively long time for the reaction to complete. For other PA, the fewer number of hydroxyl groups or the presence of methoxyl groups did not seem to promote cross-linking but instead enhanced protein unfolding (fluorescence quenching, Figure 4.3), enabling faster protein thermal aggregation.

4.4.7 Gelling properties

During heating when MP sols were transformed into viscoelastic gels, two characteristic rheological transition peaks appeared in the temperature ranges of 40–50 °C and 50–60 °C (Figure 4.6A), corresponding to the association of denatured myosin head and tail groups, respectively (Figure 4.5). The G′ reached a plateau toward the final heating temperatures, and oxidation drastically enhanced the final G′ (72 °C) compared with the non-oxidized control. The addition of all PA resulted in a further improvement of the elastic attribute, and GA and CA were the most effective, increasing the G′ by 36% and 31%, respectively. The evidence of GA promoting protein cross-linking (Figure 4.4; Figure 4.5) or CA facilitating protein unfolding (Figure 4.3) coincided with their effects on gelation, indicating that the gel elasticity development is closely related to the PA-induced physicochemical modifications of MP. Consistent with the rheological measurement, the strength of set gels was slightly increased, i.e., 13% by oxidation and 15–22 % by PA additions ($P < 0.05$, Figure 4.5B). Overall, PA-induced gel strength modifications were not as remarkable as the rheological augmentation, probably due to the less sensitivity of the gel penetration test. The puncture force is a combination of compressive, tensile, and shear forces, which is far beyond the threshold of the elastic modulus (G′) that only measures shear stress (Cao et al., 2016).

4.5 Conclusion

In conclusion, the interactions between PA and MP are affected by the structural characteristics of PA, including the number and position of hydroxyl, methoxyl, and other functional groups. Smaller PA with less structural hinderance and more reducing potential

appear to be effective in promoting protein cross-linking while larger molecules with more substituent groups are better at facilitating protein unfolding. During thermal gelation, the protein aggregation rate is affected by the PA induced physicochemical modifications of MP, which is reflected in the protein gelling properties. Further studies are needed to evaluate the effects of PA on MP gelation in the presence of other major food components, e.g., lipids or additives, which more closely resembles a composite meat product system. The macro- or micro- molecules in gelled muscle foods might also participate in the interactions with PA and modify texture related quality attributes of meat products.

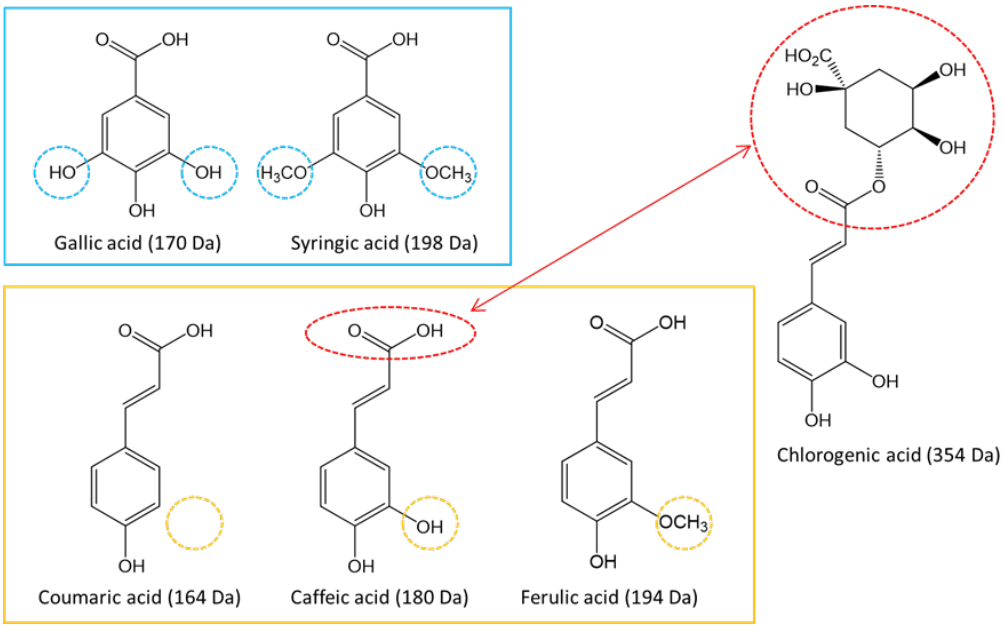


Figure 4.1. Six structurally related phenolic acids tested.

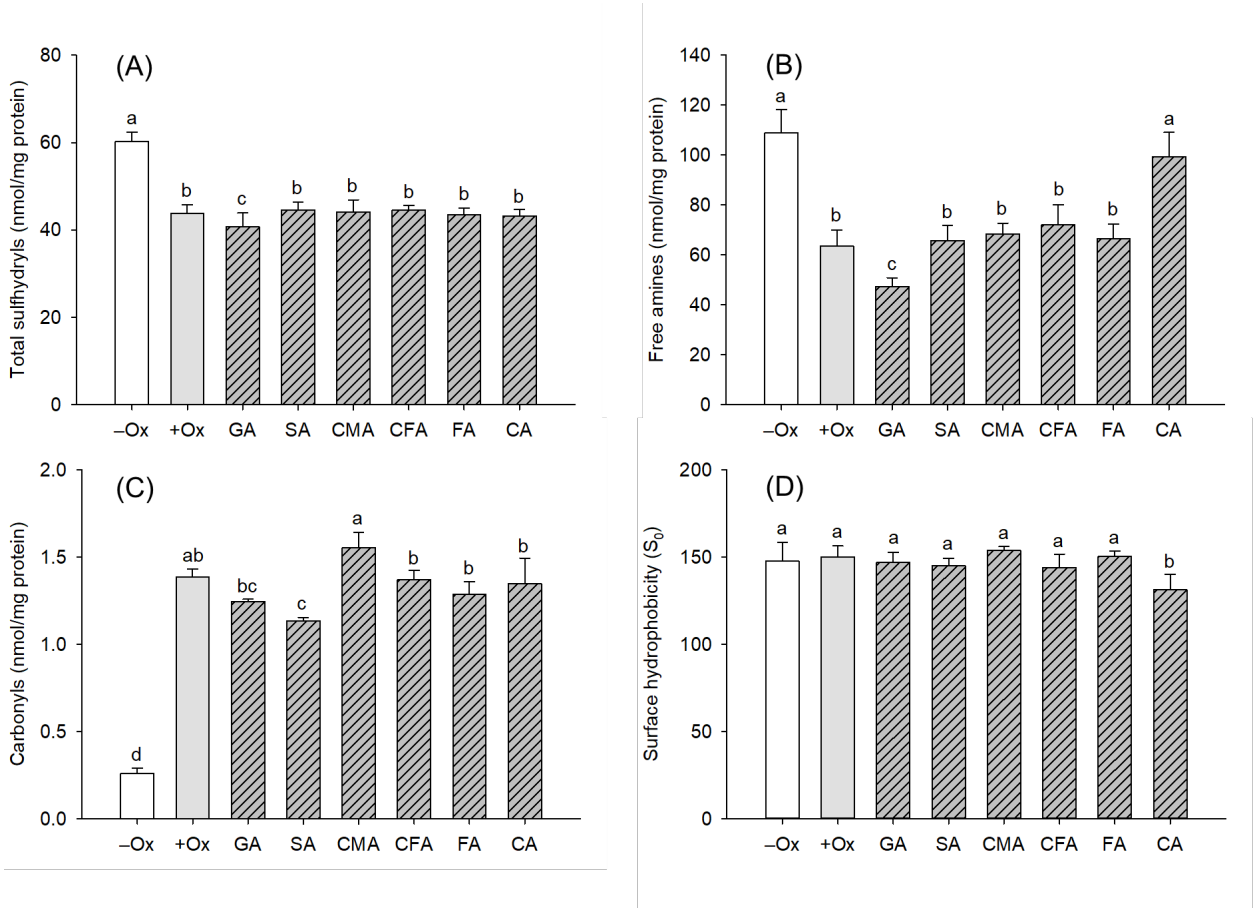
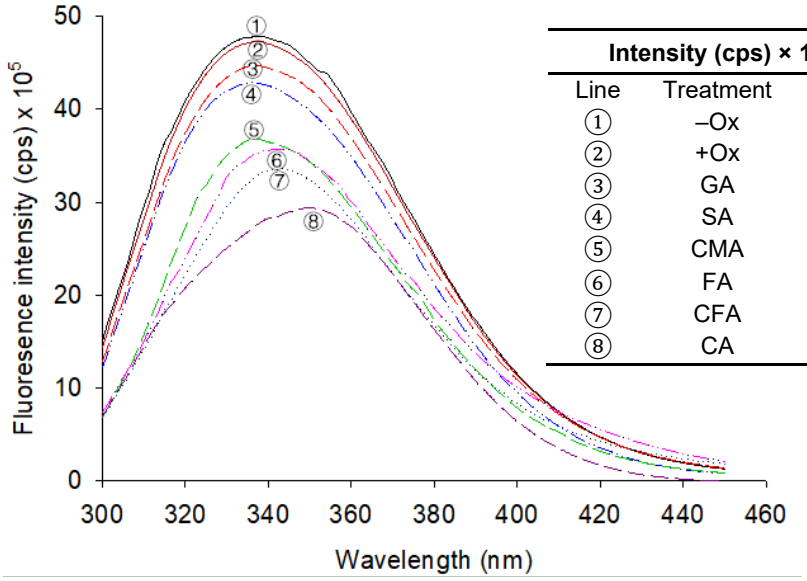


Figure 4.2. Total sulfhydryls (A), free amines (B), carbonyls (C), and surface hydrophobicity (D) of myofibrillar protein treated with different phenolics acids (60 µmol/g protein) in an oxidative environment. Control samples (no phenolic acid) included both non-oxidized (–Ox) and oxidatively tressed (+Ox) protein. GA: gallic acid, SA: syringic acid, CMA: coumaric acid, CA: caffeic acid, FA: ferulic acid, CGA: chlorogenic acid. In the same parameter, means without a common letter (a−d) differ significantly ($P < 0.05$).



Intensity (cps) × 10^5		
Line	Treatment	Max
①	–Ox	48
②	+Ox	47
③	GA	45
④	SA	43
⑤	CMA	37
⑥	FA	36
⑦	CFA	34
⑧	CA	29

Figure 4.3. Intrinsic fluorescence intensity of myofibrillar protein treated with different phenolic acids (60 µmol/g protein) in an oxidative environment. Control samples (no phenolic acid) included both non-oxidized (–Ox) and oxidatively stressed (+Ox) protein. GA: gallic acid, SA: syringic acid, CMA: coumaric acid, CA: caffeic acid, FA: ferulic acid, CGA: chlorogenic acid. Max: maximum fluorescence intensity within the emission wavelength from 300 nm to 450 nm.

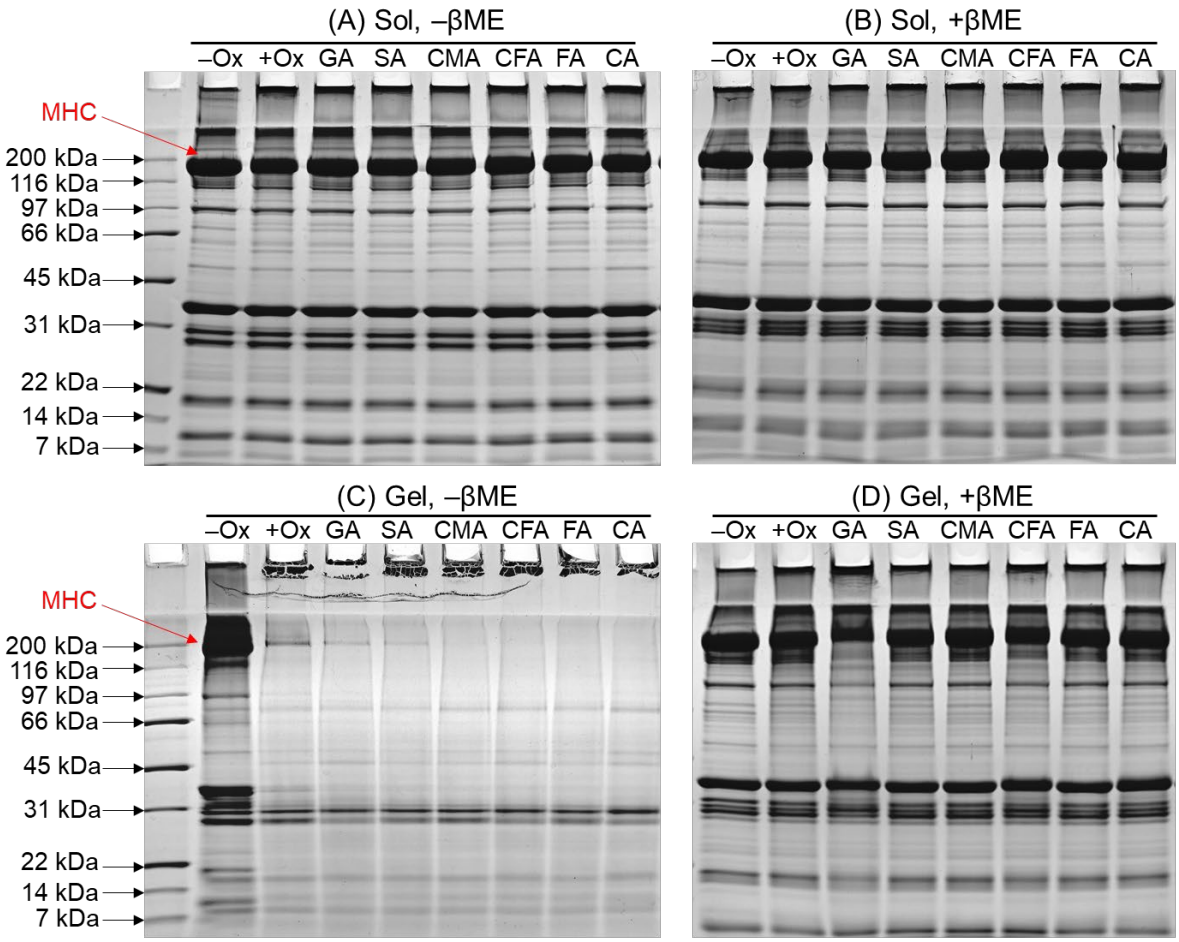


Figure 4.4. SDS–PAGE patterns of myofibrillar protein in sols (A & B) and gels (C & D) treated with different phenolic acids (60 µmol/g protein) in an oxidative environment under non-reducing (–βME) or reducing (+βME) conditions. Control samples (no phenolic acid) included both non-oxidized (–Ox) and oxidatively stressed (+Ox) protein. MHC: myosin heavy chain, GA: gallic acid, SA: syringic acid, CMA: coumaric acid, CFA: caffeic acid, FA: ferulic acid, CA: chlorogenic acid, βME: β-mercaptoethanol.

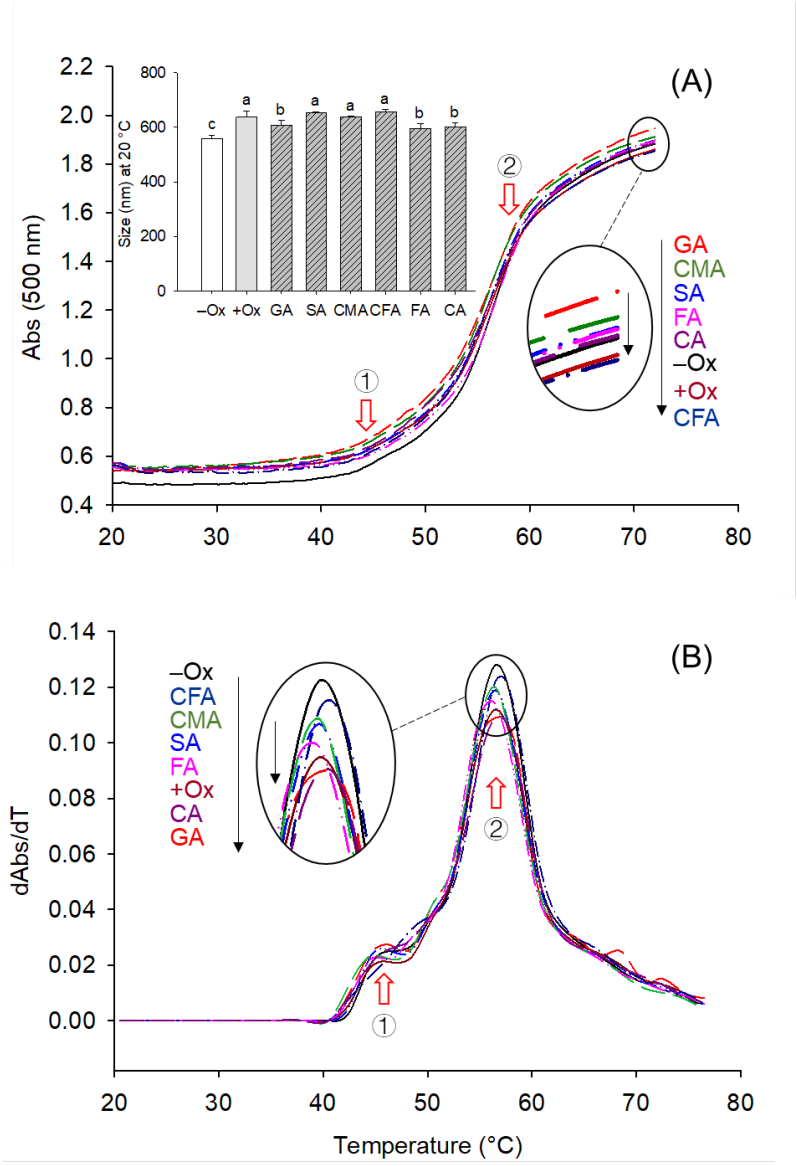


Figure 4.5. Turbidity (A) and the derivative of turbidity (B) of myofibrillar protein treated with different phenolic acids (60 µmol/g protein) in an oxidative environment. Control samples (no phenolic acid) included both non-oxidized (–Ox) and oxidatively stressed (+Ox) protein. GA: gallic acid, SA: syringic acid, CMA: coumaric acid, CFA: caffeic acid, FA: ferulic acid, CA: chlorogenic acid. The inset figure is protein particle size at 20 °C, and the two transitions are marked. Means without a common letter (a−c) differ significantly ($P < 0.05$).

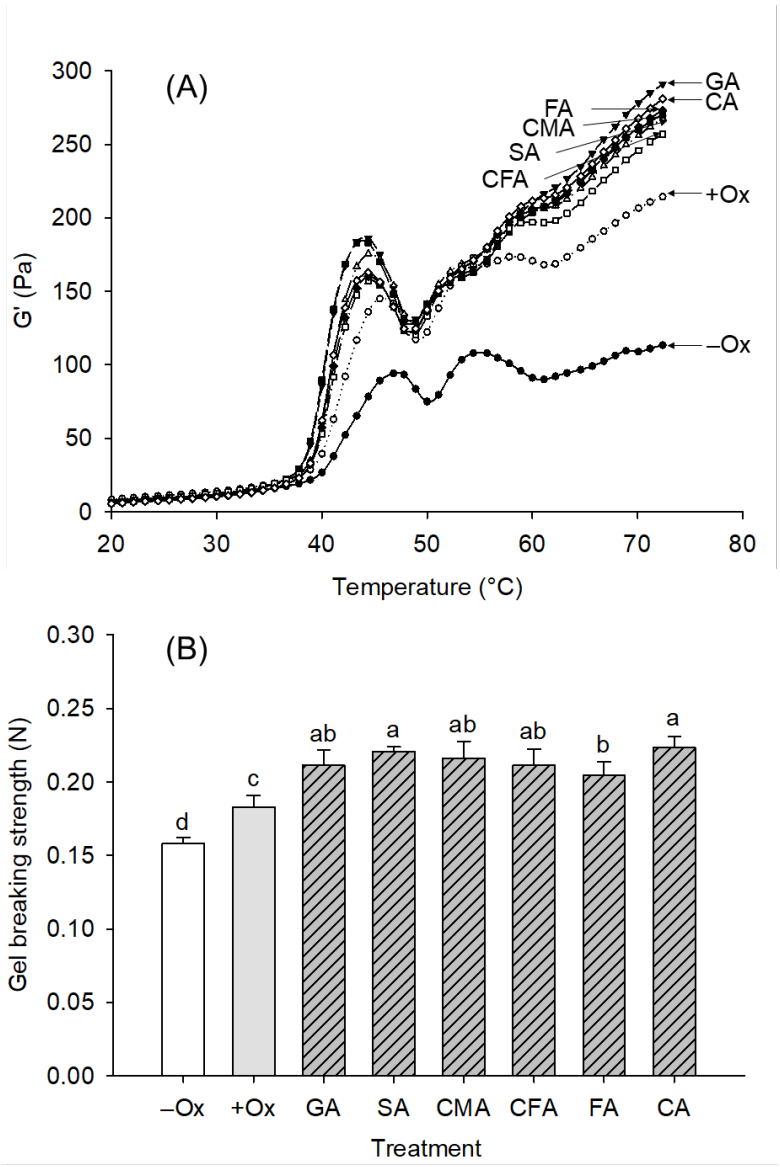


Figure 4.6. Storage modulus (G′) development (A) and gel strength (B) of myofibrillar protein treated with different phenolic acids (60 µmol/g protein) in an oxidative environment. Control samples (no phenolic acid) included both non-oxidized (–Ox) and oxidatively stressed (+Ox) protein. GA: gallic acid, SA: syringic acid, CMA: coumaric acid, CFA: caffeic acid, FA: ferulic acid, CA: chlorogenic acid. Means without a common letter (a−d) differ significantly ($P < 0.05$).

CHAPTER 5. MYOBIGRILLAR PROTEIN CROSS-LINKING AND GELLING BEHAVIOR MODIFIED BY STRUCTURALLY RELATED PHENOLIC COMPOUNDS

5.1 Summary

Protein gelation is an important phenomenon in processed meats. The present study investigated the structure−activity relationship of six phenolic compounds, that is, gallic acid (GA), chlorogenic acid (CA), propyl gallate (PG), quercetin (QT), catechin (CC), and (−)-epigallocatechin-3-gallate (EGCG) in a myofibrillar protein (MP) gelling system under controlled oxidative conditions. All phenolics induced unfolding and promoted cross-linking of MP via sulfhydryl or amine groups. At an equal molar concentration, EGCG boosted the elastic MP gel network more than other phenolics except PG. However, all three monophenols (GA, CA, and PG) and the diphenol QT increased the MP gel strength more than CC (diphenol) and EGCG (triphenol). The flavanol structure appeared to interfere with the protein gel structure development. All phenolics retarded lipid oxidation in MP−emulsion composite gels during refrigerated storage with the least polar phenolic compounds, PG and QT, showing the greatest efficacy.

5.2 Introduction

In muscle food preparation, synthetic phenolic antioxidants are commonly used to control lipid oxidation and minimize meat product quality loss, including off-flavor, discoloration, and nutrient loss (Oswell, Thippareddi, & Pegg, 2018). However, due to the increased awareness of potential toxicity of synthetic antioxidants, there is a growing demand for natural antioxidants to replace synthetic counterparts in meat product formulations. Natural phytophenols extracted from a wide range of plant materials are particularly promising due to their antioxidative efficacy, inhibition of toxicant formation in high-temperature meat processing, as well as potential health benefits (Shahidi & Ambigaipalan, 2015; Xiong, 2017). Plant-derived phenolics are a large family of chemical compounds made up of at least one phenol structure (an aromatic ring with hydroxyl groups attached) and various substituent groups. Both structural moieties play an important role in disrupting lipid oxidation chain reactions through scavenging free radicals, chelating transition metal, and stabilizing newly-formed hydroperoxides (Ribeiro et al., 2019). As such, the effectiveness of phytophenolics when acting as antioxidants is believed to be dependent upon their chemical structure (Papuc, Goran, Predescu, Nicorescu, & Stefan, 2017; Shahidi & Ambigaipalan, 2015).

One of the distinct features of meat processing, especially in the manufacture of comminuted products, is the addition of spices, herbs, and their extracts to modify the product flavor. These exogenous plant-based ingredients are an excellent source of reactive phenolics. It has been recognized that as multi-functional chemicals, phytophenolic can affect the physicochemical and structure-forming properties of proteins through both covalent and noncovalent interactions (Ozdal et al., 2013). They can interact directly with

proteins through reversible or irreversible pathways. In the reversible pathway, non-covalent interactions (hydrophobic association, hydrogen bonding, and electrostatic attraction) occur between the aromatic ring structure, hydroxyl groups, and charged hydroxyl ions in phenolics and reactive amino acid side chains in polypeptides (Le Bourvellec & Renard, 2012). In the irreversible pathway, covalent linkages (S–S, C–N, C–C, etc.) are formed. After the phenol ring structure is oxidized to electrophilic quinone derivatives, it can attack electron-dense amino acid side chain groups in proteins, particularly the amine group in lysine and sulfhydryl group in cysteine, forming protein–quinone adducts (Rohn, 2019). Phenolic-induced physicochemical modifications of meat proteins may cause altered protein functionality, such as gelation, which is the most important texture-forming property in comminuted meat products (Jiang et al., 2020; Xiong, Srinivasan, & Liu, 1997).

Several phenolics with distinct structural features have been tested for their efficacy to modify the gelling properties of myofibrillar protein. Gallic acid, the simplest phenolic compound, was reported to promote MP gelation through enhanced protein unfolding and cross-linking (Cao et al., 2016; Guo & Xiong, 2019). Catechin, a flavonoid with two phenol rings, however, impeded MP gelation due to extensive hydrophobic and covalent interactions between catechin and MP (Jia et al., 2017). On the other hand, (−)-Epigallocatechin-3-gallate, a more complex flavonoid with three phenol rings, increased cooking loss of MP gel at low concentrations (Feng et al., 2017).

The interest in structure-activity relationship has led to independent studies of the role of phenolic compound side groups in affecting protein functionalities, and some structural or size characteristics have been identified. For example, compared with ferulic

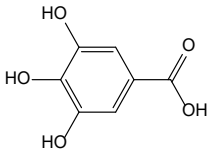
acid, catechin, and caffeic acid, tannic acid (a phenolic polymer) had the greatest impact on the film-forming properties of fish muscle proteins regarding the mechanical strength (Prodpran et al., 2012). Xiao et al. (2011) claimed that the binding affinities between dietary polyphenols and milk proteins were weakened by methylation, glycosylation, and hydrogenation of the C2=C3 double bond of flavonoids but strengthened by hydroxylation on the A and B rings of flavones and flavonols. The reactivity of flavonoids was reported to increase with a larger number of hydroxyl substituents during the interaction with soy proteins (Rawel, Czajka, Rohn, & Kroll, 2002). Balange and Benjakul (2009) noted different microstructural characteristics of fish surimi gels modified by four oxidized phenolic compounds, implying structure-dependent activity of the specific phenolics. Despite these previous findings, there is scant information on how the structure of phenolics affects the gelling properties of muscle proteins when compared on an equal molar versus equal phenol basis.

The objective of the present study was to investigate the effect of six structurally related mono-, di-, and triphenol compounds on the physicochemical (amino acid side chain groups, conformation, and cross-linking) and gelling behavior of myofibrillar protein (MP). The antioxidant efficacy of these phenolics on the MP–emulsion composite gels was subsequently evaluated. Glucose oxidase (GOx), used in the food industry to facilitate protein network formation in food through oxidative mechanisms, was applied to introduce an oxidative environment. The controllable nature of GOx catalysis would enable oxidative stress at levels that may be encountered during normal meat processing and storage (Wang et al., 2016).

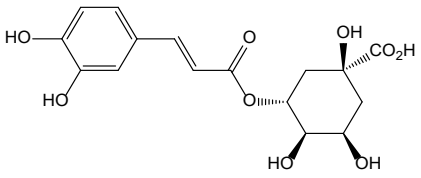
5.3 Materials and methods

5.3.1 Materials and chemicals

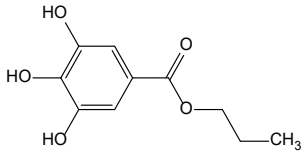
Longissimus lumborum muscle samples were collected from pork carcasses (24 h post-mortem) harvested at the University of Kentucky Meat Laboratory, a USDA-approved facility. The pigs were hybrid barrows of Large White × Duroc × Landrace raised at the university's swine farm. Individual muscle samples were vacuum-packaged and kept in a –30 ± 2 °C freezer for less than 6 months before use. GOx, an FDA-approved enzyme, was donated by Ajinomoto Co., Inc. (Kawasaki, Japan). Canola oil was purchased from a local grocery store. Gallic acid (GA, purity ≥ 98.5%), chlorogenic acid (CA, purity ≥ 98%), and (−)-epigallocatechin-3-gallate (EGCG, purity ≥ 98%) were purchased from Sinopharm Chemical Reagent (Shanghai, China); Quercetin (QT, purity ≥ 94%) was purchased from Thermo Fisher Scientific (Waltham, MA); Catechin (CC, purity ≥ 96%) and propyl gallate (PG, purity ≥ 98%) were purchased from MilliporeSigma (St. Louis, MO). All other chemicals were acquired from MilliporeSigma (St. Louis, MO), Thermo Fisher Scientific (Waltham, MA), or VWR (Radnor, PA) and were of at least analytical grade. Double-deionized water was used in all experiments. Six structurally related phenolic compounds (3 monophenols, 2 diphenols, and 1 triphenol) are shown below. PG, a widely used synthetic antioxidant in meat processing, was included for comparison with the five natural phytophenolics.



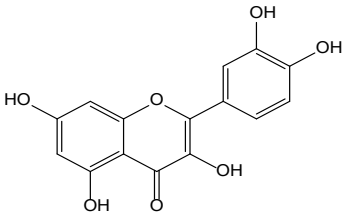
Gallic acid (GA, 170 Da)



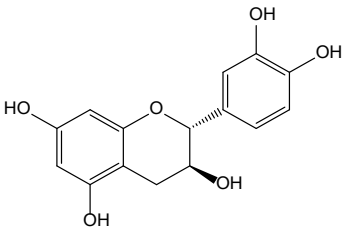
Chlorogenic acid (CA, 354 Da)



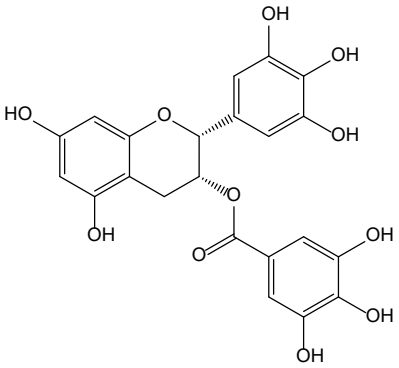
Propyl gallate (PG, 212 Da)



Quercetin (QT, 302 Da)



Catechin (CC, 290 Da)



Epigallocatechin gallate (EGCG, 458 Da)

5.3.2 MP preparation

Frozen muscle samples were tempered at 4 °C for 12 h before MP extraction using an isolation buffer of 10 mM sodium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EGTA (pH 7.0) (Park et al., 2007). The protein concentration was measured by the Biuret method using bovine serum albumin as the standard (Gornall et al., 1949). The MP pellet was kept on ice and used within 3 days.

5.3.3 Stripping of oil

The canola oil was stripped with alumina (MP Alumina N-Super I, MP Biomedicals, Graffenstaden, France) to remove tocopherols (Yang & Xiong, 2018). An aliquot of 15 g sorbent was mixed with 30 mL oil in a 50 mL-polypropylene centrifuge tube by vigorously shaking, followed by agitation in the dark at 4 °C for 24 h. The tubes were then centrifuged (2000*g*, 20 min) at 20 °C. Collected upper phase was centrifuged again under the same condition and transferred into amber glass vials after being placed under nitrogen flow for 5 min. Vials were hermetically sealed and stored at –20 °C before use (Yang & Xiong, 2018).

5.3.4 Phenolic treatment

The MP pellet was suspended in 50 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid) (PIPES) buffer containing 0.6 M NaCl (pH 6.25) to a final concentration of 20 mg/mL protein to form a viscous sol. Phenolic compounds (GA, CA, PG, QT, CC, and EGCG) were dispersed into the MP sol to obtain a final load of 60 μmol phenolics per g of protein (60 μmol phenolic/g MP). Because oxidation is commonplace in the manufacture of comminuted muscle foods (Domínguez et al., 2019), an oxidative environment was introduced by subjecting the MP-phenolic mixtures to 50 μg glucose and 8 μg GOx per mg of MP in the presence of 10 μM FeSO4 at 4 °C for 8 h (Wang et al., 2016). Moreover, the effects of different phenolics were compared on an equal phenol basis (60 μmol phenol/g MP) to determine the role of the phenol rings, and the test was conducted in a protein gel system (described later).

5.3.5 Determination of physicochemical changes of MP

Total Sulfhydryls. Total sulfhydryl content in MP samples was estimated according to Beveridge, Toma, and Nakai (1974). After being completely dissolved in a urea–SDS solution (8 M urea with 3% SDS in 0.1 M phosphate buffer, pH 7.4), the MP samples were incubated with Ellman's reagent, 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), at room temperature (20 ± 1 °C) for 15 min to develop the chromophore. The absorbance at 412 nm was read. A molar extinction coefficient of 13,600 M^{-1} cm^{-1} was applied to calculate the sulfhydryl content. Reagent blanks were run to correct for the background color.

Free Amines. Free amine content was measured using the procedure described by Adler-Nissen (1979). Before testing, MP samples were washed three times with cold deionized water and then re-suspended in 50 mM PIPES buffer containing 0.6 M NaCl (pH

6.25) to avoid color interference. The washed samples were diluted to 4 mg/mL protein and then mixed with SDS solution (1% SDS in 0.2 M phosphate buffer, pH 8.2) before reaction with 0.1% 2,4,6-trinitrobenzenesulfonic acid (TNBS) reagent in a water bath at 50 °C for 30 min. The reaction was terminated by adding 0.1 M sodium sulfite. The absorbance at 420 nm was read, and a standard curve of L–leucine was used for the amine content calculation.

Carbonyls. The carbonyl content was determined using the 2,4-dinitrophenylhydrazine (DNPH) colorimetric method as described by Levine et al. (1990). MP samples were mixed with DNPH solution and precipitated with 20% TCA. Recovered protein was washed to remove unreacted DNPH and then dissolved in 6 M guanidine hydrochloride (pH 2.3) for re-solubilization. The absorbance (at 370 nm) was read and a molar extinction coefficient of 22,000 M^{-1} cm^{-1} was used for carbonyl content calculation. The absorbance from reagent blanks (including background phenolics) was subtracted from respective samples.

Surface Hydrophobicity. MP samples were diluted to 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL protein in 50 mM PIPES buffer (pH 6.25) containing 0.6 M NaCl. To 5.0 mL of the diluted solution, 25 μL of 8.0 mM 8-anilinonaphthalene-1-sulfonic acid (ANS) were added and thoroughly mixed. After 15 min of incubation at room temperature, the fluorescence intensity (FI) was measured using a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ) with excitation and emission wavelengths set at 390 and 470 nm, respectively (slit width 5 nm). The FI of sample blanks (diluted protein samples without ANS) was measured and subtracted from the respective samples. The initial slope

of the FI–protein concentration plot was calculated by linear regression analysis and defined as surface hydrophobicity (S_0) (Cao et al., 2018).

Tryptophan Fluorescence. Dilute suspensions of MP (0.4 mg/mL in 50 mM PIPES buffer, 0.6 M NaCl, pH 6.25) were used for the analysis. The intensity of intrinsic tryptophan fluorescence, an indicator of protein structural and conformational integrity, was determined using the FluoroMax-3 fluorometer. With an excitation wavelength of 283 nm, the emission spectrum was recorded from 300 to 450 nm at a 1 nm/s scanning speed. The slit widths of both excitation and emission were set at 10 nm, and the data were collected at a 500 nm/min rate. Background spectra under the same conditions were registered and subtracted from the respective spectra of MP samples.

5.3.6 Detection of protein cross-linking

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted to determine covalent protein aggregation in both unheated (sol) and heated (gel) MP samples according to Laemmli (1970) with a slab gel system comprised of a 3% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel. To elucidate the possible involvement of disulfide bonds in oxidation-induced protein polymerization, regardless of phytophenolic treatments, 10% β-mercaptoethanol was added to the SDS–PAGE sample buffer before boiling for 3 min. Furthermore, as heating always promotes protein aggregation, the influence of temperature on phenolic-induced cross-linking was analyzed on MP samples treated with GA (as a model phenolic acid, 60 μmol phenol/g MP) after being heated to 20, 30, 45, 50, 55, 60, and 70 °C. The extent of myosin heavy chain (MHC) loss due to phenolic or heating treatments, relative to the respective MP controls, was

estimated using the UN–SCAN–IT software (Silk Scientific, Orem, UT) with the following formula:

$$\text{Relative loss (\%)} = \frac{\text{pixel intensity in control} - \text{pixel intensity in sample}}{\text{pixel intensity in control}} \times 100$$

5.3.7 Preparation of MP–emulsion composite gels

Emulsions were prepared at room temperature by homogenizing the MP suspension (20 mg/mL) and oil (25%, w/w) using a Kinematica Polytron™ PT 10-35 GT blender (Brinkmann Instruments, Inc., Westbury, NY) equipped with a PT-DA 12/2 EC-B154 probe at a speed of 17,500 rpm for 2 min. Fresh emulsions were immediately mixed with MP suspensions (40 mg/mL protein) in 50 mM PIPES buffer (pH 6.25) containing 0.6 M NaCl to obtain MP−emulsion composite sols with a final concentration of 30 mg/mL MP and 10% lipid. Phenolics at an equal molar (60 μmol phenolic/g MP) or equal phenol (60 μmol phenol/g MP) concentration basis were thoroughly mixed with the MP–emulsion composite sol before incubation under the GOx-mediated oxidation at 4 °C for 8 h.

5.3.8 Evaluation of gelling properties of MP–emulsion composite gels

Two different tests were conducted to analyze the gelling ability and gel characteristics of MP samples treated with phenolic compounds: dynamic rheological measurement (which probes protein sol-to-gel transformation) and extrusion disruption testing (which assesses the mechanical properties of set gels) (Guo & Xiong, 2019). For the former, MP–emulsion composites were subjected to oscillatory shear testing using a Bohlin CVO 100 rheometer (Malvern Instruments, Westborough, MA). Thermal gelation was achieved by heating the MP sols loaded between two parallel plates (30 mm upper plate diameter, 1 mm gap) from 20 to 72 °C at a heating rate of 1 °C/min. The exposed

sample rim was covered with a thin layer of silicon oil to prevent dehydration. During heating, samples were subjected to shearing in an oscillatory mode with a fixed frequency of 0.1 Hz and a maximum strain of 0.02. The storage modulus (G′) was recorded every 30 s.

For the extrusion test, aliquots of 5 g MP–emulsion composite sols were poured into glass vials (18 mm inner diameter), covered with aluminum foil, and then heated in a water bath from 20 to 72 °C at a 1 °C/min heating rate. After reaching the final temperature, gels were immediately chilled in an ice slurry for 30 min and stored at 4 °C overnight. The set gels were then equilibrated at room temperature for 1 h and extruded with a stainless-steel, flat-ended probe (10 mm diameter) attached to a Model 4301 Instron universal testing machine (Canton, MA) at a crosshead speed of 20 mm/min until structural failure. The puncture force required to disrupt the gels was recorded as gel strength (N).

5.3.9 Morphological examination of MP gels

The microstructure of the MP–emulsion composite gels formed in the vials was examined under light microscope as described by Wu, Xiong, Chen, Tang, and Zhou (2009). Approximate 5 mm^3 blocks of sample were excised from intact gels and fixed in 8% paraformaldehyde overnight. The samples were then dehydrated in a series of ethanol (50, 70, 90, and 100%), treated with xylol–ethanol, and embedded in paraffin wax. Sections (8 μm thick) were cut using a microtome, and the slices were stained with Ehrlich's hematoxylin. Specimen slides were observed under a MICROPHOT-FXA Nikon photomicroscope equipped with a built-in digital camera (Nikon Inc., Garden City, NY).

5.3.10 Assessment of oxidative stability

Cooked MP–emulsion composite gels were stored in the refrigerator for up to 7 days at 4 °C. Lipid oxidation during storage was checked on days 0, 3, and 7 by measuring the amount of 2-thiobarbituric acid-reactive substances (TBARS) produced (Salih, Smith, Price, & Dawson, 1987). Approximately 3 g gel samples were weighed and mixed into the assay solution containing trichloroacetic acid (TCA) and thiobarbituric acid (TBA) followed by boiling for 30 min. The supernatant of cooled solution was mixed with chloroform and centrifuged at 2000*g* for 10 min. The upper phase was further clarified with petroleum ether and centrifuged at the same conditions. Absorbance of the lower phase containing the pinkish TBA-malonaldehyde adducts was measured at 532 nm. The TBARS value, expressed as mg malondialdehyde (MDA) equivalent per kg gel sample, was calculated using the following equation:

$$\text{TBARS (mg/kg)} = (A_{532}/W_S) \times 9.48$$

where A_{532} is the absorbance at 532 nm, W_s is the sample weight (g), and the value 9.48 is a constant derived from the sample dilution and the absorption coefficient (15,600 M^{-1} cm^{-1}) of the TBA-malonaldehyde adduct.

5.3.11 Statistical analysis

Experiments were conducted with three independent trials (n = 3) on different days, each with a new batch of isolated MP. Data were subjected to the analysis of variance using a general linear model's procedure in Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA). Significant ($P < 0.05$) differences between means were identified by LSD all-pairwise multiple comparisons.

5.4 Results and discussion

5.4.1 Modification of amino acid side chains and surface hydrophobicity

Total sulfhydryl content decreased slightly, from 70 nmol/mg in the non-oxidized control sample to 63 nmol/mg in the oxidized control sample (Figure 5.1A). The addition of phenolic compounds further decreased the amount with EGCG causing the greatest reduction to 52% ($P < 0.05$). The free amine content of oxidized MP was 13% less compared with non-oxidized MP at 110 nmol/mg protein ($P < 0.05$), and samples treated with most of the phenolics displayed further amine reductions with various efficiencies (Figure 5.1B). EGCG and GA induced the largest loss (57% and 46%, respectively, $P < 0.05$). CA was the only phenolic compound that did not significantly change the content of the amine groups ($P > 0.05$). Compared with the control (non-oxidized) MP, GOx-mediated oxidation increased the carbonyl content of MP by 1.7 fold ($P < 0.05$), and this change was generally inhibited by the phenolics (Figure 5.1C). The inhibition was most effective with CC and EGCG which decreased the carbonyl production by 50% and 39%, respectively ($P < 0.05$). Moreover, under the GOx oxidation condition, protein surface hydrophobicity decreased slightly probably due to aggregation, and the presence of phenolic compounds further reduced the S_0 (Figure 5.1D) suggesting less exposures of hydrophobic groups. Of the different phenolics tested, CC and EGCG caused the greatest S_0 reductions, by 45% and 39%, respectively ($P < 0.05$). In a typical redox environment in muscle food processing, sulfhydryl groups from cysteine residues in MP can be converted to thiol derivatives, including sulfenic acid, sulfinic acid, sulfonic acid, and disulfides (Lund et al., 2011). Concurrently, some free amine groups from lysine residues are oxidized into carbonyls which subsequently react with primary amines to form the Schiff's

base adduct (Levine et al., 1990). Phenolic compounds can protect MP from oxidative modification and inhibit the formation of carbonyls (Estévez & Heinonen, 2010). Their oxidized quinone derivatives, however, are electrophilic and will bind with –SH and $–NH_2$ to form protein–phenol complexes, thus, promoting the loss of these reactive amino acid side chain groups (Cao, Ma, Huang, & Xiong, 2020). Phenolic-induced modification of amino acid side chains can lead to stronger protein cross-linking through two possible pathways (Figure 5.8). One is quinones acting as a bridge to connect two peptides (protein–quinone–protein) (Strauss & Gibson, 2004), and the other is the oxidative transformation of sulfhydryls to disulfide bonds.

5.4.2 Changes in protein structure

As shown in Figure 5.2, phenolic compounds quenched the fluorescence of MP and induced a red shift in λ_{max}, indicating the occurrence of MP unfolding (exposures to a more polar environment) and protein–phenolic binding. In general, the fluorescence quenching effect followed the order of triphenol > diphenol > monophenol. GA, the smallest phenolic compound of all, reduced the fluorescence intensity by merely 8.7% while the largest, EGCG, caused a 34% reduction. Interestingly, CA, a medium-sized monophenol as an ester of caffeic acid and quinic acid, caused the most reduction in fluorescence intensity at 52%, and the largest red shift at 22.5 nm.

Protein–phenolic interactions, both covalent and non-covalent, lead to unfolding, which can be measured by assessing quenching of intrinsic tryptophan fluorescence (Rawel, Frey, Meidtner, Kroll, & Schweigert, 2006; Royer, 2006). Tryptophan residues in native MP is buried in the hydrophobic core. When the protein tertiary structure is disrupted by phenolic binding, tryptophan residues are exposed to a more polar environment, leading to

a reduced fluorescence intensity and a larger λ_{max} (red shift) as seen in Figure 5.2. Moreover, the conceivable stacking of the benzene ring(s) in phenolics and the indole structure in tryptophan is likely to contribute to the fluorescence quenching activity. Surface hydrophobicity is an important property of MP as it is closely related to protein functionalities (Li-Chan et al., 1985). It was measured based on the principle that the fluorescent probe ANS binds to the aromatic hydrophobic residues on the protein surface to emit strong fluorescence when excited (Alizadeh-Pasdar & Li-Chan, 2000). The interaction of MP with phenolics may result in the blockage of exposed hydrophobic sites available for ANS, thus, decrease S_0. In addition, the attenuated S_0 can result from phenolic-induced protein aggregation, which limits ANS binding to proteins by reducing the surface area as well as lowering the effective concentration of MP (i.e., free molecules).

5.4.3 Protein cross-linking

Polymers were formed in oxidatively stressed MP with or without the presence of phenolic compounds (Figure 5.3). Some of the polymers were conspicuously derived from myosin heavy chain (MHC), the major gelling component of MP, because of its concomitant diminishments. For MP sols, MHC bands were recovered almost completely after disulfide bonds were cleaved by +βME, so the results are not shown in Figure 5.3. For MP gel samples, covalently linked protein aggregates were too large to enter either the stacking gel or the separation gel during SDS–PAGE, hence, a reducing agent (+βME) was used to dissolve the gels and assess the role of non S–S linkages. Moreover, the relative loss of MHC induced by oxidation and phenolic treatments, as well as the impact of temperature, were analyzed to estimate the extent of protein cross-linking (displayed as inset tables beneath the SDS–PAGE images). In non-oxidized MP sols (Figure 5.3A), the

GOx-mediated oxidation significantly decreased the intensity of MHC, by 42 % ($P < 0.05$), and the addition of phenolics did not promote this change. Upon heating (Figure 5.3B), however, the MHC band in the oxidized MP gel samples under the reducing condition (+βME) were further diminished by the addition of phenolics. GA and EGCG induced the greatest attenuation, up to 64 % ($P < 0.05$). As to the temperature effect (evaluated in a GA model), heating from 20 °C to 70 °C (the common temperature range for meat processing) caused significant losses of MHC in reduced MP samples, i.e., from 12 % to 27 % ($P < 0.05$, Figure 5.4A). When assessed under the reducing condition (+βME), within the same temperature range, a higher amount of MHC loss was observed in the GA-treated MP samples, i.e., from 29 % to 45 % ($P < 0.05$) (Figure 5.4B). The extra loss of MHC in +βME samples than in –βME samples was attributed to MP polymerization due to non-disulfide bonds.

5.4.4 MP gelling properties

All MP–emulsion composite samples displayed a typical rheological pattern during heating, which is highlighted by a transitional peak within 38–48 °C due to the transient hydrophobic association and molecular rearrangement of the myosin globular head (Figure 5.5A). However, the development of G′ (the elastic modulus of the gel) varied between sample treatments. Oxidized MP had a markedly higher final G′ than the non-oxidized MP control (567 Pa vs. 279 Pa), and the presence of phenolics further increased this elasticity parameter. On an equal molar concentration basis (60 μmol phenolic/g MP), PG and the triphenol EGCG produced higher final G′ values than other monophenols (GA and CA) and the diphenols (QT and CC). Yet, at an equal phenol concentration (60 μmol phenol/g MP), MP samples with monophenols (same as 60 μmol phenolic/g MP) exhibited

consistently higher final G′ values than diphenols (30 μmol phenolic/g MP) while EGCG (20 μmol phenolic/g MP) remained to be a strong promotor of MP network formation.

For set MP gels, there was no noticeable cooking loss for control and all treatments except for the EGCG-treated gels that exhibited considerable syneresis (data not shown). As to the measured strength of these set gels, all three monophenols, along with the diphenol QT, positively influenced the mechanical property of the MP gels with the gel strength improvements reaching 29–56 % when compared with oxidized MP (Figure 5.5B). However, MP samples treated with CC and EGCG either produced a weaker gel or had a similar gel strength when compared with the phenolic-free MP control, which was different from the effect on G′. Visible coagulation and, as aforementioned, syneresis, were noticed in gels treated with EGCG.

5.4.5 Morphology of MP gels

In the microstructure of MP–emulsion composite gels, lipid droplets were surrounded by a protein membrane and embedded in a proteinaceous gel matrix (Figure 5.6). Compared with the larger and round-shaped oil droplets found in the non-oxidized control sample, the gels made from oxidatively stressed MP displayed a denser and more uniform protein matrix, and lipid particles were smaller and irregularly shaped. Similar but more pronounced irregularities in the oil droplets morphology were noticeable when GA, CA, PG, and QT were present. For samples treated with CC and EGCG, however, fat droplets coalesced, leading to the occurrence of discontinuous patches.

5.4.6 Lipid oxidative stability

The formation and accumulation of TBARS as secondary lipid oxidation products in emulsion gel samples throughout the storage period of 0 to 7 days were monitored. As

presented in Figure 5.7, the concentration of TBARS in the non-oxidized control sample on day 0 was approximately 0.9 mg/kg, and the level increased to 3.6 mg/kg by day 7 ($P < 0.05$). In comparison, in gel samples made from oxidatively stressed MP, there was less TBARS production (2.0 mg/kg by day 7) than in the non-oxidized control ($P < 0.05$). As expected, the presence of phenolic compounds, irrespectively of structures, significantly suppressed TBARS production. This phenomenon was most remarkable for the two highly nonpolar compounds, PG and QT, which reduced the TBARS content up to 94 % and 95 % on day 7, respectively ($P < 0.05$), when compared with the non-oxidized MP overall control gel. The 2,3-double bond in the C ring and the 4-oxo group in QT are known to contribute to its strong antioxidant activity (Minatel et al., 2017).

5.4.7 Structure-activity relationship of phenolic compounds

Compared with the oxidized phenolic-free control sample, the monophenols effectively improved the gel strength to a similar extent, but PG gave rise to a higher elasticity than GA and CA (Figure 5.5). The aliphatic propanol moiety in PG, combined with the lack of a carboxyl unit, might have promoted its hydrophobic partitioning at the interface of the emulsion, resulting in a stronger interfacial membrane and higher storage modulus (G′) of the MP–emulsion composite gel. Consistent with the gelling property improvement, the morphology of these monophenol-treated gels displayed a heterogeneous membrane of the emulsion particles, which may be attributed to MP accumulation beyond the myosin monolayer at the oil surfaces (Wang et al., 2017).

The two diphenolic flavonoids (QT and CC) showed a similar effect on –SH and –NH_2 as well as efficacy for increasing the gel elasticity (G′) of MP whether on an equal molar (60 μmol phenolic/g MP) or equal phenol (30 μmol phenol/g MP) concentration

basis (Figure 5.5). However, CC decreased the surface hydrophobicity (Figure 5.1D) and induced stronger protein unfolding (Figure 5.2) and cross-linking (Figure 5.3), indicating that the flavanol structure (with asymmetrical C2 and C3) in CC had a higher binding affinity for MP than the flavonol structure in QT. The stronger CC–MP interaction led to a weaker gel when compared with the QT treatment, which was not surprising because coagulated oil droplets and a somewhat degraded gel structure were present in the CC-treated gel samples (Figure 5.6). The latter effect could originate from extensive MP aggregation in CC treated samples, which generated insoluble aggregates and impeded emulsification. Similar results have been published where CC reportedly induced severe textural deterioration in MP gels (Jia et al., 2017).

On the other hand, being the largest phenolic compound tested, EGCG (with three aromatic rings) induced the greatest loss of –SH and $–NH_2$ groups. It is likely that the high electrophilicity of the pyrogallol structure of the B ring (epigallocatechin) as well as the phenol ring in the gallate unit of EGCG facilitated quinone adductions to MP through both electron dense protein sidechain groups. This premise is supported by several previous findings that EGCG was strongly reactive with amine and sulfhydryl groups (Cao et al., 2018; Lv et al., 2019; Mori, Ishii, Akagawa, Nakamura, & Nakayama, 2010). Furthermore, the gallate moiety with multiple hydroxyl groups in EGCG are likely responsible for the triphenol's strong efficacy in reducing the protein carbonyl content, since antioxidant activity of phenolic compounds usually increases with an increase in the number of hydroxyl groups (Fukumoto & Mazza, 2000). In addition, the triphenol is capable of providing multiple protein binding sites, contributing to enhanced cross-linking by acting as a bridge. As a result, a more elastic (G′) protein network was produced by EGCG-treated

MP than the monophenol (except for PG) and diphenol treatments. However, the interaction between MP and EGCG, which possesses trice (GA, CA, and PG) and twice (QT and CC) as many phenol rings on an equal molar concentration, was so strong that the MP gel structure became porous and discontinuous (Figure 5.6). The partial recovery of the lost MP gel strength in the equal-phenol EGCG treatment (as well as in the CC treatment) (Figure 5.5), where the molar concentration of the triphenol was reduced 3 fold, strongly suggests the deleterious gel structure-breaking effect at high concentrations. Similar results have been obtained from other studies where EGCG caused extensive protein aggregation at the emulsion interface, leading to the disruption of lipid globules and the migration of water, thus, an unstable gel structure (Cao et al., 2018; Lv et al., 2019).

5.5 Conclusion

In conclusion, the effect of phenolics on the physiochemical and gelling properties of MP is dependent on their structural attributes, including number of phenol rings, size, and polarity. For monophenols, molecules with less structural hinderance are more effective in promoting protein cross-linking whereas compounds with larger substituent groups are better at facilitating protein unfolding. Polyphenols with multiple phenol structures are likely to react more strongly with MP than phenolics with fewer phenol rings due to the presence of more reactive groups in their structures. Phenolic-induced physiochemical changes in MP are well reflected in the textural properties and oxidative stability of MP–emulsion composite gels. Further studies are needed to evaluate the cooperative effects of combined phenolic compounds on the properties of gelled muscle foods, because composition-optimized mixtures of different compounds with various

structures and activities are likely to provide a broad base for developing products with the most desirable characteristics.

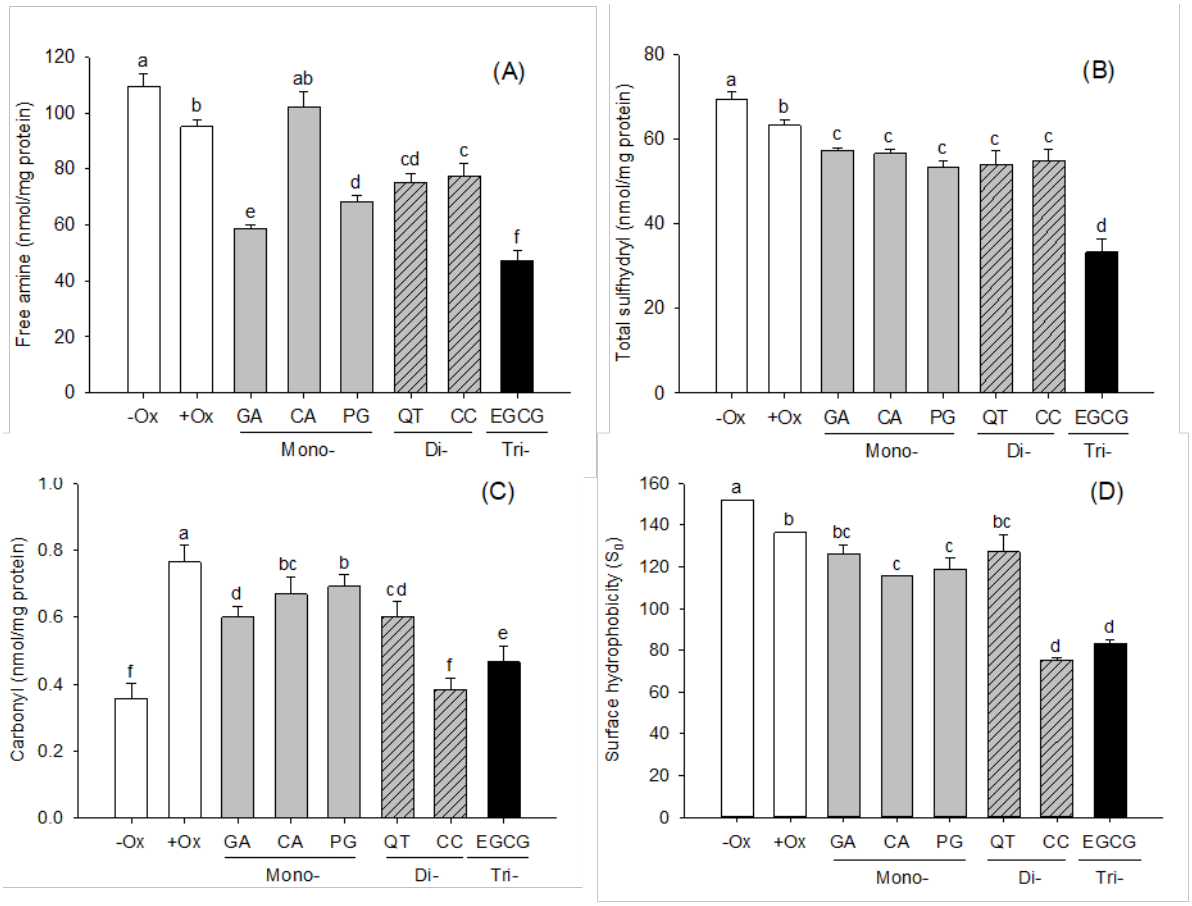
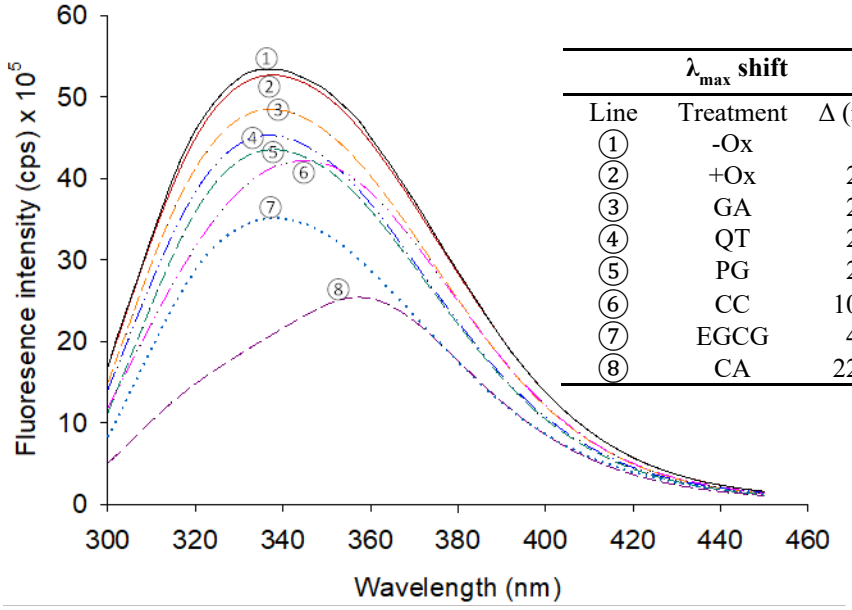
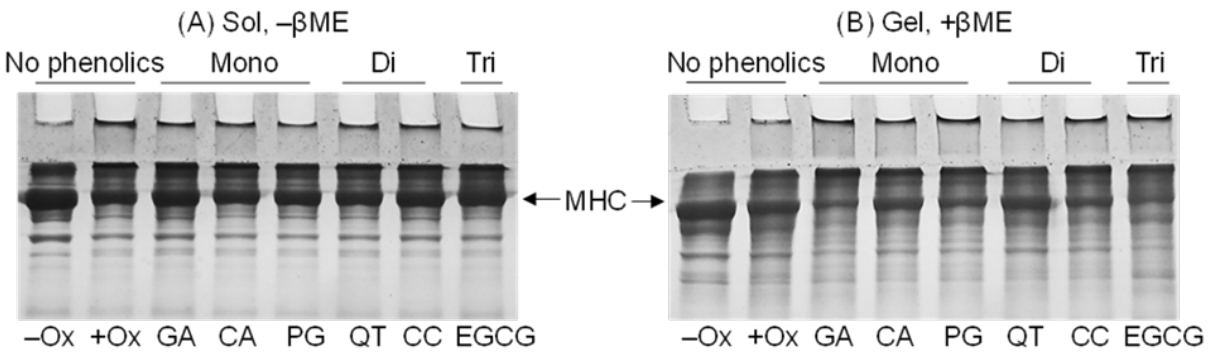


Figure 5.1. Total sulfhydryl (A), free amine (B), carbonyl (C), and surface hydrophobicity (D) in MP treated with different phenolics (60 µmol/g MP) in an oxidative environment. Control samples (without phenolics) included both nonoxidized (–Ox) and oxidatively stressed (+Ox) MP. Monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); diphenols: quercetin (QT) and catechin (CC); triphenol: (−)-epigallocatechin-3-gallate (EGCG). Means with different letters (a–f) differ significantly ($P < 0.05$).



λ_{max} shift		
Line	Treatment	Δ (nm)
①	-Ox	0
②	+Ox	2.5
③	GA	2.0
④	QT	2.0
⑤	PG	2.5
⑥	CC	10.5
⑦	EGCG	4.5
⑧	CA	22.5

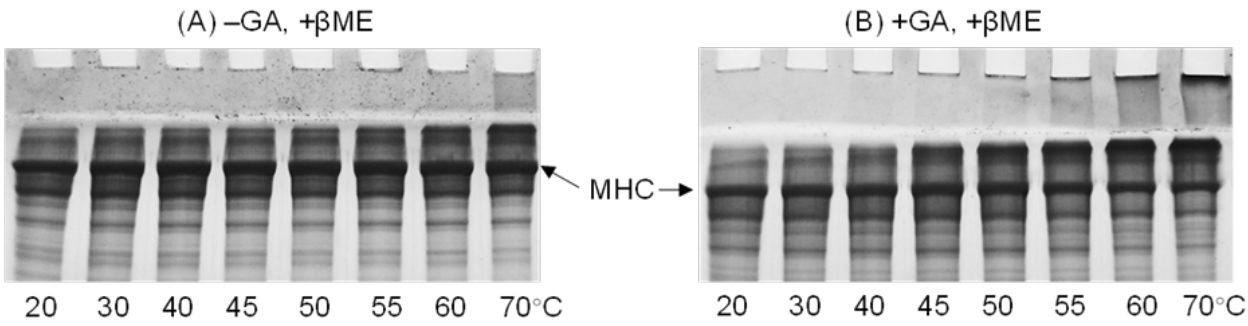
Figure 5.2. Tryptophan fluorescence intensity of MP treated with different phenolics (60 µmol/g MP) in an oxidative environment. Control samples (without phenolics) included both nonoxidized (–Ox) and oxidatively stressed (+Ox) MP. Monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); diphenols: quercetin (QT) and catechin (CC); triphenol: (−)-epigallocatechin-3-gallate (EGCG). Δ: maximum wavelength (λ_{max}) shift from nonoxidized control.



Relative loss of MHC

Gel	Phenolic compound							
	–Ox	+Ox	GA	CA	PG	QT	CC	EGCG
(A)	0.0	42a	29c	43a	42a	43a	35b	39ab
(B)	0.0	28c	64a	36c	48b	27c	46b	64a

Figure 5.3. Representative SDS–PAGE patterns of MP in sols (A) and gels (B) focusing on the changes in myosin heavy chain (MHC) induced by the interaction with phenolics. MP was treated with different phenolics (60 μmol/g MP) in an oxidative environment. Control samples (without phenolics) included both nonoxidized (–Ox) and oxidatively stressed (+Ox) MP. Monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); diphenols: quercetin (QT) and catechin (CC); triphenol: (−)-epigallocatechin-3-gallate (EGCG). βME: β-mercaptoethanol. Means with different letters (a−c) within the same row differ significantly ($P < 0.05$).



Relative loss of MHC

Gel	Temperature (°C)							
	20	30	40	45	50	55	60	70
(A)	0.0	12c	12c	18b	19b	18b	21b	27a
(B)	0.0	29c	29c	27c	28c	35bc	38ab	45a

Figure 5.4. Representative SDS–PAGE patterns of MP focusing on changes in myosin heavy chain (MHC) induced by the interaction with gallic acid (GA, 60 μmol/g MP) at different temperatures. βME: β-mercaptoethanol. Means with different letters (a–c) within the same row differ significantly ($P < 0.05$).

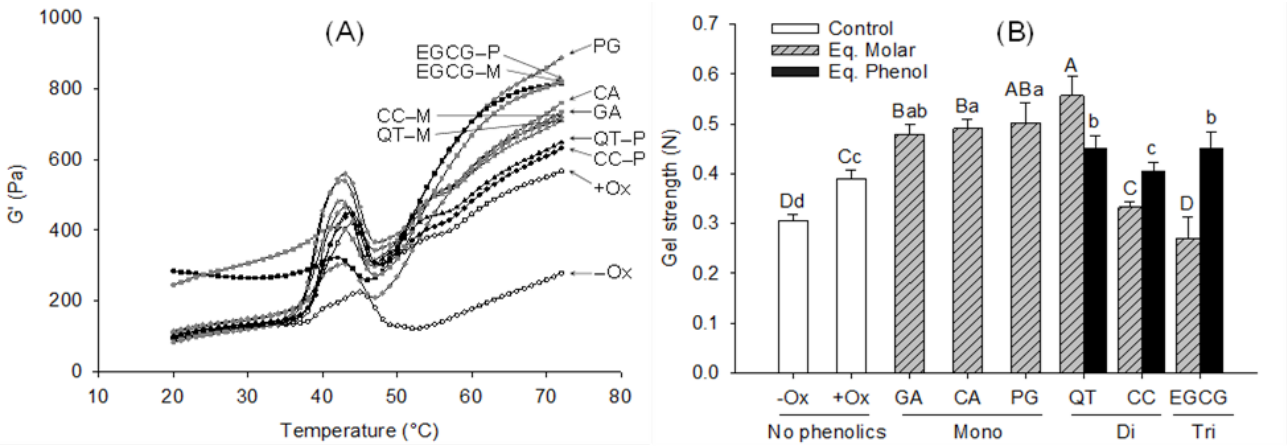


Figure 5.5. Storage modulus (G′) development during thermal gelation (A) and the gel strength (B) of the MP–emulsion composite (30 mg/mL protein, 10% fat) treated with different phenolics in an oxidative environment. Control samples (without phenolics) included both nonoxidized (–Ox) and oxidatively stressed (+Ox) MP. Monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); diphenols: quercetin (QT) and catechin (CC); triphenol: (−)-epigallocatechin-3-gallate (EGCG). Phenolics were added at an equal molar concentration (–M; 60 μmol/g MP) or an equal phenol concentration (–P; 60, 30, and 20 μmol/g MP for mono-, di- and triphenols). Means with different letters (A–D for equal molar; a−d for equal phenol) differ significantly ($P < 0.05$).

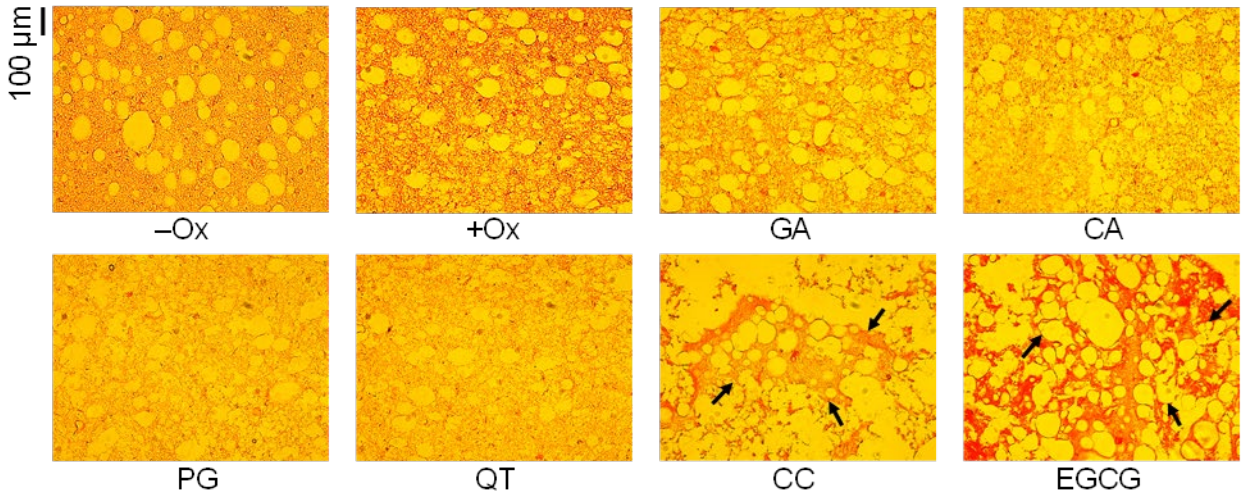


Figure 5.6. Light microscope images of MP−emulsion composite gels (30 mg/mL protein, 10% fat) treated with different phenolics (60 µmol/g MP) in an oxidative environment. Control samples (without phenolics) included both nonoxidized (–Ox) and oxidatively stressed (+Ox) MP. Monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); diphenols: quercetin (QT) and catechin (CC); triphenol: (−)-epigallocatechin-3-gallate (EGCG). The arrows point to the gel network discontinuity.

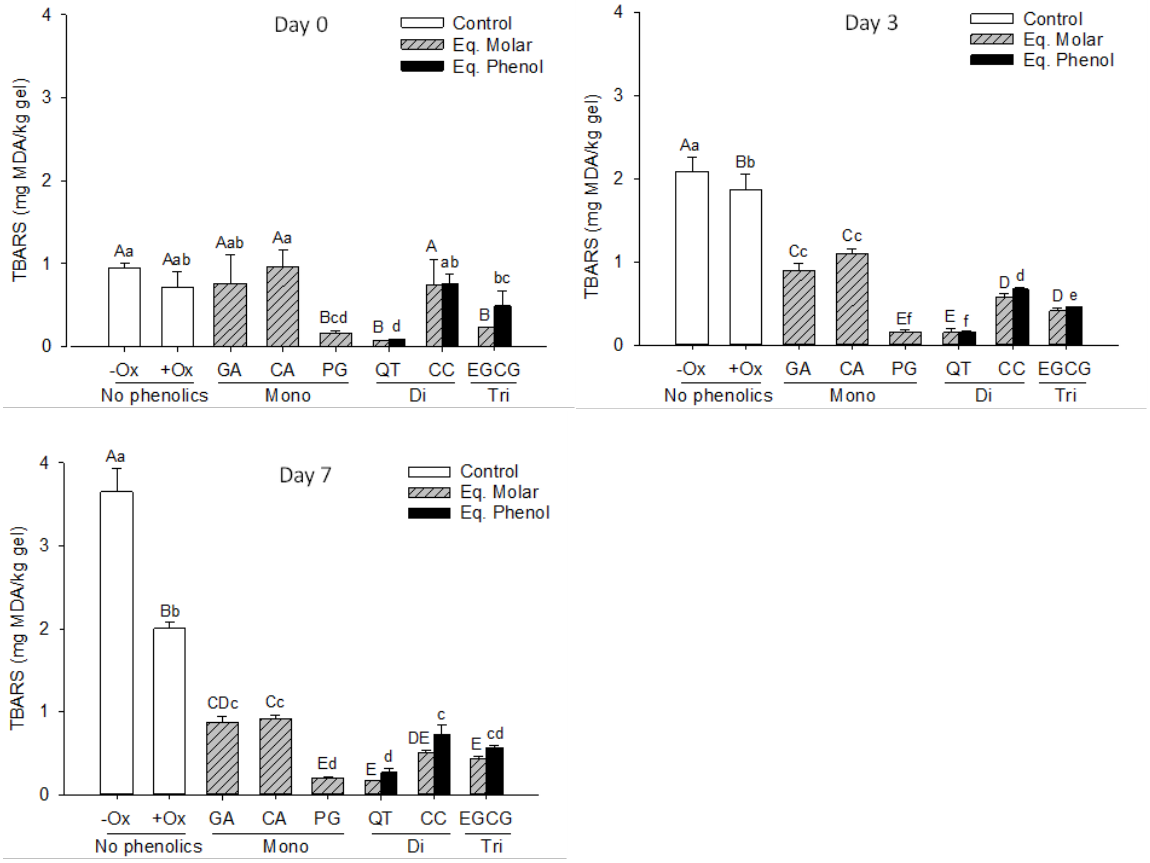


Figure 5.7. Lipid oxidation (TBARS) in MP–emulsion composite gels (30 mg/mL protein, 10% fat) treated with different phenolics in an oxidative environment during refrigerated storage (4 °C) up to 7 days. Control samples (without phenolics) included both nonoxidized (–Ox) and oxidatively stressed (+Ox) MP. Monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); diphenols: quercetin (QT) and catechin (CC); triphenol: (−)-epigallocatechin-3-gallate (EGCG). Phenolics were added at an equal molar concentration (60 µmol/g MP) or an equal phenol concentration (60, 30, and 20 µmol/g MP for mono-, di- and triphenols). In the same day, means with different letters (A–E for equal molar; a–e for equal phenol) differ significantly ($P < 0.05$).

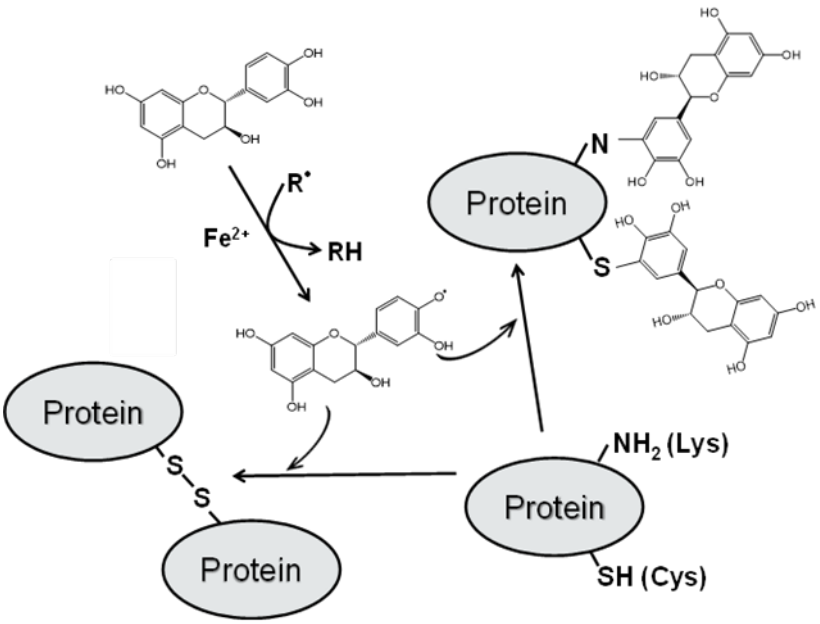


Figure 5.8. Proposed reactions of phenolics with MP under oxidative conditions.

CHAPTER 6. ELECTRICAL CONDUCTIVITY: A SIMPLE AND SENSITIVE METHOD TO DETERMINE EMULSIFYING CAPACITY OF PROTEINS

6.1 Summary

Emulsifying capacity (EC) of proteins is a benchmark standard widely used to evaluate the quality of protein ingredients in emulsion foods. EC (mL of oil emulsified per g of protein) is usually measured by a sudden drop in electrical resistance (phase transition) with the continuous addition of oil to a specific protein solution. However, little is known about electrochemical mechanisms behind this process because resistance, measured with an ohmmeter, is not sensitive enough to monitor changes in the concentration of protein electrolytes. In this study, pea (PPI), myofibrillar (MPI), and whey (WPI) protein isolates were vigorously homogenized with oil at a series of oil/protein ratios to prepare emulsions with different final protein concentrations. The conductivity was closely monitored using a conductivity meter. A linear relationship was discovered between conductivity and the final protein concentrations. At higher oil fractions, the migration of proteins from the aqueous phase to the oil–water interface limited protein mobility, leading to a conductivity drop. EC was calculated from the regression lines; when the starting protein concentration was raised from 0.5% to 2.0%, the EC of PPI, MPI, and WPI decreased from 717, 782, and 1339 to 219, 303, and 540 mL oil/g protein, respectively. The dependence of EC on the initial protein concentration and the sensitivity of conductivity to the depleting protein electrolytes suggest that protein concentration is an important factor to consider when determining EC for a given protein or comparing EC among different proteins.

6.2 Introduction

Oil-in-water (O/W) emulsions are extremely important in the food industry because they are present in a wide range of food products, such as cream liqueur, ice cream, salad dressing, mayonnaise, and bologna meats. O/W emulsions are thermodynamically unstable. Hence, to facilitate the dispersion and prevent the separation into two immiscible phases (water and oil), a strong surfactant is required (Dickinson, 2019). As amphiphilic macromolecules, proteins can act as emulsifiers to lower the interfacial tension and form a protective membrane (Gohtani & Yoshii, 2018). A distinct advantage of protein emulsifiers when compared with small-molecule surfactants (phospholipids, Tween, etc.) is that protein-coated emulsion droplets are less susceptible to oxidation due to steric (shielding) effects and radical scavenging activity of the protein membrane (Hu, McClements, & Decker, 2003; Yang & Xiong, 2015). Protein-stabilized emulsion has drawn a copious amount of attention in scientific research, and this is well reflected in the number of recent publications. In 2020 alone, 15,700 papers related to "protein-stabilized emulsion" were registered in Google Scholar search.

The efficiency of proteins to act as emulsifiers vary among different proteins due to inherent structural and surface activity differences (McClements, 2015). Emulsifying activity index (EAI) is commonly used to evaluate emulsifying properties of proteins (Pearce & Kinsella, 1978). However, it only describes the behavior of proteins at the interface and does not predict the potential of protein that can be applied to create a stable O/W emulsion. Therefore, emulsifying capacity (EC), expressed as the amount of oil (mL) emulsified per g of protein, is a valuable attribute of proteins relating to their potential for stabilizing oil in an emulsion system.

A common approach to assessing the EC of a protein involves dropwise addition of oil to a protein-water dispersion under constant mixing until phase inversion occurs which is indicated by a sudden change in electrical resistance (Webb, Ivey, Craig, Jones, & Monroe, 1970). While widely used for EC determination, this convenient method has its limitations since inconsistent results can derive from several factors in the procedure, such as the oil addition rate, blending speed, and homogenization duration (Akintayo, Esuoso, & Oshodi, 1998; Wang & Maximiuk, 2015). Moreover, dynamic electrical changes caused by increasing the oil content are not accurately reflected because resistance is not sensitive to ion concentrations. As evidence, the electrical resistance (in ohm or Ω) before the inversion point shows little change even though the protein content is largely diluted by oil titration (Webb et al., 1970). In comparison, electrical conductivity (in micro siemens per centimeter or μS/cm) is a direct function of dissolved ions, including charged proteins (macromolecular electrolytes) in the case of protein-based emulsions. Therefore, electrical conductivity may offer an insight into the underlying mechanisms of the phase inversion phenomenon.

In this study, a simple and consistent method for EC determination was invented by measuring electrical conductivity of emulsions prepared with different oil/protein ratios and the mechanism of protein-induced conductivity change was elucidated. Various concentrations of representative proteins from different food groups were tested, i.e., isolated pea protein (PPI), myofibrillar protein (MPI), and whey protein (WPI).

6.3 Materials and methods

6.3.1 Materials

Longissimus lumborum muscle samples were collected from pork carcasses (24 h post-mortem) harvested at the University of Kentucky Meat Laboratory, a USDA-approved facility. The loin muscle samples were cut into 1 cm chops before being individually vacuum-packaged and stored in a −30 °C freezer until use. Frozen muscle samples were thawed at 4 °C overnight and then chopped into small pieces. MP was isolated from the minced muscle using an extraction buffer consisting of 10 mM sodium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EGTA at pH 7.0 (Park et al., 2007). In the last washing step, the pH of MP suspended in 0.1 M NaCl was adjusted to 6.25. The MP pallet was kept on ice and utilized within 48 h. The preparation was conducted in a 4 °C walk-in cooler. Protein concentration was determined by the Biuret method using bovine serum albumin as a standard (Gornall et al., 1949). Pea protein isolate (PPI) was donated by Roquette America Inc. (Geneva, IL, USA). Whey protein isolate (WPI) was donated by Davisco (Le Sueur, MN, USA). Sunflower oil was purchased from Kroger Supermarket (Lexington, KY, USA). All chemicals used were reagent or a higher grade obtained from VWR (Radnor, PA, USA) or Thermo Fisher Scientific (Waltham, MA, USA). Double-deionized water was used in all experiments.

6.3.2 Preparation of protein stabilized emulsions

MPI was suspended in 0.6 M NaCl (to ensure solubility) while PPI and WPI were dissolved in water to make 0.5, 1.0, 1.5, and 2.0% (w/v) protein solutions, and the pH was adjusted to 6.5. To each of the protein solutions, eight aliquots of oil at 20, 50, 100, 200, 400, 600, 800, and 1000 mL oil/g protein were added, i.e., the same eight oil to proteins

ratios were prepared for proteins with different concentrations. The mixtures were homogenized using a PolytronTM PT 10-35GT homogenizer (Brinkmann Instruments, Inc., Westbury, NY, USA) with a PT-DA 12/2 EC-B154 dispersing aggregate at a speed of 17,500 rpm for 1 min. Freshly prepared emulsions were immediately subjected to EC measurement.

6.3.3 Measurement of emulsifying capacity

EC measurement by conductivity. Electrical conductivity of the emulsions was measured using a Model B30PCI benchtop multi parameter meter (VWR, Radnor, PA, USA). To process the data, electrical conductivity (μS/cm, y axis) was plotted against the corresponding final protein concentrations (mg/mL, x axis) calculated based on the initial protein concentration and the amount of oil added. A regression line was drawn within the linear region (before the deviating inflection point), which was then extrapolated to the x axis to approximate the final protein concentration (mg/mL) when the conductivity reached zero (Figure 6.1). Once the theoretical inversion point was established, the following formulas were used to calculate EC. Based on the protein volume as well as initial and final protein concentrations, V_{oil} can be expressed as a function of V, c_1, and c_2 (eq. 1), from which EC value can be derived (eq. 2).

$$c_2 = \frac{c_1 V}{V+V_{oil}}, \text{ or } V_{oil} = \frac{(c_1-c_2)V}{c_2} \quad (1)$$

$$\text{EC (mL oil/g of protein)} = \frac{V_{oil}}{m} = \frac{V_{oil}}{c_1 V} * 1000 = \frac{(c_1-c_2)}{c_1 * c_2} * 1000 \quad (2)$$

where V_{oil} is the amount of oil added (mL), V is the starting volume of protein solution (mL), m is the protein weight (g), c_1 is the initial protein concentration (mg/mL), and c_2 is the final protein concentration (mg/mL) derived from the regression formula when conductivity becomes zero.

EC measurement by resistance. To compare the new conductivity method with the conventional resistance method, two copper electrodes of a Model Multiview 110 digital multimeter (Extech Instruments, Nashua, NH, USA) were suspended into the freshly prepared emulsions to measure electrical resistance.

6.3.4 Microstructure of emulsion droplets

The microstructure of emulsions made up with initially 1% protein (PPI, MPI, and WPI) and 20, 200, and 1000 mL oil/g protein was examined. A drop of each freshly prepared emulsion was placed in the center of a specimen slide and observed under a MICROPHOT-FXA Nikon photomicroscope equipped with a built-in digital camera (Nikon Inc., Garden City, NY, USA).

6.3.5 Statistical analysis

Experiments were conducted with two independent trials on different days, each with a new batch of protein isolates. Data were subjected to the analysis of variance using a general linear model's procedure in Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA). Significant ($P < 0.05$) differences between means were identified by LSD all-pairwise multiple comparisons.

6.4 Results and discussion

6.4.1 Emulsifying capacity measured by conductivity

When the electrodes of a conductivity meter are submerged in a sample, a current is passed through the sample solution and ions carry the charge from one electrode to the appositively charged electrode to establish the current flow. The conductivity is determined by measuring the current which is proportional to the ion concentration because change in

the number of ions alters the amount of charge that can be carried between electrodes (Al-Malah, Azzam, & Omari, 2000). As shown in Figure 6.2A, at pH 6.5 PPI was able to conduct electricity before the phase transition because it was present in the continuous phase and negatively charged. When the PPI concentration decreased progressively with the oil addition, a linear relationship between conductivity and final PPI concentration was observed. Since conductivity of the protein-stabilized emulsions was induced by the presence of mobile and charged proteins, the conversion from the oil-in-water (O/W) to the water-in-oil (W/O) emulsion was indicated by a theoretical zero conductivity value. The final protein concentration was therefore calculated from the regression equation derived for EC (mL oil/g protein). Compared with the resistance method, the advantages of the conductivity method include simplified equipment, easy procedure, and improved accuracy; it does not require the complex setup of the resistance method. The conductivity method, performed on quiescent emulsions, also eliminates variations inherent to continuous oil delivery and mixing in the resistance method that is operated on non-equilibrium emulsifying solutions and hence prone to erroneous results. For example, the rate of oil addition and the blending speed must be controlled to a relatively low level (e.g., 3,000 rpm) to ensure the turbulence does not impede the resistance measurement (Akintayo et al., 1998). As a result, un-emulsified oil could be dispersed in the continuous protein matrix phase resulting in an overestimation of EC (Wang & Maximiuk, 2015). Moreover, with the present conductivity method, which uses a high shear force homogenization (17,500 rpm) for emulsion formation, the change in conductivity due to varying the oil/protein ratio enables the monitoring and tracing of the electrolyte behavior leading to

the emulsion phase transition. Such information is not provided by the resistance method because of its insensitivity to the changes in emulsion composition.

The electrical resistance method is based on the principle that oil is a nonconductor whereas the combination of protein and water is a good conductor. Therefore, when proteins are present in the continuous phase, the electrical resistance of the O/W emulsion remains relatively low until the protein-coated oil droplets begin to aggregate, at which time the resistance rises abruptly and a phase inversion point is reached (Firebaugh & Daubert, 2005; Wang & Maximiuk, 2015). As shown in Figure 6.2B, the resistance remained steady in the early stages of oil titration and lasted until the continuous phase of PPI solutions failed to support the dispersed oil phase. At this point, the system collapsed and the resistance of the emulsion increased drastically (exceeding the ohmmeter maximum range). Based on the oil content at the turning point (the inversion point), the estimation of EC is displayed in Figure 6.2. From the resistance test, the EC values of PPI at all four protein concentrations were lower compared to the results obtained using electrical conductivity. This was probably because the point of sudden increase in measured resistance was less affected by the residual free proteins than by the bulk of the added oil. Additionally, the number of samples tested (eight) was likely insufficient to reflect the actual inversion point. A continuous oil addition with timely resistance monitoring is necessary for EC determination using this method (Kato, Fujishige, Matsudomi, & Kobayashi, 1985). However, the less rigorous homogenization process that necessitates continuous emulsification may lead to an inaccurate EC estimation because when electrical resistance is recorded in a micro or macroscopically heterogeneous sample under turbulent conditions, non-emulsified flowing oil mixed in the continuous phase

would still account for resistance. This phenomenon has been described by Akintayo et al. (1998): when the emulsifying speed was increased from 1,500 rpm to 4,500 rpm, significant reductions in EC measured by the electrical resistance method were registered for legume proteins.

Since electrical conduction results from ion movement, charge-carrying and mobile proteins in the continuous phase would exert superior conductivity over the proteins immobilized at the emulsion interface (Kaci et al., 2014). Consequently, at the same initial PPI concentration, the linear decline in conductivity with reducing PPI concentration implies that the interfacial protein load was proportional to the final protein content. On the other hand, at the same final PPI concentration, the emulsions prepared with a higher amount of initial protein exhibited a lower conductivity. This is because at the equivalent final protein concentration, more oil is stabilized than in samples with less concentrated initial protein, i.e., more protein became immobilized by virtue of covering the larger area of oil surfaces created.

6.4.2 Emulsifying capacity of three proteins

The conductivity plot for each of the tested protein concentrations consisted of 8 data points corresponding to oil/protein ratios of 20, 50, 100, 200, 400, 600, 800, and 1000 mL oil/g protein to allow for the comparison between different initial protein concentrations (Figure 6.3). At the same low oil/protein ratios, a similar pattern of conductivity change was observed between PPI and WPI. Namely, an emulsion with a lower initial protein concentration had a lower conductivity than those with a higher initial protein concentration (trended by the arrowed dash line). For instance, at the 20 mL oil/g protein ratio, by lowering the initial protein concentration from 2.0% to 0.5%, the

conductivity decreased from 184 μS/cm to 80 μS/cm for PPI and from 119 μS/cm to 53 μS/cm for WPI ($P < 0.05$). However, the opposite trend was observed for MPI: at an equal 20 mL oil/g protein, for example, the conductivity of the emulsions increased from 7.1 mS/cm to 11.7 mS/cm ($P < 0.05$) when the initial protein concentration was lowered from 2.0% to 0.5%. The lower conductivity likely resulted from the deposition of additional proteins onto the interfacial monolayer of myosin or actomyosin (Gordon & Barbut, 1990). Compared with MPI, the smaller and less flexible structure of globular WPI and PPI may limit their efficiency in covering the interfacial area created during emulsification by multilayer protein membranes (Hinderink, Münch, Sagis, Schroën, & Berton-Carabin, 2019; Hunt & Dalgleish, 1994). It is noted that overall MPI had much higher conductivity values than the other two proteins throughout the process because it was suspended in ion rich NaCl solution whereas PPI and WPI were solubilized in double deionized water.

The EC of proteins reported in the literature has been largely variable and the concentrations used seem to be arbitrary. Thus, it is important to establish the effect of initial protein concentration used for EC measurement. As shown in Figure 6.4, regardless of protein type, a higher initial concentration always produced a lower EC value. The same trend could be found in published reports on myofibrillar protein and pulse proteins from pigeon pea, lima bean, and yam bean when examining the specific protein concentrations used in those studies (Akintayo et al., 1998; Borderias, Jiménez-Colmenero, & Tejada, 1985). In the estimation of EC in the present study and many others (Acton & Saffle, 1972; Eisele & Brekke, 1981), the maximum amount of oil that can be emulsified and stabilized is calculated based on per unit weight of proteins. Therefore, the initial amount of protein is an important factor which should be specified in EC-related research. The EC value of

the three proteins tested herein follows the order of WPI>MPI>PPI, which applied across all the four initial concentrations (0.5%, 1.0%, 1.5%, and 2.0%) (Figure 6.4).

6.4.3 Microstructure of emulsions

The accuracy of the conductivity method for EC measurement can be validified by the microstructure of the emulsions (Figure 6.5). At 20 mL oil/g protein, emulsions prepared with all the three proteins were far away from their inversion points so that round shaped oil droplets were distributed evenly in the matrix. When the emulsion-containing tubes were inverted, only MPI emulsion showed some residues adhering to the glass wall, indicating its higher viscosity than PPI and WPI emulsions due to differences in molecular size and structure. The fibrous myosin with a subunit molecular weight of 220 kDa is the major component in MPI, whereas in PPI and WPI, the major protein factions are β-lactoglobulin and 11S/7S globulins of much smaller sizes, i.e., ~60 kDa and 18 kDa, respectively (Dreizen, Gershman, Trotta, & Stracher, 1967; Guo & Wang, 2016; Lu, He, Zhang, & Bing, 2020). When the oil content was increased to 200 mL oil/g protein, all emulsions became visibly less fluid; for WPI emulsions, some adhesions to the wall of the glass tube were noticed upon inversion, suggesting a viscosity effect. The maximum viscosity is usually found at the point of phase transition with the attainment of fine emulsions (Allouche, Tyrode, Sadtler, Choplin, & Salager, 2004). Therefore, the viscous emulsions with fine and crowded oil droplets dominated the microstructure (Figure 6.5). At the highest oil/protein ratio tested, 1000 mL/g, which appeared to have exceeded the capacity of any of these three proteins, the O/W emulsions presumably changed into a W/O type. The phase transition was also implicated by the color change from white to a slightly yellow appearance. The conversion from the O/W emulsion to a vastly different W/O

system occurred as a result of oil droplet fusion due to inadequate interfacial coverage by proteins, at which point the oil/protein ratio is equivalent to EC. For PPI, which had the lowest EC (351 mL oil/g protein, Figure 6.4) at the initial protein concentration of 1.0%, the 1000 mL oil/g protein ratio for emulsification was farther away from the structure-breaking inversion point than it was for MPI (612 mL oil/g protein) and WPI (965 mL oil/g protein). This would explain why the apparent W/O emulsion for PPI was more homogeneous, in contrast to the structural disarray in the other two emulsions.

6.4.4 Graphic illustration of emulsion conductivity change

To further explain the oil-induced electrical conductivity change in a protein-stabilized emulsion, a mechanistic event is proposed (Figure 6.6). When a current is applied between the electrodes, the movement of negatively charged proteins in the continuous phase would move towards the anode, enabling current conduction. In an O/W emulsion, protein molecules are either free in the aqueous phase to carry the electricity or immobilized or adsorbed in the oil–water interfacial phase with little conduction capability. Therefore, the conductivity of the emulsion is largely dependent on the amount of mobile protein in the dispersed phase. By increasing the oil portion, more interfacial areas will be covered, i.e., mobile protein electrolytes will migrate from the aqueous phase to the oil–water interface for the system's free energy reduction and stability. This dynamic process continues until all mobile proteins are consumed. When the conductivity reaches zero, a phase inversion occurs at which point all the proteins have presumably partitioned at the interface. It is also possible that at high initial protein concentrations, some proteins are still present in the dispersed phase and their mobility is restricted due to high viscosity.

6.5 Conclusion

The electrical conductivity method described herein to measure protein emulsifying capacity enables the sensitive examination of dynamic protein-oil interaction leading to phase inversion. The method takes into consideration the mobility of electrical carriers (free polypeptides vs. low-mobility interfacial proteins); variations between proteins in molecular size, structure, and partitioning potential are recognized factors. Moreover, the protein concentration as an important viscosity effector is explained. Because the EC value is negatively related to the initial protein concentrations, food processors should consider this influencing factor when making meaningful comparisons between different proteins and attempting to choose appropriate proteins as oil emulsifiers.

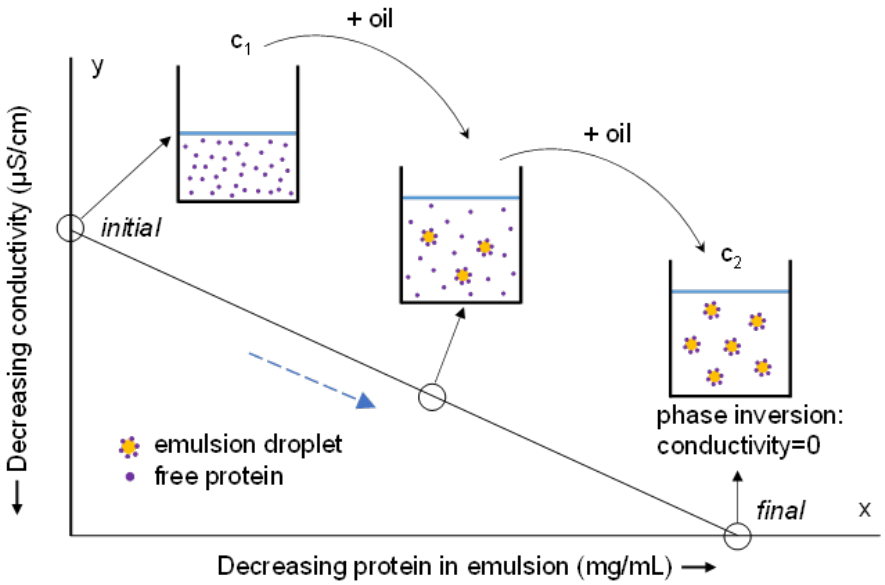
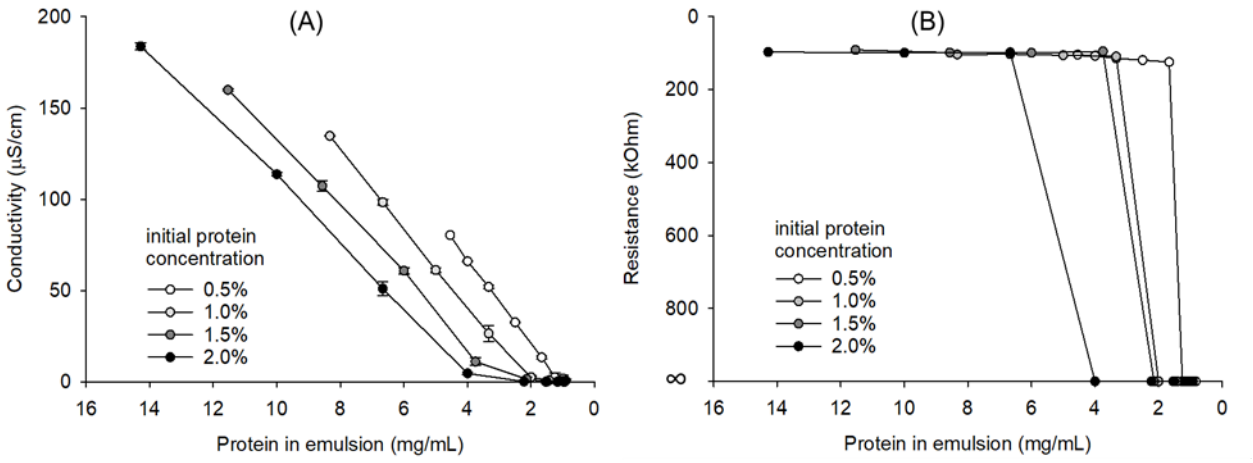


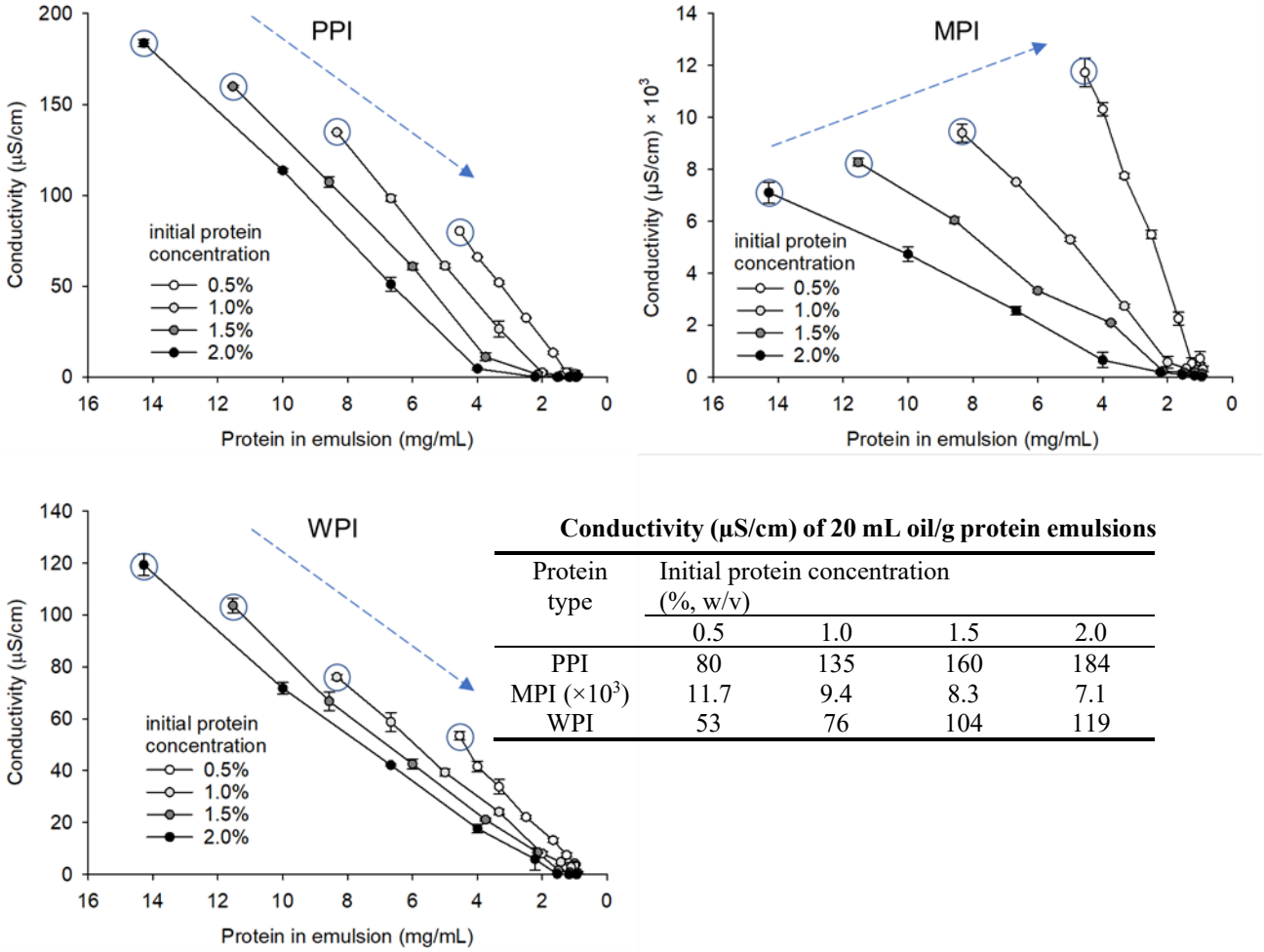
Figure 6.1. Illustrative electrical conductivity change with decreasing protein concentration in O/W emulsions. Emulsifying capacity (EC) can be calculated from the initial (c_1) and final (c_2) protein concentrations where c_2 is derived from the x-y regression line that intercepts at the x-axis (zero conductivity).



EC (mL oil/g protein) of PPI

Initial PPI concentration (%, w/v)	0.5	1.0	1.5	2.0
Conductivity method	717	351	269	219
Resistance method	400	200	200	100

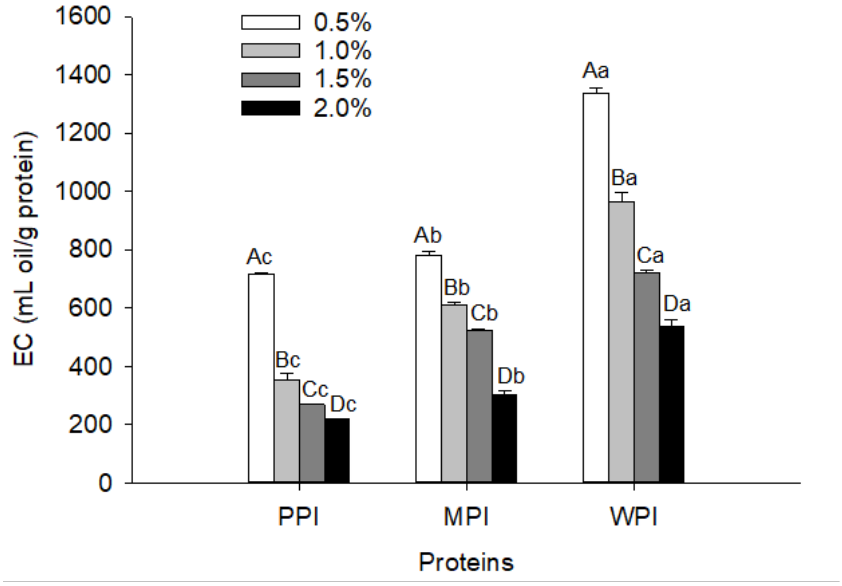
Figure 6.2. Electrical conductivity (A) or resistance (B) change with final pea protein isolate (PPI) concentration and emulsifying capacity (EC, mL oil/g protein) calculated at different initial protein concentrations. Eight aliquots of oil at 20, 50, 100, 200, 400, 600, 800, and 1000 mL oil/g protein were added to yield 8 different final protein concentrations.



Conductivity (µS/cm) of 20 mL oil/g protein emulsions

Protein type	Initial protein concentration (%, w/v)			
	0.5	1.0	1.5	2.0
PPI	80	135	160	184
MPI (×10³)	11.7	9.4	8.3	7.1
WPI	53	76	104	119

Figure 6.3. Conductivity change with final protein concentrations of pea protein isolate (PPI), myofibrillar protein isolate (MPI), and whey protein isolate (WPI) at different initial protein concentrations. For each, eight aliquots of oil at 20, 50, 100, 200, 400, 600, 800, and 1000 mL oil/g protein were added to yield 8 different final protein concentrations (%, w/v). The dash line points to descending (PPI, WPI) or ascending (MPI) conductivity of the 20 mL oil/g protein ratio emulsions (circled) prepared with initial protein concentrations of 2.0, 1.5, 1.0, and 0.5 % (w/v).



EC (mL oil/g protein) of different proteins

Protein type	Initial protein concentration (%, w/v)			
	0.5	1.0	1.5	2.0
PPI	717	351	269	219
MPI	782	612	524	303
WPI	1339	965	724	540

Figure 6.4. Emulsifying capacity (EC, mL oil/g protein) of pea protein isolate (PPI), myofibrillar protein isolate (MPI), and whey protein isolate (WPI) at different initial protein concentrations. Means with different letters (A–D for the same protein; a–c for the same initial protein concentration) differ significantly ($P < 0.05$).

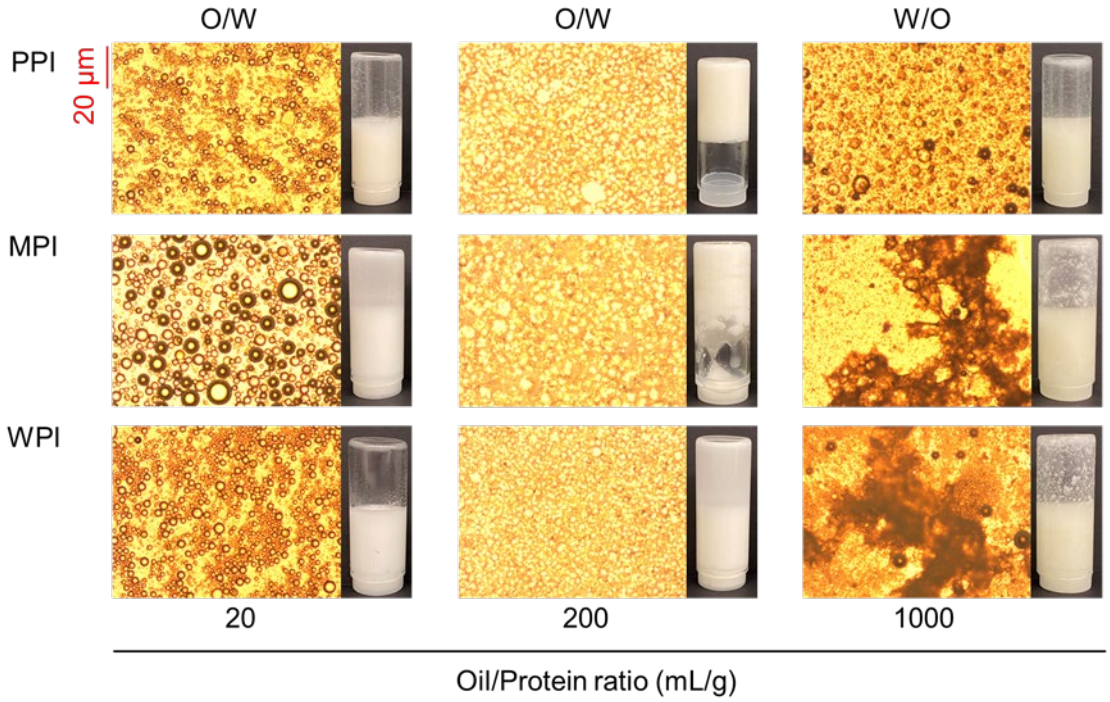


Figure 6.5. Morphology of representative emulsions made with pea protein isolate (PPI), myofibrillar protein isolate (MPI), and whey protein isolate (WPI) at the same initial protein concentration of 1% (w/v) but different oil/protein ratios of 20, 200, and 1000 mL/g. Phase inversion (O/W→W/O) occurred between 200 and 1000 oil/protein (mL/g) based on EC results (Figure 6.4).

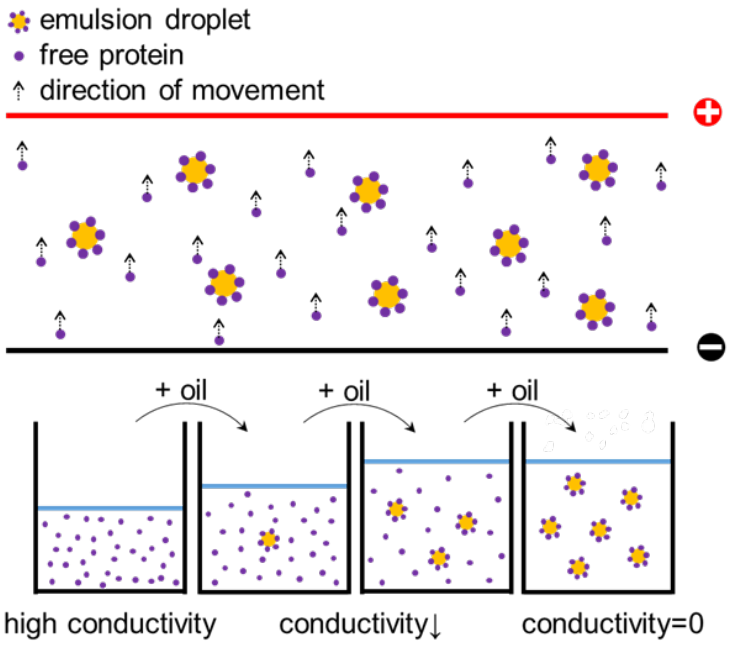


Figure 6.6. Proposed mechanism of electrical conduction in protein-stabilized emulsions and the conductivity change with oil addition.

CHAPTER 7. EFFECT OF STRUCTURALLY RELATED PHENOLIC COMPOUNDS ON THE EMULSIFYING PROPERTIES OF MYOFIBRILLAR PROTEIN

7.1 Summary

Modifications of the emulsifying properties of myofibrillar protein (MP) by phenolic structures were investigated under glucose oxidase-induced oxidative condition. Six phenolic compounds with structural relevance were selected and compared, i.e., gallic acid (GA), chlorogenic acid (CA), propyl gallate (PG), quercetin (QT), catechin (CC), and (–)-epigallocatechin-3-gallate (EGCG). The polar monophenols, GA and CA, caused little change in protein emulsification, whereas the hydrophobic monophenol, PG, and two hydrophobic diphenols, QT and CC, induced more protein partition in the oil/water interface by 16%, 50% and 65%, respectively ($P < 0.05$). Consistent with the partition result, these three phytophenols also improved the emulsifying capacity of MP. However, the largest phenolic molecule, EGCG, was detrimental for protein emulsification due to the occurrence of extensive phenolic-induced MP aggregation and insolubility. TBARS analysis showed that the phenolic compounds tested had little effect on inhibiting lipid oxidation, except for QT, which significantly decreased TBARS content by 54% ($P < 0.05$).

7.2 Introduction

Comminuted muscle foods, e.g., Frankfurters, bologna, and luncheon meats, have a common microstructure of fat particles dispersed within a protein matrix (Acton et al., 1983). Within these products, the most abundant and functional muscle protein component, myofibrillar protein (MP), acts as the amphiphilic emulsifier and readily adsorbs to the oil/water interface. The protein membranes formed around the lipids could reduce surface tension efficient enough to stabilize the emulsion system (Gordon et al., 1992). The emulsifying properties of MP play an important role in the quality of emulsified meat products because it is responsible for preventing the separation of water and fat.

Fats in emulsified meat products are especially susceptible to oxidation because larger surface area is created during the comminution process, making lipids more accessible to the reactive oxidative species (ROS). Simultaneously, the oxidative stress, oxygen, is constantly introduced in the chopping, grinding, and blending steps (Domínguez et al., 2019). Extensive lipid oxidation is responsible for the development of discoloration, undesirable flavors, and rancidity, which negatively affects product quality and shelf-life (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013). To counteract the quality deterioration caused by lipid oxidation, phenolic compounds as antioxidants are commonly incorporated into meat products. In recent years, the use of naturally occurring phenolic antioxidants has attracted tremendous interest in the meat industry due to customers' concerns over the safety of synthetic antioxidants and the potential health benefits of phytophenols (Kumar, Yadav, Ahmad, & Narsaiah, 2015). Plant derived phenolic compounds have been commonly incorporated into meat products in the form of herbs and spices in home cooking for a long period of time. To improve their potency, plant extracts containing higher

concentrations of phenolic compounds than that in the plant materials have been being developed and some of which have become commercially available (Shah et al., 2014). Apart from being antioxidants, phenolic compounds are reported to be interactive with another major macromolecule, MP, and responsible for changes in its emulsifying properties.

Several studies have been conducted to investigate the effect of phenolic compounds on MP emulsification, but various efficacies were discovered. EGCG at high concentrations (500 and 1000 mg/L) significantly reduced the emulsifying activity of MP by causing extensive protein–protein aggregation (Cao et al., 2018). Similarly, a large quantity of rosmarinic acid (300 μM/g) disrupted the emulsion structure in the MP composite gel and increased cooking loss from 58% to 77% (Wang et al., 2018). In another study where several mulberry polyphenols were tested for their effects on the emulsifying properties of MP (Cheng et al., 2020), most mulberry derived phenolic compounds, i.e., g cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside, caffeic acid, and quercetin, jeopardized protein emulsification, whereas rutin improved emulsifying activity index (EAI) by 38%. The structural diversity in the large family of phytophenols could be responsible for their different efficacies in modifying MP.

The objective of this present study was to investigate the effect of phenolic structures on their modifications of the emulsifying properties of MP. Six phenolic compounds with structural relevance were selected, i.e., three monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); two diphenols: quercetin (QT) and catechin (CC); and one triphenol: (–)-epigallocatechin-3-gallate (EGCG).

7.3 Materials and methods

7.3.1 Materials

Longissimus lumborum muscle samples were collected from pork carcasses (24 h post-mortem) harvested at the University of Kentucky Meat Laboratory, a USDA-approved facility. The food-grade glucose oxidase (GOx) was donated by Ajinomoto Co., Inc. (Kawasaki, Japan). Canola oil was purchased from Kroger Supermarket (Lexington, KY, USA). Gallic acid (GA, purity ⩾ 98.5%), chlorogenic acid (CA, purity ⩾ 98%), and (−)-epigallocatechin-3-gallate (EGCG, purity ⩾ 98%) were purchased from Sinopharm Chemical Reagent (Shanghai, China); Quercetin (QT, purity ⩾ 94%) was purchased from Thermo Fisher Scientific (Waltham, MA); Catechin (CC, purity ⩾ 96%) and propyl gallate (PG, purity ⩾ 98%) were purchased from MilliporeSigma (St. Louis, MO). All other chemicals were of at least analytical grade from MilliporeSigma (St. Louis, MO), Thermo Fisher Scientific (Waltham, MA), or VWR (Radnor, PA). Double-deionized water was used in all experiments.

7.3.2 Stripping of oil

Tocopherols were removed from the canola oil with alumina (MP Alumina N-Super I, MP Biomedicals, Graffenstaden, France). An aliquot of 15 g sorbent was mixed with 30 mL oil and vigorously shaken in the dark at 4 °C for 24 h. The mixtures were then centrifuged (2000g, 20 min) at 20 °C. The upper phase was collected and centrifuged again under the same condition. The aqueous phase, i.e., stripped canola oil, was immediately used for emulsion preparation (Yang & Xiong, 2018).

7.3.3 Emulsion sample preparation and treatment

Extraction of MP. Frozen muscle samples were tempered at 4 °C overnight before MP isolation using an extraction buffer of 10 mM sodium phosphate, 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM EGTA (pH 7.0) as described by Park et al. (2007). The protein concentration was measured by the Biuret method (Gornall et al., 1949).

Phenolic treatment. The MP pellet was diluted with 50 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid) (PIPES) buffer containing 0.6 M NaCl (pH 6.25) to a final concentration of 10 mg/mL protein. Phenolic compounds, i.e., GA, CA, PG, QT, CC, and EGCG, were dispersed into the MP sol to obtain a final load of 60 μmol phenolics per g of protein. An oxidative environment was introduced by subjecting the MP-phenolic mixtures to a GOx-catalyzed oxidation at 4 °C for 8 h. The oxidation system was composed of 50 μg glucose and 8 μg GOx per mg of MP in the presence of 10 μM $FeSO_4$.

Preparation of emulsions. After MP were incubated with phenolic compounds for 8 h under oxidative stress, MP-stabilized emulsions were prepared by homogenizing 2 mL canola oil with 18 mL MP samples in 50 mM PIPES buffer containing 0.6 M NaCl (pH 6.25) for 1 min at the speed of 17,500 rpm using a PolytronTM PT 10-35GT homogenizer (Brinkmann Instruments, Inc., Westbury, NY, USA) coupled with a low-foaming probe (PT-DA 12/2 EC-B154).

7.3.4 Characteristics of MP partition

Protein partition in the aqueous phase. The freshly prepared emulsions were centrifuged at 10,000g for 15 min at 4 °C and the cream layer was carefully removed. The amount of serum was recorded, and protein concentrations of both the whole emulsion and

centrifuged serum were determined. The MP partition in aqueous phase (%) was calculated using the following formula:

$$\text{MP in aqueous phase (\%)} = \frac{C_s \times V_s}{C_e \times V_e} \times 100$$

where C_e and V_e are the protein concentration and total volume of the whole emulsion, respectively; C_s and V_s are the protein concentration and volume of the centrifuged serum, respectively.

Detection of protein cross-linking. After emulsion samples were centrifuged at 10,000g for 15 min at 4 °C, the serum layer was collected and dissolved in the SDS–PAGE sample buffer at a 1:4 ratio. A 5% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel system were used to determine protein patterns in the serum phase (Laemmli, 1970).

7.3.5 Measurement of emulsion particle size and morphology

Particle size. The freshly prepared emulsions were diluted 50 times with 50 mM PIPES buffer containing 0.6 M NaCl (pH 6.25) after which the emulsion particle size was immediately analyzed using a ZetaSizer Nano-ZS (Malvern Instruments, Worcestershire, UK) as described by Yang and Xiong (2015).

Microstructure of emulsions. A drop of freshly prepared emulsion sample was placed in the center of a specimen slide and the microstructure of MP-stabilized emulsions was examined under a MICROPHOT-FXA Nikon photomicroscope equipped with a built-in digital camera (Nikon Inc., Garden City, NY, USA).

7.3.6 Emulsifying capacity of phenolic modified MP

A Model B30PCI benchtop multi parameter meter (VWR, Radnor, PA, USA) was used to measure electrical conductivity of the with oil to protein ratios at 20, 50, 100, and

200 mL oil/g protein. Electrical conductivity (μS/cm, y axis) was plotted against the corresponding final protein concentrations (mg/mL, x axis). A regression line was drawn and then extrapolated to the x axis to approximate the final protein concentration (mg/mL) when the conductivity reached zero. The emulsifying capacity (EC) was calculated using the following formula:

$$\text{EC (mL oil/g of protein)} = \frac{V_{oil}}{m} = \frac{V_{oil}}{c_1 V} * 1000 = \frac{(c_1 - c_2)}{c_1 * c_2} * 1000$$

where V_{oil} is the amount of oil added (mL), V is the starting volume of protein solution (mL), m is the protein weight (g), c_1 is the initial protein concentration (mg/mL), and c_2 is the final protein concentration (mg/mL) derived from the regression formula when conductivity becomes zero.

7.3.7 Evaluation of lipid oxidation

Lipid oxidation of the emulsions was evaluated by measuring the amount of 2-thiobarbituric acid-reactive substances (TBARS) produced (Salih et al., 1987). The assay solutions containing trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were mixed with 3 g emulsion samples and boiled in dark for 30 min. The supernatant of cooled solution was mixed with chloroform and centrifuged at 2000g for 10 min. The upper phase was further clarified with petroleum ether and centrifuged at the same conditions. Absorbance of the lower phase was measured at 532 nm and the TBARS value and calculated using the following equation:

$$\text{TBARS (mg/kg)} = (A_{532}/W_S) \times 9.48$$

where A_{532} is the absorbance at 532 nm, W_s is the sample weight (g).

7.3.8 Statistical analysis

Experiments were conducted with three independent trials (n = 3). Data were subjected to the analysis of variance using a general linear model's procedure in Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA). Significant ($P < 0.05$) differences between means were identified by LSD all-pairwise multiple comparisons.

7.4 Results and discussion

7.4.1 Protein partition

Phenolic compounds are known to be interactive with MP through both non-covalent and covalent pathways and alter the physicochemical properties of protein (Guo & Xiong, 2021), which would affect its partition in an emulsion system. As shown in Figure 7.1, the two polar monophenols, GA and CA, had little effect on protein partition, whereas the other less polar monophenol PG, and the two hydrophobic diphenols, QT and CC, introduced significant lower protein partitions in the aqueous phase than the control sample by 16, 50, and 65%, respectively ($P < 0.05$), indicating more proteins were present in the oil/water interface. MP-stabilized emulsions have a typical thick membrane with multilayers of protein molecules (Gordon et al., 1992), so a stronger protein–protein association induced by phenolics could be responsible for more protein disposition to the interfacial area. Phenolic compounds could facilitate protein aggregation by facilitating MP structural unfolding, which exposes the interior hydrophobic or reactive amino acid sidechain groups and enhance protein–protein interactions. The more hydrophobic nature of PG, QT, and CC than GA and CA would lead to their preferable distribution onto the O/W interface (Sasaki et al., 2010). Therefore, the larger amount of PG, QT, and CC

present in the protein membrane could promote protein aggregation in the interface, resulting in lower MP partition in the aqueous phase.

In contrast to the other phenolic compounds, EGCG caused severe protein aggregation with visible coagulation and syneresis. As the largest triphenol with multiple functional groups in its structure, it is plausible that EGCG interacted with the protein so strongly that extensive protein aggregation occurred, and the protein membrane structure was disrupted. As a result, the emulsifying properties of MP were impaired. Similar results have been reported where EGCG drastically destabilized MP emulsions (Feng et al., 2017). However, EGCG treated MP had the lowest partition in aqueous phase (Figure 7.1A), corresponding to the least amount of protein displayed in the SDS-PAGE pattern (Figure 7.1B). The discrepancy between compromised MP emulsification and less protein partition in the aqueous phase of the EGCG-treated sample might be attributed to the uneven protein distribution in the samples or the entrapment of large protein aggregates in the microcentrifuge tube. Both errors could result in less representative sampling, even though vigorous mixing was conducted in advance.

7.4.2 Emulsion size and emulsifying capacity of MP

As displayed in Figure 7.2A, all phenolic compounds tested, except for EGCG, produced emulsions with similar particle size ($P > 0.05$). On the other hand, the emulsifying capacity (EC, Figure 7.2B) of MP was significantly improved by PG, QT and CC from 704 mL oil/g protein to 1092, 1652, and 1957 mL oil/g protein, respectively ($P < 0.05$). The increase was likely due to their higher partition rate in the oil/water interface (Figure 7.1A) because the charge-carrying phenolic compounds in protein membrane could introduce electrical repulsion between oil droplets, which prevents oil flocculation and

stabilizes the emulsions (Li et al., 2019). The multiple -OH groups in diphenols, QT and CC, could be readily deprotonated and carry more charges than the monophenols, contributing to the higher EC value of protein. Between the two diphenols, the flavanol structure in CC tends to have had a higher binding affinity for MP than the flavonol structure in QT, which might explain the slightly higher EC of CC-treated MP (Guo et al., 2021).

Among all samples tested, EGCG-treated emulsions had the largest droplet size of 6783 nm, 91% higher than that of the control ($P < 0.05$), which could be attributed to the formation of large protein aggregates induced by strong EGCG–MP interactions (Cao et al., 2018).

7.4.3 Microstructure of MP-stabilized emulsions

Morphology of the MP-stabilized emulsions revealed a homogeneous texture with oil droplets distributed evenly in the continuous phase (Figure 7.3), indicating the formation of stable emulsions under the experimental conditions. Consistent with little difference in particle size between most samples (Figure 7.2A), the diameter of oil droplets and their range of variations in emulsions containing phenolic compounds appeared to be similar, with the exception of EGCG. The EGCG-treated emulsion sample had a less homogeneous morphology with a higher portion of large oil droplets than the other emulsions, corresponding to its largest particle size.

7.4.4 Lipid oxidation

Lipid oxidation of emulsions was evaluated by measuring the content of TBARS-secondary by-products from lipid peroxidation (Figure 7.4). The non-oxidized control emulsion had an initial TBARS value of 0.24 mg/kg, which could be attributed to the

oxidative stress introduced during the homogenization process. Among all the phenolic compounds tested, QT was the only effective phenolic compound in inhibiting lipid oxidation which reduced TBARS content by 54% ($P < 0.05$). Since the partition of antioxidants in an emulsion system plays an important role in their efficiency, the stronger antioxidative activity of the hydrophobic QT may result from its more distribution in the oil/water interface (Costa, Losada-Barreiro, Paiva-Martins, & Bravo-Díaz, 2021). In addition, its strong radical scavenging and metal chelating activities could also contribute to the antioxidant activity (Afanas′ev, Dcrozhko, Brodskii, Kostyuk, & Potapovitch, 1989).

7.5 Conclusion

In conclusion, phenolic compounds had different effect on the emulsifying properties of MP and such modifications are related to the polarity, size, and structure of specific phenolic molecules. Before being applied to muscle foods for desirable product quality, the role of phytophenols in altering the emulsifying properties of MP can be predicted based on the phenolic strucutres. As multi-funtional ingredients, the antioxidant efficacy of polyphenols should also be carefully evaluted in addition to the altered MP functionalities. Future studies are warranted to explore methods to avoid phenolic-induced impairment of MP emulsification and assess the storage stability of phenolic treated emulsions. Furthermore, the combined effect of more than one phenolic compound is worth investigating because phytophenols are mostly used in the format of mixtures with the presence of multiple typeps of molecules, e.g., in plant extracts and seasonings.

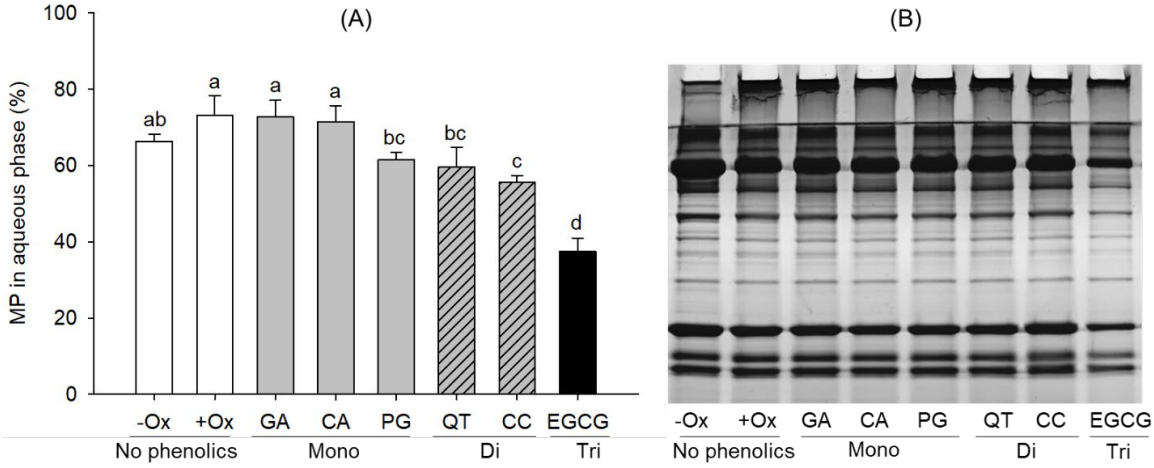


Figure 7.1. MP partition (A) and SDS–PAGE patterns (B) of the aqueous phase. MP was treated with different phenolics (60 μmol/g protein) in an oxidative environment. Control samples (no phenolics) included both nonoxidized (–Ox) and oxidatively stressed (+Ox) protein. Monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); diphenols: quercetin (QT) and catechin (CC); triphenol: (−)-epigallocatechin-3-gallate (EGCG). Means with different letters (a−d) differ significantly ($P < 0.05$).

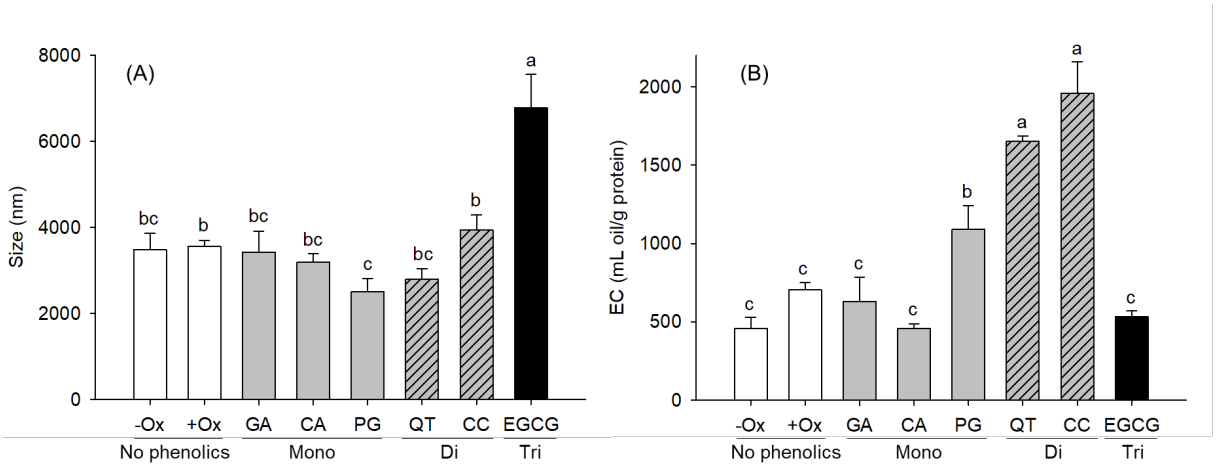


Figure 7.2. Particle size of emulsions (A) and emulsifying capacity of MP (B). MP was treated with different phenolics (60 µmol/g protein) in an oxidative environment. Control samples (no phenolics) included both nonoxidized (–Ox) and oxidatively stressed (+Ox) protein. Monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); diphenols: quercetin (QT) and catechin (CC); triphenol: (−)-epigallocatechin-3-gallate (EGCG). MHC: myosin heavy chain. In the same parameter, means with different letters (a−c) differ significantly ($P < 0.05$).

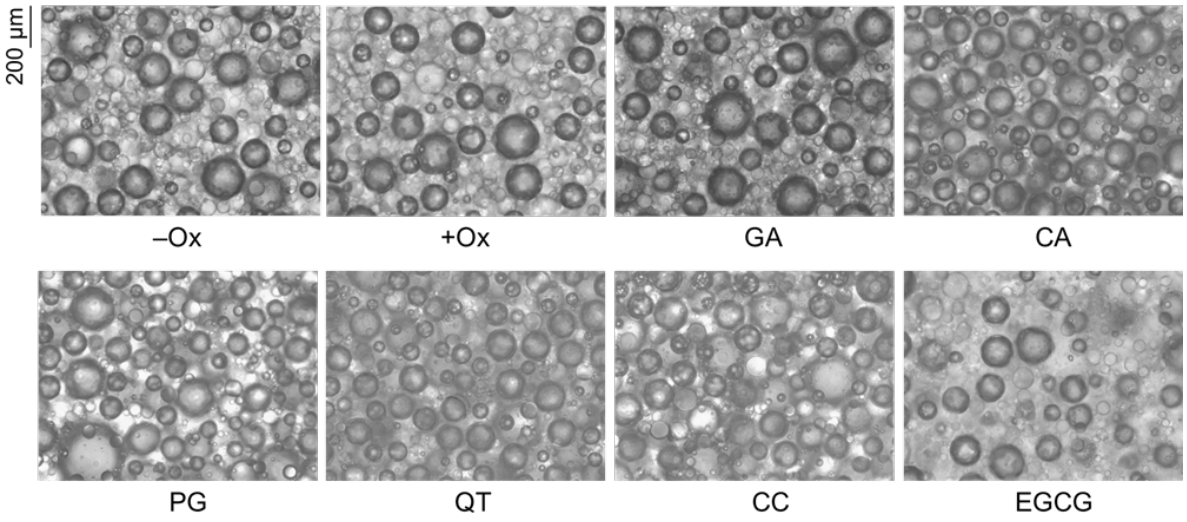


Figure 7.3. Light microscope images of MP-stabilized emulsions treated with different phenolics (60 µmol/g protein) in an oxidative environment. Control samples (no phenolics) included both nonoxidized (–Ox) and oxidatively stressed (+Ox) protein. Monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); diphenols: quercetin (QT) and catechin (CC); triphenol: (−)-epigallocatechin-3-gallate (EGCG).

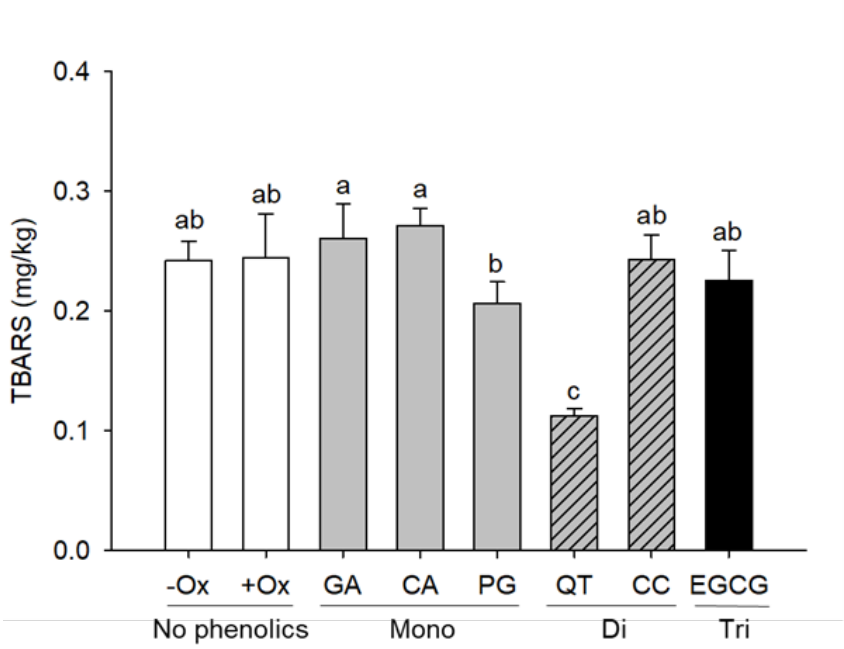


Figure 7.4. Lipid oxidation (TBARS) in MP-stabilized emulsions treated with different phenolics (60 µmol/g protein) in an oxidative environment. Control samples (no phenolics) included both nonoxidized (–Ox) and oxidatively stressed (+Ox) protein. Monophenols: gallic acid (GA), chlorogenic acid (CA), and propyl gallate (PG); diphenols: quercetin (QT) and catechin (CC); triphenol: (−)-epigallocatechin-3-gallate (EGCG). Means with different letters (a–c) differ significantly ($P < 0.05$).

CHAPTER 8. OVERALL CONCLUSIONS

Under the oxidative condition created using glucose oxidase (GOx), phenolic compounds exhibited strong interactions with myofibrillar protein (MP) and promoted protein structural unfolding and cross-linking. The phenol-induced modifications of protein functionalities, i.e., gelation and emulsification, were influenced by the structural attributes of specific phytophenols: The lack of structural hinderance in a smaller molecular would favor protein cross-linking and gel formation; A larger monophenol with more hydroxyl and hydrocarbon groups improved MP gelation by promoting protein unfolding; Phenolic compounds with less polarity tended to increase the emulsifying capacity of MP through more protein partition in the oil/water interface; Phytophenols with more than one phenol structures suppressed both emulsification and gelation due to extensive protein aggregation.

Of the six phenolic acids tested for their effects on MP gelation, gallic acid induced the most increase in gel strength and elasticity due to its strong reactivity with functional amino acid side chains through covalent bonds. As a result, protein cross-linking was promoted by the formation of protein–phenol adducts. In the emulsion composite gel system where several phytophenols were compared, quercetin was the best at improving the overall quality of the model emulsion-gel products, because it not only enhanced protein gelation but also exhibited strong inhibition effect on lipid oxidation. Both the textural properties and oxidative conditions should be considered when applying phenolic compounds into comminuted muscle foods since antioxidant is another important role they play in these commodities.

Furthermore, the amount of phenolic added was an important factor affecting the outcome of protein modifications based on the dose-dependent effect observed in this study. At 60 μmol/g protein, all phenolic acids (monophenols) promoted gelation; for MP samples treated with different amount of GA, the gelling potential was improved at higher GA concentrations. In contrast, catechin (diphenol) and EGCG (triphenol) were detrimental for gelation and emulsification of MP at the same addition level. To prepare emulsion composite gels with less textural hindrances, it was necessary to reduce the quantity of catechin and EGCG to less than 30 μmol. Protein aggregation and insolubility caused by these large phenols, especially at higher concentrations, were responsible their less tolerance amount for MP gelation.

The efficacy of phytophenols for improving MP functionality depends on oxidative conditions and specific phenolic molecules. Both texture-related properties and antioxidant activities should be considered when plant phenols are used in protein matrix-based and fat-containing processed meat. Because protein functionality changes are phenol concentration-dependent, it is essential to apply them at appropriate dosage levels to maximize the benefits and avoid unwanted consequences. Further application research is required to examine the behavior of phenolic compounds in real comminuted muscle food systems. Moreover, the combination effects of phenolic compounds with different structural characteristics should be investigated as in most processed meat products, total plant extracts or spices instead of individual compounds are utilized.

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VITA

Anqi Guo grew up in Taixing, China. She was majored in Food Science and Engineering in Jiangnan University (Wuxi, China) from 2011 to 2013 before continuing her undergraduate study as an exchange student joining the Food Science program in the University of Reading (Reading, UK). She received Bachelor's Degrees in Food Science from both Universities in 2015. She then entered graduate school in the University of Kentucky in 2016 in pursuing of a degree of Doctor of Philosophy in Food Science.

Anqi has published four papers from her research conducted at the University of Kentucky and presented her work four times at the Annual Meeting of Institute of Food Technologists (IFT) from 2017 to 2020. She was honored 1st place in the 2018 IFT Graduate Research Competition Muscle Foods Division and received an Outstanding Ph.D. Student award in 2021. After the completion of her study, she will work as a Post-Doctoral Scholar at Shanghai Jiaotong University (Shanghai, China).