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RELATIVE REACTIVITY OF PROTEIN AND LIPID TO OXIDANTS IN DIFFERENT BI-PHASIC SYSTEMS AND ITS IMPLICATION IN SAUSAGE QUALITY

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RELATIVE REACTIVITY OF PROTEIN AND LIPID TO OXIDANTS IN DIFFERENT BI-PHASIC SYSTEMS AND ITS IMPLICATION IN SAUSAGE QUALITY

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

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Lexington, Kentucky

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Lexington, Kentucky

2016

ABSTRACT OF DISSERTATION

RELATIVE REACTIVITY OF PROTEIN AND LIPID TO OXIDANTS IN DIFFERENT BI-PHASIC SYSTEMS AND ITS IMPLICATION IN SAUSAGE QUALITY

This study investigated the progression of protein and lipid oxidation in different bi-phasic model systems: simple aqueous dispersions, liposome, and oil-in-water (O/W) emulsions. Varied concentrations of isolated pork myofibrillar protein (MFP) were mixed with free fatty acids (C18:1, C18:2, C18:3), lecithin, or vegetable oil to construct different lipid-protein dispersion systems, then subjected to hydroxyl radical stress at 4 °C. Comparison of the evolution of lipid oxidation with protein modification markers in all dispersion systems showed noteworthy attenuation of tryptophan fluorescence, protein carbonyl formation, and extensive polymerization of myosin in 2 h. This process preceded lipid oxidation which exhibited notable accumulations of thiobarbituric acid-reactive substances (TBARS) only after 2 h.

The study further explored the steric role of MFP in the oxidative stability of emulsions. Oxidized MFP in the continuous phase stimulated lipid oxidation in 24 h; sharply contrasting with interface-adsorbed MFP that inhibited TBARS formation nearly 90% in 24 h. Interfacial MFP from 2 h-oxidized samples exhibited attenuated tryptophan fluorescence but more pronounced myosin polymerization than MFP in the continuous phase. Due to the site distribution, interface-adsorbed MFP in general and myosin in particular provided accentuated protection of emulsions against oxidation. Similarly, soy protein isolate (SPI) and sodium caseinate (SC) acted as antioxidant barriers in O/W emulsions.

The effect of replacing pork fat by protein-stabilized soybean oil pre-emulsion on physical characteristics and oxidative stability of fresh sausages was subsequently investigated. Substitution (60%) of SC or partially denatured SPI pre-emulsified oil for fat improved sausage water-binding capacity ($P < 0.05$). During storage at 4 °C, cooked sausage formulated with partially denatured SPI-emulsified oil displayed a slower lipid oxidation rate throughout 14 days compared with control sausage, and SC-emulsified oil sausage had the lowest TBARS produced in the first 5 days. There was no significant difference ($P > 0.05$) in texture attributes (e.g., hardness, deformability, cohesiveness, and rupture force) between different formulations.

In summary, proteins as emulsifiers at the O/W interface are kinetically preferred targets of radicals compared to unsaturated lipids and proteins in the continuous phase. Such locality effect proves to be important for the physicochemical stability of emulsion-type foods.

KEYWORDS: Protein oxidation, Lipid oxidation, Emulsion, Interfacial protein, Sausage

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CHAPTER 1

INTRODUCTION

Oxidation is considered one of the main causes for functional, sensory, and nutritional quality deterioration in meat and meat products due to the insolubilization of proteins, development of off-flavors, and formation of free radicals or other oxidized compounds (Morrissey, Sheehy, Galvin, Kerry, & Buckley 1998). Initiators of oxidation can be classified into radical, non-radical, and exogenous agents (e.g., X-ray, UV, gamma rays). Radicals are compounds such as superoxide, hydroxyl, peroxyl, alkoxyl, and hydroperoxyl that that contain one or more unpaired electrons. Non-radicals are species that have strong oxidizing potential (hydrogen peroxide, hypochlorous acid, ozone, singlet oxygen, peroxynitrite, etc.). These reactive oxygen species (ROS) may cause crosslink or cleavage of proteins, and produce low molecular volatile aldehydes, alcohols, and hydrocarbons from lipid oxidation (Choe & Min, 2005).

Lipids and proteins are two main targets in muscle food for ROS. There is now good evidence that both protein and lipid oxidation contributes to meat quality. Mild oxidation is beneficial for flavor development as well as protein functionality (Xiong, Blanchard, Ooizumi, & Ma, 2010) whereas severe oxidation is detrimental to food quality (Xiong, 2000).

Lipid and protein oxidation may occur independently or in conjunction; however, the relative susceptibility of lipids and proteins to oxygen species in different food systems is not well understood. In general, two hypotheses have been proposed. Some researchers believe that lipid oxidation occurs faster than the modification of protein

(Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008). Proteins present in food are then attacked by secondary products resulting from lipid oxidation, such as MDA and HNE. Another hypothesis suggests that proteins are able to produce free radicals and transfer them to lipids or other proteins (Elias, Kellerby, & Decker, 2008). The specific oxidation sequence in each food system also depends on the target concentration, the rate constant for the reaction of oxidant with target (Davies, 2005), and the construction of the system (the distribution of lipids and proteins).

Protein can act as an anti- or pro-oxidant depending upon its concentration and distribution in the system. Recent studies have demonstrated the antioxidant effect of different kinds of proteins which could delay lipid oxidation in liposome system, oil-in-water (O/W) emulsions, and other model systems (Hu, McClements, & Decker, 2003; Lethuaut, Metro, & Genot, 2002; Ries, Ye, Haisman, & Singh, 2010). The antioxidant activity of proteins is attributed to the cooperative effect of a variety of properties, including scavenging free radicals, chelating metal ions, and ability to alter the physical properties of food systems (Díaz & Decker, 2004; Levine, Berlett, Moskovitz, Mosoni, & Stadtman, 1999; Kaul, Sharma, & Mehta, 2008). As consumer demands for all natural foods increase, the antioxidant functionality of protein allows it to become a new substitute for synthetic antioxidants to control lipid oxidation in fatty foods. However, more kinetic studies about protein oxidation and the exact mechanism of lipid and protein interaction need be further studied. In different bi-phasic model systems (lipids and proteins), the distribution of proteins and lipids contributes to their susceptibility to ROS. When fatty acids and proteins amorphously exist in an aqueous phase, it is supposed that they physically have a similar chance to be attacked by radicals. In contrast, the lipid

bilayer in liposome systems and the protein membrane at the oil-water interface in emulsion systems made lipids and proteins response differently to radicals in an aqueous phase. It is believed that protein or protein hydrolysates could provide a physical barrier against liposome oxidation (Viljanen, Kivikari, & Heinonen, 2004; Zhang, Xiong, Chen, & Zhou, 2013). While in protein-stabilized emulsions, the membrane formed at the oil-water interface may serve as a physical shield to prevent radical attack of the interior lipid (Hu, McClements, & Decker, 2003; Jiang, Zhu, Liu, & Xiong, 2014). Both chemical (radical neutralization) and physical (steric) protection needs to be considered when comparing the reactivity of lipids and proteins subjected to radicals.

This paper is devoted to investigating the relative reactivity of protein and lipid to ROS, with special interest to their implications in the quality of comminuted meat products. The establishment of the susceptibility of proteins and lipids in a complex oxidizing environment provides guidance in selecting the appropriate antioxidants to manipulate oxidation, storage and processing conditions, and feeding strategies in order to improve meat quality. The specific objectives of my dissertation research are:

1. To monitor the progress of oxidative changes in a mixed lipid–protein system so as to clarify the reactivity of lipids and proteins subjected to ROS.
2. To test the hypothesis that by virtue of steric hindrance, interfacial MFPs, when compared with that in the continuous phase, can be more effective in protecting oil droplets against oxidative changes.
3. To explore different types of protein sources for the construction of antioxidative membranes in formulated emulsions or meat batter products.
4. To investigate the effects of replacing fat with protein-stabilized emulsions on the

physical characteristics and oxidative stability of comminuted meat products (fresh sausages).

CHAPTER 2

LITERATURE REVIEW

2.1. Mechanism of oxidation

2.1.1. Mechanism of lipid oxidation

Food lipids, including triacylglycerides, phospholipids, and sterols, are found in most biological materials consumed as food or added as functional ingredients in processed food. Meanwhile, lipids are also one of the most chemically unstable components under oxidative stress. Lipid oxidation can be conveniently divided into three distinct stages: initiation, propagation, and termination (Nawar, 1996; Frankel, 1998). A simplified scheme explaining the mechanism of autoxidation is given in Figure 2.1 (Shahidi & Zhong, 2010). The most prevalent initiators in food systems are ROS, such as OH· and HOO·. Initiation is the step in which fatty acid radicals are produced by ROS attack. Fatty acid radicals are unstable and react readily with an oxygen species to form peroxyl radicals, which are also unstable. Peroxyl radicals further react with other fatty acids and create lipid hydroperoxides and different fatty acid radicals. The cycle continues, as new fatty acid radicals react in a similar way. Lipid hydroperoxides decompose by cleavage of the fatty acid chain adjacent to alkoxy radicals. These decomposition products include complex mixtures of aldehydes, ketones, alcohols, hydrocarbons, esters, furans, and lactones (Frankel, 1984). The secondary oxidation products can also break down and undergo further degradation to form epoxides, cyclic peroxides, and bicyclic endoperoxides (Enser, 1987). The radical reaction terminates when two radicals react and produce a non-radical species. The progress of lipid oxidation can be monitored by determination of (i) primary products as lipid

hydroperoxides, (ii) secondary products as malondialdehydes (MDA) or 4-hydroxynonenal (HNE), (iii) reaction products between the oxidized lipids and other substances, and (iv) radical species produced during the course of the reactions.

2.1.2. Mechanism of protein oxidation

Protein oxidation was first investigated in cell biology and medicine. Studies about free radical-mediated oxidation of proteins started at the beginning of the early 20th century: oxidation of amino acids by Fenton systems was first reported in 1906; and in 1925, it was found glutathione could serve as an anti- and pro-oxidant (Dean, Fu, Stocker, & Davies, 1997). Currently in the field of food chemistry, oxidation of proteins is one of the most innovative topics of study. Protein oxidation undergoes similar processes to those in lipid oxidation, involving initiation, propagation, and termination stages (Schaich & Pryor, 1980). Oxidation of the protein backbone is initiated by oxidative attack on the α-hydrogen atom of an amino acid residue to form a carbon-centered radical; this radical may react with another carbon-centered radical to form a protein-protein crosslinking derivative, or reacts with oxygen to form an alkylperoxyl radical, followed by alkoxyls radical. The generation of alkoxyl radicals sets the stage for cleavage of the peptide bond by either the diamide or α-amidation pathways (Berlett & Stadtman, 1997). The most common consequences of protein oxidation are shown in Figure 2.2 (Lund, Heinonen, Baron, & Estévez, 2011). Remarkable and measurable changes caused by protein oxidation in muscle foods may be generalized as (i) formation of protein carbonyls, (ii) loss of sulfhydryl groups, (iii) secondary and tertiary structural changes, and (iv) formation of protein cross-linking.

2.2. Comparative time course of protein and lipid oxidation

2.2.1. Reaction rate of lipid oxidation

The oxidative stability of lipids depends on a number of intrinsic and extrinsic factors. The chemical structure of a lipid molecule is one of the determination factors on its susceptibility to oxidation, particularly the number and location of double bonds (Miyashita, Azuma, & Ota, 1995). Main unsaturated fatty acids comprising the lipids of animal tissues are oleic, linoleic, linolenic, and arachidonic acid. The free fatty acids are surface-active compounds; they are more polar than triacylglycerol due to the presence of unesterified carboxylic acid groups. Fatty acids with more double bonds have higher rate constants for oxidation: arachidonic acid > linolenic acid > linoleic acid > oleic acid (Table 2.1, Bielski, Cabelli, Arudi, & Ross, 1985; Wilkinson, Helman, & Ross, 1995; Neta, Huie, & Ross, 1990).

The oxidation rate of lipids also depends upon the type of oxidants. The rate constants for the reaction of a range of reactive radicals, such as the singlet oxygen, superoxide radicals, and Trichloromethyl peroxyl radical with free fatty acids are shown in Table 2.1. As may be expected, there is a large variation in the rate constants between different oxidants.

2.2.2. Reaction rate of protein oxidation

Proteins present in foods are susceptible to attack by free radicals, hydroperoxides, singlet oxygen, and secondary lipid oxidation products leading to the modification of amino acids, formation of protein-protein cross-linking, and protein

fragmentation (Xiong, 2000). The amino acids most susceptible to oxidation are shown in Table 2.2 (Berlett & Stadtman, 1997; Stadtman, 1992; Buxton, Greenstock, Helman, & Ross, 1988; Wilkinson, Helman, & Ross, 1995; Pattison, Hawkins, & Davies, 2003). Different oxidants have varied behaviors in protein oxidation. In general, the most reactive radicals tend to be the least selective. For example, the hydroxyl group reacts with most amino acid side-chain sites; a slightly different reaction rate was found among these side-chain sites (Table 2.2), though the aromatic and sulfur-containing side-chains are expected to be depleted much more rapidly. Less reactive oxidants are more selective in the site they oxidize, for example, the rate constants for reaction of hypochlorous acid (HOCl) radical with amino acid side-chains vary from 3.0×10^7 for Cysteine residues to below the measurable detection limit (Table 2.2), consistent with much more selective damage by this oxidant. For this reason, a number of relatively weak oxidants can give rise to selectively damaged proteins.

Kinetic data is of great use in determining the reaction rate of a particular oxidant with a target. The rate constants for reaction with a range of reactive radicals with biological macromolecules (shown in Table 2.3) offer an indication of the potential importance of proteins as targets for oxidants (Buxton, Greenstock, Helman, & Ross, 1988). Aromatic residues, though often buried in the hydrophobic core of molecules, are usually more susceptible to oxidation than aliphatic residues and hence are commonly used as a marker of protein oxidation (Dean, Fu, Stocker, & Davies, 1997). However, in a mixed system, the overall oxidation rate of proteins and lipids is affected by many other factors besides their rate constants with oxidants, especially the way they interact with each other. For example, the protein conformation affects how it would interact with

lipids (Aynié, Meste, Colas, & Lorient, 1992): casein tends to crosslink in the presence of oxidized lipids more readily than whey protein due to its disordered random-coil and flexible structure (Sharma, Zakora, & Qvist, 2002). Many environmental factors such as pH, temperature, water activity, and the presence of catalysts or inhibitors may also be involved.

2.2.3. Inter-relationship of protein and lipid oxidation

The sequence of lipid and protein oxidation has been the subject of much debate. The two phenomena were described as "correlated" (Dalsgaard et al., 2010), "concomitant" (Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008), or "simultaneous" (Hidalgo & Zamora, 2002; Soyer, Özalp, Dalmış, & Bilgin, 2010). It seems, therefore, obvious that lipid and protein oxidation are linked, but it is difficult to determine which of the phenomena initiates.

The ability of lipid radicals to damage proteins was first demonstrated by the observation of fluorescent cross-links between lipid peroxidation products and proteins (Roubal, & Tappel, 1966). Peroxyl radicals formed during lipid oxidation can abstract hydrogen atoms from protein molecules leading to a radical-mediated chain reaction similar to that of lipid oxidation (Stadtman & Levine, 2003). Hydroperoxides are generated from fatty acid decomposition early during oxidation (Viljanen, Halmos, Sinclair, & Heinonen, 2005). It has been also suggested that proteins present in food are susceptible to attack by secondary products resulting from lipid oxidation, such as MDA and HNE, leading to the formation of various reaction products (Mestdagh, Kerkaert, Cucu, & De Meulenaer, 2011). This would predict that protein oxidation occurs

subsequent to the evolution of lipid hydroperoxides. However, proteins and amino acids can oxidize without lipid radicals by reacting with metals and oxygen. They are also able to bear free radicals and transfer them to other macromolecules such as lipids, DNA, starch, or other proteins (Østdal, Davies, & Andersen, 2002). It was reported that fatty acid oxidation could be induced by bovine serum albumin (BSA) radicals (Østdal, Davies, & Andersen, 2002), and amino acid residues of β-lactoglobulin were oxidized prior to the propagation of lipid oxidation in O/W emulsions (Salminen, Heinonen, & Decker, 2010). Therefore, it is pertinent to compare the kinetics of the evolution of lipid hydroperoxides with specific markers of protein oxidation.

2.3. Implications of lipid and protein oxidation for meat quality

2.3.1. Lipid oxidation and meat quality

Palatable or undesirable flavors may arise from different extents of lipid oxidation. On one hand, hydrolysis of some of the fat and a certain degree of oxidation produce volatile compounds that produce a pleasant flavor in most dry-cured country hams and fermented sausages (Carrapiso, Ventanas, & García, 2002). Additional work has shown that the interactions between lipids and Maillard reaction products are very important for the development of desirable meaty flavors in cooked meat (Mottram & Edwards, 1983). In general, Maillard reaction mixtures containing lipids produce several lipid-derived volatile compounds such as aldehydes, furans, hydrocarbons, alcohols, and ketones. What's more, the reaction mixtures also contain the Maillard products such as H_2S, NH_3, etc. All of them are able to interact with each other to form new heterocyclic aroma volatile compounds as the consequences of the interactions between lipids with Maillard

products (Van, Hwang, Jeong, & Touseef, 2012).

On the other hand, lipid oxidation and physicochemical changes are the major causes of meat deterioration; it involves changes in flavor, texture, color, and nutritive value as well as the formation of potentially toxic compounds (Kanner, 1994). The decomposition of lipid hydroperoxides produces low molecular weight volatile compounds causing rancidity (Frankel, 1984). The secondary oxidation products can further break down to form volatile materials and dialdehydes contributing to flavor deterioration. Cooked meat held in the refrigerator develops rancid odors and flavors that are particularly noticeable after reheating, this rapid development of oxidized flavor in refrigerated cooked meat has been termed “warmed-over flavor” (Tims & Watts, 1958). During frozen storage, the oxidation processes are slowed but not completely halted. In fact, some lipid-soluble radicals may even be more stable at lower temperatures and thereby propagate oxidation (Kanner, 1994).

2.3.2. Protein oxidation and functionality

Protein oxidation is closely related to its functionality. In some cases, mild protein oxidation is necessary for protein-protein interaction and the formation of gel networks. Studies have shown that mild oxidation promotes protein network formation (through protein-protein crosslinking) and enhances gelation of MFP in meat processing (Xiong, Blanchard, Ooizumi, & Ma, 2010). Protein oxidation has also been found to be important for the development of characteristic organoleptic properties of salted herring (Andersen, Andersen, & Baron, 2007).

Nevertheless, intense protein oxidation is commonly linked to a decrease in

muscle protein functionality, reducing tenderness and juiciness, and promoting flavor deterioration and discoloration in fresh meat and meat products (Rowe, Maddock, Lonergan & Huff-Lonergan, 2004a; Xiong, 2000). Researchers found that increased protein oxidation in both sarcoplasmic and myofibrillar protein extracts was negatively correlated to tenderness (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004b). Another study also reported that high-oxygen atmospheres cause significantly less tender meat compared to no oxygen packaging during storage (Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007). Utrera and Estévez (2012) recently elucidated the potential impact of chemical modification occurring during carbonylation on the functional properties of MFP oxidized by Cu^{2+}, Fe^{3+}, and Mb in combination with H_2O_2. According to their findings, intense protein carbonylation quickly led to severely impaired water holding, foaming, and gelling capacities. Liu, Xiong, and Chen (2010) also reported that decreased water holding and higher drip losses were associated with increases of protein carbonyl content and cross-linkage among both myofibrillar and sarcoplasmic proteins. In addition, protein oxidation induces protein polymerization and aggregation, thus, changing its digestibility, which negatively impacts the nutritional values of muscle foods. The recent studies supported that oxidative modification of MFP led to the formation of protein aggregates and a decreased susceptibility to undergo proteolytic degradation, which may have an effect on protein digestibility (Morzel, Gatellier, Sayd, Renerre, & Laville, 2006; Sante-Lhoutellier, Engel, Aubry, & Gatellier, 2008; Baron, Hyldig, & Jacobsen, 2009).

The interactions of lipid hydroperoxides and their secondary products with proteins or amino acids have a considerable impact on flavor stability during processing,

cooking, and storage. The form of these interactions is determined at the point when proteins enter the lipid oxidation reaction chain, depending upon whether radicals or secondary products predominate. Radical induced cross-linking or scission of the proteins may be responsible for most nutritional losses; hydroperoxide radicals are very reactive with sulfur and amine functional groups of amino acids, whereas aldehydes and epoxides, secondary products of lipid oxidation, react with thiols from cysteine (Gardner, 1979).

2.4. Application of antioxidants to control oxidation

2.4.1. Antioxidants used in meat processing and storage

Antioxidant strategy has been successfully employed in the food industry for quality preservation of food products (Nawar, 1996; Frankel, 1998). Antioxidants work by a variety of mechanisms, including control of oxidative substrates (for example, oxygen and lipids), control of pro-oxidants (for example, ROS and pro-oxidant metals), and inactivation of free radicals. Compared with synthetic antioxidants (BHA: Butylated hydroxy anisole, BHT: butylated hydroxy toluene, TBHQ: tertiary butylated hydroxy quinone), natural antioxidants such as vitamin E (α-tocopherol), vitamin C (ascorbic acid), carotenoids (β-carotene and vitamin A), and plant derived phenolics are of great interest because of their safety and health characteristics (Barden & Decker, 2013). Natural antioxidants sometimes perform distinctively in different food systems. The addition of natural antioxidants that prevent lipid oxidation may not inhibit or delay oxidation of proteins, indicating a difference in their mechanism for the oxidation reaction and their interaction with antioxidants (Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007).

2.4.2. Protein and peptides as antioxidants in meat systems

Depending upon their ability to scavenge free radicals or chelate metals, proteins can be either pro- or anti-oxidative (Davies, Dean, Davies, & Davies, 1997). Many studies focus on the antioxidant effect of proteins such as BSA (Heinonen, Rein, Satué-Gracia, Huang, German, & Frankel, 1998; Viljanen, Kylli, Kivikari, & Heinonen, 2004b), lactalbumin (Viljanen, Kivikari, & Heinonen, 2004a), lactoglobulin (Elias, McClements, & Decker, 2007), and casein (Hu, McClements, & Decker, 2003) against lipid oxidation. It has been reported that hydrolyzed potato protein is able to improve the oxidative stability of cooked ground beef during refrigerated storage (Wang & Xiong, 2005). Rice protein hydrolysates can also effectively inhibit lipid oxidation in food models and may be used as an effective natural antioxidant to retard lipid oxidation and improve shelf life of meat products (Zhou, Canning, & Sun, 2013). The antioxidant properties of proteins are attributed to the cooperative effect of a variety of properties including the ability of aromatic and sulfur-containing amino acids to scavenge free radicals, and the capacity to act as metal-ion chelators. Certain proteins with specific iron binding sites including transferrin, phosvitin, and lactoferrin are considered to be effective antioxidants in lipid dispersions (Waraho, McClements, & Decker, 2011a).

Food emulsions contain an oil-water interface that has a major impact on the lipid oxidation pathway by influencing the location and reactivity of prooxidative transition metals, lipid hydroperoxides, minor lipid components, free radical scavengers, and metal chelators. Oxidation in a finely chopped meat batter occurs rapidly at the oil-water interface due to the large surface area that facilitates interactions between lipids and

water-soluble prooxidants. When proteins coexist in O/W emulsion, they either act as emulsifiers and locate at the oil-water interface, or exist in the continuous phase as unadsorbed protein. In protein-stabilized emulsions, the membrane formed around the oil droplets offers resistance to mechanical disruption, and, in some cases, serves as a physical shield to prevent radical attack of the interior lipid (Hu, McClements, & Decker, 2003; Jiang, Zhu, Liu, & Xiong, 2014). Moreover, many amino acid side chain groups, including the imidazole ring (histidine), the indole group (tryptophan), and the sulfur moiety (cysteine and methionine), contribute to oxidative stability via scavenging free radicals or sequestering prooxidative metal ions (Díaz & Decker, 2004; Kaul, Sharma, & Mehta, 2008). Besides radical protection, the idea of oxygen blockage by a protein interface was brought forth by Toikkanen, Lähteenmäki, Moisio, Forssell, Partanen, and Murtomäki (2014) who showed that a well-formed protein viscoelastic layer was able to effectively reduce the oxygen permeability. Therefore, the inhibition of oxygen transport by the protein interface may be considered as another reason to protect lipids from oxidation. This premise was also discussed in caseinate-stabilized emulsion systems: the cross-linking of sodium caseinate at the emulsion interface improved oxidative stability of emulsions (Ma et al., 2012). For continuous-phase proteins, it has been reported that whey protein isolate, soy protein isolate, and sodium caseinate could inhibit lipid oxidation as well (Faraji, McClements, & Decker, 2004). Comparing interfacial proteins with unadsorbed proteins in the continuous phase, Rampon, Lethuaut, Mouhous-Riou, and Genot (2001) and Berton, Ropers, Guibert, Solé, and Genot (2012) studied emulsions stabilized by BSA/β-lactoglobulin/β-casein and demonstrated that interfacial proteins were more readily modified.

Table 2.1. Rate constants k (L /mol^{-1}×s^{-1}) for reaction of singlet oxygen/superoxide/ Trichloromethyl peroxyl radical with lipids (Bielski, Cabelli, Arudi, & Ross, 1985; Wilkinson, Helman, & Ross, 1995; Neta, Huie, & Ross, 1990).

Substrate	Singlet oxygen with olefins in carbon tetrachloride	Superoxide radicals in aqueous solution	Trichloromethyl peroxyl radical
Oleic acid	1.7×10^4	no reaction	1.7×10^6
Linoleic acid	4.2×10^4	1.18×10^3	3.9×10^6
Linolenic acid	8×10^4	1.70×10^3	7.0×10^6
Arachidonic acid	1×10^5	2.05×10^3	7.3×10^6
Lecithin	10^5	-	-

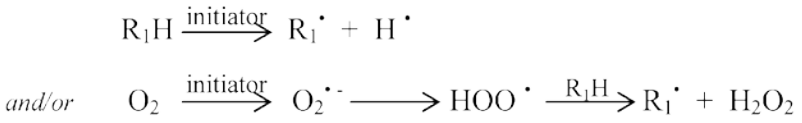
Table 2.2. Reaction rate of oxidant-susceptible amino acids (Berlett & Stadtman, 1997; Stadtman, 1992; Buxton, Greenstock, Helman, & Ross, 1988; Wilkinson, Helman, & Ross, 1995; Pattison, Hawkins, & Davies, 2003).

Amino acids	Hydroxyl k ($L/mol^{-1}\times s^{-1}$)	Singlet oxygen pH=7 k ($L/mol^{-1}\times s^{-1}$)	$HOCl^-$ k ($L/mol^{-1}\times s^{-1}$)	Oxidation products
Cysteine	3.4×10^{10}	1×10^5 in D_2O	3.0×10^7	Disulfides, cysteic acid
Tryptophan	1.3×10^{10}	3.2×10^7	1.1×10^4	2-, 4-, 5-, 6-, and 7-Hydroxytryptophan, nitrotryptophan, kynurenine, 3-hydroxykynurinine, formylkynurinine
Tyrosine	1.3×10^{10}	3.8×10^7 in H_2O pH=10	44	3,4-Dihydroxyphenylalanine, tyrosine-tyrosine cross-linkages, Tyr-*O*-Tyr, cross-linked nitrotyrosine
Histidine	1.3×10^{10}	9×10^7	1.0×10^5	2-Oxohistidine, asparagine, aspartic acid
Methionine	8.3×10^9	2.1×10^7	3.8×10^7	Methionine sulfoxide, methionine sulfone
Phenylalanine	6.5×10^9	D_2O:EtOH (75:25) 7×10^5	-	2,3-Dihydroxyphenylalanine, 2-, 3-, and 4-hydroxyphenylalanine
Arginine	3.5×10^9	$<=1\times10^6$	26	Glutamic semialdehyde

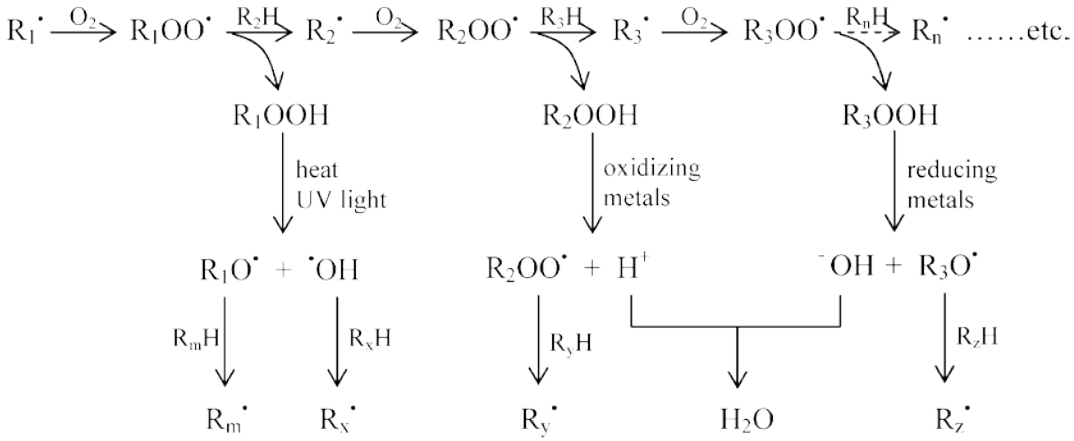
Table 2.3. Rate constants for reaction of hydroxyl group with macromolecules at pH 7 (Buxton, Greenstock, Helman, & Ross, 1988).

Substrate	Rate constant k (L /mol^{-1}×s^{-1})
Linoleic acid	9×10^{9}
Albumin	7.8×10^{10}
Collagen	4×10^{11}
Ascorbate	1×10^{10}
Glutathione	1.4×10^{10}
Trolox C (water-soluble vitamin E analogue)	6.9×10^{9}

Initiation:



Propagation:



Termination:

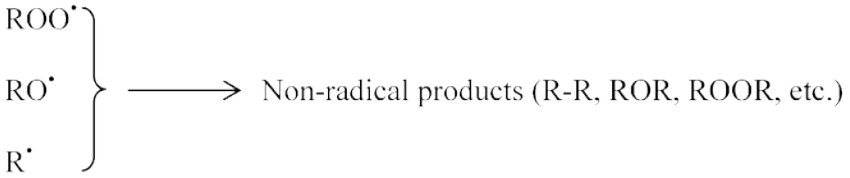


Figure 2.1. Lipid autoxidation pathways (Shahidi & Zhong, 2010).

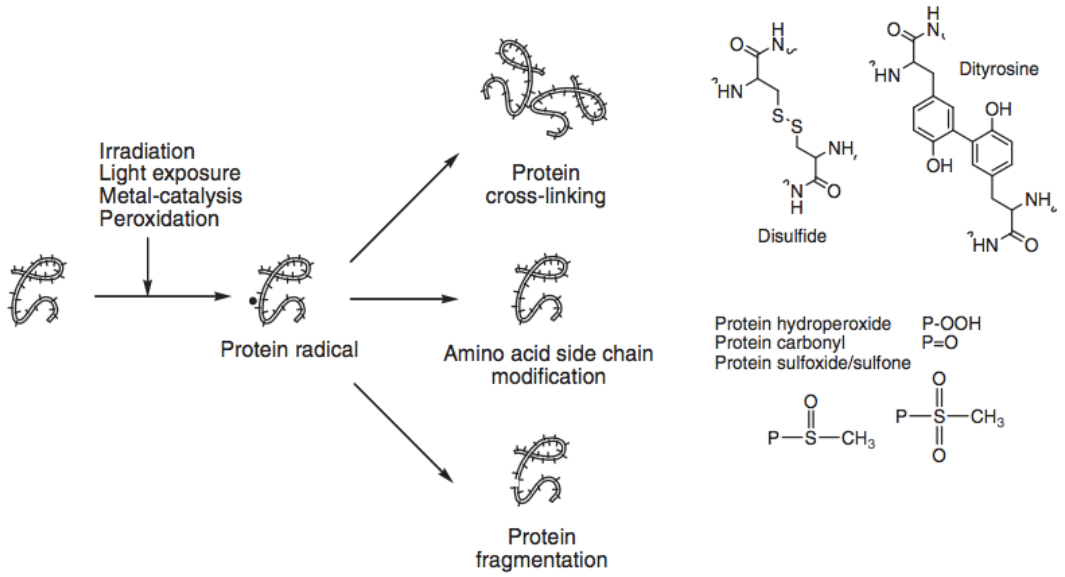


Figure 2.2. The most common consequences of oxidation of proteins (Kanner, 1994).

CHAPTER 3

PROGRESSION OF LIPID AND PROTEIN OXIDATION IN A MIXED MYOFIBRILLAR PROTEIN–FREE FATTY ACID AQUEOUS DISPERSION UPON OXIDATIVE STRESS

3.1. Summary

Free fatty acid–protein dispersions prepared from varying concentrations (0.1–2.0% w/v) of isolated pork myofibrillar protein (MFP) mixed with different free fatty acids (0.8% w/v oleic acid C18:1; linoleic acid C18:2; linolenic acid C18:3) in 0.15 and 0.6 M NaCl (pH 6.25) were subjected to hydroxyl radical stress at 4 °C for 0, 2, and 24 h. Indexes of oxidation products from lipids (hydroperoxides, TBARS) and proteins (intrinsic tryptophan fluorescence, carbonyls, sulfhydryls, SDS–PAGE) were monitored over time. The evolution of protein oxidation markers showed substantial losses (up to 65%, $P < 0.05$) for sulfhydryls and tryptophan fluorescence and increased carbonyl formation within 2 h, irrespective of NaCl concentrations. This process preceded lipid oxidation which exhibited notable accumulation (two-fold) of TBARS only after 2 h. A higher protein:lipid ratio was associated with slower hydroperoxide and TBARS production suggesting an antioxidant role of MFP to protect free fatty acids in the mixed systems. Protein conformational analysis indicated that the hydrophobic groups initially exposed might react with radicals thereby sparing lipids.

3.2. Introduction

Fatty acids, both free and as constituents within the triacylglycerol complex, play an important role in biological systems as structural components and metabolic substances. Fatty acids are carbon chains mostly containing 12~22 carbon atoms and a carboxyl group at the other end. According to the number of double bonds, they can be divided into three groups: saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids. Essential fatty acids can be found in various kinds of oil and fat. Oleic acid (18:1, omega-9) is abundant in animal fat, as well as vegetable oil and certain microorganisms. Linoleic acid (18:2, omega-6) is a major fatty acid in soybean, rapeseed, and sunflower oils. And fish, nut, and certain plant oils are the main source of linolenic acid (18:3, omega-3). Polyunsaturated fatty acids may provide a wide range of health benefits, including a lower risk of coronary heart disease and an improvement in cholesterol levels. In addition, free fatty acids are closely related to the quality and commercial value of fats and oils (Frega, Mozzon, & Lercker, 1999). They oxidize more rapidly than their corresponding methyl esters (Miyashita & Takagi, 1986). The carboxylic acid groups of free fatty acids are able to form complexes with transition metals and increase metal solubility; in addition, their acid groups can accelerate the decomposition of lipid hydroperoxides. Therefore, free fatty acids can act as prooxidants in both bulk and emulsified oils which increases lipid oxidation (Waraho, McClements, & Decker, 2011b).

During the storage of fat/oil, there is an induction period where very few lipid oxidation products are formed. When this induction period has expired, a steep rise in the oxidative reaction rate occurs. The induction period and the reaction rate of oxidation for

free fatty acids are affected by the degree of unsaturation, temperature, ultraviolet light, and the presence of oxidants and antioxidants. Belitz, Grosch, and Schieberle (2009) reported the induction period and relative oxidation rate for different fatty acids (shown in the following table). Bielski, Arudi, and Sutherland (1983) measured the reaction rate constants of the perhydroxyl radical (HO_2) with linoleic, linolenic, and arachidonic acids in aqueous ethanolic solutions which were 1.2 x 10^3, 1.7 x 10^3, and 3.0 x 10^3 $M^{-1}s^{-1}$, respectively; and no reaction was found for oleic acid. The relative oxidative stability of different polyunsaturated fatty acids catalyzed by Fe^{2+}-recycling solution in an aqueous phase (pH 7.4, 37 °C) was measured by Miyashita, Nara, and Ota (1993) since formulated foods often contain a lipid phase dispersed in an aqueous medium. They found that the characteristic oxidative stability of polyunsaturated fatty acids in an aqueous phase was strongly associated with their individual conformation and differed from that in the neat phase.

Fatty acid	Number of allyl groups	Induction period (h)	Oxidation rate (relative)
18:0	0		1
18:1 (9)	1	82	100
18:2 (9, 12)	2	19	1,200
18:3 (9, 12, 15)	3	1.34	2,500

Myofibillar protein (MFP) is abundant in sulfhydryl groups (from cysteine and methionine), which are susceptible to hydroxyl radicals and readily converted to disulfide bonds, mixed disulfides, and thiol radicals (Shacter, 2000). Moreover, hydroxyl radicals produced by a Fe^{2+}-recycling solution lead to potent site-specific oxidation. For example, histidine, as a good ligand for ferric, is vulnerable to convertion into oxo-histidine or aspartic acid (Carpenter, 1979). Other amino acyl moieties, especially lysine, arginine,

proline, and threonine, cause the formation of carbonyl groups (aldehydes and ketones) on the side chains (Shacter, 2000). In addition, aromatic residues, though often buried in the hydrophobic core of molecules, are typically more prone to oxidation than aliphatic residues and hence are commonly used as a marker of protein oxidation (Dean, Fu, Stocker, & Davies, 1997; Waraho, McClements, & Decker, 2011a).

Unsaturated fatty acids and MFP are two main targets of oxidants during the processing and storage of meat products. Their interaction has been biologically investigated including the binding of long chain fatty acids with protein and post-translational modification (oxidation) of proteins by fatty acids (Magee, 1990). However, there were few discussions on the interaction of proteins and fatty acids in food systems. Li, Jiang, Zhang, Mu, and Liu (2008) suggested the antioxidant properties of protein in protecting linoleic acid from oxidation. And Østdal, Davies, and Andersen (2002) reported that fatty acid oxidation could be induced by bovine serum albumin (BSA) radicals (Østdal, Davies, & Andersen, 2002). The relative susceptibility of protein and fatty acids to reactive oxygen species (ROS) under meat processing and storage conditions needs to be studied further.

Free fatty acids are distributed within triacylglycerol as their acyl components in food systems. Most triacylglycerol are unsymmetrical, being derived from mixtures of fatty acids that vary in their susceptibilities to oxidation due to different degrees of unsaturation. Therefore, it is difficult to directly measure the individual effect of fatty acids on the oxidative stability of triacylglycerol. In order to clarify the oxidative stability of fatty acids esterified at the glycerol moieties in an aqueous phase, oleic, linoleic, and linolenic acids were tested individually with MFP to monitor the progress of oxidative

changes in a mixed lipid–protein system. This knowledge also assists with the development of novel antioxidant technologies to allow deliberate control of oxidation for flavor and texture purposes.

3.3. Materials and methods

3.3.1. Materials

Myofibrillar protein was isolated from Longissimus muscle collected from three pork carcasses (48 h-post-mortem). Collected muscle samples were cut into 1 cm thick slices, individual vacuum-packaged, and stored in a freezer (−30 °C) until use within 6 months. Frozen samples were tempered at 4 °C for 4 h and then used for MFP extraction using an isolation buffer (pH 7.0) of 100 mM sodium chloride (NaCl), 10 mM sodium phosphate, 2 mM $MgCl_2$, and 1 mM EGTA (Park, Xiong, & Alderton, 2006). The pH of MFP suspension in 0.1 M NaCl was adjusted to 6.25 during the last wash. The suspension was then centrifuged at 2,000*g* for 15 min to produce MFP pellet. The MFP pellet was kept on ice and used within two days. Oleic acid (18:1, 99%+), linoleic acid (18:2, 99%+), and linolenic acid (18:3, 99%+) were purchased from Sigma-Aldrich. Co. (St. Louis, MO, USA).

3.3.2. Preparation of bi-phasic systems

Protein with free fatty acid systems were prepared by mixing oleic, linoleic, or linolenic acids (0.8 wt% final) with 0.1%, 0.8% and 2% (w/v) MFP in 25mM phosphate buffer (PBS) containing 0.6 M NaCl or 0.15 M NaCl (pH 6.25), respectively. The mixtures were stirred continuously.

3.3.3. Oxidation treatment

Three systems were oxidized for 0, 2, and 24 h at 4 °C with hydroxyl radicals produced by a Fe^{2+}-recycling solution comprised of 10 μM $FeCl_3$/100 μM ascorbic acid/5 mM H_2O_2 (Levine, Oliver, Fulks, & Stadtman, 1981; Martinaud, Mercier, Marinova, Tassy, Gatellier, & Renerre, 1997; Park, Xiong, & Alderton, 2006). Propyl gallate, EDTA, and Trolox (1 mM each) were used to terminate the oxidative reaction (Park, Xiong, & Alderton, 2006).

3.3.4. Measurement of lipid oxidation

Lipid oxidation in different dispersions was monitored by the formation of conjugated diene hydroperoxides, and 2-thiobarbituric acid-reactive substances (TBARS). For the measurement of conjugated diene (CD) (Srinivasan, Xiong, & Decker, 1996), samples (0.5 mL) from different systems were mixed with 5 mL of extracting solvent (3:2 v/v hexane:isopropanol) and vortexed for 1 min. The mixtures were then centrifuged at 2,000*g* for 10 min and the supernatants read at 233 nm. The molar extinction coefficient (25,200 $M^{-1}cm^{-1}$) was used to calculate CD (Juntachote, Berghofer, Siebenhandl, & Bauer, 2007).

The ferric thiocyanate method was used to measure the amount of peroxide at the beginning of the lipid peroxidation, in which peroxide reacts with ferrous chloride and form ferric ion (Sakanaka, Tachibana, Ishihara, & Raj Juneja, 2004). Sample mixtures of 100 μL were mixed with 4.7 mL of 75% ethanol, 50 μL of 30% ammonium thiocyanate, and 100 μL of 20 mM ferrous chloride solution in 1 M HCl. After incubation at room

temperature for 3 min, the absorbance was read at 500 nm.

For TBARS (Sinnhuber & Yu, 1977), varying samples were mixed with trichloroacetic acid (TCA) and thiobarbituric acid (TBA) followed by boiling for 30 min. The supernatant of the cooled solutions were mixed with chloroform. After centrifugation at 2,000*g* for 10 min, the absorbance (532 nm) of the upper phase was recorded and the value converted to TBARS content using the molar extinction coefficient of 152,000 $M^{-1}cm^{-1}$ (Witte, Krause, & Bailey, 1970).

3.3.5. Determination of protein modification

Evaluation of protein modification in different dispersion systems was performed by monitoring the loss/formation of sulfhydryls and carbonyls, tryptophan fluorescence intensity, and the band intensity after sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Free sulfhydryl content in different dispersion systems was measured using the Ellman's reagent [DNTB: 5,5′-dithio-bis (2-nitrobenzoic acid)] colorimetric method (Liu, Xiong, & Chen, 2009). A reagent blank was run with 25 mM phosphate buffer (pH 6.25) containing 0.6 M/0.15 M NaCl. A molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ was used for the sulfhydryl content computation.

The content of protein carbonyl derivatives in samples was estimated by the reaction with 2,4-dinitrophenylhydrazine (DNPH) as detailed by Levine (1990). In brief, the DNPH-reacted samples were precipitated using TCA (20%) and then washed with ethanol:ethyl acetate (1:1 v/v) three times to remove unbound pigments. The final pellets were then dissolved in 6.0 M guanidine hydrochloride. Protein concentrations were

measured at 280 nm and the carbonyls content was calculated using a molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$.

Tryptophan fluorescence intensity was monitored to detect protein structural changes. Samples (100 μL) were diluted in 5 mL of 25 mM PBS (pH 6.25) containing 0.6 M/0.15 M NaCl. After the addition of 2 mL of petroleum ether, the mixtures were vortexed then centrifuged at 2,000*g* for 5 min. The lower phases were collected for fluorescence testing using a Fluromax-3 fluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with a 283 nm excitation wavelength and a 300–400 nm emission wavelength at a 1 nm/s scanning speed (Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008).

SDS–PAGE was performed using a 4% polyacrylamide stacking gel and a 10% polyacrylamide separating gel. Samples with 20 mg/mL MFP were diluted to 2 mg/ml before mixing with SDS–PAGE sample buffer (1:1) with or without 10% β–mercaptoethanol (βME). The mixtures were then boiled for 3 min. Into each well of the gel, 30 μL of prepared sample was loaded.

3.3.6. Statistical analysis

Data were obtained from three independent experiments with separate MFP preparations. In each trial, duplicate or triplicate samples were analyzed. And the results were processed using a general linear model of Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA). Significant differences ($P < 0.05$) between means were identified by least significant difference (LSD) all-pair wise multiple comparisons.

3.4. Results and discussion

3.4.1. Lipid oxidation

The peroxidation of fatty acids in a MFP aqueous solution was measured with respect to primary (hydroperoxides) and secondary (ketones-like and aldehydes-like) oxidation products, detected by CD and TBARS tests, respectively. In both, high salt (0.6 M) and low salt concentration (0.15 M) systems, the oleic acid with MFP system maintained relatively lower CD values during oxidation. The oxidation of oleic acid involves the formation of a hydroperoxide group at positions 8, 9, 10, or 11 of oleic acid with a single double bond. Therefore, there was barely any CD formed from oleic acid oxidation. The small volume of CD may arise from the intense oxidative desaturation of oleic acid (Brenner, 1974) or the impurity of MFP. CD content in linoleic acid samples did not change significantly in the first two hours of oxidation, but showed a remarkable increase ($P < 0.05$) from 2 to 24 h (Figure 3.1). In addition, the CD values in linoleic acid systems were higher under lower salt concentration. Oxidation of linolenic acid samples generates conjugated diene hydroperoxide groups, and the formation of CD exhibited a similar trend as linoleic acid: no significant change in 2 h ($P > 0.05$), while a nearly two-fold increase at 24 h. Comparing different fatty acids with MFP samples, CD content in linolenic acid samples was the highest. The result is in accordance with Miyashita and Takagi (1986)'s finding that linolenic acid has the highest oxidative rate, followed by linoleic and oleic acids according to their peroxide value. There were no significant differences noted between different concentrations of MFP in most of the systems ($P > 0.05$). And the trend of conjugated dienes formation during oxidation was similar in different salt concentrations (0.6 M and 0.15 M).

The secondary oxidation products derived from lipid hydroperoxides were tested by TBARS (Figure 3.2). In oleic acid with MFP systems (0.6 M NaCl/0.15 M NaCl), the TBARS content gradually increased but was relatively small (less than 0.5 μM), which may have resulted from impurity of the MFP. Linoleic acid with MFP systems (0.6 M NaCl/0.15 M NaCl) possessed a much higher TBARS content than oleic acid. And linolenic acid showed nearly fourfold TBARS values than that of linoleic acid during oxidation (Figure 3.2). From Figure 3.2, the formation of TBARS in the fatty acids with MFP samples is not directly related to the salt concentration. Their differences can be attributed to the different chemical structures and physical properties of the fatty acids. Linolenic acid has one more double bond than linoleic acid which makes it more susceptible to ROS compared to linoleic acid or oleic acid. It is also reported that polyunsaturated fatty acids such as linolenic acid, with low melting points, are extremely susceptible to oxidation. And monounsaturated oleic acid with a higher melting point (13.4 °C) (no longer a clear liquid at refrigerated temperatures) is relatively oxidatively stable (O'Brien, 2008).

The scavenging effect of MFP on lipid oxidation also depends on MFP concentration. For samples with 0.8% or higher concentrations of MFP, their TBARS did not change significantly in 2 h ($P > 0.05$), but increased remarkably from 2 to 24 h. In comparison, TBARS content in systems with 0.1% MFP rose continuously during 24 h (the major increase still occurs from 2 to 24 h) (Figure 3.2). It can be concluded that higher concentrations of MFP could better inhibit lipid oxidation in mixed protein:lipid systems. As a favorite target of the hydroxyl radical, MFP exhibited a consequential

"sparing" effect to protect lipids. This hypothesis is consistent with the development of protein oxidation (described below).

3.4.2. Antioxidant activity of MFP

The ferric thiocyanate method evaluates lipid oxidation by peroxide values (denoted by absorbance at 500 nm); it could further characterize the role of MFP in preventing fatty acids from oxidation. As shown in Figure 3.3, oxidation in oleic, linoleic, and linolenic acids with MFP samples was markedly inhibited by the increased concentration of MFP, regardless of the salt concentration. Among the three fatty acids, the highest antioxidant activity of MFP was found in oleic acid, which exhibited a nearly seventy percent reduction in oxidation at 24 h when the concentration of MFP increased from 0.1% to 0.8%, and another eighty percent reduction when further increased to 2% (Figure 3.3). These results reinforced the conclusion from the TBARS results (Figure 3.2) that MFP played a protective role in fatty acid oxidation. A higher concentration of MFP in meat products could confer desirable functional properties.

3.4.3. Protein oxidation

To compare the oxidation susceptibility of MFP when coexisting with fatty acids in an aqueous system and to confirm the hypothesis that MFP could act as a sacrificing antioxidant to protect fatty acids from oxidation, oxidative modification of MFP was measured.

3.4.3.1. Sulfhydryl and carbonyl derivatives

Modification of amino acid side chain groups is the primary consequence of oxidative attack of proteins. The loss of sulfhydryls and formation of carbonyls are two common measurements of protein changes in oxidized muscle foods (Díaz & Decker, 2004; Li, Xiong, & Chen, 2012). As presented in Table 3.1, the sulfhydryl content decreased while the carbonyl continuously increased during oxidation ($P < 0.05$). For the thiol groups, most of the loss occurred within the first 2 h, preceding the onset of lipid oxidation which took place mostly between 2 and 24 h (Figure 3.2). The extent of sulfhydryl reduction depends upon different salt levels, fatty acids, and protein concentrations. The free sulfhydryl content of MFP was higher in low salt concentrations and with a slower reduction rate during oxidation. It could be explained by the fact that MFP has a low solubility in 0.15 M NaCl but solubilizes in 0.6 M NaCl which makes it more exposed to hydroxyl radicals in an aqueous phase. Moreover, a more rapid decline in sulfhydryls was found in samples of fatty acids with less double bonds and lower concentrations of MFP. Linolenic acid with three double bonds is more likely to be oxidized. Therefore, MFP, when incorporated with linolenic acid, displayed a slower rate of oxidative changes. This again reinforced the competitive relationship of proteins and fatty acids. The sulfhydryl changes varied from different protein concentrations, higher concentrations of MFP had less reduction in sulfhydryls most likely due to the concentration effect.

Table 3.1 also summarizes the protein carbonyl content in different oxidizing systems. Oxidative stress resulted in a gradual rise of the carbonyl content in all systems. It is noteworthy to point out that freshly prepared samples already had measurable amounts of carbonyls produced during the MFP isolation and sample preparation. Among

samples prepared with different fatty acids, MFP with linolenic acid had a higher initial carbonyl value but also maintained a lower rate of increase during oxidation: at 24 h of oxidation, carbonyls of MFP in oleic acid systems increased by 65% and 68% (average) in 0.6 M and 0.15 M sodium chloride, respectively; while linolenic acid samples increased by 17% and 27% (average) under high and low level of salt concentrations, respectively. At the high salt concentration, significant increases ($P < 0.05$) in carbonyls were noticed in 2 h–oxidized samples except for samples with 0.1% or 2% MFP and linolenic acid, which substantiated the theory that modification of the primary structure of MFP occurred prior to fatty acid oxidation. Similar trends were found in samples with low salt concentration.

3.4.3.2. Conformational changes of MFP

Tertiary structural changes of MFP were detected by the quenching of tryptophan fluorescence (Figure 3.4 & Figure 3.5). Tryptophan fluorescence intensity reduced over time in all groups. Within 2 h, there were great losses of fluorescence (Figure 3.4 & Figure 3.5) especially in samples with higher MFP concentration and lower unsaturation degree of fatty acids. From 2 to 24 h, tryptophan fluorescence intensity continued decreasing, especially in samples with highly unsaturated fatty acids. Higher salt levels were related to a more rapid reduction rate of the fluorescence intensity that could be explained by the higher solubility of MFP.

The aggregation and cross-linking of MFP (2%) were detected by SDS–PAGE (Figure 3.6). In all non-reducing samples, the intensity of myosin bands remarkably decreased in 2 h, and the bands were barely perceptible at 24 h (Figure 3.6a, c). Myosin heavy chain (MHC) in MFP with oleic acid samples completely vanished within 2 h of

oxidation (Figure 3.6a, c), suggesting a more extensive polymerization compared with the MFP in the linoleic or linolenic acid samples. When oxidized samples were treated with β-mercaptoethanol (βME), a reducing agent, MHC in 0.6 M sodium chloride systems was mostly recovered (Figure 3.6b), indicating a disulfide-dependent polymerization of myosin. In contrast, at low salt concentrations, myosin bands were just partially recovered (Figure 3.6d). These polymers were formed not only by disulfide bonds but also other covalent bonds, such as tyrosine–tyrosine or carbonyl–NH_2 interactions (Li, Xiong, & Chen, 2012). During oxidation, the intensity of actin bands reduced in a similar pattern but was less affected than MHC (Figure 3.6b).

The hydroxyl radical is one of the most reactive free radicals and can be formed from ascorbic acid, transition metals, and hydrogen peroxide. The reaction of ascorbic acid and ferrous yields ferric ions continuously and produces hydroxyl radicals when reacted with hydrogen peroxide in solution (Gutowski & Kowalczyk, 2013). Hydroxyl radicals may add onto the double bonds in lipids and undergo further chain reactions or attack the R groups of proteins, resulting in protein radicals, or directly attack amino acid side chain groups and generate protein radicals (Halliwell, 2006; Shanlin, Stocker, & Davies, 1997). When exposed to hydroxyl radicals, the great loss of fluorescence (Figure 3.4 & Figure 3.5) and myosin band intensity (Figure 3.6) within 2 h, along with the substantial loss of sulfhydryl and the accumulation of carbonyl (Table 3.1), confirmed an earlier protein modification in a mixed MFP-free fatty acid system. Comparing these protein oxidation markers with TBARS values, the time lag in TBARS values at the first 2 h signified that the onset of oxidation stress upon MFP took place earlier than fatty acids, regardless of the salt concentration. These results are in accordance with the rate

constants reported by Buxton, Greenstock, Helman, and Ross (1988); the hydroxyl radical would react faster and preferentially with some kinds of proteins such as collagen (rate constant: 4×10^{11} $ml^3mol^{-1}s^{-1}$), albumin (rate constant: 8×10^{10} $ml^3mol^{-1}s^{-1}$), and GSH (rate constant: 1.4×10^{10} $ml^3mol^{-1}s^{-1}$) than linoleic acid (rate constant: 9×10^{9} $ml^3mol^{-1}s^{-1}$). When both MFP and fatty acids existed in an oxidized system, MFP tended to move toward fatty acids and possessed a preferable location to be attacked by radicals in an aqueous phase because of its amphipathy.

3.5. Conclusion

The susceptibility of lipids and proteins to ROS largely depends upon the degree of unsaturation of the fatty acids and concentration of MFP. In general, MFP oxidation preceded unsaturated fatty acid oxidation in a mixed MFP–free fatty acid dispersion system. This may be attributed to the higher rate constant of protein when reacting with hydroxyl radicals and its preferred location during oxidation. Moreover, MFP was more prone to oxidation when coexisting with fatty acids with a lower degree of unsaturation. And the exposed hydrophobic groups in MFP at the early stage of oxidation may serve as antioxidants to neutralize radicals. In addition, highly unsaturated fatty acids could likewise partially spare proteins without accentuating protein oxidation.

Table 3.1. Sulfhydryl and carbonyl content in free fatty acid with MFP systems upon high salt (0.6 M) concentration.

	Sulfhydryl (nmol/mg protein)			Carbonyl (μmol/g protein)		
Oxidation systems	0 h	2 h	24 h	0 h	2 h	24 h
Oleic (18:1)						
MFP 0.1%	21.23 ± 1.91^{a}	7.46 ± 1.09^{b}	6.69 ±1.69^{b}	3.24 ± 0.14^{b}	5.21 ± 0.57^{a}	5.30 ± 1.18^{a}
MFP 0.8%	34.87 ± 1.46^{a}	18.14 ± 3.82^{b}	10.04 ± 0.73^{b}	3.02 ± 0.17^{b}	3.98 ± 0.21^{ab}	4.84 ± 0.52^{a}
MFP 2.0%	36.54 ± 1.46^{a}	30.11 ± 1.09^{b}	23.93 ± 0.36^{c}	2.57 ± 0.05^{b}	3.53 ± 0.09^{ab}	4.42 ± 0.68^{a}
Linoleic (18:2)						
MFP 0.1%	26.76 ± 1.01^{a}	6.18 ± 1.09^{b}	6.00 ± 1.09^{b}	2.55 ± 0.14^{c}	4.79 ± 0.37^{b}	8.82 ± 0.38^{a}
MFP 0.8%	26.79 ± 0.82^{a}	6.43 ± 0.36^{b}	5.66 ± 0.36^{b}	4.17 ± 0.67^{c}	5.91 ± 0.22^{b}	7.73 ± 0.32^{a}
MFP 2.0%	26.12 ± 0.55^{a}	23.68 ± 1.44^{ab}	22.32 ± 0.46^{b}	3.61 ± 0.13^{c}	4.53 ± 0.44^{b}	6.29 ± 0.12^{a}
Linolenic (18:3)						
MFP 0.1%	22.56 ± 1.46^{a}	12.35 ± 0.00^{b}	11.97 ± 0.91^{b}	8.96 ± 0.88^{a}	8.93 ± 0.48^{a}	9.43 ± 0.07^{a}
MFP 0.8%	27.15 ± 0.18^{a}	22.52 ± 2.00^{a}	12.10 ± 2.91^{b}	6.61 ± 0.23^{c}	7.13 ± 0.12^{b}	8.71 ± 0.08^{a}
MFP 2.0%	28.95 ± 1.64^{a}	24.71 ± 0.36^{b}	15.44 ± 0.00^{c}	5.45 ± 0.47^{a}	5.56 ± 0.21^{a}	6.23 ± 0.05^{a}

$^{a–c}$ Means within the same system between different oxidation times with different lowercase letters differ significantly ($P < 0.05$).

Table 3.2. Sulfhydryl and carbonyl content in free fatty acid with MFP systems upon low salt (0.15 M) concentration.

	Sulfhydryl (nmol/mg protein)			Carbonyl (μmol/g protein)		
Oxidation systems[1]	0 h	2 h	24 h	0 h	2 h	24 h
Oleic (18:1)						
MFP 0.1%	38.35 ± 0.73^{a}	35.13 ± 0.18^{b}	4.38 ± 0.73^{c}	3.29 ± 0.58^{a}	6.02 ± 0.09^{a}	6.4 ± 1.68^{a}
MFP 0.8%	36.93 ± 0.18^{a}	32.26 ± 0.18^{b}	32.17 ± 2.18^{b}	3.19 ± 0.52^{b}	4.21 ± 0.11^{a}	4.67 ± 0.01^{a}
MFP 2.0%	41.31 ± 0.55^{a}	35.90 ± 0.18^{b}	34.49 ± 2.18^{b}	2.51 ± 0.11^{c}	3.37 ± 0.02^{b}	4.09 ± 0.1^{a}
Linoleic (18:2)						
MFP 0.1%	33.22 ± 1.33^{a}	5.28 ± 0.18^{b}	5.90 ± 0.73^{b}	2.32 ± 0.46^{b}	4.43 ± 0.18^{a}	5.75 ± 0.64^{a}
MFP 0.8%	37.83 ± 1.82^{a}	19.64 ± 1.09^{b}	19.17 ± 1.27^{b}	4.36 ± 0.54^{b}	5.89 ± 0.19^{a}	5.28 ± 0.07^{ab}
MFP 2.0%	38.99 ± 0.18^{a}	32.17 ± 1.82^{b}	26.38 ± 0.55^{c}	3.99 ± 0.04^{c}	4.56 ± 0.11^{b}	6.13 ± 0.01^{a}
Linolenic (18:3)						
MFP 0.1%	25.94 ± 0.18^{a}	16.48 ± 0.18^{b}	9.28 ± 0.18^{c}	6.79 ± 1.74^{a}	7.32 ± 0.09^{a}	8.68 ± 1.66^{a}
MFP 0.8%	32.56 ± 0.55^{a}	26.25 ± 0.73^{b}	13.64 ± 0.36^{c}	6.34 ± 0.18^{b}	6.11 ± 0.03^{b}	8.21 ± 0.15^{a}
MFP 2.0%	33.07 ± 0.55^{a}	32.04 ± 0.55^{a}	27.28 ± 1.46^{b}	5.06 ± 0.05^{a}	5.86 ± 0.12^{a}	6.20 ± 0.20^{a}

$^{a–c}$ Means within the same system between different oxidation times with different lowercase letters differ significantly ($P < 0.05$). Oxidation systems[1] denoted different compositions of protein with free fatty acid systems. Oleic: different concentrations of MFP (0.1%, 0.8% and 2%) with oleic acid system; linoleic: different concentrations of MFP (0.1%, 0.8% and 2%) with linoleic acid system; linolenic: different concentrations of MFP (0.1%, 0.8% and 2%) with linolenic acid system.

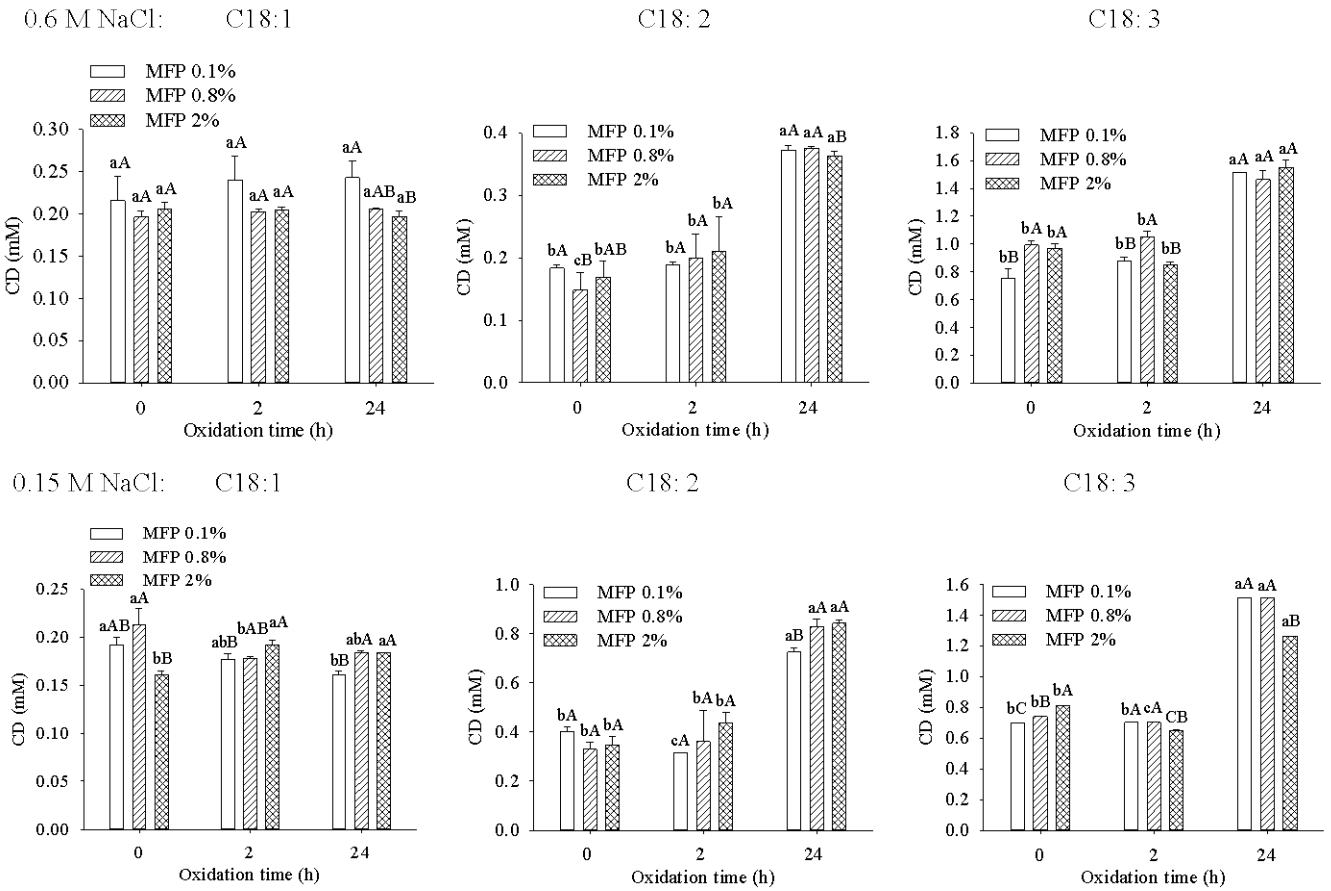


Figure 3.1. Formation of conjugated dienes (CD) in mixed free fatty acid (0.8%) and MFP (0.1%, 0.8%, or 2%) systems upon high salt (0.6 M) and low salt (0.15M) concentrations.

(a-c) Means within the same system (concentration) between different oxidation times with different lowercase letters differ significantly ($P < 0.05$).

(A-E) Means within the same oxidation time between different systems with different uppercase letters differ significantly ($P < 0.05$).

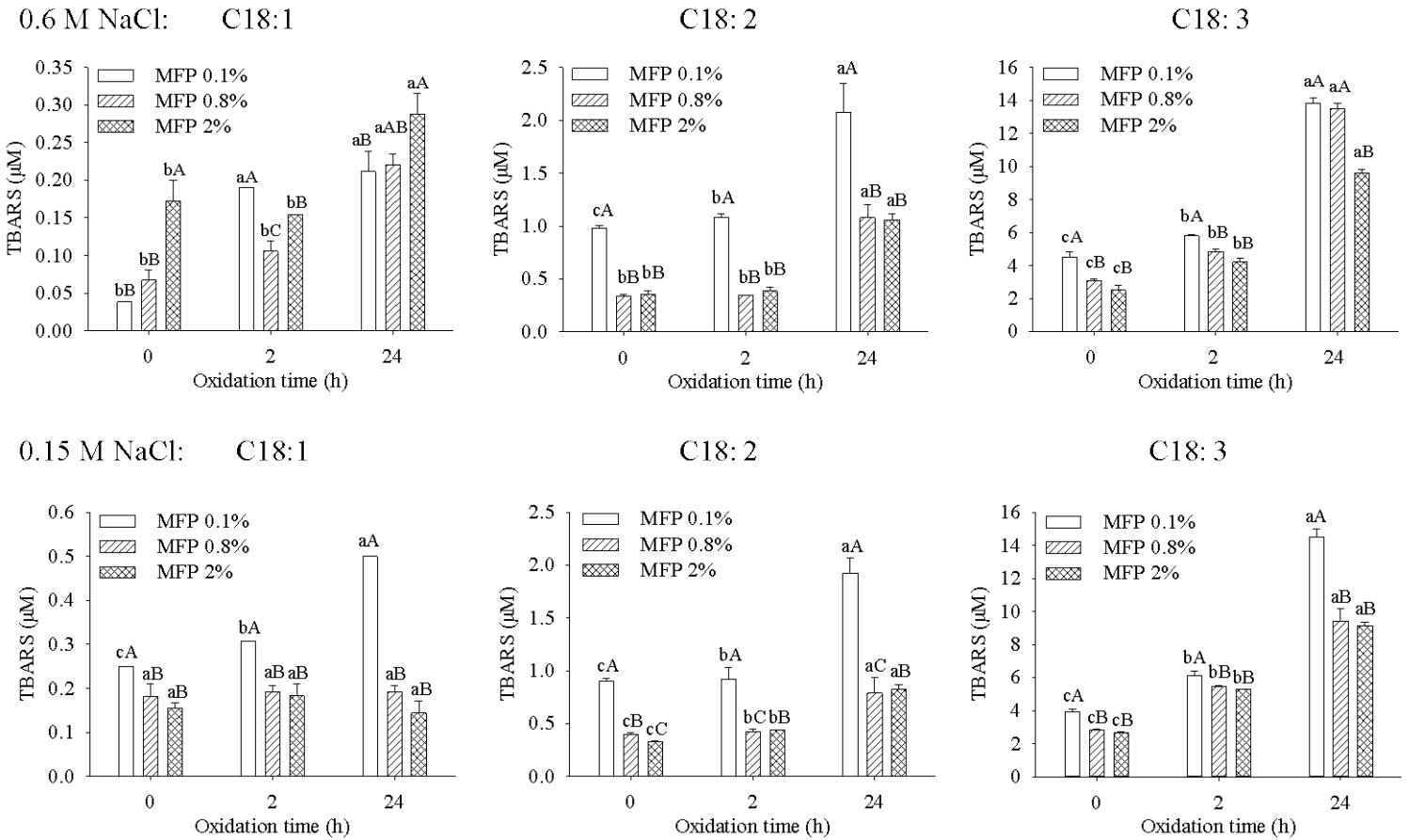


Figure 3.2. Formation of thiobarbituric acid-reactive substances (TBARS) in mixed free fatty acid (0.8%) and MFP (0.1%, 0.8%, or 2%) systems upon high salt (0.6 M) and low salt (0.15M) concentrations.

(a-c) Means within the same system (concentration) between different oxidation times with different lowercase letters differ significantly ($P < 0.05$).
(A-E) Means within the same oxidation time between different systems with different uppercase letters differ significantly ($P < 0.05$).

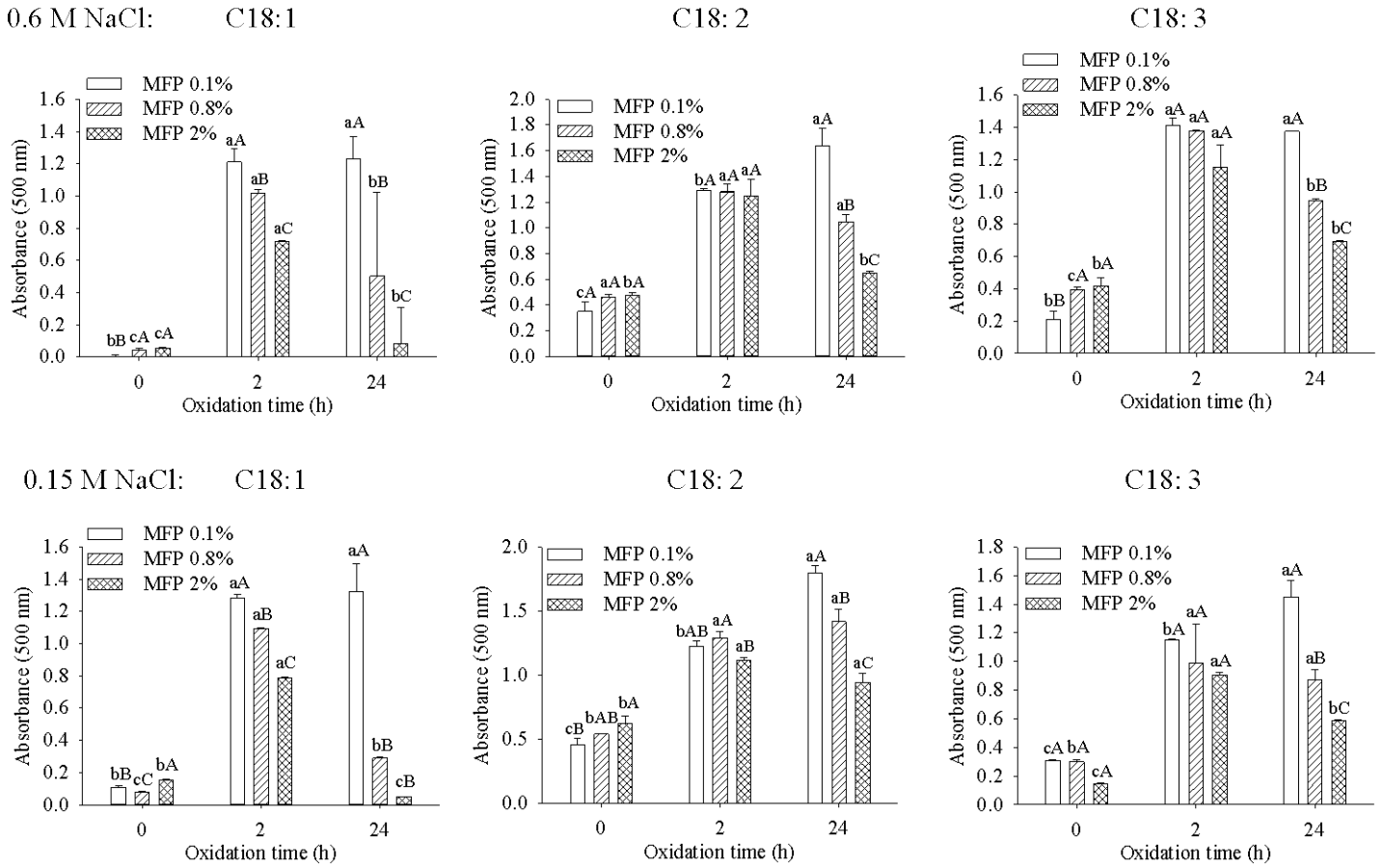


Figure 3.3. Effect of MFP (0.1%, 0.8%, or 2%) on oxidation of oleic/linoleic/linolenic acid upon high salt (0.6 M) and low salt (0.15M) concentrations.

(a-c) Means within the same system (concentration) between different oxidation times with different lowercase letters differ significantly ($P < 0.05$).
(A-E) Means within the same oxidation time between different systems with different uppercase letters differ significantly ($P < 0.05$).

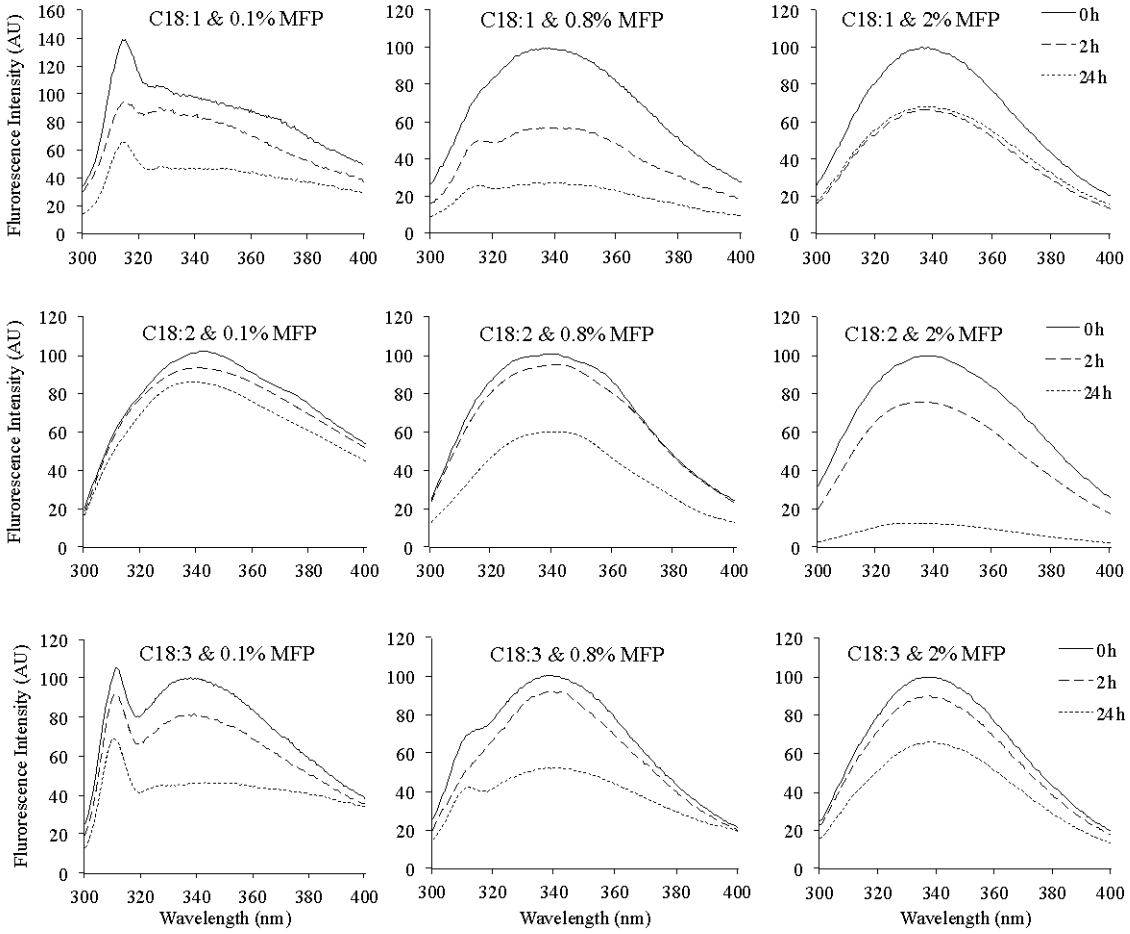


Figure 3.4. Fluorescence changes during oxidation in mixed free fatty acid and MFP (0.1%, 0.8%, or 2%) systems upon high salt (0.6 M) concentrations.

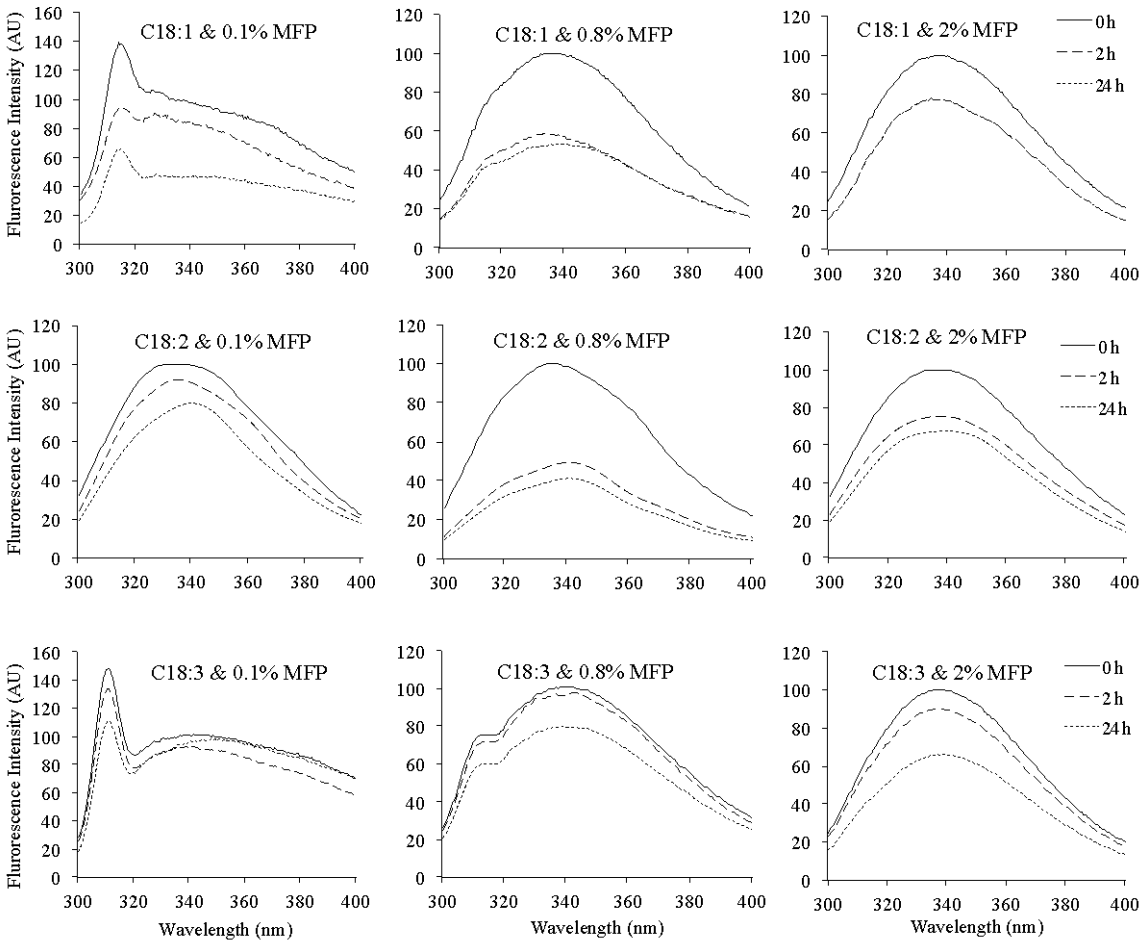


Figure 3.5. Fluorescence changes during oxidation in mixed free fatty acid and MFP (0.1%, 0.8%, or 2%) systems upon low salt (0.15 M) concentrations.

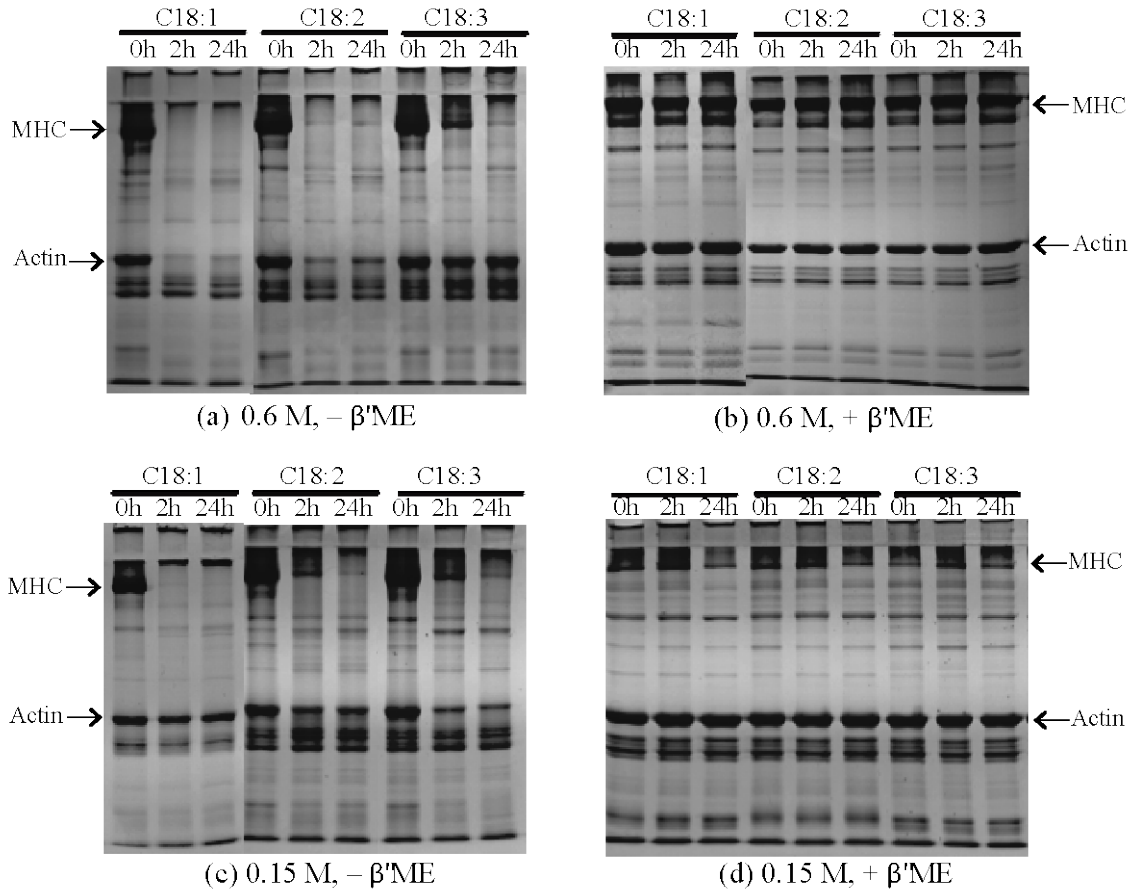


Figure 3.6. SDS–PAGE result of mixed free fatty acid (0.8%) and protein (2%) systems during oxidation in high salt (0.6 M) and low salt (0.15M) concentrations. (MHC represents myosin heavy chains.)

CHAPTER 4

COMPARATIVE TIME-COURSE OF LIPID AND MYOFIBRILLAR PROTEIN OXIDATION IN DIFFERENT BI-PHASIC SYSTEMS

4.1. Summary

This study aimed to investigate the relative reaction rate of protein and lipid oxidation in different bi-phasic model systems (linoleic acid; liposome; emulsion) containing myofibrillar protein (MFP; 0.1%, 0.8%, or 2%) under hydroxyl radical stress. Protein oxidation markers exhibited significant changes: reduction (up to 50%) in tryptophan fluorescence, carbonyl formation, and extensive polymerization of myosin in 2 h. In contrast, no detectable changes ($P > 0.05$) in lipid peroxide occurred within 2 h except for samples with 0.1% MFP which showed an early increase in TBARS. The results indicated that MFP was more susceptible to oxidative damage than lipids. Of the three bi-phasic systems, the oxidative stability of lipids followed the order of emulsion system > linoleic acid system > liposome system. The extent and rate of protein versus lipid oxidation were influenced by the protein:lipid ratio and varied between bi-phasic systems. In general, a higher protein:lipid ratio was associated with slower TBARS production and more rapid protein oxidation, suggesting a sacrificing role of MFP to protect lipids.

4.2. Introduction

Lipid and protein oxidations are the primary causes for quality deterioration in muscle foods. These two processes can be inter-related or occur independently; however, the relative susceptibility of lipid and protein to oxygen species in different food systems is not well understood. On one hand, the decomposition of lipid hydroperoxides generates alkoxyl and peroxyl radicals that may promote protein oxidation by abstracting hydrogen atoms from protein (Stadtman & Levine, 2003). Aldehydes produced from further chain reactions can initiate protein oxidation as well and are known to be involved in protein-lipid adducts (Gardner, 1979; Karel, Schaich, & Roy, 1975; Li & King, 1999; Xiong & Decker, 1995). On the other hand, protein radicals are able to react with double bonds in unsaturated lipids and susceptible side chain groups of other proteins (Elias, Kellerby, & Decker, 2008). In general, the characteristic oxidative stability of lipid and protein in an aqueous phase differs from that in the non-aqueous phase and largely depends upon their distribution in the medium (Miyashita, Nara, & Ota, 1993). It is probably also influenced by their intermolecular packing with each other in an aqueous medium. Therefore, the sequence of lipid and protein oxidation needs to be studied case by case.

Many formulated foods contain a lipid phase dispersed in an aqueous medium. In our previous study, we discussed the progress of oxidative changes in a mix system composed of MFP and different free fatty acids (Chapter 3). However, fatty acids are always present in triacylglycerols or phospholipids as their acyl components in food systems. Therefore, it is necessary to investigate the oxidative stability of fatty acids esterified at the glycerol moieties in an aqueous phase. A triacylglycerol is an ester derived from glycerol and three fatty acids. It makes up the majority of food lipids and is

one of the best sources of lipids to use for the evaluation of potency of an antioxidant in emulsions (Decker, Warner, Richards, & Shahidi, 2005). In oil-in-water (O/W) emulsions, triacylglycerols disperse in the form of small spherical droplets in an aqueous phase. Such system is thermodynamically unstable, and an emulsifier is needed to minimize the contact area between oil and water (McClements & Decker, 2000). Emulsifiers, such as amphiphilic proteins, phospholipids, and small molecule surfactants, are surface-active molecules capable of adsorbing to the surface of oil droplets during homogenization. The properties of the interfacial membrane formed by these emulsifiers in O/W emulsions could influence the rate of lipid oxidation (McClements & Decker, 2000; Jiang & Xiong, 2015).

Because of the abundant presence of unsaturated fatty acids, phospholipids are considered to be a primary source of oxidative reactants that are responsible for rancidity in meat products. Moreover, with a high degree of unsaturation, phospholipids possess around 100 times more surface area than triacylglycerols on a weight basis (Decker, Warner, Richards, & Shahidi, 2005). Lecithin (phosphatidylcholine) is a common type of phospholipid, composed of a hydrophilic phosphoric acid with a choline “head”, and two hydrophobic fatty acids “tails”, joined together by a glycerol molecule. Lecithin is rich in egg yolk and soybean oil, and is widely utilized in food processing application as an emulsifier because of its strong amphiphilic nature (Dickinson & Yamamoto, 1996). Liposomes can be made from lecithin molecules that self-assemble in aqueous media into a spherical, enclosed structure (Sabın, Prieto, Ruso, Hidalgo-Alvarez, & Sarmiento, 2006). Namely, an aqueous solution core is surrounded by a hydrophobic membrane, in

the form of a lipid bilayer (hydrophobic tails line up against one another, forming a membrane of hydrophilic heads on both sides facing the water).

In different dispersion systems, such as liposome and O/W emulsions, protein could play an antioxidant role to protect lipids from oxidation (Hu, McClements, & Decker, 2003; Lethuaut, Metro, & Genot, 2002; Ries, Ye, Haisman, & Singh, 2010; Cheng, Chen, & Xiong, 2014). In order to investigate the comparative time-courses of protein and lipid oxidation in different bi-phasic systems, this study analyzed the oxidative indicators in systems that have different ratios of MFP to lipids (oil/lecithin/free fatty acids). A better understanding of the relative susceptibility of protein and lipid oxidation may help with food product formulation, processing, and storage quality controls.

4.3. Materials and methods

4.3.1. Preparation of MFP

Longissimus muscle was collected from three pork carcasses (48 h-post-mortem). Samples were prepared by cutting into 1 cm thick pieces. Individual samples were vacuum-packaged and stored in a freezer (−30 °C) until use (less than 6 months). Frozen samples were tempered at 4 °C for 4 h and then used to extract MFP by the isolation buffer (pH 7.0) of 100 mM sodium chloride (NaCl), 10 mM sodium phosphate, 2 mM $MgCl_2$, and 1 mM EGTA (Park, Xiong, & Alderton, 2006). The pH of MFP suspension in 0.1 M NaCl was adjusted to 6.25 at the last wash. The suspension was then centrifuged at 2,000*g*, 15 min to get MFP pellet. The MFP pellet was kept on ice and used within two days.

4.3.2. Preparation of stripped soybean oil

Commercially refined soybean oil was bought from a local store and stripped by alumina (MP Alumina N-Super I, MP Biomedicals, France) to remove tocopherols (Berton, Genot, & Ropers, 2011b). Briefly, approximately 15 g sorbent and 30 ml oil were vigorously mixed in a 50 ml-polypropylene centrifuge tube, and then shook in the dark at 4 °C for 24 h. The tubes were then centrifuged (2,000*g*, 20 min) at 20 °C to separate the sorbent from stripped oil. Collected upper phase was centrifuged again under the same conditions and finally transferred into amber glass vials after being placed under nitrogen flow for 5 min. Vials were hermetically sealed and stored at –20 °C. HPLC analysis confirmed the tocopherol content of the stripped oil was approximately 1 mg/100 g (α-tocopherol 0.45 mg/100 g, γ-tocopherol 0.62 mg/100 g; δ-tocopherol non-detected).

4.3.3. Preparation of bi-phasic systems

O/W emulsions were prepared from the mixture of 10% (v/v) stripped soybean oil and 90% (w/v) MFP in 25 mM phosphate buffer (PBS) at pH 6.25 containing 0.6 M NaCl. The mixture was pre-chilled and then homogenized (17,500 rpm, 2 min) using a Polytron blender (Brinkmann Instruments, Inc., Westbury, NY, USA) with a low-foaming probe (PTA-20TS). The pre-homogenized crude emulsion was then finely emulsified with a laboratory scale jet homogenizer (NanoDeBee, B.E.E. International Inc., Easton, MA, USA) at a pressure of 35 MPa for 1 pass. During emulsification, the high-pressure pipeline was pre-chilled with cold water and covered with ice to ensure the temperature of outlet emulsion was under 15 °C. Three MFP concentrations (0.1, 0.8, and 2%, w/v)

were used to allow the determination of protein concentration effect.

The liposome systems were prepared as described by Huang and Frankel (1997) with modifications. Lecithin (L-α-Phosphatidylcholine, from soybean) was hydrated in 0.6 M NaCl, 25 mM PBS (pH 6.25) and sonicated for 30 min using a sonic dismembrator (model 300, Fisher Scientific, Farmingdale, NY, USA) at 4 °C. MFP with a final concentration of 0.1%, 0.8%, or 2% (w/v) were suspended in the fresh liposome solution (0.8 wt% lecithin, final) as liposome with protein system.

Protein with free fatty acid systems were prepared by mixing linoleic acid (0.8 wt% final) with 0.1%, 0.8%, and 2% (w/v) MFP in 25 mM PBS containing 0.6 M NaCl (pH 6.25), respectively. The mixtures were kept in cold (4 °C) under continuous stirring.

4.3.4. Oxidation treatment

Three systems were oxidized for 0, 2, and 24 h at 4 °C with hydroxyl radicals produced by a Fe^{2+}-recycling solution comprised of 10 μM $FeCl_3$ / 100 μM ascorbic acid / 5 mM H_2O_2 (Levine, Oliver, Fulks, & Stadtman, 1981; Martinaud, Mercier, Marinova, Tassy, Gatellier, & Renerre, 1997; Park, Xiong, & Alderton, 2006). Propyl gallate, EDTA, and Trolox (1 mM each) were used to terminate the oxidative reaction (Park, Xiong, & Alderton, 2006).

4.3.5. Morphology

Confocal laser scanning microscopy (CLSM) was applied to visualize the microstructural changes in emulsions, liposomes, and free fatty acid systems over oxidation. The oil phase was stained with Nile Red dye (0.1%, w/v), and the protein was

stained with fluorescein isothiocyanate dye (0.1%, w/v). The dye-treated samples were kept in the dark for 20 min and then photographed with an Olympus FV1000 microscope (Olympus America) with 488 nm and 543 nm lasers, respectively (Xu, Lin, & Nagy, 2014). Two sets of images were merged using Olympus FLUOVIEW 1.5 software.

4.3.6. Measurement of lipid oxidation

Lipid oxidation in different dispersions was monitored by the formation of 2-thiobarbituric acid-reactive substances (TBARS) (Sinnhuber & Yu, 1977). Varying samples were mixed with trichloroacetic acid (TCA) and thiobarbituric acid (TBA) followed by boiling for 30 min. The supernatant of the cooled solution was mixed with chloroform. After centrifugation at 2,000*g* for 10 min, the absorbance (532 nm) of the upper phase was recorded and the value converted to TBARS content using the molar extinction coefficient of 152,000 $M^{-1}cm^{-1}$ (Witte, Krause, & Bailey, 1970).

4.3.7. Determination of protein modification

Evaluation of protein modification in different dispersion systems was performed by monitoring the formation of sulfhydryl and carbonyl, tryptophan fluorescence intensity, and the band intensity from sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Free sulfhydryl content in different dispersion systems was measured using the Ellman's reagent [DNTB: 5,5′-dithio-bis (2-nitrobenzoic acid)] colorimetric method (Liu, Xiong, & Chen, 2009). Reagent blank was run to correct for the color. A molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ was used for the sulfhydryl content computation.

The content of protein carbonyl derivatives in samples was estimated by the reaction with 2,4-dinitrophenylhydrazine (DNPH) as detailed by Levine, Berlett, Moskovitz, Mosoni, and Stadtman (1999). In brief, the DNPH-reacted samples were precipitated using TCA (20%) and then washed with ethanol:ethyl acetate (1:1 v/v) three times to remove unbound pigments. The final pellets were then dissolved in 6 M guanidine hydrochloride. Protein concentration was measured at 280 nm and the carbonyl content was calculated using a molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$.

Intrinsic tryptophan fluorescence was followed to observe fluorescence intensity and pattern of samples to detect protein structural changes. Samples (100 μL) were diluted in 5 mL of 25mM PBS (pH 6.25) containing 0.6 M NaCl. After adding 2 mL of petroleum ether, the mixture was vortexed and then centrifuged at 2,000*g* for 5 min. The lower phase was collected for fluorescence testing using a Fluromax-3 fluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with a 283 nm excitation wavelength and a 300–400 nm emission wavelength at a 1 nm/s scanning speed (Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008).

SDS–PAGE was conducted with a 4% polyacrylamide stacking gel and a 10% polyacrylamide separating gel. Samples with 8 mg/mL and 20 mg/mL MFP were diluted to 2 mg/ml before mixing with SDS–PAGE sample buffer (1:1) while samples with 1 mg/mL protein were directly dissolved in the sample buffer (1:1) with or without 10% β–mercaptoethanol (βME). The mixture was then boiled for 3 min. In each well of the gel, 30 μL of prepared sample was loaded.

4.3.8. Statistical analysis

Data were obtained from three independent experiments with different batches of MFP preparation. In each trial, duplicate or triplicate samples were analyzed. And the results were processed using a general linear model's procedure of Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA). Significant ($P < 0.05$) differences between means were identified by LSD all-pair wise multiple comparisons.

4.4. Results and discussion

4.4.1. Microstructure of dispersions

The microstructure of emulsion, liposome, and free fatty acid systems differed and showed morphological changes upon oxidative stress up to 24 h (Figure 4.1). The emulsion system appeared to have a continuous protein network (green) with oil droplets evenly distributed (Figure 4.1a). During homogenization, MFP adhered to the interface of oil droplets and then underwent unfolding and rearrangement to form a stabilizing layer (Das & Kinsella, 1990; Dickinson, 1997). Small fat droplets surrounded by protein membrane dispersed homogeneously in the liquid matrix at 0 h. The microstructure of the emulsion system was relatively stable after 2 h of oxidation while small oil droplets flocculated and coalesced apparently at 24 h.

Liposome is a phospholipid bilayer colloidal system. It encapsulates a region of aqueous solution inside the hydrophobic membrane. The lecithin stained by red and protein dyed by green interweaved with each other at 0 h after incorporating MFP into the liposome solution (Figure 4.1b). This phenomenon was not obvious at 2 and 24 h, indicating the localization of MFP on the liposome might result from electrostatic interaction (Xin, Xiong, Chen, & Zhou, 2013), which no longer played a leading role in

the latter stage of oxidation. Overall, the lipid and protein distributions in liposome systems were relatively stable during oxidation.

Free fatty acid systems have different behavior: fatty acids are neither surrounded by a surfactant, such as a protein membrane in an emulsion, nor form a lipid bilayer as in the case of a liposome, but rather, are freely dispersed in an aquatic system at 0 h when agitated (Figure. 4.1c). Therefore, at the beginning, free fatty acids and MFP, in theory, have an equal probability to react with hydroxyl radicals when constantly agitated. However, the fatty acid-MFP system was not as physically stable as emulsion and liposome when oxidized. In 2 h, protein began to flocculate, and linoleic acid moved with the protein and adhered to the protein hydrophobic sites. In 24 h, as more hydrophobic groups became exposed, severe protein aggregation occurred. Such aggregation complexes entrapped free fatty acids inside thus making MFP a more susceptible target due to its location.

4.4.2 Lipid oxidation

The progression of lipid oxidation in different systems was assessed by monitoring the production of TBARS during oxidation (Figure 4.2). There were no significant changes ($P > 0.05$) in TBARS values within 2 h except for samples with 0.1% MFP that presented an early increase in TBARS (Figure 4.2a, b). The notable accumulation (two-fold) of TBARS appeared mainly from 2 to 24 h in emulsion samples (Figure 4.2a). In liposome systems, the significant accumulation ($P < 0.05$) of these secondary products showed in samples with 0.1% of MFP at 2 h and samples with 0.8% of MFP at 24 h. The TBARS value in liposomes with 2% MFP remained the same ($P >$

0.05) over 24 h of oxidation (Figure 4.2b). With regard to linoleic acid systems, TBARS did not change significantly in the first 2 h (Figure 4.2c) but increased markedly from 2 to 24 h.

The mechanism of lipid oxidation does not change much in the presence of proteins (Hidalgo & Zamora, 2002). Nevertheless, the results from the TBARS assay differed among samples with different protein:lipid ratios. In general, systems with higher concentrations of MFP (0.8% and 2%) prolonged the lag phase, thereby slowing the formation of the secondary product of lipid oxidation (Figure 4.2). Higher protein concentrations facilitated the formation of a more compact protective network around lipid (Tong, Sasaki, McClement, & Decker, 2000).

Emulsion system possessed the lowest TBARS value (average 0.9 μM at 24 h, Figure 4.2a) among the three systems. Compared with liposome and fatty acid systems, the MFP viscoelastic layer formed during high-pressure homogenization in emulsion provided additional physical shielding for lipids and effectively reduced the oxygen permeability. It was difficult for hydroxyl radicals or other water-soluble oxidants to gain access to the oil to initiate and accelerate lipid peroxidation. The TBARS in linoleic acid samples was higher than emulsion samples but lower than liposome samples (Figure 4.2). Free fatty acid system showed a loose structure of proteinaceous network that entrapped most of linoleic acid (Figure 4.1c). Oxidative damage was unavoidable but reduced when it came to large protein scales in the system. In liposome system, the fatty acid profile of soy lecithin (11.4% C18:1, 56.6% C18:2, and 7.1% C18:3) rendered it very susceptible to oxidation (Wang & Wang, 2008). The stabilizing effect of proteins or protein hydrolysates in a liposomal model system was suggested by Viljanen, Kivikari, and

Heinonen (2004a) and Zhang, Xiong, Chen, and Zhou (2013). It is believed protein/protein hydrolysates provided a physical barrier against liposome oxidation. However, the protection was no longer effective when oxidized because of the separation of MFP and lipid showed by Figure 4.1b.

4.4.3 Protein oxidation

The modification of amino acid side-chain groups is the primary consequence of oxidative stress in proteins. The changes of sulfhydryl and carbonyl content in emulsion, liposome, and free fatty acid with MFP systems were monitored during oxidation (Table 4.1). In most samples, the major loss of free sulfhydryl content occurred within the first 2 h, and there was no noteworthy decrease ($P > 0.05$) from 2 to 24 h (Table 4.1). In comparison, their formation of carbonyls increased continuously during 24 h. Samples with a lower protein concentration displayed a rapid modification rate.

Conformational alteration of protein was also detected by the intensity change of tryptophan fluorescence (Figure 4.3). Tryptophan fluorescence intensity reduced during oxidation in all groups. In emulsion, there was a loss of 15–40% in tryptophan fluorescence at the first 2 h and this followed by a slower second phase so that approximately 55–70% fluorescence remained by 24 h (Figure 4.3a). The intensity loss of tryptophan fluorescence was principally due to the break of protein tertiary structure (unfolding). Protein unfolding exposed more hydrophobic sites and hence, protein solubility decreased and hydrophobic interactions actuated causing aggregation of protein molecules (Nakai, Hori, Kagamiyama, Nakazawa, & Nozaki, 1983). The theory corresponded with the captured microstructure images in Figure 4.1. Compared to

emulsion samples (Figure 4.3a), the extent of fluorescence intensity reduction in liposome samples was less significant (Figure 4.3b). This was probably due to the location of protein in solution. MFP in emulsion samples had already borne certain conformational changes when adsorbed to the interface of oil droplets and exhibited more tertiary stuctural changes during oxidation. While protein in the aqueous phase of liposome systems underwent more oxidative changes in its primary structure (Table 4.1) than spatial structure. In comparison, fluorescence intensity of fatty acid with protein samples gradually reduced during oxidation (Figure 4.3c). In particular, there was a large loss in tryptophan fluorescence with high concentration of MFP (2%). This suggested more unfolding and structural change in fatty acid system, which was also depicted in Figure 4.1c.

The bands intensity change in SDS–PAGE indicated the aggregation and cross-linking of MFP (Figure 4.4). In emulsion systems, the intensity of myosin heavy chains (MHC) in all samples remarkably decreased in 2 h, and the bands were barely perceptible at 24 h (Figure 4.4a). When oxidized samples were treated with βME, MHC bands were partially recovered (Figure 4.4a). Therefore, these polymers were formed not only by disulfide bonds but also other covalent bonds, such as Tyrosine–Tyrosine and carbonyl–NH_2 interactions (Li, Xiong, & Chen, 2012); in particular, malonaldehyde that derived from lipid oxidation reacted with protein resulting in irreversible cross-linking. During oxidation, the intensity of actin bands reduced in a similar pattern but was less affected than MHC. The delayed attenuation of myosin and actin bands in emulsion samples with 0.8% and 2% MFP resulted from the concentration effect.

SDS–PAGE results of liposome samples were given by Figure 4.4b. Liposome samples exhibited a gradual disappearance of MHC during 24 h, and these bands were mostly recovered when treated with βME, indicating the formation of disulfide bonds played a predominant role in protein aggregation. In 2 h, the band intensity of MHC significantly reduced; the convertion of sulfhydryl groups to disulfide bonds caused significant myosin cross-linking, in accordance with the sulfhydryl results in Table 4.1. The actin appeared to be more resistant upon oxidation according to the SDS–PAGE results. Comparing the SDS–PAGE results of liposome oxidation systems with the emulsion, the intensity reduction of MHC bands in liposome samples (Figure 4.4b) was less intense than emulsion samples with the same concentrations in 2 h (Figure 4.4a). And the MHC bands in liposome samples have better recovery when subjected to βME. It can be concluded that more sulfhydryl–disulfide bonds conversion was involved in the modification of MFP in liposome system.

The pattern of SDS–PAGE in fatty acid with MFP system (Figure 4.4c) was similar as liposome and emulsion system. In 2 h, the amount of MHC drastically decreased in that high molecular polymers derived from myosin cross-linking and aggregation barely entered the stacking gel. The loss of MHC bands in fatty acid with protein samples (with 0.8% and 2% protein) (Figure 4.4c) was slower than emulsion samples (Figure 4.4a) but faster than liposome samples (Figure 4.5e) in a non-reducing environment when comparing their band intensities at 2 h. The MHC bands in fatty acid with protein samples were largely reversible in reducing environment. Actin in free fatty acid system underwent minor conformational changes compared with myosin.

4.5. Conclusion

Comparing the development of lipid oxidation with specific oxidative markers of protein, significant changes in most of protein oxidation markers occurred prior to the onset of lipid oxidation products in all three bi-phase systems. It indicated MFP was more susceptible to oxidative damage (hydroxyl radicals) than lipids when protein located at the surface of oil droplets, adhered to lipids, or dispersed in aquatic phase. Moreover, the susceptibility of lipid and protein to reactive oxygen species largely depended upon the dispersion of lipid and protein in multiphasic systems. The emulsion systems had the lowest TBARS accumulation (Figure 4.2a) and more rapid protein oxidation within 24 h (Figure 4.3a & Figure 4.4a) due to the unique MFP barrier. In addition, the lipid:protein ratio modulated the extent of reaction. Higher lipid:protein ratio was related to more severe oxidation indicating the role of MFP as an antioxidant.

The study of the three typical bi-phasic systems not only provides information about the oxidative stability of different food lipids (free fatty acid, phospholipid, and triacylglycerol) when coexisting with MFP, but also considers the difference of the physical characteristics (steric role) as making a comparison of the susceptibility of lipids and proteins subjected to ROS. With the understanding of the oxidation preference of lipids and MFPs in these bi-phasic systems, their interactions in a more complicated food system would be predicted. The knowledge may lead to the development of novel antioxidant strategies in that some antioxidants are more effective in preventing lipid from oxidation while others target toward proteins. Choosing appropriate antioxidants could better protect the oil/protein against oxidation and then increase shelf life, as well as allow food manufacturers to include more nutritionally beneficial lipids in their

products.

Table 4.1. Sulfhydryl and carbonyl content in protein-lipid systems during oxidation.

Oxidation systems[1]	Sulfhydryl (nmol/mg protein)			Carbonyl (μmol/g protein)		
	0 h	2 h	24 h	0 h	2 h	24 h
Emulsion						
MFP 0.1%	22.12 ± 1.22^{a}	13.90 ± 0.31^{b}	14.04 ± 0.94^{b}	0.71 ± 0.00^{b}	1.69 ± 0.63^{ab}	2.65 ± 0.22^{a}
MFP 0.8%	28.90 ± 0.94^{a}	19.04 ± 1.43^{b}	18.34 ± 1.92^{b}	1.83 ± 0.31^{c}	4.10 ± 0.18^{b}	5.98 ± 0.78^{a}
MFP 2.0%	33.53 ± 1.36^{a}	26.47 ± 1.67^{b}	16.54 ± 1.18^{c}	2.52 ± 0.13^{c}	3.98 ± 0.11^{b}	4.94 ± 0.02^{a}
Liposome						
MFP 0.1%	71.67 ± 1.27^{a}	33.20 ± 0.36^{b}	26.75 ± 0.35^{c}	1.58 ± 0.23^{c}	3.04 ± 0.09^{b}	4.48 ± 0.07^{a}
MFP 0.8%	44.91 ± 0.18^{a}	29.34 ± 1.27^{b}	26.38 ± 1.64^{b}	2.27 ± 0.22^{b}	3.71 ± 0.19^{a}	3.99 ± 0.10^{a}
MFP 2.0%	39.50 ± 1.73^{a}	25.35 ± 0.18^{b}	23.81 ± 0.55^{b}	2.18 ± 0.03^{b}	3.39 ± 0.18^{a}	3.54 ± 0.10^{a}
Linoleic acid						
MFP 0.1%	26.76 ± 1.01^{a}	6.18 ± 1.09^{b}	6.00 ± 1.09^{b}	2.55 ± 0.14^{c}	4.79 ± 0.37^{b}	8.82 ± 0.38^{a}
MFP 0.8%	26.79 ± 0.82^{a}	6.43 ± 0.36^{b}	5.66 ± 0.36^{b}	4.17 ± 0.67^{c}	5.91 ± 0.22^{b}	7.73 ± 0.32^{a}
MFP 2.0%	26.12 ± 0.55^{a}	23.68 ± 1.44^{ab}	22.32 ± 0.46^{b}	3.61 ± 0.13^{c}	4.53 ± 0.44^{b}	6.29 ± 0.12^{a}

Oxidation systems[1] denoted different compositions of protein-lipid systems. Emulsion: different concentrations of MFP with oil (10%) system; Liposome: different concentrations of MFP with liposome (0.8%) system; Linoleic acid: different concentrations of MFP with linoleic acid system.

[a–c] Means ± standard deviations within the same system (row) between different oxidation times sharing no common lowercase letter differ significantly ($P < 0.05$).

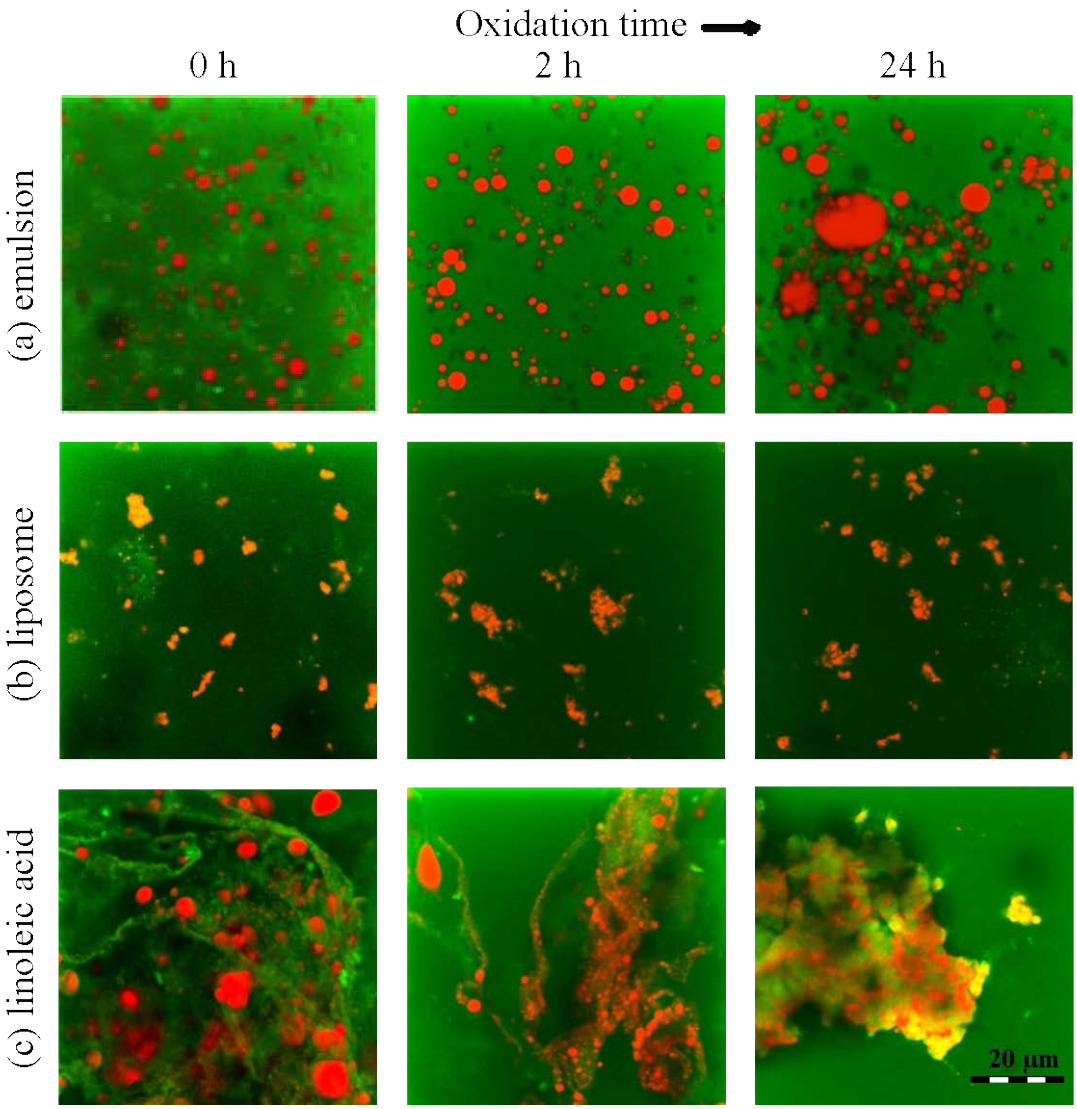


Figure 4.1. Oxidation induced changes in microscopic images of (a) emulsion, (b) liposome, and (c) linoleic acid dispersion containing 2% MFP. (Oxidation was performed with a Fe^{2+}-recycling solution for 0 (control), 2, and 24 h.)

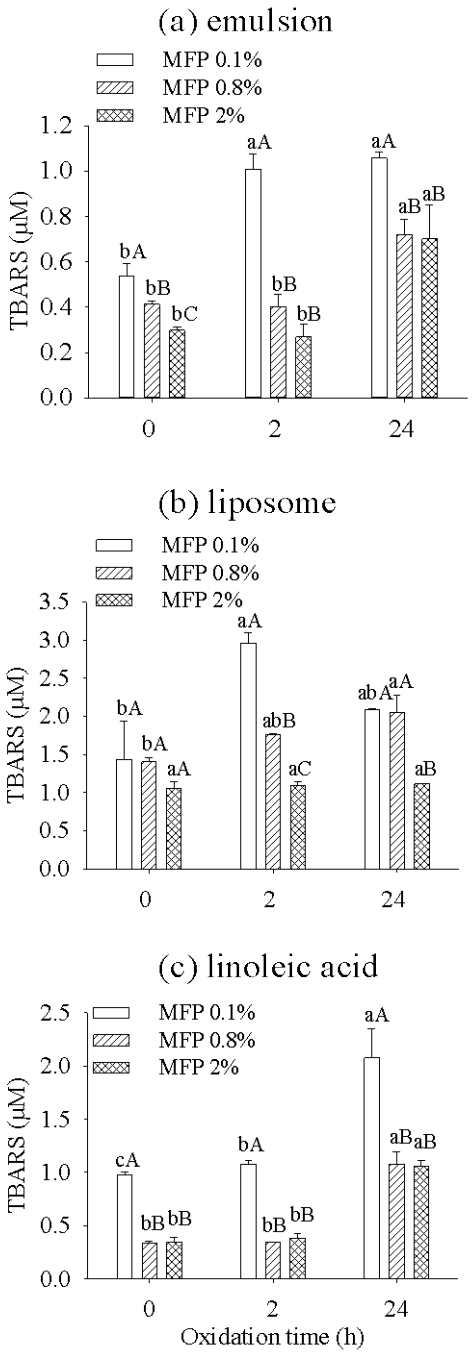


Figure 4.2. Formation of thiobarbituric acid-reactive substances (TBARS) in (a) emulsion (10% oil), (b) liposome (0.8% lecithin), and (c) linoleic acid (0.8%) systems with different concentrations of MFP (0.1, 0.8, and 2%) during oxidation (0, 2, 24 h).

(a–c) Means within the same system between different oxidation times with different lowercase letters differ significantly ($P < 0.05$).
(A–C) Means within the same oxidation time between different systems sharing no common uppercase letter differ significantly ($P < 0.05$).

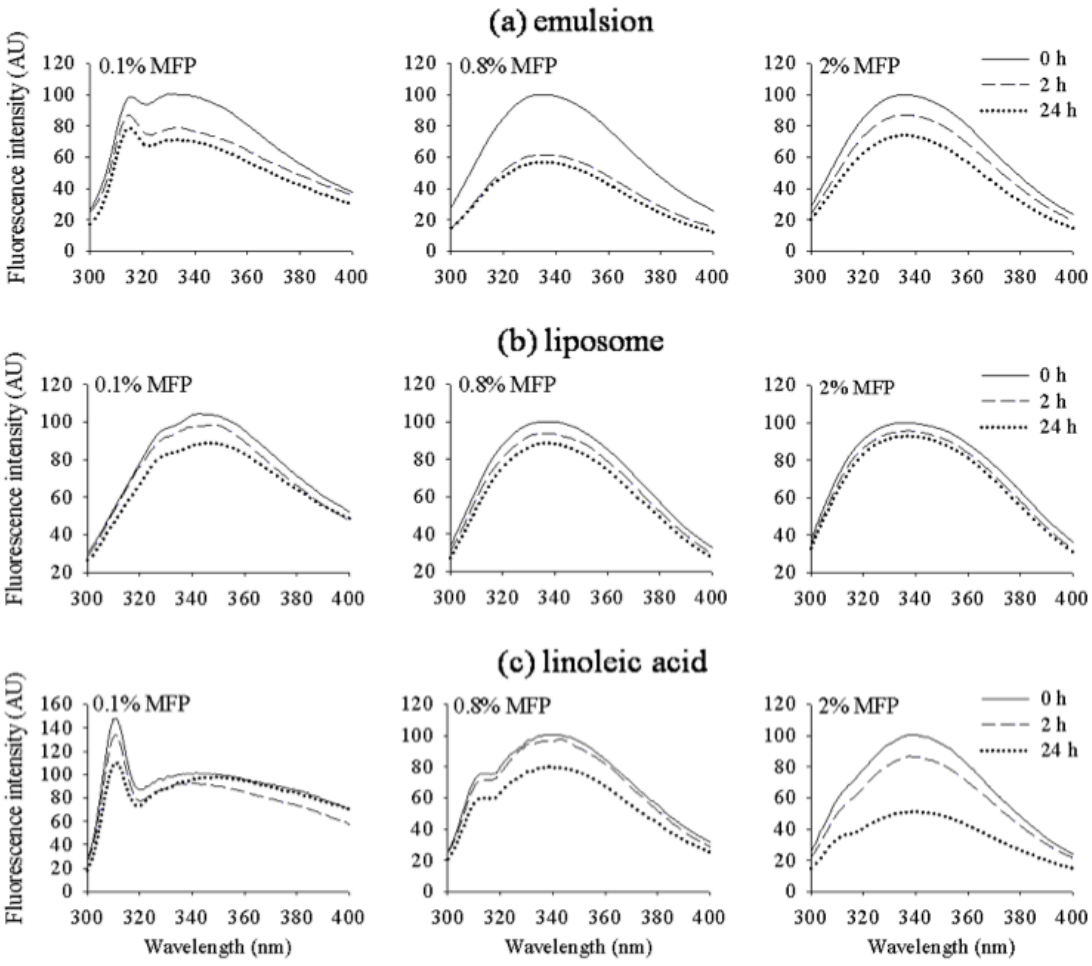


Figure 4.3. Changes in fluorescence spectra in (a) emulsion, (b) liposome, and (c) linoleic acid systems with different concentrations of MFP (0.1, 0.8, and 2%) upon oxidation for 0, 2, and 24 h.

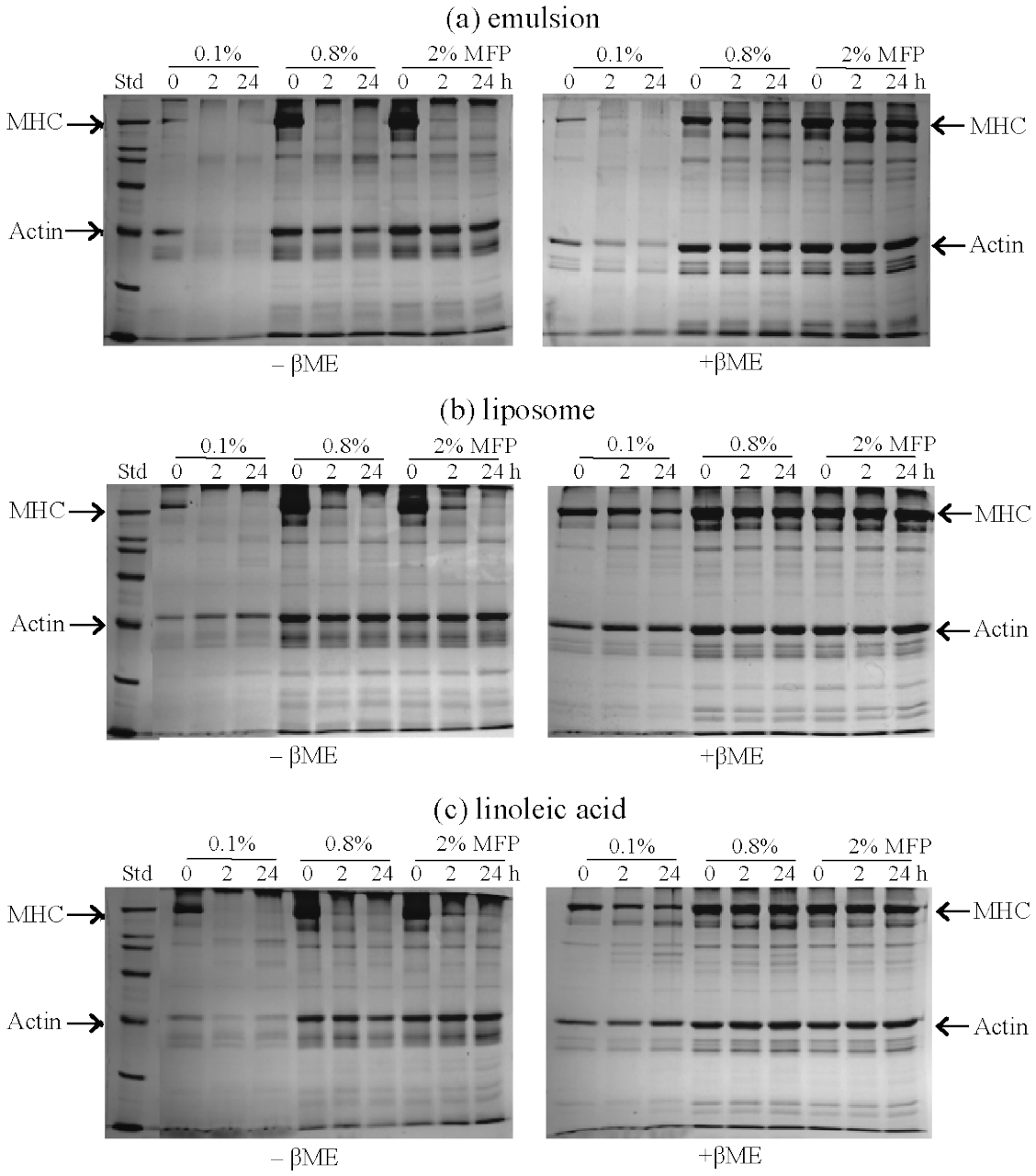


Figure 4.4. SDS−PAGE of (a) emulsion, (b) liposome, and (c) linoleic acid systems with different concentrations of MFP (0.1, 0.8, and 2%) during oxidation. βME, β-mercaptoethanol (a disulfide-bond- breaking agent); MHC, myosin heavy chain.

`CHAPTER 5

INHIBITION OF LIPID OXIDATION IN OIL-IN-WATER EMULSIONS BY INTERFACE-ADSORBED MYOFIBRILLAR PROTEIN: EVIDENCE OF PHYSICAL PROTECTION

5.1. Summary

This study aimed to investigate the steric role of myofibrillar protein (MFP) in the oxidative stabilization of meat emulsions. Emulsions with 10% oil were prepared using either 2% (w/v) Tween 20 or 0.25, 0.5, and 1% (w/v) MFP and then subjected to hydroxyl radical oxidation at 4 °C for 0, 2, and 24 h. MFP was more readily oxidized (intrinsic fluorescence quenching; sulfur losses; carbonyl formation) than oil (conjugated dienes; TBARS). However, oxidized MFP in the continuous phase stimulated lipid oxidation after 24 h, sharply contrasting with interface-adsorbed MFP that inhibited TBARS formation nearly 90% ($P < 0.05$). Interfacial MFP from 2 h-oxidized samples exhibited greater losses of fluorescence and more extensive polymerization of myosin (detected by SDS–PAGE) than MFP present in the continuous phase. Results indicated that interface-adsorbed MFP in general and myosin in particular provided both chemical (radical neutralization) and physical (steric) protection of emulsions against oxidation.

5.2. Introduction

Emulsions are dispersed systems consisting of at least two immiscible phases (continuous and discontinuous) with the discontinuous phase being stabilized by surface-active compounds known as “surfactants” (Walstra & van Vliet, 2008). Emulsifiers vary not only in their ability to disperse oil droplets but also the potential to enhance the oxidative stability of emulsions. In food, small-molecule surfactants and proteins are two most important types of emulsifiers. Polyoxyethylene sorbitanmonolaurate (Tween 20) is a non-ionic emulsifier widely used in the food industry (Eskandani, Hamishehkar, & Dolatabadi, 2013). Tween 20 has also been found to be antimicrobial when used as a spray on meat surfaces (Calicioglu, Kaspar, Buege, & Luchansky, 2002). Generally, it forms a thin film at the interface of oil and water to reduce surface tension.

In protein-stabilized emulsions, the membrane formed around the oil droplets offers resistance to mechanical disruption, and, in some cases, serves as a physical shield to prevent radical attack of the interior lipid (Hu, McClements, & Decker, 2003; Jiang, Zhu, Liu, & Xiong, 2014). Moreover, many amino acid side chain groups, including the imidazole ring (histidine), the indole group (tryptophan), and the sulfur moiety (cysteine and methionine), contribute to oxidative stability via scavenging free radicals or sequestering prooxidative metal ions (Díaz & Decker, 2004; Kaul, Sharma, & Mehta, 2008).

The physicochemical stability of protein emulsions depends on the structure of adsorbed protein layers and whether other surfactants are also present (Dickinson, 1992). The specific structure of the interfacial protein layer(s), thus, the emulsifying properties, is derived from the inherent structural characteristics of the protein. These include the

relatively open and flexible structure in β-casein (Evers, Andersson, Lund, & Skepö, 2012), elongated fibrous configuration in myosin (Galluzzo & Regenstein, 1978), and compact globular shape in β-lactoglobulin (Zhai, Wooster, Hoffmann, Lee, Augustin, & Aguilar, 2011). In a real food system, competition for the interface can come from other surface-active components, especially small molecule surfactants that can readily diffuse toward the interface. Small gaps present in the interfacial layer of a protein-stabilized emulsion allow the penetration of small molecule surfactants, leading to rapid displacement of protein segments from the interface (Damodaran, 2005; Dickinson, 1992).

Comminuted meats are a complex emulsion-type batter system in which animal fat plays a major role in the flavor, juiciness, rheological, and structural properties (Gordon & Barbut, 1992). However, the high proportion of saturated fatty acids and cholesterol existing in animal fat may cause cardiovascular health concerns. Therefore, there is a growing interest in the reformulation of traditional comminuted meat products (frankfurters, fermented sausage, etc.) by partial or total replacement of animal fat with vegetable oils to improve the products' nutritional value (Asuming-Bediako, Jaspal, Hallett, Bayntun, Baker, & Sheard, 2014; Brewer, 2012). Such formulation approaches usually involve the preparation and incorporation of pre-emulsions stabilized by muscle proteins with and without the aid of small surfactants (Wu, Xiong, & Chen, 2011). Furthermore, gelled pre-emulsions prepared with a polysaccharide gum matrix have been used as animal fat replacements (Jiménez-Colmenero, Triki, Herrero, Rodríguez-Salas, & Ruiz-Capillas, 2013).

As with most oil-in-water (O/W) emulsions, oxidation in a finely chopped meat batter occurs at the oil-water interface. Aside from the chemical role, steric hindrances of the protein membrane, though not well investigated, must be a contributing factor to a product's overall oxidative stability. The objective of the present study was to test the above hypothesis. In doing so, model pre-emulsions were created in which vegetable oil droplets were physically stabilized by a MFP coating, then subjected to oxidative stress. The consequential oxidative changes in interface-adsorbed MFP and that dispersed in an aqueous phase, in comparison with the occurrence of lipid oxidation, were assessed.

5.3. Materials and methods

5.3.1. Preparation of MFP

Longissimus muscle (pH 5.6−5.9) was collected from three pork carcasses (48 h postmortem). Individual samples (~90 g each) were vacuum-packaged and kept in a freezer (−30 °C) until use (less than 6 months). Frozen muscle samples were tempered at 4 °C for 5 h prior to MFP extraction using an isolation buffer of 0.1 M sodium chloride (NaCl), 10 mM sodium phosphate, 2 mM $MgCl_2$, and 1 mM EGTA (pH 7.0) (Park, Xiong, & Alderton, 2006). The pH of MFP suspension in 0.1 M NaCl at the last wash was adjusted to 6.25 before centrifugation (2,000*g*, 15 min). The protein concentration of MFP pellet was measured by the Biuret method using bovine serum albumin (BSA) as a standard. The MFP pellet was kept on ice and used within two days.

5.3.2. Preparation of emulsions

Commercial refined soybean oil was purchased from a local grocery; the HPLC analysis confirmed its tocopherol content was slightly greater than 100 mg/100 g (α-tocopherol 18.6 mg/100 g, γ-tocopherol 87.6 mg/100 g; δ-tocopherol 26.3 mg/100 g) agreeing with the reported literature (Carpenter, 1979). Tween 20-stabilized emulsions were prepared from the mixture of 10% (v/v) soybean oil and 90% (v/v) prechilled buffer (25 mM phosphate, pH 6.25, containing 0.6 M NaCl, 4 °C) with 2% (w/v) Tween 20 by homogenization for 2 min with a Polytron blender (Brinkmann Instruments, Inc., Westbury, NY, USA). A low-foaming probe (PTA-20TS) and a speed of 17,500 rpm were used. The pre-homogenized crude emulsion was then finely homogenized with a laboratory scale jet homogenizer (NanoDeBee, B.E.E. International Inc., Easton, MA, USA) at an overall pressure of 35 MPa for 1 pass. During emulsification, the high-pressure pipeline was kept cool with ice pouches so the temperature of outlet emulsion did not exceed 15 °C.

MFP-stabilized emulsions were prepared from the mixture of 10% (v/v) soybean oil and 90% (v/v) MFP in 25 mM phosphate, pH 6.25, containing 0.6 M NaCl. Three MFP concentrations (0.25, 0.5, and 1%, w/v) were used to allow the determination of protein concentration effect. The same two-stage homogenization process as above was utilized for the emulsion preparation.

5.3.3. Oxidation treatment

To oxidize, Tween 20-stabilized and MFP-stabilized emulsions were incubated at 4 °C with hydroxyl radicals produced by an Fe^{2+}-recycling solution comprised of 10 μM $FeCl_3$/100 μM ascorbic acid/5 mM H_2O_2 for 0 h, 2 h, and 24 h (Levine, Oliver, Fulks, &

Stadtman, 1981; Martinaud, Mercier, Marinova, Tassy, Gatellier, & Renerre, 1997; Park, Xiong, & Alderton, 2006). The oxidative reaction was terminated by propyl gallate/Trolox/EDTA (1 mM each) (Park, Xiong, & Alderton, 2006). In addition, MFP at a final concentration of 0.25, 0.5, or 1% (w/v) was added to the Tween 20 emulsion by gently mixing followed by oxidation as described above. Because proteins would not adsorb at the interface in Tween emulsion, it was deemed appropriate to determine the antioxidative efficacy of MFP in the continuous phase as compared to the other emulsion systems.

5.3.4. Measurement of emulsion particle size and morphology

Emulsion samples for droplet size distribution were diluted with 25 mM phosphate buffer (pH 6.25) at a 1:50 emulsion/buffer ratio. Emulsion particle size was analyzed by ZetaSizer Nano-ZS (Malvern Instruments, Worcestershire, UK) at room temperature. The morphology of emulsified oil droplets was observed under a MICROPHOT-FXA Nikon photomicroscope equipped with a built-in digital camera (Nikon Inc., Garden City, NY, USA).

Fluorescence microscopy was applied to visualize the microstructure of emulsions and evidence the formation of protein coating around lipid droplets. The oil phase in the emulsions was stained with Nile Red dye (0.1%, v/v) and protein stained with fluorescein isothiocyanate dye (0.1%, v/v). The dye-treated samples were placed in small cylindrical containers (10 μL) and incubated at room temperature in the dark for 20 min. Fluorescence was produced by excitation with a blue light (488 nm) and emission in the green region (514 nm) using a model Nikon Eclipse E800 fluorescent microscope.

Furthermore, total internal reflection fluorescence (TIRF) was measured to probe dynamic morphological changes in protein-coated emulsion particles. An EMCCD (Andor) camera was mounted on the top of a Nikon Ti-U inverse microscope with a 100× oil type objective. To detect the fluorescence of the fluorescein dye (emission wavelength: 514 nm), a 532 nm excitation filter and a 575 nm emission filter were used. An Olympus ZDC2 auto focus module was applied in order to limit focal drift. All the images were obtained with a 100 ms frame rate.

5.3.5. Recovery of adsorbed and unadsorbed proteins

Control and oxidized Tween 20-stabilized emulsions with added MFP (1%) were centrifuged (35,000*g*, 60 min, 4 °C); the serum phase was collected and the cream phase was discarded. Care was taken to avoid any cream or oil residue in the collected serum samples. First, the separated cream layer was removed using a spatula, then a hole was pricked at the bottom of the plastic centrifuge tube using a needle in order to collect the clear part of serum phase. The upper part of serum phase was discarded to avoid oil residues. When adding MFP to Tween-stabilized emulsion, most proteins stayed in the continuous phase because thermodynamically it was difficult for them to displace the small surfactant (Tween 20).

Similarly, MFP-stabilized emulsions (1%) with or without the oxidative stress were centrifuged (35,000*g*, 60 min, 4 °C) to separate serum phase from cream phase that contained oil and adsorbed protein. The serum phase was carefully collected as described above to obtain unadsorbed MFP. The cream layer was washed two times with deionized water then subjected to the recovery of adsorbed protein using Tween 20 as the desorbing

(displacing) agent (Lee, Lefèvre, Subirade, & Paquin, 2009). To do so, washed cream was dispersed in 25 mM phosphate buffer (pH 6.25, same volume as the removed serum phase) containing 2% (v/v) Tween 20 by stirring at 4 °C for 3 h then centrifuging at 35,000*g* for 60 min at 4 °C. The cream layer was removed with a spatula, and the aqueous solution containing originally adsorbed MFP was collected. The concentration of adsorbed and unadsorbed protein was measured by the Bradford method (Bradford, 1976).

5.3.6. Determination of lipid oxidation

Evaluation of oxidative changes in control and oxidized emulsion samples was performed by monitoring the formation of conjugated diene hydroperoxides (Srinivasan, Xiong, & Decker, 1996) and 2-thiobarbituric acid-reactive substances (TBARS) (Sinnhuber & Yu, 1977). For conjugated dienes, emulsion samples (0.5 mL) were mixed with 5 mL of extracting solvent (hexane:isopropanol, 3:2, v/v) and vortexed for 1 min. After centrifugation at 2,000*g* for 5 min, the absorbance (233 nm) of the supernatant was recorded and the value converted to conjugated dienes using the molar extinction coefficient of 25,200 $M^{-1}cm^{-1}$ (O'Brien, 1969).

For the measurement of TBARS, emulsion samples were 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 treated with chloroform and centrifuged at 2,000*g* for 5 min. The absorbance of the upper phase containing the pinkish TBA-malonaldehyde adducts was measured at 532 nm and the molar extinction coefficient of 152,000 $M^{-1}cm^{-1}$ was used to calculate TBARS content (Witte, Krause, & Bailey, 1970).

5.3.7. Measurement of protein oxidation

Carbonyl. The content of protein carbonyl derivatives in control and oxidative stressed emulsion samples was estimated by using the 2,4-dinitrophenylhydrazine (DNPH) colorimetric method (Levine et al., 1990). Briefly, the DNPH-reacted MFP samples after 20% TCA precipitation were recovered by centrifugation (11,000*g* for 10 min) then washed with ethanol:ethyl acetate (1:1, v/v) solvent to remove unbound pigments. The final MFP pellets were dissolved in guanidine hydrochloride (6.0 M), and the absorbance at 370 nm and 280 nm were recorded for the determination of carbonyl and protein concentration, respectively. A molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$ was used for carbonyl content computation.

Sulfhydryl. Free sulfhydryl content in emulsion samples was determined by reaction with Ellman's reagent [DNTB: 5,5′-dithio-bis (2-nitrobenzoic acid)] as detailed by Beveridge, Toma, and Nakai (1974). Blanks were run with 25 mM phosphate buffer (pH 6.25) containing 0.6 M NaCl. The sulfhydryl concentration was calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$.

Electrophoresis. SDS–PAGE was performed with a 4% polyacrylamide stacking gel and a 10% polyacrylamide separating gel. Adsorbed and unadsorbed (continuous-phase) proteins prepared as described above were dissolved in the SDS–PAGE sample buffer with and without 10% β-mercaptoethanol (βME) to obtain a final protein concentration of 2 mg/mL and then boiled for 3 min. Into each well, 60 μg of prepared sample was loaded.

Circular dichroism. Far-UV (190–260 nm) circular dichroism spectra of collected MFP samples were obtained on a Jasco J-810 spectropolarimeter (Hachioji City, Tokyo, Japan) using a 0.1 cm path length cuvette. The protein samples were concentrated on a centrifugal filter device (Amicon Ultra-15, Billerica, MA, USA) with a molecular weight cutoff of 10 k and then diluted to 0.2 mg/mL with a 0.45 M NaCl, 25 mM phosphate buffer (pH 7.0). After filtering through a 0.22 μm Millipore filter (PVDF) to reduce light scattering, the spectrum was obtained immediately (Greenfield, 2007). The results were expressed as ellipticity, θ (mdeg). The same phosphate buffer without adding MFP was used as the control. The scan rate, response, bandwidth, and step resolution were, respectively, 100 nm/min, 0.25 s, 1.0 nm, and 0.5 nm. Values from three scans were averaged to obtain one spectrum.

Intrinsic tryptophan fluorescence. The procedure described by Estévez, Kylli, Puolanne, Kivikari, and Heinonen (2008) with slight modifications was followed to observe fluorescence pattern and intensity of protein samples. Recovered adsorbed and unadsorbed MFP (100 μL) were diluted in 5 mL of 25 mM phosphate buffer (pH 6.25) containing 0.6 M NaCl, and then subjected to fluorescence testing in a Fluromax-3 fluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA). The excitation and emission wavelengths were 283 nm and 300–400 nm, respectively, and the scanning speed was 1 nm/s.

5.3.8. Statistical analysis

Data were collected from two independent experimental trials (replicates) with different batches of MFP isolation. All analyses were performed on triplicate samples,

and the results were subjected to the analysis of variance using the general linear model's procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA). All-pair wise multiple comparison (LSD) was used to identify significant differences ($P < 0.05$) between means.

5.4. Results and discussion

5.4.1. Structural characteristics and physical stability of emulsions

The light microscopy of freshly made emulsions is shown in Figure 5.1A. In Tween 20-stabilized emulsion (a), nano-level oil droplets exhibited a highly homogenous distribution, while MFP-stabilized emulsions (b, c, d) were comprised of larger (micro-level) particles that were less evenly dispersed, depending on the protein concentration. The emulsion with 1% protein (d) contained the smallest oil droplets ($d_{4,3}$ =706 nm) that appeared better distributed in the continuous phase when compared with the emulsion ($d_{4,3}$ =1220 nm) prepared with 0.25% MFP (b). At lower protein concentrations, for example, 0.25% MFP, most MFP was found to accumulate at the interface as adsorbed protein with a minimal amount being present in continuous phase (result not shown). While a higher protein concentration allowed greater surface loading to achieve a strong steric effect as well as provided excess unadsorbed proteins in the continuous phase to restrict the mobility of the oil droplets (Damodaran, 2005). Other researchers have reported similar phenomenon that decreasing the initial concentration of emulsifier resulted in increased average droplet size and rapid physical destabilization of emulsions (Berton, Ropers, Viau, & Genot, 2011). In contrast, Tween 20 as a more surface-active small surfactant can effectively adsorb to the oil droplet surface under high pressure. This

thin surfactant film surrounding nano-level oil droplets lowered surface tension and provided long-term stability with respect to droplet flocculation, coalescence, and creaming.

To illustrate the steric role of the interfacial membrane in the oxidative stability of emulsions (which will be described later), morphological changes of emulsions prepared with 1% MFP or Tween 20 with added MFP (1%) during oxidation up to 24 h were compared (Figure 5.1B). The Tween emulsions with added MFP appeared to be relatively stable over the entire storage period (upper panel), while droplets present in the MFP emulsions exhibited notable flocculation in 2 h and aggregates in 24 h (lower panel). This morphological shift for the latter emulsion system was a dynamic process characterized by the partial release of protein from the oil-water interface which was initially non-appreciable (2 h) but became remarkable (24 h): the adsorbed MFP was reduced to 2.86 mg/mL (or 72% of total protein) from its fresh state at 4.30 mg/mL (or 89% of total protein). Oxidant-induced structural changes and aggregation of membrane proteins facilitated the release of protein and consequently, destabilization of the emulsion. Although flocculation and partial protein detachment were also observed in non-oxidized MFP emulsions during storage due to gravity against non-covalent forces (Dickinson, 1992), the results are not included due to the irrelevance to oxidation. This phenomenon was not seen in the Tween emulsions even in the presence of MFP added to the continuous phase due to the lack of protein as an interfacial constituent. Despite its less physical stability, the MFP emulsion retained much of the membrane structure which may serve as a barrier against radical attack.

To visualize in greater detail the effect of protein coating on the emulsion droplet morphology and distribution, fluorometry and TIRF microscopy (Figure 5.2) were employed. The fluorescence image of MFP-stabilized emulsions illuminated with two different fluorescent dyes demonstrated the coverage of oil droplets by MFP and the oil droplets' rearrangement during oxidation (Figure 5.2a). In 2 h, the MFP emulsion displayed a broad droplet size distribution with an obviously larger mean diameter when compared with the Tween emulsions due to flocculation and coalescence of small droplets. Interestingly, after 24 h of oxidation, many of the small droplets coated with protein migrated to the surface of the large, coalesced oil droplets forming neo-interfacial clusters (pointed by arrow), resembling a milk fat emulsion stabilized by casein micelles (Xiong, Aguilera, & Kinsella, 1991). In comparison, a different morphological image was observed for emulsions prepared with Tween 20 where not only oil droplets were much smaller but also well-separated, showing no apparent flocculation after 24 h, which was in accordance with light microscopy imaging (Figure 5.1b).

The above microscopic observation was corroborated by the image captured with TIRF (Figure 5.2b). The TIRF technique produces a live microscopic profile of dynamic aggregation of protein in MFP emulsions and reversible Brownian flocculation in Tween emulsions. Isolated fluorescent dots in the TIRF image (Figure 5.2b) were attributed to the emission of protein dye in the emulsion allowing the differentiation of morphological variations between MFP emulsions and Tween emulsions with added MFP under oxidative stress. In the MFP emulsions, protein flocculation occurred within 2 h, followed by partial coalescence in 24 h. The Tween emulsion samples with added MFP also showed a few fluorescent dots, and their presence exhibited no remarkable change.

These were protein aggregates rather than oil droplets because of the weaker surface activity of proteins relative to Tween 20 (Dimitrova & Leal-Calderon, 1999), therefore, less likely for the continuous phase MFP to displace the adsorbed Tween at the interface.

5.4.2. Lipid oxidation

The time course of oxidative reaction was monitored by the measurement of primary (conjugated dienes) and secondary (TBARS) products of lipid oxidation. Conjugated dienes, which mirror the production of hydroperoxides, are unstable, especially in an intense oxidative environment. Their content typically increases for a short time then decreases due to spontaneous degradation into secondary products (Frankel, 2005). In Figure 5.3a, conjugated dienes decreased within 24 h oxidation in all emulsion samples except for 1% MFP-stabilized emulsion. The relatively high protein concentration in the 1% MFP emulsion conspicuously prolonged the lag phase, thereby slowing down the production of hydroperoxide conjugated dienes, hence, the increasing pattern within 24 h. The protein concentration effect was also observed by Kiokias, Dimakou, Tsaprouni, and Oreopoulou (2006) who reported that increasing whey protein concentration led to a decrease in emulsion droplet size but an increase in lipid oxidative stability. At an equal protein concentration, conjugated diene content in MFP-stabilized emulsions was approximately a half of Tween 20-stabilized emulsions with added MFP (Figure 5.3a). This was strong evidence that MFP, when acting as an emulsifier surrounding the oil droplets, as opposed to being in the aqueous phase, could effectively delay lipid oxidation. It should be noted that initial emulsion

samples (0 h) had a relatively high conjugated diene value, which appeared to be a product of the emulsifying process.

The TBARS results are depicted in Figure 5.3b. The Tween emulsions with or without added MFP (0.25, 0.5, or 1%) both showed remarkably higher ($P < 0.05$) TBARS values, including time 0, when compared with MFP-generated emulsions. Therefore, membrane protein acted as an effective mechanism for the inhibition of lipid oxidation during the homogenization process as well as during prolonged oxidative stress. The result was consistent with the conjugated diene inhibition (Figure 5.3a). For each emulsion sample, oxidation up to 2 h caused no significant increase in TBARS ($P > 0.05$). After 24 h of oxidation, the TBARS content rose significantly in Tween emulsions compared to MFP emulsions that had minimal changes.

The remarkable oxidative protection by interfacial MFP can be attributed to multiple mechanisms. First, MFP surrounding the lipid droplets can prevent direct contact between radicals and the enclosed lipid, which has been demonstrated in other protein-stabilized O/W emulsions (Hu, McClements, & Decker, 2003; Jiang, Zhu, Liu, & Xiong, 2014). Furthermore, induced cross-linking of the membrane proteins from radical attack would contribute to a stronger coverage on lipid droplets which, in turn, would reinforce the physical shielding effect. On the other hand, due to the adsorption to the oil droplets, the secondary and tertiary structures of a protein will unfold (Damodaran, 2005), exposing the susceptible amino acid side chain groups that can more readily scavenge radicals and donate electrons than unsaturated fatty acids. The reaction rate constant of many proteins, such as albumin and collagen, with hydroxyl radical has been reported to be a magnitude higher than that of linoleic acid (Buxton, Greenstock, Helman, & Ross,

1988). The influence of oil droplet size and interfacial area (larger for MFP-emulsions than for Tween-emulsions) on lipid oxidation would be negligible based on recent evidence (Dimakou, Kiokias, Tsaprouni, & Oreopoulou, 2007, Osborn & Akoh, 2004).

While membrane proteins consistently demonstrated an antioxidative effect, the proteins present in the continuous phase exhibited a rather complex behavior. Comparison between Tween emulsions with and without 0.25% added MFP indicated considerable reductions of TBARS at 0 and 2 h when MFP was present in the aqueous phase (Figure 5.3b). This was probably due to the radical-stabilizing effect of free (unadsorbed) MFP, for example, myosin (Lund, Luxford, Skibsted, & Davies, 2008). As a favorite target of the hydroxyl radical, MFP had a consequential "sparing" effect to protect lipids. This hypothesis is consistent with the extent of protein oxidation (described below) because, at the equal 10% oil, the net amount (μmol) of carbonyl derivatives produced increased ($P < 0.05$) with the concentration of MFP, i.e., the higher the MFP concentration the more oxidation (carbonyl net amount) (Table 5.1). The accumulation of protein oxidation products during oxidation (notably during the initial 2 h), which was moderately significant for Tween emulsion samples with 0.25% added MFP and strongly significant in samples with higher levels of MFP addition (Table 5.1), appeared to stimulate lipid oxidation (TBARS formation).

5.4.3. Protein oxidation

To confirm the role of MFP as a sacrificing antioxidant to protect protein-stabilized emulsions and delineate the specific actions of interfacial and continuous-phase MFP, oxidative changes at the primary, secondary and tertiary levels of the emulsions

and separated proteins are presented below. The results obtained when compared with lipid oxidation were applied to explain in detail the sequential oxidative change of the two main emulsion constituents.

5.4.3.1. Sulfhydryl and carbonyl derivatives

Modification of amino acid residue side chain groups is the primary consequence of oxidative attack of proteins. The loss of sulfhydryls and formation of carbonyls are two common measures of protein changes in oxidized muscle foods (Díaz & Decker, 2004; Li, Xiong, & Chen 2012). As presented in Table 5.1, the sulfhydryl content decreased while the carbonyls increased continuously during oxidation ($P < 0.05$). For the thiol groups, almost all the loss occurred within the first 2 h, preceding the onset of lipid oxidation which took place mostly between 2 and 24 h (Figure 5.3). However, the extent of sulfhydryl reduction was quite different between the two types of emulsions, i.e., 21.1–49.0% for Tween emulsions with added MFP compared to 60.1–71.0% for MFP-generated emulsions, irrespective of protein concentration. Thus, membrane-forming MFP was more prone to hydroxyl radicals than continuous-phase MFP.

Table 5.1 also summarizes the protein carbonyl content in different emulsions. It is noteworthy that freshly prepared emulsions already had measurable amounts of carbonyls produced during the MFP isolation and emulsion preparation. Oxidative stress resulted in a gradually rise of the carbonyl content in MFP in both emulsion systems. For Tween emulsions, the content of carbonyls on a protein basis (μmol/g protein) generally was not affected by the amount of MFP added ($P > 0.05$), indicating that proteins in the continuous phase were equally susceptible to radicals. However, when calculated based on the volume, the net amount of carbonyl derivatives in the emulsion (μmol per 100 mL)

increased with the level of MFP addition. The latter value suggests that the extra oxidized proteins may serve as initiators for lipid oxidation as previously described.

In comparison, the MFP emulsions had a more rapid carbonyl formation during oxidation with the net increase in 24 h ranging from 3.68 to 6.09 μmol/g protein versus about 1.5 μmol/g protein for Tween emulsions ($P < 0.05$) (Table 5.1). This result again substantiates the premise that interfacial protein in MFP-stabilized emulsions played a sacrificing role as antioxidant to protect lipids. Interestingly, the pattern of sulfhydryl reduction (completed in 2 h) was not in parallel with the increase in carbonyls (progressive throughout 24 h), suggesting an independent nature of the two oxidative processes. During oxidation, free thiol groups can be readily converted to disulfide bonds while the formation of carbonyl derivatives appeared to be progressive, which may involve lysine, arginine, proline, and threonine side chain groups (Stadtman, 2001). In strong agreement, results from the SDS–PAGE showed disulfide-dependent polymerization of myosin since the polymers were dissociated when oxidized samples were treated with β-mercaptoethanol, a reducing agent (Figure 5.4). Moreover, myosin heavy chain in adsorbed MFP completely vanished within 2 h of oxidation (Figure 5.4a; see arrow), suggesting more extensive polymerization compared with the MFP in the continuous phase (Figure 5.4b). The observation further confirms the high reactivity of membrane proteins relative to their counterpart in the bulk phase.

5.4.3.2. Secondary and tertiary structures

The characteristic circular dichroism spectra of MFP in the far UV region are depicted in Figure 5.5a and Figure 5.5b. The adsorbed protein recovered from MFP-stabilized emulsions exhibited a negative band near 208 nm and 222 nm and a strong

peak at 200 nm (Figure 5.5a), resembling a typical α-helix curve. While the pattern of the circular dichroism spectrum changed very little, the ellipticity attenuated with the oxidation time, indicating destabilization of the secondary structures. Yet, the results suggest that even after oxidation, α-helix was still a major structural component in adsorbed MFP which can be largely ascribed to myosin helical rod. In contrast, oxidation significantly disrupted the secondary structure of proteins present in the continuous phase of Tween emulsions with added MFP. The conformational changes were evidenced by the reduced ellipticity, the shift of the wavelength, and roughness of the circular dichroism curves (Figure 5.5b).

Intrinsic tryptophan fluorescence was used to assess oxidative changes in the tertiary structure of adsorbed and unadsorbed MFP, and the results are shown in Figure 5.5c and Figure 5.5d. Desorbed MFP from 2 h-oxidized emulsion samples exhibited greater losses of fluorescence than continuous-phase MFP, confirming that interface-adsorbed proteins were more oxidizable than unadsorbed protein. This finding was in accordance with Rampon, Lethuaut, Mouhous-Riou, and Genot (2001) who studied BSA-stabilized emulsion and demonstrated that BSA located at the oil droplet surface was more readily modified than unadsorbed BSA in the aqueous phase.

5.5. Conclusion

In O/W emulsions, MFP present either at the interface or in the continuous aqueous solution, notably myosin, proves to be a kinetically preferred target of free radicals when compared with lipids. This is due to the high reactivity of protein thiol groups. The physical location of the proteins within the complex emulsion systems

dictates their antioxidative efficacy: interfacial MFP compared with continuous-phase MFP imparts strong protection, giving rise to a remarkable oxidative stability of emulsified lipids. Hence, capable of building a cohesive interfacial membrane, MFP can be used to prepare oil-based pre-emulsions suitable for functional sausage products. Further research is needed to explore other kinds of protein sources for the construction of antioxidative membranes in formulated meat emulsions or batter products.

Table 5.1. Sulfhydryl and carbonyl content in two different emulsion systems as affected by oxidation (0, 2, and 24 h).

	Sulfhydryl (nmol/mg protein)			Carbonyl (μmol/g protein)		
Emulsion	0 h	2 h	24 h	0 h	2 h	24 h
Tween + MFP						
0.25% protein	85.0 ± 1.3^{aA}	67.1 ± 1.6^{bA}	66.6 ± 3.4^{bA}	3.01 ± 0.91^{aA}	4.24 ± 0.50^{aAB}	4.78 ± 0.08^{aCD}
	(21.3)*	(16.8)	(16.7)	(0.75)	(1.06)	(1.20)
0.5% protein	81.2 ± 0.1^{aC}	46.3 ± 0.3^{bB}	46.0 ± 4.0^{bC}	2.66 ± 0.15^{bAB}	2.90 ± 0.07^{bC}	4.03 ± 0.17^{aD}
	(40.6)	(23.2)	(23)	(1.33)	(1.45)	(2.02)
1% protein	83.0 ± 2.5^{aB}	42.4 ± 1.9^{bB}	42.1 ± 2.2^{bB}	2.76 ± 0.06^{bAB}	3.26 ± 0.11^{aBC}	4.51 ± 0.99^{aCD}
	(83.0)	(42.4)	(42.1)	(2.76)	(3.26)	(4.51)
MFP						
0.25% protein	76.1 ± 0.3^{aCD}	22.1 ± 3.1^{bD}	21.0 ± 2.8^{bE}	2.32 ± 0.29^{cABC}	4.46 ± 0.78^{bA}	8.41 ± 0.10^{aA}
	(19.0)	(5.5)	(5.3)	(0.58)	(1.12)	(2.10)
0.5% protein	74.0 ± 7.0^{aE}	24.3 ± 0.9^{bE}	17.0 ± 4.4^{bDE}	1.61 ± 0.02^{cC}	3.15 ± 0.45^{bC}	6.10 ± 0.12^{aB}
	(37.0)	(12.2)	(8.5)	(0.81)	(1.58)	(3.05)
1% protein	77.9 ± 3.4^{aC}	31.1 ± 0.3^{bC}	27.4 ± 4.4^{bCD}	1.62 ± 0.07^{cC}	2.71 ± 0.27^{bC}	5.30 ± 0.36^{aBC}
	(77.9)	(31.1)	(27.4)	(1.62)	(2.71)	(5.30)

$^{a–c}$ Means ± standard deviations within the same emulsion (row) between different oxidation times without a common lowercase letter differ significantly ($P < 0.05$).

$^{A–E}$ Means ± standard deviations within the same oxidation time (column) between different emulsions without a common uppercase letter differ significantly ($P < 0.05$).

*Values in parentheses denote net sulfhydryl (nmol) and carbonyl (μmol) contents on an equal volume basis (100 mL emulsion).

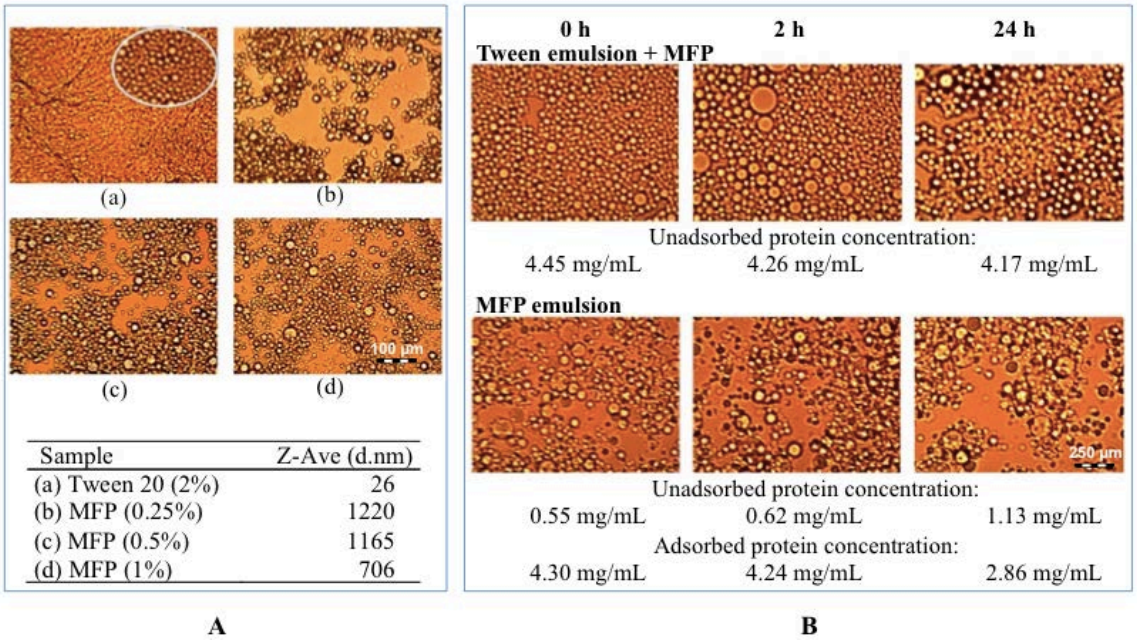


Figure 5.1. Microscope images of freshly prepared emulsions (A): Tween emulsion (a), 0.25% MFP emulsion (b), 0.5% MFP emulsion (c), and 1% MFP emulsion (d). The particle sizes (inset table) and a portion of magnified Tween emulsion (circle) are shown. Microscope images and protein distributions of emulsions during oxidation (B): Tween emulsions with 1% added MFP (upper panel), and 1% MFP emulsions (lower panel). Note the bar scale of 100 µm in A and 250 µm in B.

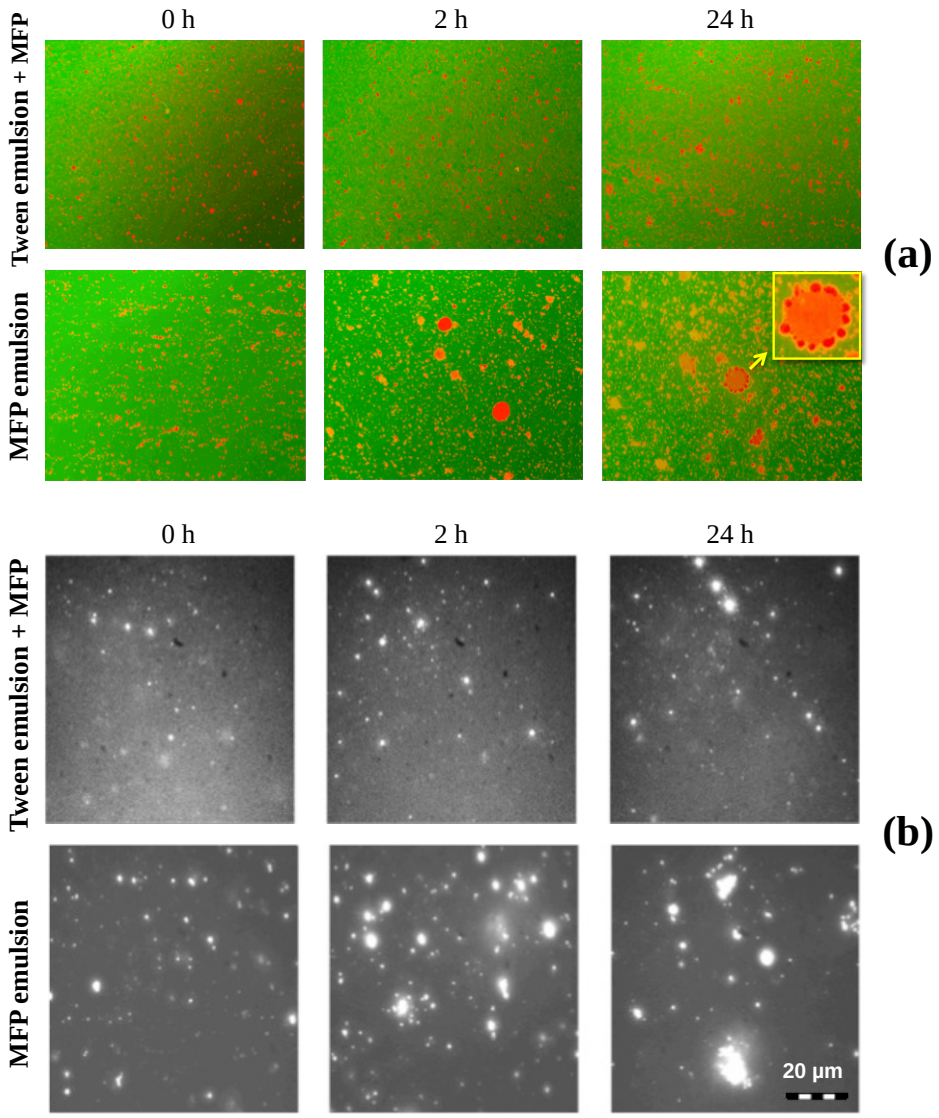


Figure 5.2. Fluorescence microscopy (a) and TIRF (b) images of Tween-stabilized emulsion with 1% added MFP (upper panel) and those of 1% MFP-stabilized emulsion (lower panel) with different oxidation times. In "a", a large particle surrounded by small droplets in 24-h oxidized MFP emulsion is enlarged for visibility.

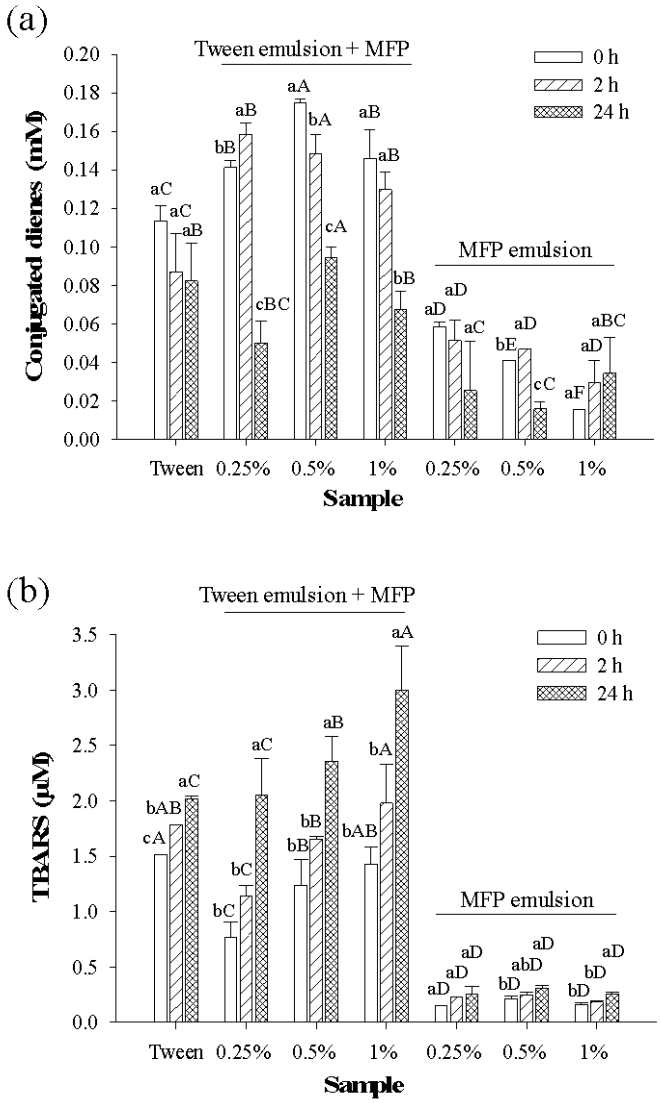


Figure 5.3. Formation of conjugated dienes (a) and thiobarbituric acid-reactive substances (TBARS) (b) in emulsions during oxidation.

(a–c) Means within the same emulsion between different oxidation times with different lowercase letters differ significantly ($P < 0.05$).
(A–E) Means within the same oxidation time between different emulsions with different uppercase letters differ significantly ($P < 0.05$).

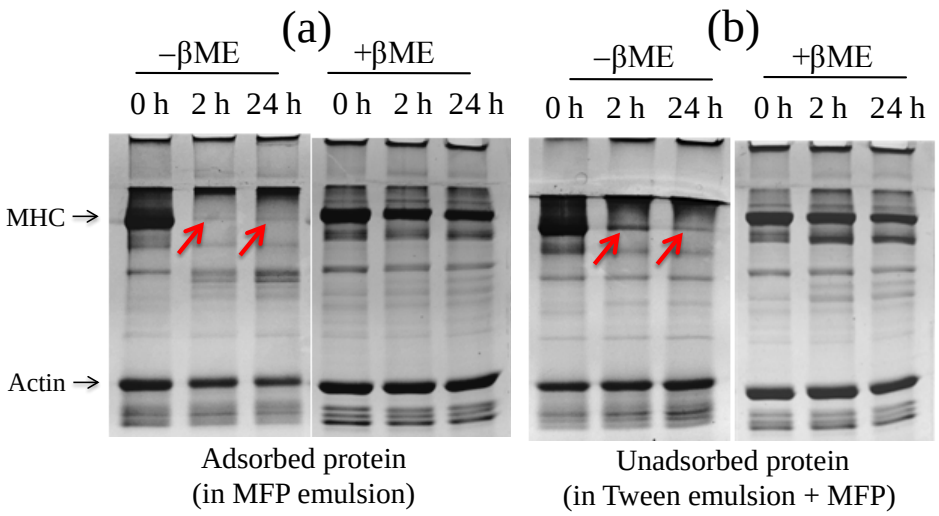


Figure 5.4. SDS–PAGE of adsorbed protein in MFP emulsion (a) and unadsorbed protein in Tween emulsion with added MFP (b) during oxidation. βME: β-mercaptoethanol (a disulfide bond breaking agent). MHC: myosin heavy chain.

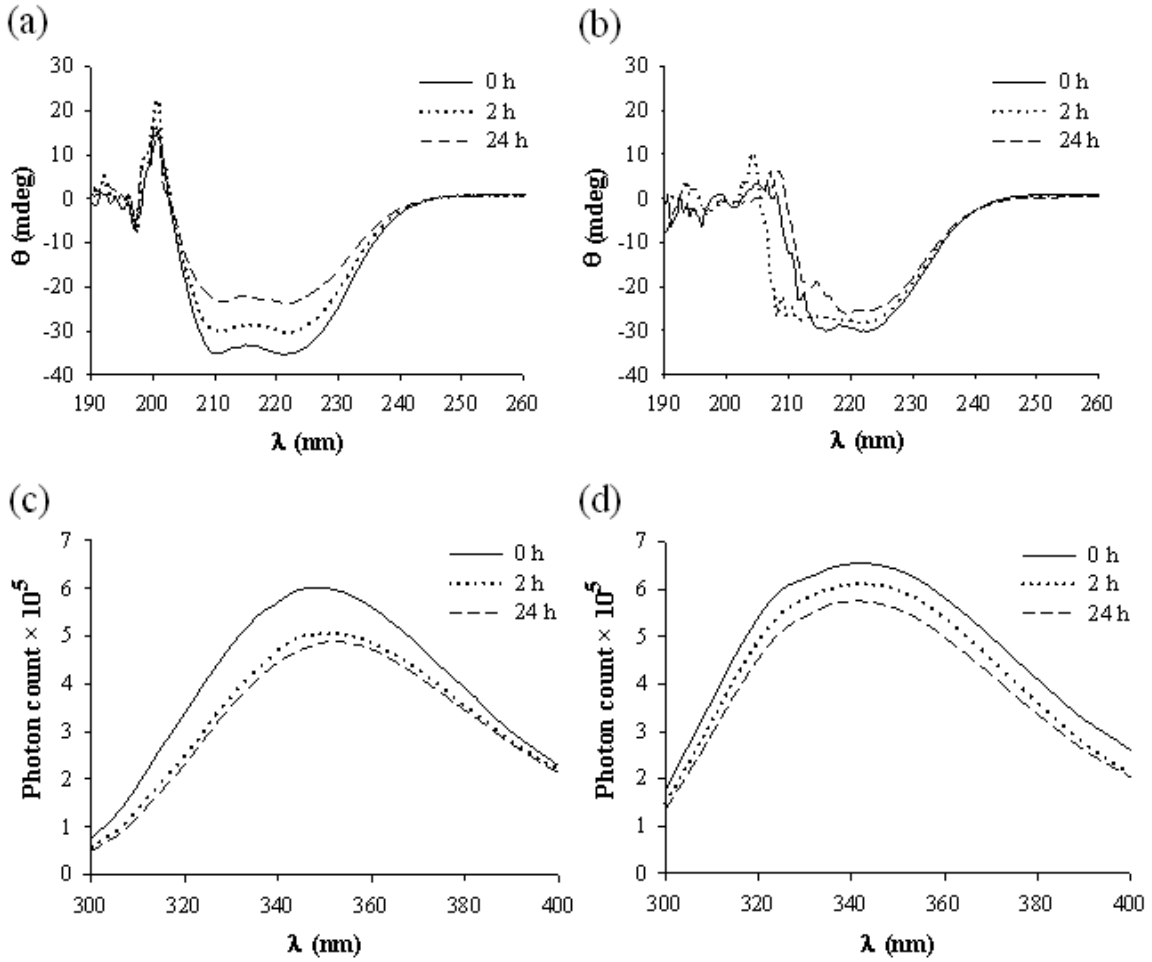


Figure 5.5. Circular dichroism spectra of adsorbed protein in MFP emulsion (a) and unadsorbed protein in Tween emulsion with added MFP (b) during oxidation; fluorescence of adsorbed protein in MFP emulsion (c) and unadsorbed protein in Tween emulsion with added MFP (d) during oxidation.

CHAPTER 6

CONSTRUCTION OF ACTIVE PROTEIN MEMBRANES FOR THE FABRICATION OF FUNCTIONAL OIL-IN-WATER FOOD EMULSIONS

6.1. Summary

Native soy protein isolate (SPI), denatured SPI, and sodium caseinate (SC) were used individually to construct interfacial membranes of oil-in-water (O/W) emulsions. Emulsions with 10% soybean oil were prepared using either 0.25, 0.5, and 1% (w/v) proteins or 2% Tween 20 as emulsifiers, then subjected to hydroxyl radical oxidation at 4 °C for 0, 2, and 24 h. A direct association between reduced TBARS production and interfacial protein membrane formation was observed, suggesting that interfacial proteins acted as a physical barrier to free radicals in addition to scavenging radicals. Different degrees of protein oxidation were evaluated by the content sulfhydryl, carbonyl, and fluorescence intensity. In 2 h, interfacial proteins were oxidized rapidly (detected by carbonyl formation and fluorescence intensity), and with the progression of lipid peroxidation, both interfacial and continuous-phase proteins were oxidized in 24 h.

6.2. Introduction

Soy protein isolate (SPI) and sodium caseinate (SC) are important food ingredients used in a wide variety of formulated food such as milk products and comminuted and restructured meats for their moisture, fat-binding, thickening, emulsifying, and emulsion stabilizing properties. Functional as emulsifiers, they could decrease the interfacial tension in O/W emulsion systems and prevent coalescence of lipid droplets through the formation of a physical barrier at the interface (Jiang & Xiong, 2015). Surface and emulsifying properties of proteins are strongly correlated with their structures. For example, native SPI is of limited functionality, therefore, many attempts have been made to alter its native structure to maximize the emulsifying capacity. Partial unfolding before emulsification has been concluded as a useful way and it could be achieved by heating (Feng, Xiong, & Mikel, 2003), high-pressure treatment (Molina, Papadopoulou, & Ledward, 2001; Wang, Tang, Li, Yang, Li, & Ma, 2008), pH shifting (Jiang, Chen, & Xiong, 2009), isoelectric precipitation (Chove, Grandison, & Lewis, 2001), and enzymatic treatments. Moderate oxidization could generate soluble protein aggregates with more flexible structure and resulted in smaller droplet size and better emulsion stability (Chen, Zhao, Sun, Ren, & Cui, 2013).

Among food proteins, the soluble caseins are of particular importance as an emulsifier because of their ability for rapidly conferring a low interfacial tension during emulsification (Dickinson & Golding, 1997). Sodium caseinate is composed of a family of related phosphoproteins, αS1, αS2, β, κ, all relatively small molecules of about 20,000 – 24,000 Daltons (Creamer & Berry, 1975). All of the individual caseins, except perhaps κ-casein, show a strong tendency to adsorb to oil/water interface to provide long-term

emulsion stability. At neutral pH, caseinates are charged and conformationally flexible, which attributes to their high proline content. The charge prevents close approach of the emulsion droplets, and the flexibility allows it to adopt an extended conformation after it is adsorbed.

As SPI and SC are widely used in many lipid-enriched food systems, the interaction of lipid and protein oxidation has aroused scientific attention. Lipid is sensitive to oxidation and will produce lipid peroxidation-derived free radicals as well as lipid hydroperoxides and reactive aldehydes which will interact with protein, leading to protein oxidation (Refsgaard, Tsai, & Stadtman, 2000). Proteins, similar to lipids, are vulnerable to reactive oxygen species (ROS) during processing and storage. Protein oxidation is the structural modification induced directly by reactive oxygen species or indirectly by reaction with the by-products of lipid peroxidation (Shacter, 2000). Oxidative modification can trigger a number of changes in amino acid residue side-chains and protein polypeptide backbone, resulting in protein fragmentation, cross-linking, unfolding, and conformational changes (Davies, 2005; Stadtman & Berlett, 1998). Radical oxidant reacts with most kinds of amino acid side chains while aromatic and sulfur-containing amino acid side chains are particularly sensitive to oxidation (Davies, 2005).

Besides the emulsification capacity of SPI and SC, their antioxidant abilities were further investigated. It was suggested that SPI possesses appreciable antioxidant activity in lipid-aqueous systems (Pratt & Birac, 1979). Such antioxidant property may derive from its polyphenolic compounds including isoflavones, chlorogenic acid isomers, caffeic acid, and ferulic acid (Pratt & Birac, 1979). SC is also able to inhibit oxidative

reactions by chelating iron and scavenging free radicals (Díaz, Dunn, McClements, & Decker, 2003), thus making it potential antioxidant in food systems. Caseins and tryptic digests of caseins showed inhibitory properties in the oxidation of linoleate by lipoxygenase, peroxyl radicals, and hemoglobin (Rival, Fornaroli, Boeriu, & Wichers, 2001). Casein hydrolysates had the ability to scavenge the ABTS radical cation (Chin & Kitts, 2004). SC films could considerable retard lipid oxidation in cooked turkey breast meat slices, as demonstrated by TBARS and hexanal analyses (Caprioli, O'Sullivan, & Monahan, 2009). The antioxidant mechanisms allow SPI and SC to be used as natural antioxidants in a variety of food systems (Díaz & Decker, 2004).

The potential reasons for differences in emulsification capacity and antioxidant ability of proteins in O/W emulsions may include differences in protein concentration, protein solubility and hydrophobicity, protein chelating properties (differences in free radical scavenging amino acids), and the location of protein (interfacial or continuous). The oxidative stability of the different protein-stabilized emulsions was in the order of casein > WPI > SPI as determined by monitoring both lipid hydroperoxide and headspace hexanal formation (Hu, McClements, & Decker, 2003). And our previous study revealed that interface-adsorbed myofibrillar protein could provide both chemical (radical neutralization) and physical (steric) protection of emulsions against oxidation (Chapter 5). This study aims to explore other kinds of protein sources, such as SPI and SC, for the construction of antioxidative membranes in formulated emulsions or meat batter products. The results are expected to give guidance to better use SPI/SC in industry processing and storage.

6.3. Materials and methods

6.3.1. Preparation of native and heat-denatured SPI

Soybeans were dried in oven at 35°C overnight, briefly pulsed in a food processor for hull removal, then finely milled. The flour was treated with hexane/ethanol (10:1, v/v) to remove fat; the extraction was repeated twice. The defatted flour was dispersed in deionized water at a ratio of (1:10, w/v) and adjusted to pH 8.0. The solution was stirred for 2 h and then centrifuged at 10,000g for 30 min, the supernatant was adjusted to pH 4.5 and further centrifuged at 3,300g for 20 min. The resulting pellet was washed twice using 5-fold (w/v) deionized water and followed by centrifuging at 6,000g for 10 min. The precipitate was dispersed into 5-fold deionized water and adjusted to pH 7.0. The samples were freeze dried and stored at 4 °C until use.

Ten fractions of 300mL of SPI dispersions in deionized water (10%, w/v) were placed in beakers and heated in water bath at 90°C for 10 min to get partially denatured SPI (PDSPI) or 2 h for fully denatured SPI (FDSPI). Then the beakers were immediately immersed in ice water and equilibrated to room temperature. The heated SPI solutions were combined and freeze-dried.

Freeze dried native SPI (NSPI)/PDSPI/FDSPI (10%) and commercial sodium caseinate (5%) were pre-dissolved in 25 mM phosphate, pH 6.25, containing 0.6 M NaCl and stirred overnight at a walk-in cooler (4 °C) before used.

6.3.2. Differential scanning calorimetry (DSC)

DSC thermograms were recorded on a 2920 modulated DSC (TA Inc., New Castle, DE, USA) with a heating rate of 5°C/min and temperature ranges of 25–120°C.

The instrument was calibrated for temperature and enthalpy using indium. NSPI/PDSPI/FDSPI samples were filled in hermetic aluminum pan with 15 mg of 10% (w/w) soy protein dispersions in 25 mM phosphate, pH 6.25, containing 0.6 M NaCl, and sealed. An empty pan was used as a reference. The enthalpy of denaturation (ΔH) and the temperature of denaturation (T_D) were calculated using the DSC software after manually setting the start and end points of the endothermic peak.

6.3.3. Preparation of stripped soybean oil

Commercially refined soybean oil was purchased from a local store and stripped with alumina (MP Alumina N-Super I, MP Biomedicals, France) to remove tocopherols (Berton, Genot, & Ropers, 2011a). Briefly, approximately 15 g sorbent and 30 ml oil were vigorously mixed in a 50 ml-polypropylene centrifuge tube, and then placed on a shaker in the dark at 4 °C for 24 h. The tubes were then centrifuged (2,000 g, 20 min) at 20 °C to separate the sorbent from stripped oil. Collected upper phase was centrifuged again under the same conditions and finally transferred into amber glass vials after being placed under nitrogen flow for 5 min. Vials were hermetically sealed and stored at – 20 °C. HPLC analysis confirmed the tocopherol content of the stripped oil was approximately 1 mg/100 g (α-tocopherol 0.45 mg/100 g, γ-tocopherol 0.62 mg/100 g; δ-tocopherol non-detected).

6.3.4. Preparation of Tween- or protein- stabilized emulsion

Tween 20-stabilized emulsions were prepared from the mixture of 10% (v/v) stripped soybean oil and 90% (v/v) pre-chilled buffer (25 mM phosphate, pH 6.25,

containing 0.6 M NaCl, 4 °C) with 2% (w/v) Tween 20 by homogenization for 2 min with a Polytron blender (Brinkmann Instruments, Inc., Westbury, NY). A low-foaming probe (PTA-20TS) and a speed of 17,500 rpm were used. The pre-homogenized crude emulsion was then finely homogenized with a laboratory scale jet homogenizer (NanoDeBee, B.E.E. International Inc., Easton, MA) at an overall pressure of 35 MPa for 1 pass. During emulsification, the high-pressure pipeline was kept cool with ice pouches so the temperature of outlet emulsion did not exceed 15 °C. In addition, proteins (NSPI/PDSPI/FDSPI/SC) at a final concentration of 0.25, 0.5, or 1% (w/v) were added to the Tween 20 emulsion by gently mixing. Because proteins would not adsorb at the interface in Tween emulsion, it was deemed appropriate to determine the antioxidative efficacy of proteins in the continuous phase as compared to the protein-stabilized emulsion systems.

Protein-stabilized emulsions were prepared from the mixture of 10% (v/v) stripped soybean oil and 90% (v/v) NSPI/PDSPI/FDSPI/SC in 25 mM phosphate, pH 6.25, containing 0.6 M NaCl. Three protein concentrations (0.25, 0.5, and 1%, w/v) were used to allow the determination of protein concentration effect. The same two-stage homogenization process as above was utilized for the emulsion preparation.

6.3.5. Oxidation treatment

To oxidize, Tween 20-stabilized and protein-stabilized emulsions were incubated at 4 °C with hydroxyl radicals produced by a Fe^{2+}-recycling solution comprised of 10 μM $FeCl_3$/100 μM ascorbic acid/5 mM H_2O_2 for 0 h, 2 h, and 24 h (Levine, Oliver, Fulks, & Stadtman, 1981; Martinaud, Mercier, Marinova, Tassy, Gatellier, & Renerre, 1997; Park,

Xiong, & Alderton, 2006). The oxidative reaction was terminated by propyl gallate/Trolox/EDTA (1 mM each) (Park, Xiong, & Alderton, 2006).

6.3.6. Morphology

The morphology changes of protein-stabilized emulsions over oxidation time were observed using confocal laser scanning microscopy (CLSM). The oil phase was stained with Nile Red dye (0.1%, w/v), and the proteins were stained with fluorescein isothiocyanate dye (0.1%, w/v). The dye-treated samples were kept in the dark for 20 min and then photographed with an Olympus FV1000 microscope (Olympus America) with 488 nm and 543 nm lasers, respectively (Xu, Lin, & Nagy, 2014). Two sets of images were merged using Olympus FLUOVIEW 1.5 software.

6.3.7. Measurement of emulsion stability

Emulsion samples for droplet size distribution were diluted with 25 mM phosphate buffer (pH 6.25, refractive index: 1.34) at a 1:50 emulsion/buffer ratio (v/v). Emulsion particle size was analyzed by ZetaSizer Nano-ZS (Malvern Instruments, Worcestershire, UK) at 22.5 °C with the following settings: measurement angle, 90°; equilibration time, 120 s; number of runs, 3; run duration, 10 s; number of measurements, 3; delay between measurements, 10 s.

Because the O/W emulsions made with NSPI/PDSPI/FDSPI/SC would have different particle sizes, they may vary in flocculation behavior during storage and affect the oxidative stability. To test the tendency to flocculate, emulsion samples were placed in test tubes (22 mm diameter × 43 mm height) sealed with Teflon-coated caps and kept at 4 °C to track the formation of the cream and serum layers during oxidation. The

volume of the serum phase (H) was quantified and the creaming index was reported as Creaming index (%) = (H/Ho) × 100, where Ho represents the initial height of the emulsion.

6.3.8. Determination of lipid oxidation

Evaluation of oxidative changes in control and oxidized emulsion samples was performed by monitoring the formation of conjugated diene hydroperoxides and 2-thiobarbituric acid-reactive substances (TBARS) (Sinnhuber & Yu, 1977; Srinivasan, Xiong, & Decker, 1996). For conjugated dienes, emulsion samples (0.5 mL) were mixed with 5 mL of extracting solvent (3:2 v/v hexane: isopropanol) and vortexed for 1 min. After centrifugation at 2,000*g* for 5 min, the absorbance (233 nm) of the supernatant was recorded and the value converted into conjugated dienes using the molar extinction coefficient of 25,200 $M^{-1}cm^{-1}$ (O'Brien, 1969).

For the measurement of TBARS, emulsion samples were mixed into the assay solution containing trichloroacetic acid (TCA) and thiobarbituric acid (TBA) followed by boiling for 30 min. The supernatant of the cooled solution was treated with chloroform and centrifuged at 2,000*g* for 5 min. The absorbance of the aqueous phase containing the pinkish TBA-malonaldehyde adducts was measured at 532 nm and the molar extinction coefficient of 152,000 $M^{-1}cm^{-1}$ was used to calculate TBARS content (Witte, Krause, & Bailey, 1970).

6.3.9. Measurement of protein oxidation

Protein modifications were examined over time through monitoring the oxidative and structural changes using the following analytical indexes.

Carbonyl. The content of protein carbonyl derivatives in control and oxidatively stressed emulsion samples was estimated by using the 2,4-dinitrophenylhydrazine (DNPH) colorimetric method (Levine et al., 1990). Briefly, the DNPH-reacted MFP samples after 20% TCA precipitation were recovered by centrifugation (11,000*g* for 10 min) then washed with ethanol: ethyl acetate (1:1, v/v) solvent to remove unbound pigments. The final MFP pellets were dissolved in guanidine hydrochloride (6.0 M), and the absorbance at 370 nm and 280 nm were recorded for the determination of carbonyl and protein concentration, respectively. A molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$ was used for carbonyl content computation.

Sulfhydryl. Free sulfhydryl content in emulsion samples was determined by reaction with Ellman's reagent [DNTB: 5,5′-dithio-bis (2-nitrobenzoic acid)] as detailed by Liu, Xiong, and Chen (2009). Blanks were run with 25 mM phosphate buffer (pH 6.25) containing 0.6 M NaCl. The sulfhydryl concentration was calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$.

Intrinsic tryptophan fluorescence. The procedure described by Estévez, Kylli, Puolanne, Kivikari, and Heinonen (2008) with slight modifications was followed to observe fluorescence pattern and intensity of protein samples. Recovered adsorbed and unadsorbed MFP (100 μL) were diluted in 5 mL of 25 mM phosphate buffer (pH 6.25) containing 0.6 M NaCl, and then subjected to fluorescence testing using a Fluromax-3 fluorometer (Horiba Jobin Yvon Inc., Edison, NJ). The excitation and emission

wavelengths were 283 nm and 300–400 nm, respectively, and the scanning speed was 1 nm/s.

6.3.10. Statistical analysis

Data were collected from two independent experimental trials (replicates) with different batches of protein isolate. All analyses were performed on triplicate samples, and the results were subjected to the analysis of variance using the general linear model's procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL). All-pair wise multiple comparison (LSD) was used to identify significant differences ($P < 0.05$) between means.

6.4. Results

6.4.1. Heat-induced SPI structural modification

DSC was applied to measure the extent of structural change (unfolding) of SPI. Three thermograms corresponding to isolates have marked differences in their degrees of denaturation (Figure 6.1). In agreement with previous work on soy protein, peaks corresponding to endothermic transitions are attributed to β-conglycinin (75.8 °C for NSPI and 72.8 °C for PDSPI) and glycinin (90.7 °C for NSPI and 92.6 °C for PDSPI) (Sorgentini & Wagner, 1999). PDSPI has a wider and steadier peak for β-conglycinin compared to NSPI; the short-time heating may lead to certain structural changes of β-conglycinin. More importantly, the thermogram curve of PDSPI showed an increased transition temperature (To, Tp, Tc) and a relatively lower ΔH for glycinin peak as compared to NSPI. It suggested the occurrence of dissociation or partial denaturation and

the formation of more stable glycinin conformation upon thermal pretreatment. The thermogram of FDSPI does not exhibit clear transition peak during heating (Figure 6.1). After thermal pretreatment at 95 °C for 2 h, the peaks of SPI completely disappeared, indicating the complete denaturation of β-conglycinin and glycinin components in FDSPI.

6.4.2. Emulsion droplet dispersions

Figure 6.2 shows CLSM images for emulsions stabilized by NSPI/PDSPI/FDSPI/SC. Using a combination of double labeling technique, the protein network can be visualized jointly with lipid globules in the same sample. For the unoxidized emulsions (0 h), it can be observed that most of the droplets were present in a separated and unflocculated form. Droplet size gradually increased during the oxidation of SPI emulsions. In 2 h, smaller droplets gathered together and exhibited in a clustered form due to their hydrophobic nature. Relatively larger oil droplets were shown in 2 h-oxidized NSPI emulsion and 24 h-oxidized PDSPI emulsion, probably due to droplet aggregation. As expected, emulsions stabilized by different proteins have varied microstructures. For NSPI emulsions, a homogenous protein network around the oil droplets were observed at time 0. The flocculated state of droplets in fresh emulsions was dependent upon the denaturation degree of the SPIs (Shao & Tang, 2014). Preheating treatment of SPI enhanced the formation of a more structured protein matrix. With more hydrophobic SPIs aggregated close to oil droplets, emulsions prepared with PDSPI or FDSPI showed many brilliant fluorescent protein dots in the continuous phase. In FDSPI emulsions (2 h and 24 h), some well-defined interfacial films surrounding oil droplets could be noticed due to the increased thickness of the interfacial protein film. In addition,

larger protein aggregates were shown in 24 h-oxidized FDSPI emulsion. These more structured emulsions also presented very high apparent viscosities (Puppo, Beaumal, Speroni, de Lamballerie, Añón, & Anton, 2011).

In comparison, the close-packing of oil droplets in SC-stabilized emulsion were homogenously entrapped within its network. The smaller droplets fit within the spaces among larger droplets. No significant flocculation occurred during SC emulsion oxidation. At neutral pH, SC layer provides good stability against coalescence due to a combination of electrostatic and steric inter-particle repulsions (Dickinson & Golding, 1997). These CLSM observations are basically consistent with the droplet size results (Figure 6.3).

6.4.3. Particle size and emulsion stability

The particle size and coalescence stability of emulsions made with NSPI/PDSPI/FDSPI/SC were needed before comparisons of oxidative stability could be made. They can be evaluated by the size distribution for a specific oxidation period. A 1% protein concentration was chosen for these comparison studies, as this concentration is sufficient to cover the interface of the emulsion droplets and results in a monomodal distribution of sizes (Figure 6.3). In Figure 6.3, emulsions prepared with 1% NSPI/PDSPI/FDSPI/SC showed a monomodal distribution of particle size with different diameters: the initial mean particle diameters of NSPI-, PDSPI-, FDSPI-, and SC-stabilized O/W emulsions ranged from 255 nm to 460 nm, from 458 nm to 825 nm, from 955 nm to 1718 nm, and from 78 nm to 295 nm, respectively. The sizes of protein-stabilized emulsion droplets depend upon the relative amounts of protein and oil and the

homogenization technique. Initial particle size decreased with increasing protein concentration for all samples (data not shown). The particle size distributions and characteristics of the emulsions changed significantly during the oxidation of NSPI and FDSPI emulsions (Figure 6.3A, C). Emulsions prepared with 1% NSPI/FDSPI showed a remarkable shift in the average particle size to larger sizes, suggesting limited stability of these emulsion systems. Moreover, these oxidized suspensions were characterized by a wider distribution, in particular to NSPI emulsions. Large increases in particle size distributions for PDSPI and SC emulsions were not observed in 24 h of oxidation, exhibiting a good physical stability (Figure 6.3B, D). The droplet size profile of all unoxidized/oxidized emulsions remained monomodal during 24 h of oxidation (Figure 6.3).

Denaturation caused disruption of the original quaternary structure of SPI, and led to the dissociation of its subunits and an increase in protein-protein interactions (Keerati-u-rai & Corredig, 2009). The stable size distribution for oil droplets prepared with PDSPI may indicate that the dissociation of β-conglycinin, together with the limited denaturation of glycinin, increase its capacity to lower interfacial tension and cause an improvement of the emulsification properties of SPI.

Under quiescent conditions, the most obvious manifestation of emulsion instability is creaming (visible separation of bulk dispersed phase), and there is a close correlation of the rate of creaming with the emulsifier used. Figure 6.4 shows the changes in percentage of creaming index (CI%) for all the tested emulsions upon oxidation up to 48 h. The CI% (NSPI/PDSPI/FDSPI) progressively increased over oxidation time; and increasing the degree of denaturation resulted in large decreases in the creaming rate as

well as the CI% at the end of the storage. After oxidized for 48 h, the CI% of FDSPI emulsion (approximately 20%) was half that of PDSPI emulsion (approximately 40%) and considerably lower than NSPI emulsion (approximately 59%). In this case, the serum layer of FDSPI was much less turbid than that of PDSPI emulsion. For NSPI emulsion, the serum phase separated more quickly but less clear than the others (Figure 6.4). In contrast, there was no distinct creaming for the SC emulsions. The creaming behavior of emulsions was particularly sensitive to the surface hydrophobicity of proteins that affects the inter-droplet interactions of separated or flocculated droplets. The observation is well in accordance with Shao and Tang (2014) who found that heat pretreatment of SPI remarkably decreased CI% at the end of storage.

6.4.4. Assessment of lipid oxidation

Table 6.1 and Figure 6.5 show the formation of lipid conjugated diene hydroperoxide and TBARS in O/W emulsions stabilized by various proteins with a series of concentrations (0.25%, 0.5%, 1%) or stabilized by 2% Tween 20 (then added 0.25%, 0.5%, 1% of various proteins). By comparing the lipid oxidation indexes between protein-stabilized emulsion and Tween-stabilized emulsion, we are able to evaluate the antioxidant ability of interfacial protein membrane and continuous phase protein in O/W emulsions. And the different lipid oxidation extents among different protein-stabilized emulsions allows us to determine the most protective proteins.

In the intense oxidative environment, conjugated dienes are unstable. Their content typically increases for a short time and then decreases as a result of spontaneous degradation into secondary products (Frankel, 2005). In Table 6.1 (NSPI), conjugated

dienes did not change significantly within 24 h of oxidation in all NSPI samples, except for 0.5% NSPI-stabilized emulsion. For PDSPI samples, conjugated dienes decreased significantly ($P < 0.05$) after 24 h oxidation in all emulsion samples, except for 0.25% PDSPI-stabilized emulsion. For FDSPI samples (Table 6.1), the content of conjugated dienes in FDSPI-stabilized emulsion was lower than other protein-stabilized emulsions. In SC samples (Table 6.1), higher SC concentration resulted in lower conjugated dienes value after 24 h oxidation ($P < 0.05$). Although it is difficult to track the changes of unstable conjugated dienes, their contents in Tween-stabilized emulsions with added proteins (in average) were 16.5% higher than protein-stabilized emulsion (Table 6.1). The results indicated that protein used as an emulsifier surrounding the oil droplets, as opposed to being in the aqueous phase, might delay lipid oxidation. Moreover, the Tween emulsion mixed with proteins had relatively higher conjugated diene values at 0 h compared to protein emulsion samples, suggesting the early protective role that proteins played in the emulsifying process.

The TBARS results are depicted in Figure 6.5. Except FDSPI samples (Figure 6.5C), the Tween emulsions with added proteins (0.25, 0.5, or 1%) showed distinctly higher ($P < 0.05$) TBARS values, including time 0, when compared with protein-stabilized emulsions (Figure 6.5A, B, D). It demonstrated that interface-adsorbed NSPI/PDSPI/SC was effective against lipid oxidation during the homogenization process and upon prolonged oxidative stress. Compared to the control (Tween emulsion without protein), the interfacial protein in protein-stabilized emulsions conspicuously prolonged the lag phase, thereby slowing the production of TBARS in 2 h (Figure 6.5A, B, C, D). For PDSPI emulsion samples, oxidation up to 2 h caused no significant increase in

TBARS (P > 0.05). Interface-adsorbed PDSPI decelerated the increasing pattern of TBARS. Moreover, in Tween emulsions with added NSPI/PDSPI/SC (Figure 6.5A, B, D), their lipid oxidation (denoted by TBARS values) was considerably inhibited by increasing the protein concentration to 1%. The SC samples (Figure 6.5D) had the smallest TBARS values compared to the others (Figure 6.5A, B, C). In SC-stabilized emulsions, it showed that TBARS formation was not sensitive to the amount of SC used to prepare the emulsion (P > 0.05, Figure 6.5).

6.4.5. Measurement of protein oxidation

Sulfhydryl and carbonyl derivatives. The denaturation degree of SPIs is directly related to their initial sulfhydryl content. At time 0, NSPI samples had an average of 41.7 nmol sulfhydryl groups/mg protein, PDSPI samples maintained 88% of the sulfhydryl groups in NSPI samples while FDSPI samples maintained 38% (Table 6.2). As presented in Table 6.2, the sulfhydryl content showed a decreasing trend in most emulsion samples during oxidation. The loss of thiol groups occurred within the first 2 h, and continuously during oxidation in NSPI and PDSPI samples. In most FDSPI and SC samples, there were no significant differences in sulfhydryl content during oxidation (P > 0.05), since they have relatively small sulfhydryl contents at the beginning. The major caseins (αS1 and β caseins) contain no cysteine, and the minor caseins (αS2 and κ) each contain two cysteinyl residues per molecule. Therefore, they have limited capacity to form intra- or intermolecular disulfide bonds. Both heat denaturation and oxidation caused decline in free sulfhydryl groups. Free sulfhydryl groups can be oxidized to reversible form (protein disulfide and sulfenic acid) or irreversible form (sulfinic and sulfonic acids) in different

oxidative environments (Eaton, 2006). Cysteine residues and disulfide bonds had an important influence on the structure of proteins (Visschers & De Jongh, 2005).

As Figure 6.6, the carbonyl content increased continuously during oxidation ($P < 0.05$). The extent of carbonyl formation was different between protein-stabilized emulsions and Tween-stabilized emulsions. Protein-stabilized emulsions had a more rapid carbonyl formation during oxidation. In 2 h, the carbonyl content in NSPI-, PDSPI-, or FDSPI-stabilized emulsions increased by 1.38 μmol/g, 0.46 μmol/g, and 0.39 μmol/g (avg.), respectively, compared to a net increase of 0.23 μmol/g, 0.20 μmol/g (avg.), and 0.31 μmol/g in Tween emulsion with added NSPIs, PDSPIs, or FDSPIs (Figure 6.6A, B, C). Interface-adsorbed protein exposed more rapid carbonyl formations probably because the location was more vulnerable to be oxidized. It also showed more carbonyl formation in native SPI than denatured SPIs (Figure 6.6), suggesting native SPI was more susceptible to the oxidant. It is most likely because the heat denaturation process inactivates lipoxygenase activity and eliminates oxidation. Protein oxidation was noticed in Tween emulsions with added proteins as well (Figure 6.6). The results, along with TBARS results (Figure 6.5), substantiate that interfacial protein in protein-stabilized emulsions played a sacrificing role as an antioxidant to protect lipids. In contrast, SC samples exhibited a slightly different behavior; both interface and continuous phase SC have similar increasing patterns in carbonyl formation (Figure 6.6D).

Intrinsic tryptophan fluorescence. Tryptophan residues are able to emit fluorescence in the range 300–400 nm when excited at 280 nm. Tryptophan fluorescence intensity is an indicator of changes in protein tertiary structure because it denotes the

relative position of the tryptophan residues within proteins (Keerati-u-rai, Miriani, Iametti, Bonomi, & Corredig, 2012).

The quenching of fluorescence intensity was shown in Table 6.3. Its decreasing trend was very similar to the increasing pattern of the carbonyl formation (Figure 6.6). Greater losses of fluorescence were exhibited in SPI-stabilized emulsions than Tween emulsions (with SPIs). And in 2 h, fluorescence intensity in NSPI-stabilized emulsions decreased the most among all SPI samples (Table 6.3). These results again confirmed that interface-adsorbed SPIs in protein-stabilized emulsions were more oxidizable than unadsorbed SPIs in Tween-stabilized emulsions. For SC samples, most fluorescence intensity reduction occurred from 2 to 24 h. However, even though the SC oxidation was not significant ($P > 0.05$) in 2 h from the results of sulfhydryl content (Table 6.2) and tryptophan fluorescence (Table 6.3), it could effectively inhibit oil droplets from oxidation (Figure 6.5). Interface or continuous phase SC has similar extent of oxidation, but a thick membrane formed by flexible caseins may physically help prevent oil droplets from oxidation.

6.5. Discussion

Proteins are vulnerable to oxidation because of their high abundance in biological systems and high rate constants for reaction of oxidants (Davies, 2005). Protein oxidation is the structural modification induced directly by ROS or indirectly by reaction with the by-products of lipid peroxidation (Shacter, 2000). Hydroxyl radical-mediated protein oxidation has received the most attention because hydroxyl radicals show the highest rate constants for the reactions with protein (Hawkins & Davies, 2001). Peroxyl radicals also

play an important role in protein oxidation. They can be considered an important link between protein oxidation and lipid peroxidation because the oxidant is regarded as the central species of the lipid peroxidation chain reactions and a ROS for protein oxidation (Duggan, Rait, Platt, & Gieseg, 2002; Gieseg, Pearson, & Firth, 2003). Oxidative stress triggers multiple structural changes of target proteins, resulting in oxidation of side chain groups, backbone fragmentation, cross-linking, unfolding, and changes in conformation (Davies, 2005; Stadtman & Berlett, 1998). In this study, significant changes ($P < 0.05$) in most protein oxidation markers occurred within the first 2 h, preceding TBARS accumulation in SPIs/SC-stabilized emulsion systems. It signified that interfacial SPIs/SC, similar to MFP (Chapter 5), was more susceptible to oxidative damages (hydroxyl radicals) than lipids. The mechanism of the oxidative protection by interfacial non-meat protein is similar to interfacial MFP (a physical protection, which described in Chapter 5).

Higher protein concentration in protein-stabilized emulsions resulted in a thicker protein membrane aiding in its protective role. Also, with higher protein concentration in continuous aqueous phase, it is possible for unadsorbed protein to spare lipid oxidation through scavenging of free radicals, as well as chelation of metal ions (Coupland & McClements, 1996; Hu, McClements, & Decker, 2003). On the other hand, the accumulation of protein oxidation products appeared to stimulate lipid oxidation (TBARS formation) and the remaining lipoxygenase in SPI may catalyze lipid peroxidation (Wu, Wu, & Hua, 2010). Lipid peroxidation happened later and its by-products may be another reason that caused protein oxidation during the storage of emulsions (Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008; Hu, McClements, & Decker, 2003).

The oxidative stability of the emulsions is also influenced by the properties of the proteins. Recent research has found that particle size was not related to oxidation rates, suggesting that surface area was not a major factor in determining oxidative stability of the emulsions made with different proteins (Dimakou, Kiokias, Tsaprouni, & Oreopoulou, 2007; Osborn & Akoh, 2004). Kato, Osako, Matsudomi, and Kobayashi (1983) reported a good correlation of emulsifying activity index/emulsion stability with surface hydrophobicity of proteins using ovalbumin, soy 7S globulin, κ-casein, β-lactoglobulin, and bovine serum albumin. The surface hydrophobicity of SPI was found to increase with heat denaturation. Denaturation of SPI results in the unfolding of its polypeptide chain, which could achieve a molten globule-like conformation that maintained most of the original secondary structure and overall compactness but lost some tertiary structure. These structural modifications improved both emulsifying activity and emulsion stability (Jiang, Chen, & Xiong, 2009). It was reported that preheat treatment at 90 °C or 95 °C for 3 min denatured β-conglycinin and reduced the 11S acidic-basic subunit aggregation, which improves its functionality especially in muscle foods (Feng & Xiong, 2002). Hence, PDSPI possessed increased emulsifying activity, which helped maintain the physical structure of protein-stabilized emulsions.

SC could cover the interface as little as 1 mg/m^2 and to the extent of 2 – 3 mg/m^2 as the proteins change conformation due to its flexibility (Fang & Dalgleish, 1993). At lower concentrations, casein spreads widely over the oil/water interface (thin layer). And when casein is at higher concentrations, each molecule is not required to spread as widely and can protrude into solution (Damodaran, 1997). The ability of casein to form a thick layer around the emulsion droplets and its unique iron chelating properties (Hu,

McClements, & Decker, 2003) could help to explain why the SC-stabilized emulsions had the greatest oxidative stability (Figure 6.5).

6.6. Conclusion

When SPIs/SC were used as emulsifiers in O/W emulsions, active protein membranes could physically inhibit hydroxyl radical-induced lipid oxidation. At the beginning, interfacial protein oxidized quickly by direct reaction with hydroxyl radicals, and with the process of lipid peroxidation, the lipid oxidation products could further aggravate protein oxidation. In addition, NSPI with more free sulfhydryl groups compared to other denatured SPIs and SC, was particularly vulnerable to oxidation.

Table 6.1. Formation of conjugated dienes (mM) in different emulsions stabilized by proteins or Tween (with added proteins) during oxidation.

	Protein emulsion			Tween emulsion + Protein		
Emulsion	0 h	2 h	24 h	0 h	2 h	24 h
NSPI						
0.25%	0.159 ± 0.014^{aB}	0.137 ± 0.002^{aE}	0.133 ± 0.002^{aD}	0.185 ± 0.005^{aA}	0.191 ± 0.003^{aA}	0.191 ± 0.000^{aA}
0.5%	0.151 ± 0.002^{aBC}	0.137 ± 0.000^{bE}	0.118 ± 0.002^{cE}	0.159 ± 0.004^{aB}	0.163 ± 0.001^{aB}	0.165 ± 0.005^{aB}
1%	0.163 ± 0.003^{aB}	0.148 ± 0.002^{aC}	0.149 ± 0.007^{aC}	0.143 ± 0.006^{aC}	0.143 ± 0.002^{aD}	0.147 ± 0.001^{aCD}
PDSPI						
0.25%	0.106 ± 0.000^{cF}	0.133 ± 0.002^{bD}	0.166 ± 0.000^{aC}	0.226 ± 0.000^{aB}	0.214 ± 0.010^{abAB}	0.201 ± 0.000^{bA}
0.5%	0.157 ± 0.002^{aE}	0.153 ± 0.002^{aCD}	0.134 ± 0.000^{bF}	0.232 ± 0.001^{aA}	0.233 ± 0.019^{aA}	0.184 ± 0.002^{bB}
1%	0.170 ± 0.002^{aD}	0.162 ± 0.002^{aC}	0.141 ± 0.005^{bE}	0.198 ± 0.000^{aC}	0.195 ± 0.004^{aB}	0.159 ± 0.001^{bD}
FDSPI						
0.25%	0.088 ± 0.003^{bE}	0.106 ± 0.003^{aE}	0.114 ± 0.001^{aB}	0.135 ± 0.001^{bA}	0.136 ± 0.000^{bA}	0.141 ± 0.000^{aA}
0.5%	0.115 ± 0.001^{aD}	0.113 ± 0.002^{aD}	0.107 ± 0.000^{bB}	0.134 ± 0.002^{aA}	0.133 ± 0.001^{aAB}	0.112 ± 0.021^{aB}
1%	0.120 ± 0.001^{aC}	0.123 ± 0.004^{aC}	0.120 ± 0.001^{aAB}	0.129 ± 0.001^{aB}	0.129 ± 0.001^{aB}	0.125 ± 0.002^{bAB}
SC						
0.25%	0.131 ± 0.005^{aB}	0.135 ± 0.002^{aB}	0.138 ± 0.001^{aB}	0.143 ± 0.005^{aA}	0.144 ± 0.005^{aA}	0.147 ± 0.000^{aA}
0.5%	0.130 ± 0.001^{bB}	0.140 ± 0.000^{aAB}	0.134 ± 0.002^{bC}	0.128 ± 0.002^{aB}	0.126 ± 0.002^{aC}	0.118 ± 0.000^{bE}
1%	0.140 ± 0.003^{aA}	0.141 ± 0.003^{aAB}	0.123 ± 0.001^{bD}	0.117 ± 0.002^{aC}	0.107 ± 0.001^{bD}	0.102 ± 0.000^{cF}

$^{a-c}$ Means ± standard deviations within the same emulsion between different oxidation times with different lowercase letters differ significantly ($P < 0.05$).

$^{A-F}$ Means ± standard deviations within the same oxidation time between protein-stabilized emulsions and Tween-stabilized emulsions (added the same kind of protein) with different capital letters differ significantly ($P < 0.05$).

Table 6.2. Sulfhydryl contents (nmol/mg of protein) in different emulsion systems as affected by oxidation (0, 2, and 24 h).

	Protein emulsion			Tween emulsion + Protein		
Emulsion	0 h	2 h	24 h	0 h	2 h	24 h
NSPI						
0.25%	47.6 ± 1.2^{aA}	34.2 ± 2.2^{bA}	10.8 ± 1.6^{cC}	47.0 ± 2.8^{aA}	34.4 ± 2.5^{bA}	20.1 ± 2.8^{cA}
0.5%	38.6 ± 0.3^{aB}	27.8 ± 1.9^{bB}	15.9 ± 1.9^{cAB}	33.5 ± 3.7^{aC}	13.9 ± 1.6^{bD}	12.8 ± 2.5^{bBC}
1%	39.0 ± 0.3^{aB}	21.0 ± 1.6^{bC}	13.7 ± 1.2^{cBC}	35.3 ± 1.2^{aBC}	15.2 ± 0.9^{bD}	14.3 ± 0.3^{bBC}
PDSPI						
0.25%	34.2 ± 4.7^{aAB}	17.4 ± 0.3^{bAB}	14.8 ± 3.4^{bA}	34.0 ± 4.4^{aAB}	15.0 ± 1.9^{bBC}	8.6 ± 2.8^{bB}
0.5%	38.4 ± 3.1^{aA}	18.5 ± 0.6^{bA}	8.2 ± 1.6^{cB}	27.8 ± 2.5^{aB}	13.5 ± 0.3^{bCD}	8.2 ± 0.3^{cB}
1%	38.2 ± 1.6^{aA}	18.3 ± 1.6^{bA}	7.5 ± 1.9^{cB}	30.2 ± 0.3^{aB}	10.6 ± 1.9^{bD}	5.7 ± 1.9^{bB}
FDSPI						
0.25%	17.6 ± 4.6^{aAB}	14.1 ± 0.0^{aB}	10.6 ± 4.7^{aC}	20.7 ± 3.4^{aA}	18.8 ± 0.6^{aA}	19.7 ± 0.6^{aA}
0.5%	15.4 ± 0.6^{aB}	13.9 ± 0.9^{abB}	11.0 ± 1.9^{bC}	19.9 ± 0.3^{aA}	19.9 ± 0.0^{aA}	19.0 ± 0.6^{aA}
1%	14.8 ± 3.1^{aB}	11.7 ± 0.9^{aC}	11.9 ± 0.0^{aC}	14.7 ± 0.9^{aB}	15.2 ± 0.9^{aB}	13.9 ± 0.9^{aB}
SC						
0.25%	23.4 ± 1.9^{aA}	20.1 ± 4.0^{aA}	15.9 ± 0.9^{aA}	23.2 ± 3.7^{aA}	21.7 ± 1.6^{aA}	10.7 ± 0.3^{bB}
0.5%	16.8 ± 0.6^{aAB}	13.9 ± 0.6^{aBC}	11.7 ± 1.6^{aB}	17.6 ± 3.3^{aAB}	16.8 ± 1.2^{aAB}	13.0 ± 1.2^{aB}
1%	14.8 ± 4.1^{aB}	11.5 ± 0.3^{aCD}	10.8 ± 1.9^{aB}	11.5 ± 2.5^{aB}	9.7 ± 1.9^{aD}	9.3 ± 1.9^{aB}

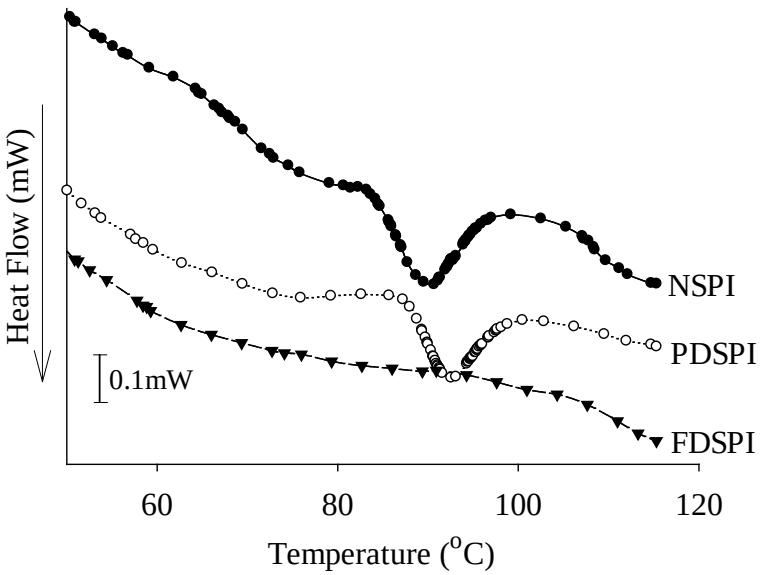
$^{a-c}$ Means ± standard deviations within the same emulsion between different oxidation times without a common lowercase letter differ significantly ($P < 0.05$).

$^{A-E}$ Means ± standard deviations within the same oxidation time between protein-stabilized emulsions and Tween-stabilized emulsions (added the same kind of protein) without a common capital letter differ significantly ($P < 0.05$).

Table 6.3. Relative fluorescence intensity of different emulsions stabilized by proteins or Tween (with added proteins).

	Protein emulsion			Tween emulsion + Protein		
Emulsion	0 h	2 h	24 h	0 h	2 h	24 h
NSPI						
0.25%	100%^{a}	64%^{b}	63%^{b}	100%^{a}	91%^{b}	87%^{b}
0.5%	100%^{a}	82%^{b}	78%^{b}	100%^{a}	93%^{a}	73%^{b}
1%	100%^{a}	81%^{b}	46%^{c}	100%^{a}	91%^{b}	78%^{c}
PDSPI						
0.25%	100%^{a}	79%^{b}	72%^{b}	100%^{a}	88%^{b}	80%^{c}
0.5%	100%^{a}	84%^{b}	80%^{c}	100%^{a}	83%^{b}	56%^{c}
1%	100%^{a}	83%^{b}	84%^{b}	100%^{a}	79%^{b}	72%^{b}
FDSPI						
0.25%	100%^{a}	90%^{b}	65%^{c}	100%^{a}	99%^{a}	98%^{a}
0.5%	100%^{a}	81%^{b}	62%^{c}	100%^{a}	94%^{a}	82%^{b}
1%	100%^{a}	87%^{b}	74%^{c}	100%^{a}	94%^{a}	64%^{b}
SC						
0.25%	100%^{a}	94%^{a}	72%^{b}	100%^{a}	97%^{a}	75%^{b}
0.5%	100%^{a}	98%^{a}	64%^{b}	100%^{a}	99%^{a}	82%^{b}
1%	100%^{a}	97%^{a}	78%^{b}	100%^{a}	98%^{a}	88%^{b}

$^{a–c}$ Means within the same emulsion between different oxidation times with different lowercase letters differ significantly ($P < 0.05$).



Sample	First Peak				Second Peak			
	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
NSPI	67.8	75.8	82.5	0.2	83.1	90.7	99.2	0.6
PDSPI	58.2	72.8	84.1	0.3	86.1	92.6	101.7	0.4
FDSPI	42.4	50.8	85.3	3.2	-	-	-	-

Figure 6.1. Differential scanning calorimetry (DSC) thermograms of 10% (w/v) soy protein isolates in distilled water with different degrees of denaturation. Inset table: Temperatures and enthalpies associated with characteristic denaturation behaviors of SPIs. T_o, onset temperature; T_p, peak temperature; T_c, conclusion temperature; ΔH, enthalpy.

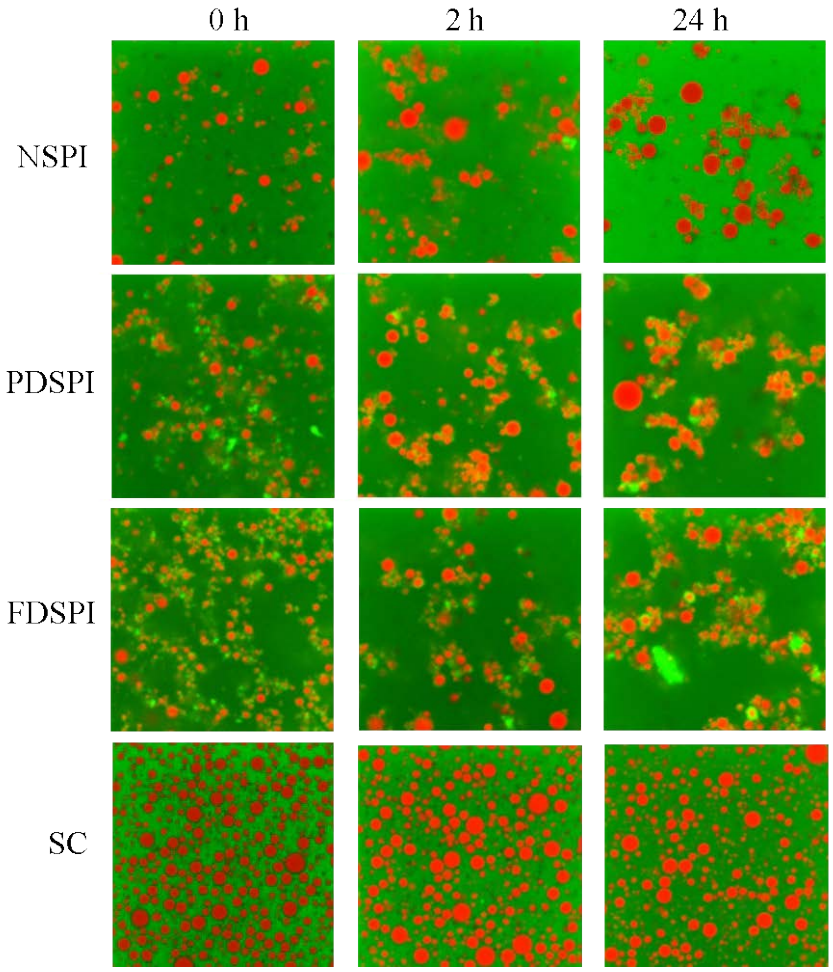


Figure 6.2. Representative confocal images of different protein-stabilized emulsions over oxidation (Fat globules: red, protein network: green).

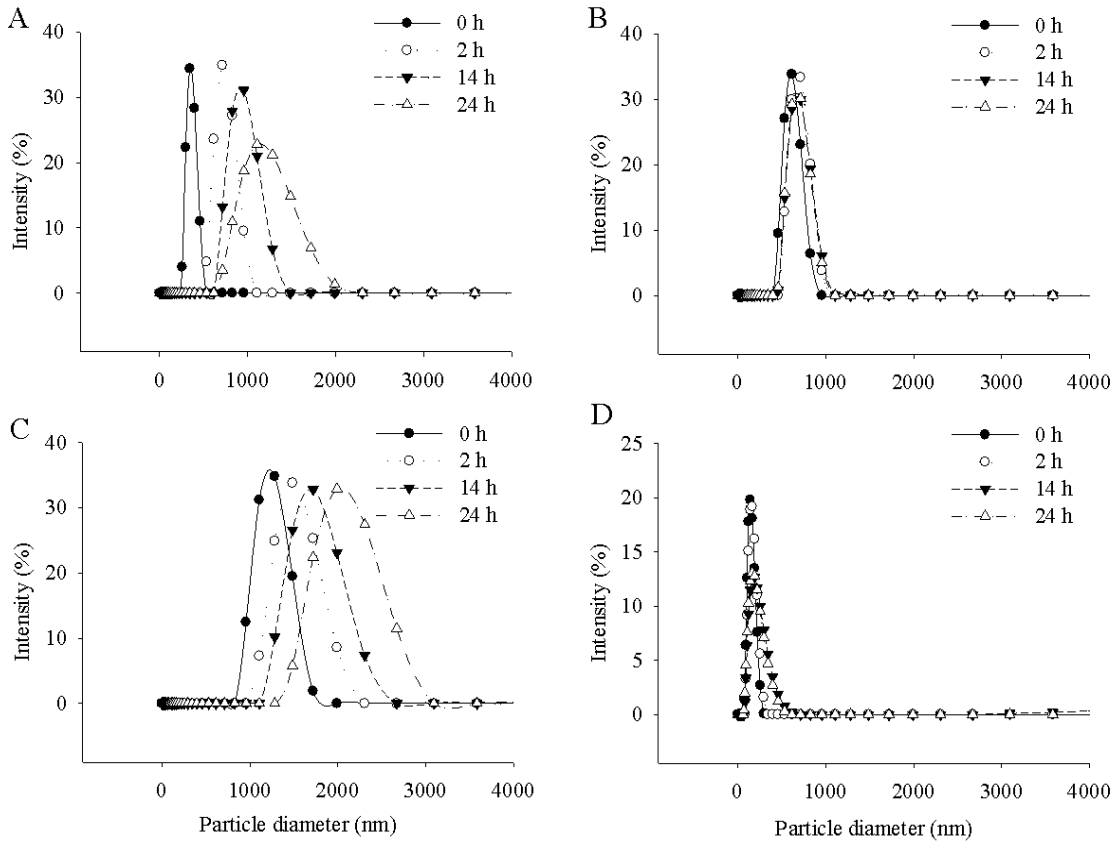


Figure 6.3. Average particle size and oxidation-induced changes of protein-stabilized emulsions prepared with (A) NSPI (B) PDSPI (C) FDSPI (D) SC.

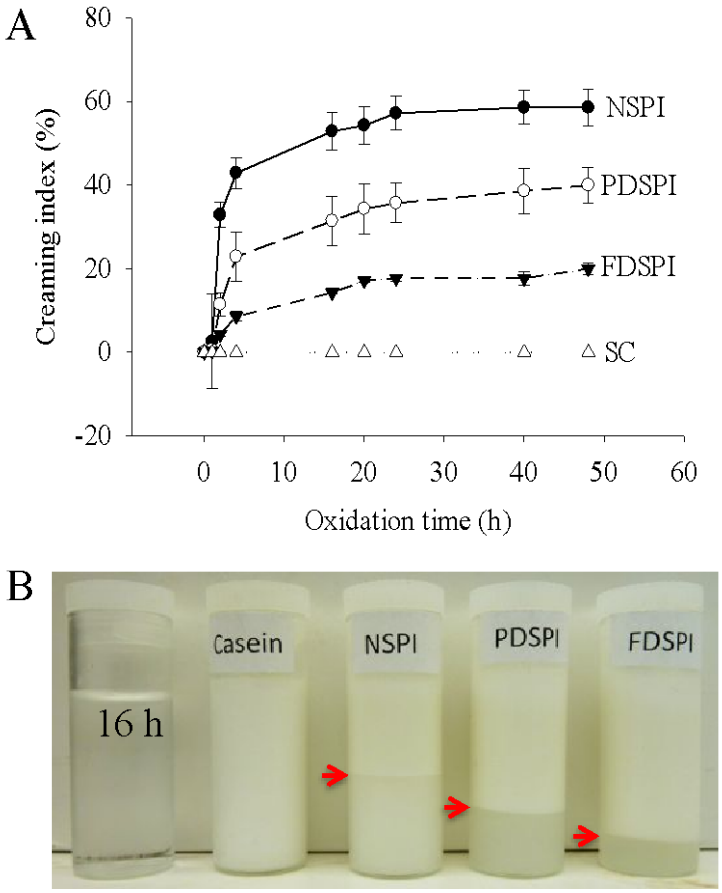


Figure 6.4. Oxidation-induced phase separation (B: below picture, 16 days) and resulting serum layer height (A: upper figure) of O/W emulsions prepared with NSPI/PDSPI/FDSPI/SC.

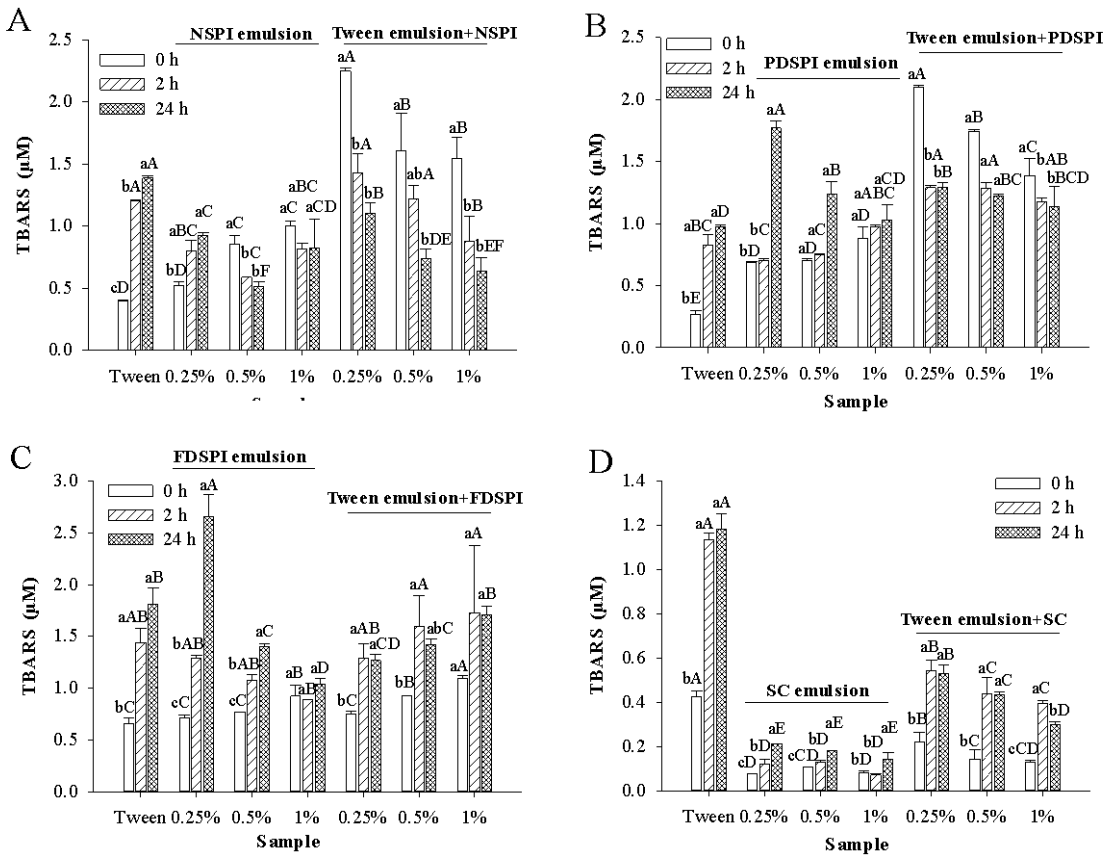


Figure 6.5. Formation of thiobarbituric acid-reactive substances (TBARS) in different emulsions stabilized by (A) NSPI (B) PDSPI (C) FDSPI (D) SC (left) or Tween with added (A) NSPI (B) PDSPI (C) FDSPI (D) SC (right) during oxidation.

(a−c) Means within the same emulsion between different oxidation times with different lowercase letters differ significantly ($P < 0.05$).
(A−F) Means within the same oxidation time between different emulsions with different capital letters differ significantly ($P < 0.05$).

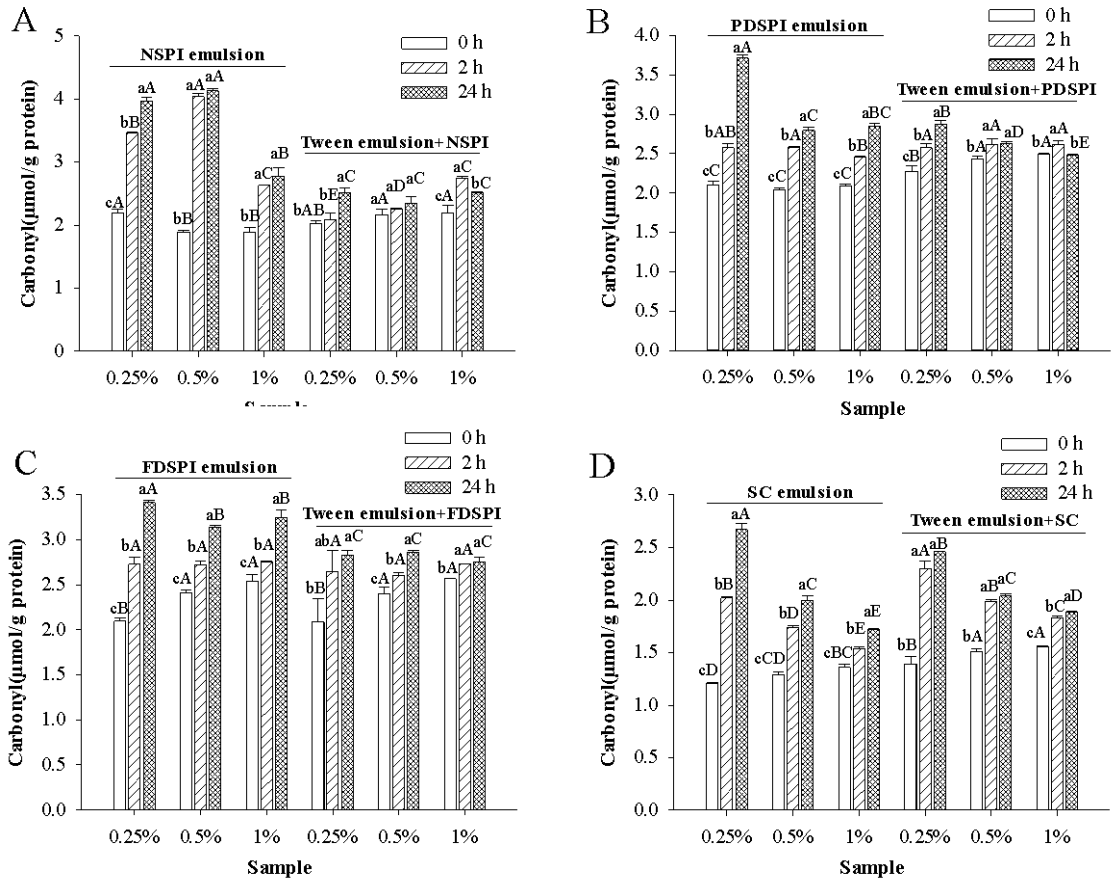


Figure 6.6. Formation of Carbonyls in different emulsions prepared by (A) NSPI (B) PDSPI (C) FDSPI (D) SC (left) or Tween with added (A) NSPI (B) PDSPI (C) FDSPI (D) SC (right) during oxidation.

(a−c) Means within the same emulsion between different oxidation times with different lowercase letters differ significantly ($P < 0.05$). (A−F) Means within the same oxidation time between different emulsions with different capital letters differ significantly ($P < 0.05$).

CHAPTER 7

EFFECTS OF PARTIAL REPLACEMENT OF FAT WITH PROTEIN-STABILIZED OIL PRE-EMULSION ON PHYSICAL CHARACTERISTICS AND OXIDATIVE STABILITY OF FRESH PORK SAUSAGE

7.1. Summary

Three types of sausage were prepared: 1) control with 10% (w/w) pork fat; 2) 60% fat replacement with sodium caseinate (SC)-stabilized soybean oil emulsion; and 3) 60% fat replacement with partially denatured soy protein isolate (PDSPI)-soybean oil emulsion. The SC sausage exhibited a higher storage modulus (G') upon cooking (20–80 °C) when compared with the control and PDSPI sausage. Substitutions with oil emulsions improved cooking yield by up to 3% over the control. During refrigerated storage (4 °C), cooked SC sausage showed lower TBARS values ($P < 0.05$) in the first 5 days, and PDSPI sausage displayed a slower lipid oxidation rate throughout 14 days compared with the control. Moreover, SC and PDSPI sausages possessed higher CIE L^* values and lower b^* values ($P < 0.05$). The a^* value of the control sausage decreased rapidly during storage while the fat replacement with pre-emulsified oil helped maintain the redness. There was no significant difference in the texture profile attributes between the treatments ($P > 0.05$). The morphology of fresh control sausage, revealed by scanning electron microscopy, showed a sponge-like texture with rough protein coats; SC and PDSPI sausages presented a denser structure with fewer cavities within the protein matrix.

7.2. Introduction

Consuming polyunsaturated fatty acids in place of saturated is recommended to reduce the risk of obesity, hypertension, coronary heart disease, and cardiovascular disease (Choi et al., 2009; Mozaffarian, Micha, & Wallace, 2010; Özvural & Vural, 2008). Vegetable oils are free of cholesterol and have a higher ratio of unsaturated fatty acids to saturated than animal fats. Hence, there is a growing interest in reformulation of traditional sausages by partial or total replacement of animal fat with vegetable oils to improve the nutritional value of meat products (Jiang & Xiong, 2015).

The different physical properties (melting point, fatty acid composition) of vegetable oils also affect the quality characteristics of the resulting meat products in terms of flavor and texture. The direct incorporation of vegetable oils in meat products had lower processing yield (5.5–6.5%) and overall palatability (Bloukas & Paneras, 1993). Therefore, several strategies, such as modifying the physical properties, adding starch or dietary fibers, are brought up to modulate the properties of reformulated meat products. Several researchers have reported that the substitution of pork fat by olive oil did not affect the textural and sensory properties after adding locust bean/xanthan gum (Lurueña-Martínez, Vivar-Quintana, & Revilla, 2004) or rice bran fiber (Choi et al., 2010). Javidipour and Vural (2002) suggested that the interesterified vegetable oils as a substitute of beef fat could offset the changes of appearance, color, texture, flavor, and sensory scores brought out by fat replacement.

O/W emulsions with the physical characteristics and stability improved by the formation of a protein network structure offer another attractive possibility for the formulation of healthier meat products (Jiang & Xiong, 2015). Pre-emulsification of

vegetable oil was stabilized with various protein systems formulated using sodium caseinate, soy protein isolate, meat protein, and microbial transglutaminase, which could possibly minimize its separating from meat matrix, provide better texture, and improve physical stability (Cáceres, García, & Selgas, 2008; Jiménez-Colmenero, 2007). When using non-meat protein as emulsifiers, more meat protein becomes available to make a contribution to gel-formation and water binding (Bishop, Olson, & Knipe, 1993). SPI and SC have been widely used in many formulated meat products as a source of functional proteins. Their interface properties, including hydrophobicity and solubility, are key factors determining their emulsification capacity (Molina, Papadopoulou, & Ledward, 2001).

It has been shown that thermal treatment of soy protein isolate induces a marked increase in viscosity and a great improvement in the emulsifying properties (Corredig, 2009). A controlled denaturation process allows partial unfolding and exposures of hydrophobic amino acid residues side chain groups while avoiding the generation of insoluble protein aggregates. Compared with native proteins, partially denatured proteins become more amphiphilic and are able to orient at the oil-water interface, leading to an increased fat-binding capacity (Voutsinas, Cheung, & Nakai, 1983). Such emulsions tend to have smaller average oil droplet size, higher adsorbed protein percentage, and lower protein surface load at the oil/water (O/W) interface (Cui, Chen, Kong, Zhang, & Hua, 2014). Heated treatment of SPI enhances its interaction with MFP and results in the smoothest and most ordered gel network in frankfurters (Feng, Xiong, & Mikel, 2003; Wang et al., 2015). Milk proteins, for example, casein, could also modify the texture and cooking behavior of sausages. It was reported that caseinate is an adequate substitute for

meat in frankfurters (Atughonu, Zayas, Herald, & Harbers, 1998). It could act as a surface-active material at the interface between meat protein and fat globules (Su, Bowers, & Zayas, 2000). Casein is unique in that it has a random coil conformation and heating does not increase its hydrophobicity significantly (Voutsinas, Cheung, & Nakai, 1983)). Its presence reduces exudate on heating and provides wider temperature stability during comminution. However, heating may impair its emulsifying properties (Voutsinas, Cheung, & Nakai, 1983).

Most research focuses on the physical characteristics of the fat replaced sausages. However, the oxidative stability of these sausages is another concern that needs more attention. Lipid oxidation in fat replacement meat products varies depending upon the nature of the product, the type, amount and means of addition of non-meat fats, and the antioxidative system used to control rancidity development (Jiménez-Colmenero, 2007). The production of sausages disrupts the integrity of lean/adipose tissue membranes, which enhances the oxidation susceptibility during the subsequent refrigerated storage (Nieto, Castillo, Xiong, Álvarez, Payne, & Garrido, 2009). In addition, vegetable oil contains more unsaturated fatty acids, which are a preferred target for radicals. Our previous research suggested proteins could form a membrane surrounding oil droplets to prevent the lipid from oxidation (Chapter 5).

In this study, commercial soybean oil was pre-emulsified using PDSPI (90 °C, 10 min) and SC. Fresh (not nitrite-cured) sausages were prepared with 10% (w/w) lipid and 18% protein by 60% substitution of pork backfat with pre-emulsified oil. Our objective was to compare processing parameters, physical characteristics, and oxidation stability of fat-replacement sausages (60% oil/40% fat) with the control sausage (100% fat). The

antioxidant and emulsifying effects of casein and partially denatured SPI (PDSPI) on meat emulsions were further investigated.

7.3. Materials and methods

7.3.1. Materials

Lean meat (moisture 73%, fat 6%) was collected from pork triceps brachii of three 48 h post-mortem carcasses by removing visible fat and connective tissues, and f(no GMO) were purchased from a local grocery store. Casein sodium salt (protein concentration: 88%) from bovine milk was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were reagent grade or higher and purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma Chemical Co. (St. Louis, MO, USA).

7.3.2. Preparation of native and heat-denatured SPI

Soybeans were dried in oven at 35°C overnight, broken into pieces for the hull removal by pulsing briefly in a food processor, and then finely milled. The flour was treated with hexane:ethanol (10:1, v/v) to remove fat; the extraction was repeated twice. The defatted flour was dispersed in deionized water at a ratio of 1:10 (w/v) and adjusted to pH 8.0. The solution was stirred for 2 h and then centrifuged at 10,000 g for 30 min. The supernatant was adjusted to pH 4.5 and further centrifuged at 3,300 g for 20 min. The resulting pellet was washed twice using 5-fold (w/v) deionized water and followed by centrifuging at 6,000g for 10 min. The precipitate was dispersed into 5-fold deionized water and adjusted to pH 7.0. Samples were freeze dried and stored at 4 °C until use.

Ten fractions of 300 mL of SPI dispersions in deionized water (10%, w/v) were placed in beakers and heated in a water bath at 90°C for 10 min. The beakers were then immediately immersed in ice water and equilibrated to room temperature. The heated SPI (PDSPI) solutions were combined and freeze dried.

7.3.3. Preparation of protein-stabilized oil pre-emulsion

Commercial soybean oil was purchased from a local store. Freeze dried PDSPI (10%) and commercial SC (5%) were pre-dissolved in 25 mM phosphate, pH 6.25, containing 0.6 M NaCl and stirred overnight in a walk-in cooler (4 °C). Protein stabilized emulsions were prepared from the mixture of 10% (v/v) soybean oil and 90% (v/v) protein (PDSPI/SC, 1% w/v) in 25 mM phosphate, pH 6.25, containing 0.6 M NaCl. The mixture was homogenized at 17,500 rpm for 2 min with a Polytron blender (Brinkmann Instruments, Inc., Westbury, NY, USA). Then the pre-homogenized crude emulsion was finely homogenized with a laboratory scale jet homogenizer (NanoDeBee, B.E.E. International Inc., Easton, MA, USA) at an overall pressure of 35 MPa for 1 pass.

7.3.4. Meat batter manufacturing

Sausage batters (1.5 kg for each treatment) were prepared in a 4 °C walk-in cooler. Lean meat and fat were ground separately through meat grinder (Model KSM90, KitchenAid Inc. St. Joseph, Michigan, USA) with 4.8 mm plate. The ground lean meat (750 g, each treatment) was blended with salt (2.1%, final), then fat (10%) in a buffer solution (25 mM phosphate with 0.6 M NaCl, pH 6.25) or emulsions (750 mL, each treatment) to make fat /PDSPI /SC sausage batters in a food processor (Black & Decker

Corporation, Towson, Maryland, USA). Mixing time was standardized to 5 min. A thermometer was used to monitor the temperature of the batter, which was maintained below 12 °C throughout preparation. The pH of the meat batter was approximately 5.80. Batters were stuffed into 19 mm diameter cellulose casings and manually tied into 15 cm length rolls with cotton twine. The sausage links were held for 16 h at 4 °C for ingredient equilibrium, then slowly cooked in a water bath from 20 to 80 °C at a heating rate of approximately 0.6 °C /min. The internal temperature of the roll centers reached 71°C (monitored using a probe thermometer).

7.3.5. pH and color measurement

The pH and color of both raw sausage batters and cooked sausages were measured. Triplicate 4 g meat samples were mixed with 20 mL of 0.15 NaCl solutions and homogenized 8 seconds (setting 3) using a Polytron blender (Brinkmann Instruments, Inc., Westbury, NY, USA). The pH of the resulting meat slurry was tested. Meanwhile, CIELAB coordinates, L^* (lightness), a^* (redness), and b^* (yellowness), of the raw batters and cooked sausages were measured, respectively, using a hand held Minolta Chroma Meter with a 10.5 cm diameter plate, illuminant D_{65} (average daylight) and a 0° angle observer (Model CR-310, Minolta Camera Co. Ltd., Osaka, JAPAN), and a LabScan XE spectrophotometer with a 0.25 inch port size, illuminant D_{65} and a 10° standard observer (Hunter Associates Laboratory, Inc., Reston, VA, USA).

7.3.6. Dynamic rheological properties

The effect of various formulas of sausages on their rheological properties was tested using a Bohlin CVO rheometer (Malvern Instruments, Inc., Westborough, MA, USA) in oscillatory mode. Uncooked sausage batters (after 16 h incubation) were loaded onto the rheometer plate and dispersed with a spatula; the exposed sample perimeter was covered with a thin layer of silicon oil to prevent evaporation. The upper plate (30 mm diameter) was parallel to the rheometer plate with 1 mm gap. The samples were heated from 20 to 80 °C at a rate of 1 °C/min, during which the sample was subjected at a fixed frequency of 0.1 Hz with a maximum strain of 0.02. Storage modulus (G′) and loss modulus (G″) were monitored during the gelling process with increasing temperatures.

7.3.7. Cooking loss and proximate analysis

The weight of each sausage was measured before and after cooking to determine the cooking loss, which was defined as: (uncooked weight – cooked weight)/uncooked weight × 100%. Proximate analysis was performed on sausages to determine their specific composition. A HFT-2000 digital fat analyzer (Data Support Co., Inc., Encino, CA, USA) was used to determine the fat and moisture content (%). The crude protein concentration was determined by a nitrogen analyzer (Elementar Americas Inc, Mt Laurel, NJ, USA).

7.3.8. Textural profile analysis

Before analysis, cooked sausage links were equilibrated at room temperature for 2 h. The discs (thickness: 19 mm) were carefully cut from the center of the sausage (diameter: 19 mm) with a sharp knife. Breaking strength and compression analysis were

performed using two sets of samples. To determine the breaking strength, 19 mm-thick sausage samples were compressed between two parallel plates on a universal testing system (Instron, Norwood, MA, USA) until the structure was ruptured, a 1000 N load cell was used at a crosshead speed of 50 mm/min. For the compression test, the samples were compressed to 80% of the original height in two consecutive cycles at a crosshead speed of 50 mm/min. As described by (Xiong, Noel, & Moody, 1999), force of the first compression peak was noted as F1, force of the second compression peak was noted as F2. Hardness = F1; Deformability = (F1/F2) ×100/F1; Cohesiveness = $(F2/F1)^2$.

7.3.9. Assessment of lipid oxidation

Cooked sausages were placed on white polystyrene trays and overwrapped with a polyvinylchloride film then stored in the dark in a 4 °C refrigerator. TBARS were measured to evaluate lipid oxidation on days 1, 3, 5, 7, 10, and 14 of storage as described by (Wang & Xiong, 2005). Briefly, cooked sausages samples (3–5 g) were mixed into the assay solution containing trichloroacetic acid (TCA) and thiobarbituric acid (TBA), followed by boiling for 30 min. The supernatant of the cooled solution was treated with chloroform and then petroleum ether (each centrifuged at 2000 g for 15 min). The absorbance of the aqueous phase containing the pinkish TBA−malonaldehyde adducts was measured at 532 nm, and the molar extinction coefficient of 152 000 M^{-1} cm^{-1} was used to calculate the TBARS content.

7.3.10. Scanning electron microscopy (SEM)

The morphology of fresh sausages was determined using a JMS-6360 LV (Hitachi Corp., Tokyo, Japan). The center of sausages were cut into pieces (size of 3×3×2 mm) and immersed in 0.1 M phosphate buffer (pH 7.0) containing 2.5% glutaraldehyde for 24 h at 4 °C. The fixed samples were rinsed with 0.1M phosphate buffer for three times (10 min per time). They were then post-fixed by 1% osmium tetroxide for 4 h and rinsed for another three times. The post-fixed samples were dehydrated in graded solutions of 50, 60, 70, 80, 90, 95, and 100% ethanol for 10 min per solution. Acetone solution (10 min) was used for further dehydration. The dried samples were mounted on an aluminum holder with a conductive carbon disk and then coated with gold in an ion sputtering apparatus. The specimens were examined and photographed with a scanning electron microscope at a voltage of 15 kV.

7.3.11. Statistical analysis

Data were collected from three independent experimental trials (replicates). All analyses were performed on triplicate samples (n = 3), and the results were subjected to the analysis of variance using the procedure of the general linear model of the Statistix software 9.0 (Analytical Software, Tallahassee, FL). All pairwise multiple comparisons [least significant difference (LSD)] were used to identify significant differences ($P < 0.05$) between means.

7.4. Results and discussion

7.4.1. pH and color of fresh raw batter

The pH value in the control raw batter was lower ($P < 0.05$) than any of the modified products (Table 7.1). It may result from the fact that the pork fat used in the control formulation had a lower pH than the O/W emulsions (pH 6.25). The differences in pH among the modified sausages were very small ($P > 0.05$). The fat-replacement batters had a different color profile than the control (all pork fat). In general, they had higher L^* value and lower a^*/ b^* values ($P < 0.05$). After incubation for 16 h, there were minor changes in L^*, a^*, and b^* values, but the trend remained the same.

7.4.2. Rheology of meat batters upon cooking

The changes in dynamic viscoelastic properties of sausage batters during thermal gelation were continuously monitored by the storage modulus (G′) and loss modulus (G″). The G′, which describes the elastic property of a gelling system, began to increase at approximately 48 °C, and then rose steadily upon subsequent heating for all formulations (Figure 7.1A), as the sausage sol was converted to a gel. The G′ of PDSPI sausage batter reached its peak first at 60 °C and then slowly decreased. Heated SPI could affect the macroscopic changes of meat emulsions during heating (Feng, Xiong, & Mikel, 2003) due to the increase of surface hydrophobicity. When temperature further increased, fat sausage batter attained its topmost G′ at approximately 67 °C. As fat became completely liquefied at higher temperatures, the G′ began to fall. The fat replacement by SC emulsion presents the highest G′ values (after 60 °C), contributing to the formation of a more cohesive and elastic structure. The oil droplets interacted with SC in the development of the sausage gel networks and gave a homogeneous distribution of fat globules in sausages. Modifications in the meat protein conformation have been found to

happen in different temperature ranges. Myosin, the main component involved in the meat emulsion gelation process, from 54° C – 58° C as observed by the sudden increase in G′. Further increases in G′ with heating may result from a network formation by aggregation and entanglement of unfolded protein molecules through nonspecific hydrophobic and sulfhydryl-disulfide interactions (Ferris, Sandoval, Barreiro, Sánchez, & Müller, 2009). Also, contribution from the connective tissue proteins in increasing G′ during heating should be acknowledged. At temperatures between 60 °C and 70 °C, the collagen and muscle fibers are longitudinally shrunk (up to a fourth of their original length if unrestricted) (Siripurapu, Mittal, & Blaisdell, 1987; Tornberg, 2005) as would be expected in a high moisture environment such as the meat emulsion considered here (Markowski, Bialobrzewski, Cierach, & Paulo, 2004).

Towards the end of heating stage (around 80 °C), a slight decrease in G′ can be observed in Figure 7.1A, which may be attributed to the softening of connective tissue and collagen at this stage. In meat emulsion product, it was observed that collagen may dissolve and transform into gelatin if fibers are not stabilized through heat-stable intermolecular bonds (Siripurapu, Mittal, & Blaisdell, 1987). Additionally, muscle fibers hardening may also occur during this late heating, and the occurrence of one or the other would depend on processing conditions upon heating (Ferris, Sandoval, Barreiro, Sánchez, & Müller, 2009).

The viscous properties, suggested by G″ (Figure 7.1B), followed a similar but more obvious trend as G′. At pre-gel low temperatures, the fat sausage batter was expected to be partially solidified. Thus, it exerted a greater resistance against shear (higher G″ at the beginning).

7.4.3. Cooking loss and proximate analysis of sausages

The modified sausages had a preferred effect on cooking loss or processing yield compared to the control sausage, characterized by 1.5% to 3% less cooking losses than the control (Table 7.2). It indicated good thermal behaviors in the meat systems. SC or heated SPI binds fat and water in an emulsion system, thereby increasing yield and reducing shrinkage, while contributing high-quality protein (Pietrasik & Jarmoluk, 2003). Replacement of pork fat by O/W emulsion improved emulsion stability and presented good fat and water binding properties of the meat matrix (Jiménez-Colmenero, Herrero, Pintado, Solas, & Ruiz-Capillas, 2010). Similar results have been observed in other studies. Lee, Jung, Lee, Kim, Lee, and Choi (2015) reported that vegetable oil replacement significantly ($P < 0.05$) increased WHC and decreased cooking loss in emulsion-type pork sausages. Youssef and Barbut (2011) found that substituting beef fat with pre-emulsified canola oil had a positive effect on cooking yield and other textural parameters. It suggested that pre-emulsification added to meat batters could help produce a stable meat matrix.

Control and modified sausages presented similar composition in proximate analysis (Table 7.2). There were no significant differences ($P > 0.05$) in the fat, moisture, and protein content of the different formulations. The pork fat in lean meat was approximately 6%, and 10% vegetable oil in emulsion was added to the sausage batters. It resulted in almost 10% lipid in modified (cooked) sausages; pre-emulsified oil constituted approximatley 60% of their lipid content. In regard to the fatty acid profile, this means a considerable increase in the proportion of unsaturated fatty acids. A study

conducted by Kang and Park (2007) reported that the total cholesterol content in pork fat is 81.45 mg/100 g. Thus, fat substitution by vegetable oils in emulsion-type pork sausages also assists reducing cholesterol content.

7.4.4. pH and color of cooked sausages

The pH of uncooked sausage batters was lower than that found for cooked sausages (Table 7.1). During heating, the pH of sausages increased as a result of the release of imidazolium, a basic group, from the amino acid histidine (Choi, et al., 2009; Kim et al., 2010). And there is no significant difference ($P > 0.05$) between the control and fat replacement sausages during storage (Figure 7.3). The consistent pH during storage (pH 6.0 – 6.2) also suggested the absence of lactic acid bacteria spoilage (Korkeala, Alanko, Mäkelä, & Lindroth, 1990).

Sausage color was affected by formulation (Figure 7.2). Control sample (100% fat) had the lowest L^* value ($P < 0.05$, Figure 7.2A). Bishop, Olson, and Knipe (1993) observed that pre-emulsifying fats increased lightness and reduced redness in bologna. However, a^* value of the control sausage decreased rapidly during storage while the fat replacement with pre-emulsified oil helped maintain the redness (Figure 7.2B). From Figure 7.2C, the control sausage possessed a higher b^* value compared to fat replacement sausages. Similar results are also observed by Muguerza, Fista, Ansorena, Astiasaran, and Bloukas, (2002): replacing 20% pork fat with olive oil produced lighter and more yellow sausages.

7.4.5. Texture profile of cooked sausages

The results of the comparative texture profile analysis of sausages in which pork fat was replaced with vegetable oil are shown in Figure 7.4. With similar fat content in cooked sausages, textural properties of sausages were not significantly ($P > 0.05$) affected by replacement of pork fat with O/W emulsion and by the type of emulsifier used in the emulsions, except a relative lower rupture force ($P < 0.05$) in PDSPI sausages at day 7. The fat content of sausage was closely related to the hardness of meat products; however, no effect of fat source was found for texture profile analysis traits of adhesiveness, cohesiveness, or resilience in fresh sausage (Baer & Dilger, 2014). Generally, the hardness and rupture force of all sausages increased ($P < 0.05$) while their deformability and cohesiveness remained the same ($P > 0.05$) during storage. That may arise from the loss of moisture during storage.

7.4.6. Lipid oxidation in cooked sausages

One of the potential problems associated with healthier lipid formulations in meat products is their influence on the rate and extent of lipid oxidation, as vegetable oil increases the concentrations of unsaturated fatty acids in their lipid profile. Lipid oxidation products may lead to the development of unpleasant flavors and aromas in sausages (Bhattacharyya, Sinhamahapatra, & Biswas, 2007), and could be detected by TBARS values (Figure 7.5).

Lipid oxidation was affected ($P < 0.05$) by the formulation and storage (Figure 7.5). TBARS values were similar between the reformulated samples and control at the onset of storage. During 14 days of storage, TBARS value of fresh sausage made with PDSPI emulsion increased at a slower rate, compared with the control ($P < 0.05$). And

interestingly, the sausages made with SC emulsion had less lipid oxidation ($P < 0.05$) at the beginning (1 – 3 days) but its TBARS value increased rapidly from 3 to 10 days, resulting in a higher lipid oxidation extent by the end of storage. The phenomenon in the present experiment could be implicated by several factors. According to Gordon and Barbut (1990), protein becomes a coat surrounding the fat globules during chopping, and the formed protein matrix aids fat stabilization during storage. In contrast, the pre-emulsified oil stabilized by PDSPI or SC with a smaller particle size created a better homogeneous matrix structure. PDSPI or SC entangled with meat protein that adhered to the surface of oil droplets could physically prevent lipid from oxidation. The protective effect of SC and PDSPI against lipid oxidation in O/W emulsions also derived from a combination of free radical scavenging and metal chelation (Faraji, McClements, & Decker, 2004). It has been reported that PDSPI has greater oxidative stability than SC, since other antioxidants associated with SPI (such as the isoflavone) could also be effective peroxyl radical scavengers (Faraji, McClements, & Decker, 2004). This would help to explain the lower rate of lipid oxidation in PDSPI sausages as compared to SC sausages (Figure 7.5). Similar results are also observed by Delgado-Pando, Cofrades, Ruiz-Capillas, Solas, Triki, and Jiménez-Colmenero (2011) and Bloukas, Paneras, and Fournitzis (1997) who have found that up to 20% of pork backfat can be replaced by olive oil in the form of pre-emulsified fat with SPI without negatively affecting the quality characteristics of dry fermented sausages (Bloukas, Paneras, & Fournitzis 1997). In addition, the natural presence of various antioxidant substances (tocopherols, phenolic compounds) in vegetable oils may also help to buffer the oxidative susceptibility of their unsaturated fatty acids (Bishop, Olson, & Knipe, 1993).

7.4.7. Microstructure of cooked sausage

The morphology of the control sausage at Day 1 showed a sponge-like appearance with rough protein coats (Figure 7.6). Compared with the control, SC and PDSPI samples (Day 1) present a denser structure with very few cavities in the nature of the protein matrix (Figure 7.6). When SC or PDSPI emulsions were incorporated into meat batters during chopping, it promoted protein–protein interactions and uniform distribution of fat globules, with smaller and closely packed fat globules well embedded in a relative smooth protein matrix.

After stored in the refrigerator approximately 10 days, the matrix generally becomes disorganized and loses some of its spongy appearance (Figure 7.6). This is particularly apparent in the control; its microstructure lost continuity and exhibited irregular formation. The clear morphological difference most likely resulted from protein aggregation. In addition, the microstructure of the control showed larger cavities compared with other samples. The formation of these cavities may have been due to expansion of a number of constituents, mainly fat, water, or air (Jiménez-Colmenero, Herrero, Pintado, Solas, & Ruiz-Capillas, 2010). The difference could also be perceived by the visual look of the cross section of the sausages at 10 days (Figure 7.7). A looser structure and more cavities (at the cross section area) were observed in the control compared with the others. And the hue of the control appeared more grey and yellow while the others maintained a pinkish color. It was also proven by Chen et al. (2015) that chicken plasma protein pre-emulsified oil could form a more homogeneous distribution

of fat globules in frankfurters when it was used as a stabilizer in pre-emulsified fat than when it was added as a powder additive.

Since all the sausages had very similar compositions (Table 7.2), the morphology of the sausages indicates that in these comminuted systems the characteristics of the continuous protein matrix and the fat globules are affected by fat replacement in the product formulation. And the difference reflected in the morphological characteristics of the sausages often relates to their textural properties and has a clear effect on the release of fat and water during cooking (Jiménez-Colmenero, Herrero, Pintado, Solas, & Ruiz-Capillas, 2010). The roughly coated fat globules were prevalent in the unstable fat batters (the control). Gordon and Barbut (1990) suggested that the interfacial protein film at the interface of fat globules has pores, and fat could exude from the pores, especially when the protein coats were rough. While in O/W emulsion systems, thread-like protein strands partitioned and immobilized the fat globules by physical binding of their interfacial protein film to the protein matrix. This complex, multilayered structure formed by protein emulsifier could prevent fat separation in meat batters.

7.5. Conclusion

In summary, the gel/emulsion systems in fat replacement sausages increased the processing yield; and the rheology curve suggested good thermal behavior in meat systems. The substitution of pork fat with O/W emulsion improved emulsion stability, providing good fat and water binding properties. The results indicate that a significant reduction in lipid oxidation and cooking loss could be achieved by using protein-stabilized emulsions as partial substitutes for fat in fresh sausages without adversely

affecting the product quality. The protein membrane at the interface of oil droplets decreases oil separation and inhibits lipid oxidation in fat-replacement sausage products.

Table 7.1. pH and color of raw sausage batters.

Raw batter	pH	0 h			16 h		
		*L**	*a**	*b**	*L**	*a**	*b**
Control	5.77 ± 0.01[b]	66.11 ± 0.70[b]	18.14 ± 0.25[a]	12.54 ± 0.11[a]	64.68 ± 0.30[c]	18.52 ± 0.17[a]	12.35 ± 0.13[a]
SC	5.83 ± 0.02[a]	76.49 ± 1.17[a]	14.72 ± 0.27[b]	11.18 ± 0.20[c]	75.46 ± 0.81[a]	14.55 ± 0.22[b]	11.19 ± 0.12[c]
PDSPI	5.84 ± 0.02[a]	75.51 ± 0.09[a]	14.24 ± 0.10[c]	11.53 ± 0.11[b]	73.99 ± 0.84[b]	14.16 ± 0.20[c]	11.56 ± 0.10[b]

[a–c] Means ± standard deviations between different formulations with different lowercase letters differ significantly ($P < 0.05$).

Table 7.2. Proximate composition and cooking loss in cooked sausages.

Content	Fat	Moisture	Protein	Cooking loss
Control (100% fat)	9.25 ± 0.41^{a}	71.12 ± 0.33^{a}	18.04 ± 1.35^{a}	32.09 ± 1.47^{a}
SC (60% oil/40% fat)	9.17 ± 1.17^{a}	71.19 ± 0.92^{a}	17.86 ± 0.47^{a}	30.59 ± 1.00^{b}
PDSPI (60% oil/40% fat)	9.39 ± 0.62^{a}	71.01 ± 0.48^{a}	18.50 ± 0.36^{a}	29.45 ± 1.95^{b}

$^{a-c}$ Means ± standard deviations between different formulations with different lowercase letters differ significantly ($P < 0.05$).

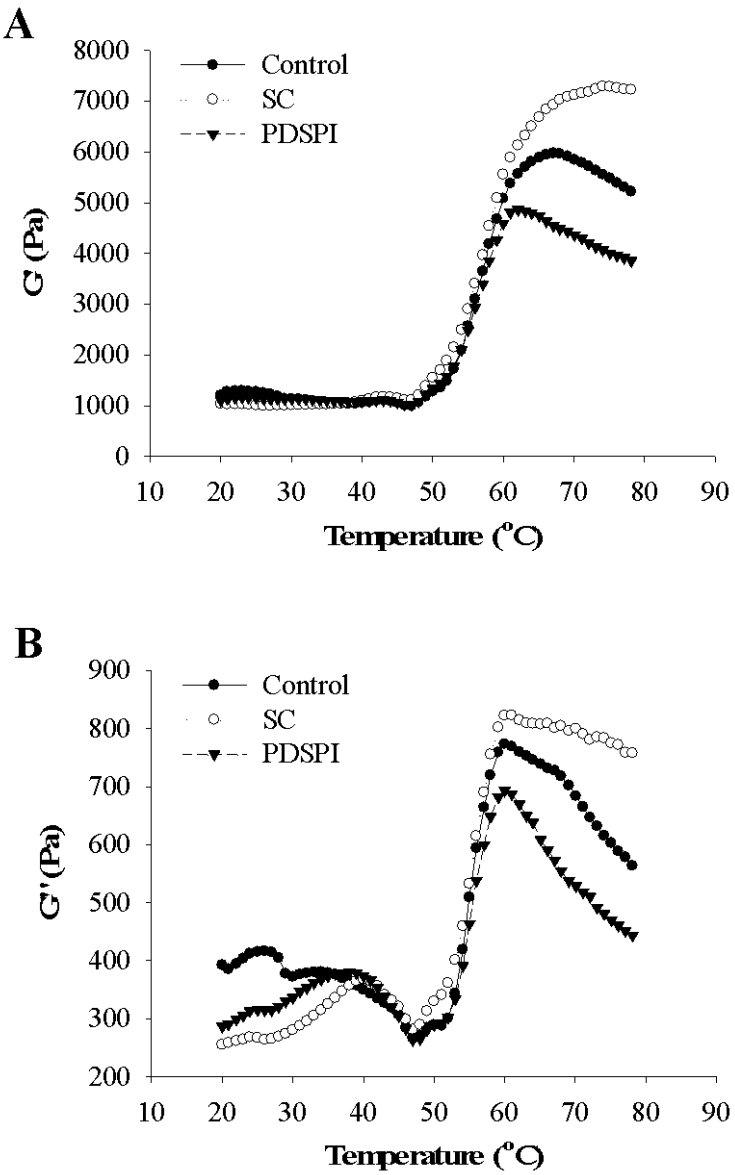


Figure 7.1. Dynamic rheological properties (**A**: storage modulus, G', and **B**: loss modulus, G") of different sausage batters.

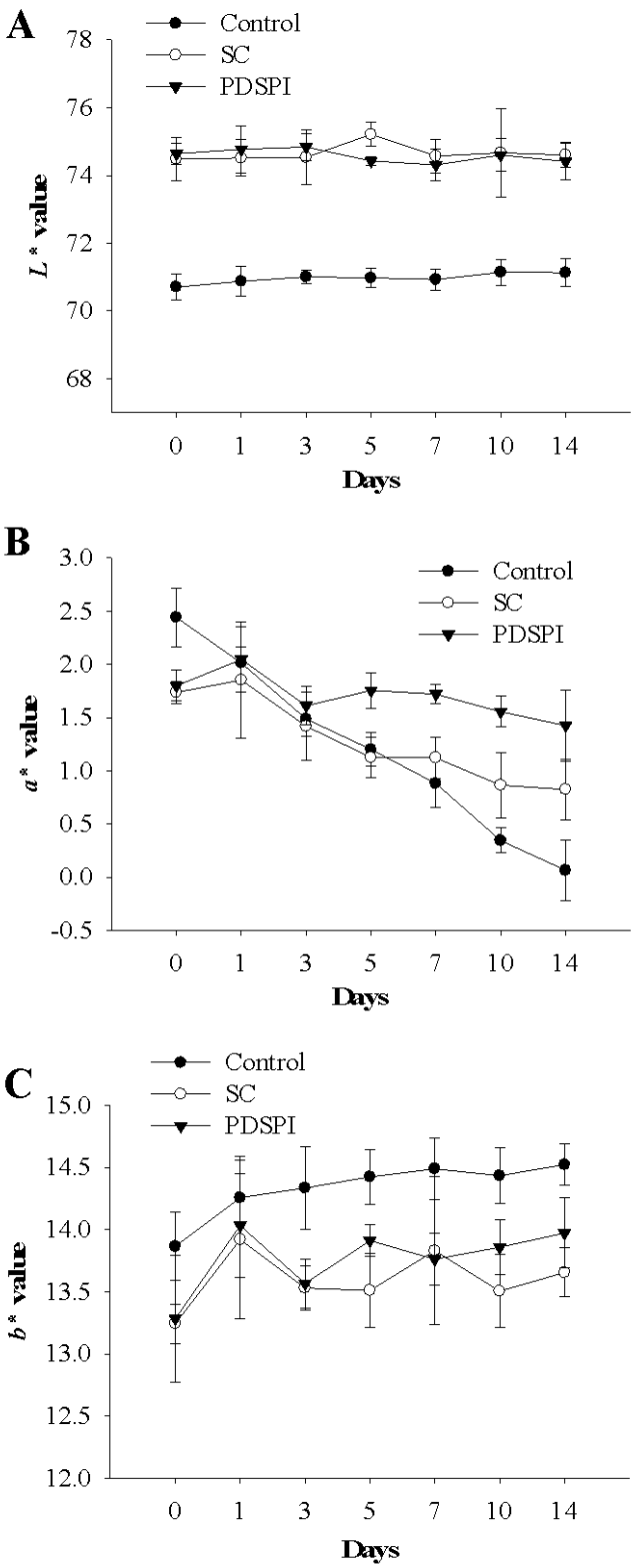


Figure 7.2. Color measurement (**A**: *L** value, **B**: *a** value, **C**: *b** value) of cooked sausages stored at 4 °C for 14 days.

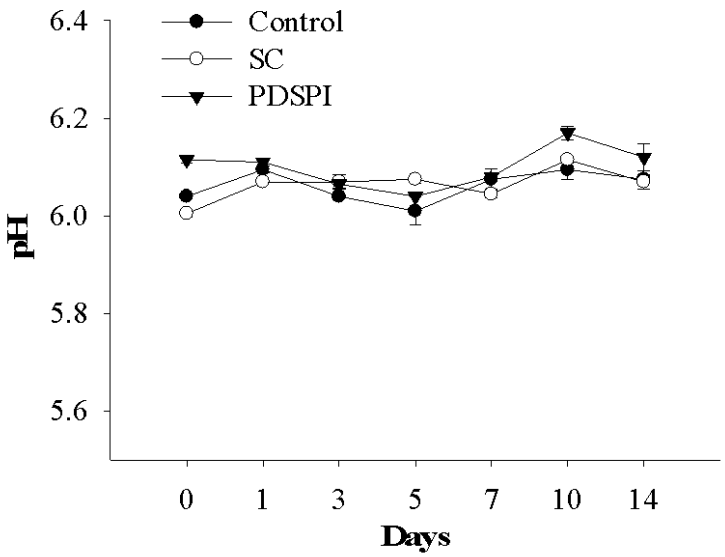


Figure 7.3. pH of cooked sausages stored at 4 °C for 14 days.

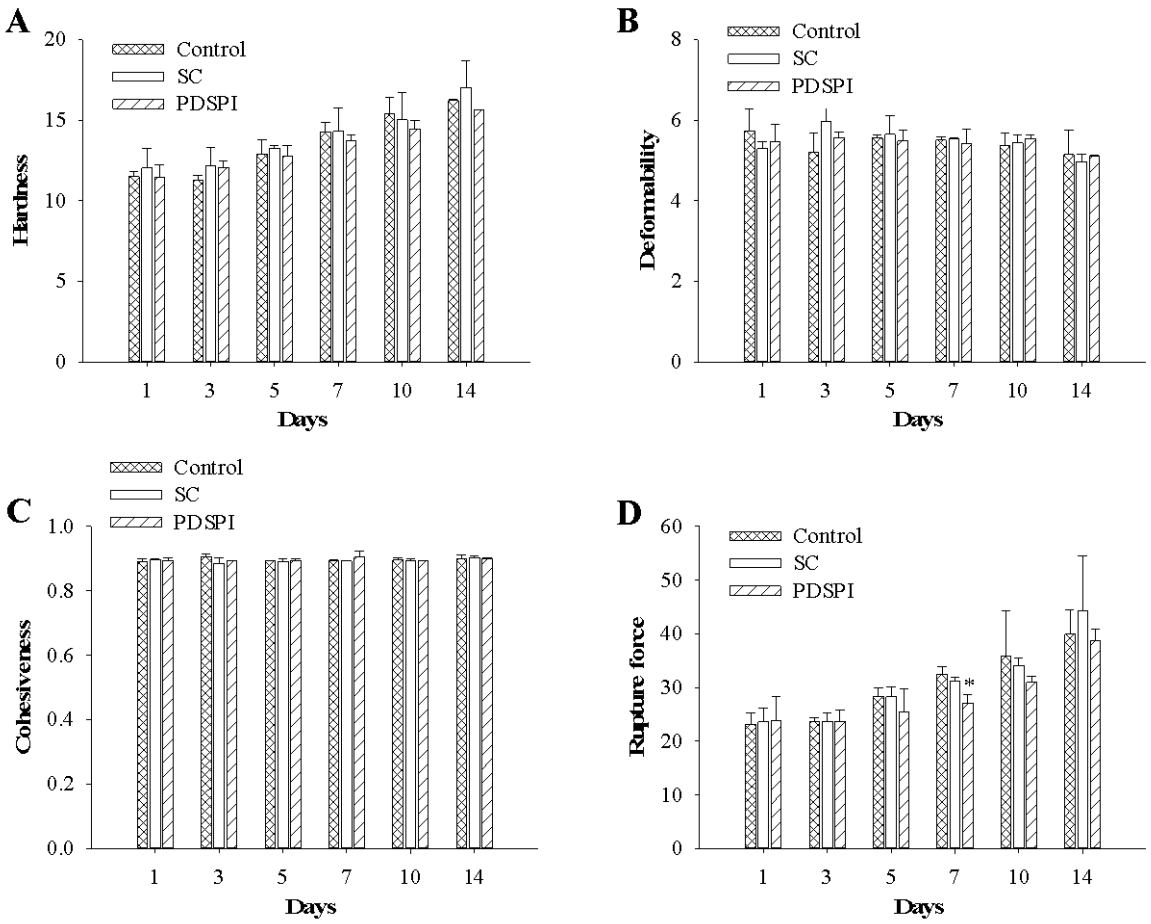


Figure 7.4. Texture profile analysis (**A**: Hardness, **B**: Deformability, **C**: Cohesiveness, **D**: Rupture force) of cooked sausages stored at 4 °C for 14 days.

* Means within the same day between different formulas denoted by * differ significantly ($P < 0.05$).

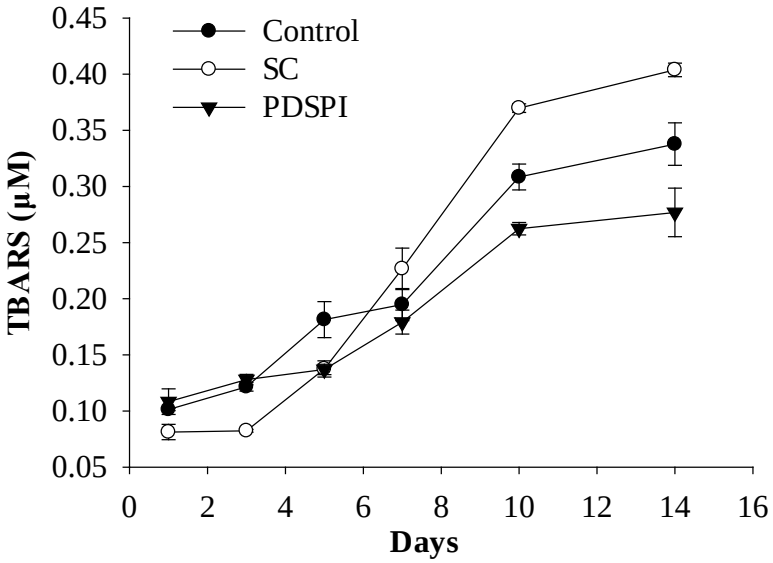


Figure 7.5. Thiobarbituric acid-reactive substances (TBARS) of cooked sausages stored at 4 °C for 14 days.

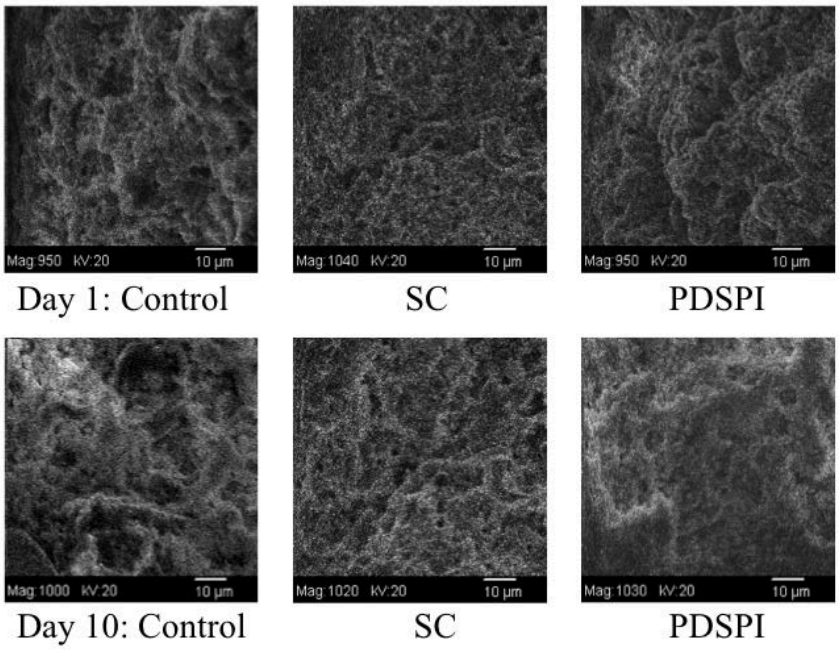


Figure 7.6. Morphology of cooked sausages at Day 1/Day 10 captured by scanning electron microscopy (SEM).

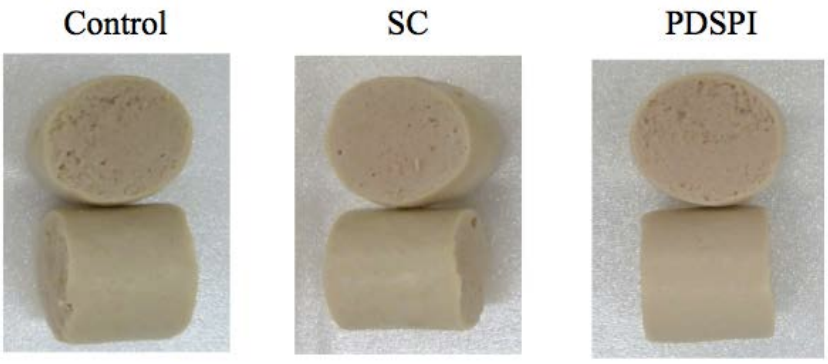


Figure 7.7. Appearance of cooked sausages stored at 4 °C for 10 days.

CHAPTER 8

OVERALL CONCLUSIONS

In conclusion, the reactivity of lipid and protein to oxidizing agents depended on a number of intrinsic and extrinsic factors, including the chemical structure of lipid and protein, lipid:protein ratio, and their physical distribution in aqueous solutions. In general, myofibrillar protein (MFP), regardless of its location and concentration, was a kinetically preferred target of free radicals compared to lipids. Presumably, as a favorite target of the hydroxyl radical, MFP was consequentially oxidized, thus, protecting lipids.

The lipid:protein ratio modulated the extent of lipid and protein oxidation. In mixed dispersion systems, a higher MFP concentration was associated with a slower hydroperoxide and TBARS production, suggesting an antioxidant role of MFP in protecting lipids. Conversely, by virtue of susceptibility, proteins in dispersion systems with abundant highly unsaturated fatty acids (e.g. C18:3 vs. C18:1) tended to be less oxidatively modified.

The physical location of the MFP within complex emulsion systems dictated the protein's antioxidative efficacy: by adsorbing on the surface of fat globules, MFP formed a cohesive interfacial membrane which imparted steric protection. This, combined with the radical scavenging effect, gave rise to a higher oxidative stability of emulsified lipids. Because MFP, sodium caseinate (SC), and soy protein isolate (SPI) as emulsifiers all decreased lipid oxidation in oil-in-water emulsions, there appears to be a universal steric function of proteins in maintaining the oxidative stability of such a bi-phasic system.

While membrane protein consistently demonstrated an antioxidative effect, the MFP present in the continuous phase exhibited a rather complex behavior. It is possible that unadsorbed MFP was able to spare lipids when subjected to oxidative attack. This is presumably due to the strong radical scavenging and metal chelation capability of protein. However, the accumulation of protein oxidation products appeared to stimulate lipid oxidation (TBARS formation) at the later stage. On the other hand, membrane-forming MFP was more prone to hydroxyl radicals than continuous-phase MFP due to its proximity and vicinity to the lipid core.

The remarkable protection of lipid against oxidation by interfacial proteins is further evidenced by the *in situ* model frankfurter validation study: cooked sausage formulated with partially denatured SPI- or SC-emulsified vegetable oil had an improved oxidative shelf-life and the textural properties were not affected by the formulation treatments. Future research is needed to study the susceptibility of lipid and protein to other reactive oxygen species (e.g., singlet oxygen, peroxyl radicals, hydrogen peroxide); and to develop antioxidant ingredients (protein antioxidant vs. lipid antioxidant) and processing technologies in order to manipulate oxidation, storage, and processing conditions for flavor and texture purposes.

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