INTERACTIONS OF 4-OXO-2-NONENAL (4-ONE) WITH BOVINE SKELETAL MUSCLE PROTEINS

by

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(Under the Direction of Anand Mohan)

ABSTRACT

Lipids are an important component of meat as they provide desirable sensory characteristics and nutritional benefits. However, lipids are susceptible to degradation through oxidation. Lipid oxidation contributes to meat quality deterioration, as well as produces toxic oxidative by-products. 4-oxo-2-nonenal (4-ONE) is an oxidative by-product that is highly reactive and cytotoxic. In this study, we investigated the influence of 4-ONE induced protein degradation on fresh and gastric digested bovine skeletal muscle proteins. Additionally, we developed an LC-MS/MS-based method to detect 4-ONE in meat samples. Results indicated that 4-ONE naturally forms in fresh muscle proteins. We report here for the first time that 4-ONE causes severe degradation of bovine skeletal muscle proteins. Future studies should assess the bioprotective role of endogenous enzymes, antioxidants, and other food ingredients for their potential to prevent the formation and/or detoxification of 4-ONE in meat.

INDEX WORDS: 4-oxo-2-nonenal, lipid oxidation, protein degradation, meat proteins

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DEDICATION

I would like to dedicate my thesis to my family and friends. Mom, thank you for always picking up my calls when I need extra love and for teaching me the importance of perseverance. Dad, thank you for challenging me to be my best self and for supporting me through all my endeavors. Aunt Marti, thank you for encouraging me to pursue a career in STEM and for your unconditional love. Brandon and Carly, thanks for always being my source of comfort and comedic relief in times of great stress, I love you both. And lastly, thank you to all my friends for always being there to pick me up and for being an amazing support system. Thank you everyone for all the love and support!

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CHAPTER 1

INTRODUCTION

Past decades have seen a considerable research effort on the role of lipid oxidation contributing to the degradation of muscle foods' quality and nutritional properties. A substantial effort has been focused on the direct influence of lipid oxidation damages to polyunsaturated fatty acids resulting in the formation of α, β-unsaturated aldehydes (Faustman et al., 2010; Ramanathan et al., 2012; Ramanathan et al., 2014). The α, β-unsaturated aldehydes have been linked with the oxidation of skeletal muscle proteins leading to meat quality deterioration (Gonzales et al., 2021; Ramanathan et al., 2014; Tatiyaborworntham et al., 2012). The majority of reactive aldehyde species originating from lipid oxidation are implicated in cellular pathologies capable of covalently modifying biological macromolecules (Omotayo et al., 2015). Additionally, aggressive reactive aldehydes, such as malondialdehyde, 4-hydroxy-2-nonenal (4-HNE), and 4-oxo-2-nonenal (4-ONE) have been reported as potent toxic species with a predisposition to cause chronic diseases (Bastide et al., 2015; Gonzales et al., 2021; Ramanathan et al., 2012; Van Hecke et al., 2015).

The accumulation of the lipid peroxidation by-product, 4-HNE or its derived adducts has been studied extensively in meat science research (Alderton et al., 2003; Faustman et al., 1999; Faustman et al., 2010; Gonzales et al., 2021; Lin et al., 2005; Naveena et al., 2010; Ramanathan et al., 2012; Ramanathan et al., 2014; Spiteller et al., 2001; Suman et al., 2006; Tatiyaborworntham et al., 2012). In meat, 4-HNE facilitates redox instability of myoglobin and inhibits glycolytic enzymes activity by covalent modification (Gonzales et al., 2021). Recent

studies have shown that 4-ONE has the potential to influence mitochondrial function and inhibit enzymatic functions (Gonzales et al., 2021; Picklo et al., 2011). Additionally, 4-ONE was depicted as more potent and aggressive than 4-HNE.

The chemical structure of 4-HNE and 4-ONE are similar with a functional group and electron localization difference, see Figure 1. 4-ONE contains a ketone functional group at the C-4 position that has been hypothesized to make the compound more reactive towards protein nucleophiles compared to 4-HNE. The ketone at the C-4 position on 4-ONE acts as an additional target for nucleophiles. Also, 4-ONE does not contain a hydroxyl group so the 4-ONE adduct cannot cyclize and may contain two free carbonyl groups, which are reactive toward the primary amines (Feeney et al., 1975). 4-ONE is known as a potent modifier of biomolecules and is more toxic than 4-HNE (Spiteller et al., 2001).

Research has shown 4-ONE to be highly cytotoxic and more reactive than 4-HNE (Doorn et al., 2006; Lin et al., 2005; Long et al., 2013). 4-HNE has the potential to migrate from the site of origin to other intracellular sites, and form 4-HNE protein adducts resulting in human disease (Smathers et al., 2012). Lee and Blair *et al*. (2000) showed that the 4-ONE is a breakdown product of lipid hydroperoxides. During the condition of oxidative stress, polyunsaturated fatty acids are oxidized to generate electrophilic aldehydes, which bind to proteins and polynucleotides, leading to cell death (Lee & Blair, 2000). Furthermore, reactions of 4-ONE with the side chains of Histidine, Lysine, and Cysteine were investigated in the absence of oxygen. The results indicated that there was very little protein-protein cross-linking for 4-ONE in the absence of oxygen. However, in the presence of oxygen, cross-linking was detected. These findings confirm that 4-ONE has a biological propensity of modifying proteins (Zhang et al., 2003). 4-ONE can cross-link α-synuclein molecules making a more stable oligomer, which is an

important compound found in Parkinson's disease (Näsström et al., 2009). Zhu *et al*. (2009) further studied 4-ONE induced cross-linking by focusing on specifically covalent cross-linking of glutathione and carnosine to proteins. 4-ONE was found to form cross-links between Cystine-Lysine and Histidine-Lysine pyrrole (Zhu et al., 2009). Although the mechanisms of 4-ONE's toxicity have yet to be studied, there is evidence that 4-ONE rapidly forms Schiff bases on proteins found in human tissues cells (Lin et al., 2005).

Recent studies in our laboratory have shown that 4-ONE is a potent, highly toxic and reactive chemical species in fresh meat (Gonzales et al., 2021). Our results showed evidence of severe vacuolization and rupturing of mitochondrial membranes following the incubation of myoglobin with 4-ONE. These findings support the claim that 4-ONE disrupts mitochondrial functions, inhibits lactate dehydrogenase activity, and induces myoglobin redox instability (Gonzales et al., 2021). It is critical to understand the mechanics of 4-ONE mediated protein oxidation in meat to understand the potential health implications of dietary oxidized meat proteins.

The objective of this study was to investigate the interactions of 4-ONE with bovine skeletal muscle proteins. Specific objectives were to assess 4-ONE induced protein degradation in a controlled modeled system with fresh *Psoas major* (PM), as well as with cooked and digested PM (experiment 1). Furthermore, isolated and detected naturally formed 4-ONE using LC-MS/MS in fresh PM (experiment 2). The lack of research focusing on the role of 4-ONE in mediated protein degradation provided the opportunity to expand our knowledge of the molecular interaction between dietary meat proteins and 4-ONE. Additionally, simulated gastric digestion was used in this study to gain a better understanding of the effects of 4-ONE induced protein degradation following cooking and digestion. Degradation of meat proteins induced by 4-

ONE may have pathological consequences in the gut and on internal organs upon intestinal uptake. The detrimental effects of the 4-ONE induced oxidation of meat proteins may further implicate aggressive oxidative reactions and a potential rise in human health disorders (Van Hecke et al., 2014).

CHAPTER 2

LITERATURE REVIEW

2.1 Meat quality

Meat is a nutritious component of the human diet. Throughout history, humans have been consuming meat due to its nutritional benefits. In the U.S. and other developed counties, meat comprises a large portion of the average human diet, contributing to more than 15% of total daily caloric intake, 40% of daily protein intake, and 20% of daily fat intake (Daniel et al., 2011). Meat consumption is projected to increase between 2022 and 2024 due to the population growth and rising incomes in developed countries (Whitnall & Pitts, 2019). Throughout history, humans have been consuming meat due to its nutritional benefits. Meat provides an important source of protein, omega-3 fatty acids, and other nutrients such as vitamin B_{12}, high levels of iron, etc. (Kausar et al., 2019). These nutritive qualities help supply energy, build body tissue, regulate vital processes, as well as aids with additional bodily functions. Furthermore, many food cultures include meat consumption for traditional purposes. Meat's distinct flavor profile and pleasing odors are often highlighted as the center of any meat-based dish. Due to the large and increasing consumption of meat around the world, it is helpful to understand the science behind the quality of meat.

2.2. Lipid oxidation in meat

Lipids can be defined as organic compounds comprised of fats and oils that yield high energy. Lipids include compounds such as triacylglycerols, fatty acids, glycerophospholipids, and sterols. They are also comprised of smaller building blocks such as fatty acids, glycerol, and

phosphoric acid (Kerth, 2013). In a standard meat sample, there is typically about 50-100mg/g of total lipids (Williams, 2007). Depending on the type of meat, the age of the animal, as well as other potential factors such as the animal's diet, there could be a large variation in total lipids and phospholipids (Kerth, 2013). Lipids are an important component of meat products as they are responsible for the desirable sensory characteristics of meats. Lipids provide the flavor and aromatic profile of meat, as well as contribute to juiciness. Additionally, lipids provide energy to the biological processes of the human body and are vital for proper human nutrition as they contain many essential fatty acids.

However, lipids are highly susceptible to degradation. Lipid oxidation is the main process that causes meat quality deterioration and is responsible for compromising meat's desirable sensory properties. Meat is susceptible to oxidation due to its high concentrations of unsaturated lipids, metal catalysts, pigments, vitamins, and oxidizing agents in the muscle tissue. Lipid oxidation in food systems can deteriorate the nutritive value and sensory quality of a food product, as well as pose potential health risks, and create analytical problems. Lipid oxidation is impacted by numerous internal and external factors including, processing techniques, water activity, temperature, the concentration of anti- and prooxidants, fatty acid composition, etc. During lipid oxidation, sensory degradation of the product occurs, as well as the nutritional loss that may lead to the formation of toxic substances. To prevent the oxidation reaction from occurring it is important to carefully control processes such as aging, processing, and storage of the meat (Love & Pearson, 1971). Most of the research on meat focuses on understanding the mechanisms behind lipid oxidation and methods to control the process.

In addition to changes in the sensory qualities of meat due to oxidation, multiple toxic compounds are produced during lipid oxidization. Several research studies have confirmed that

one of the largest problems associated with lipid oxidation is the generation of toxic compounds that have the potential to cause many health pathologies, including cancer, atherosclerosis, inflammation, and more. Angeli *et al.* (2017) concluded that lipid hydroperoxides formation can lead to cell cytotoxicity and DNA fragmentation which contributes to the accumulation of DNA lesions in colon cancer cells (Angeli et al., 2011). Several lipid oxidation by-products are responsible for producing rancid flavor and odors, as well as being highly reactive. Examples of these by-products are hexanal, propanal, malondialdehyde, and 4-hydroxynonenal (4-HNE). Additionally, the oxidation by-product, 4-oxo-2-nonenal (4-ONE) is a potent modifier of biochemical chemicals and is more toxic than 4-HNE (Spiteller et al., 2001). Considering that meat quality and nutrition are important contributors influencing consumers' food selection, lipid oxidation is an important research topic in the food industry.

2.2.1 Lipid Oxidation Mechanism

Lipid oxidation is a complex process, as many factors can affect lipid oxidation. Lipid oxidation in foods is initiated by free-radicals, lipoxygenase, and/or photooxidation (Kołakowska, 2003). Lipid oxidation can be described as a process where pro-oxidants (free radicals or nonradical species) react with polyunsaturated fatty acids to form a lipid radical. Pro-oxidants are chemicals that induce oxidative stress by inhibiting antioxidant systems, or by generating reactive oxygen species (ROS). ROS are highly reactive chemicals that have been formed from O_2. Examples of ROS include superoxide, hydroxyl radical, peroxides, and singlet oxygen. Autoxidation, a continuous free-radical chain reaction, is the main process by which lipid oxidation of meat occurs. The classical mechanism of lipid oxidation involves three stages: initiation, propagation, and termination, see Figure 2. During the initiation phase, the pro-oxidants abstract the allylic hydrogen from an unsaturated fatty acid, forming the carbon-

centered lipid radical. The propagation phase involves the lipid radical rapidly reacting with oxygen to form a lipid peroxy radical, which further reacts with another fatty acid to propagate a chain reaction (Yin et al., 2011). The propagation mechanism may occur up to 100 times before the termination phase occurs. The radical species formed during propagation may be stabilized into non-radical compounds. Furthermore, the peroxides that are formed as lipid oxidation primary products can undergo scission to form lower molecular weight volatile and nonvolatile compounds, known as secondary lipid oxidation products. Among these secondary by-products, aldehydes are one of the most abundant products found, such as 4-hydroxy-2-nonenal (4-HNE) and hexanal malondialdehyde (MDA) (Estévez, 2015). Lastly, during the termination phase, the high radical concentration causes two radicals to react and produce a non-radical species. Termination occurs when two radical species react with one another to form a non-radical adduct (Yin et al., 2011). However, lipid oxidation can often reoccur, as the non-radical species are likely to combine with another free radical and the oxidation cycle will continue. The oxidation reaction rate and activation energy of the reaction depends on the type of ROS initiator and the number of unsaturated bonds that are found in the substrate (Litwinienko et al., 1999). Additionally, free radical reactions can be terminated when a radical reacts with an antioxidant, such as Vitamin C and E. The hydrogen abstraction caused by the peroxide radical from the antioxidant molecule produces an antioxidant radical (Sikorski & Kolakowska, 2010).

Several methods can be useful for measuring lipid oxidation in a food system; these methods can be categorized as direct and indirect. The direct method measures the oxidation level based on the concentration of primary and secondary products that are present in the sample. The most common methods for determining the primary oxidation products are iodine value, diene conjugation measurement, and peroxide value. Furthermore, the thiobarbituric acid

relative substances (TBARS) test is the most common method for measuring secondary products. TBARS detects levels of malondialdehyde (MDA), the major lipid oxidation product as well as other minor related compounds. Additionally, High-Performance Lipid Chromatography (HPLC) and Gas Chromatography (GC) can also directly determine the concentration of MDA or other secondary lipid oxidation products. Indirect methods of lipid oxidation measurement in foods involve sensory evaluation, as well as fluorometric methods (Kerth, 2013).

2.2.2 Intrinsic Factors

The degradation process starts as the animal is sacrificed and then continues over time until the meat is consumed. Meat composition, specifically fatty acid composition, is one of the most important intrinsic factors affecting the development of lipid oxidation as fatty acids are the substrate of oxidation processes. Lipids in meat contain triglycerides and phospholipids, and the number of triglycerides is correlated with the amount of intramuscular fat while the amount of phospholipids is constant (Domínguez et al., 2019). Research has revealed that phospholipids are important in the initiation of the oxidation of lipids (Amaral et al., 2018). And the arrangement of lipids in the membrane facilitates the propagation phase of lipid oxidation (Erickson, 2002). Pikul *et al.* (1984) assessed the role of triglycerides and phospholipids on malondialdehyde formation, overall the results indicated that phospholipids contributed to about 90% of the malondialdehyde formation (Pikul et al., 1984).

Furthermore, the number of unsaturated fatty acids is correlated with the oxidation susceptibility of the food product. Polyunsaturated fatty acids are more prone to oxidation than monounsaturated fatty acids. This is because the removal of hydrogen from a carbon double bond requires less energy when compared to the energy required to remove hydrogen from a methyl carbon. The hydrogen that is bonded to this carbon can be easily removed, and thus lipid

oxidation is more likely to occur (Amaral et al., 2018). Although higher content of polyunsaturated fatty acids in meat can be considered desirable to consumers, it can affect the oxidative stability of meat.

Metal ions can also trigger lipid oxidation in meat by readily donating electrons, leading to increased production of free radicals (Amaral et al., 2018). The oxidative stability of meat is dependent on the amount of heme-protein present because oxidation of hemeproteins can result in rancidity, as well as the browning of meat during storage. Iron is the most likely catalyst for initiating lipid oxidation by generating most $OH^{\bullet}$ via the Fenton reaction (Min & Ahn, 2005). The Fenton reaction involves the oxidation of ferrous (Fe^{2+}) to the ferric form (Fe^{+3}) and the production of superoxide anion ($O_2^{\bullet -}$). The superoxide anion can react with unsaturated fatty acids to form an alkyl radicals and promote oxidation (Amaral et al., 2018). Furthermore, the Fenton reaction is the main mechanism for heme-protein oxidation. Several studies have found that lipid and heme-protein oxidation can occur simultaneously, and each process can enhance the other. The reactive oxygen species that are produced during oxy heme-protein oxidation can abstract hydrogen from the polyunsaturated fatty acid and further initiate lipid oxidation as well. Aldehydes produced during lipid oxidation can also alter the heme-protein redox stability, enhancing the oxy heme-protein prooxidant potential (Chaijan & Panpipat, 2017). Ferrous can further catalyze the propagation phase of oxidation by reacting with hydroperoxides and decomposing them into alkoxy and hydroxyl radicals that can then react with nearby molecules (Erickson, 2002).

There are also enzymes found naturally in meat that are found to initiate oxidation. Studies have shown that enzymatic systems that can reduce iron were found to generate active catalysts in the presence of polyunsaturated fatty lipids (Erickson, 2002). Lipoxygenase is the

main enzyme involved in the initiation phase of lipid oxidation, as it has the ability to abstract hydrogen from the allylic methylene position of polyunsaturated fatty acids, which results in a conjugated diene hydroperoxyl (Hui, 2006).

Additionally, there are antioxidant compounds found naturally in meat that can prevent oxidation and the actions of free radicals and lipid oxidation catalysts. There are three main endogenous antioxidant compounds: vitamins, peptides, and enzymes. α-Tocopherol has been proven to be a good source of an antioxidant vitamin. During oxidation, the ROS attacks the α-tocopherol quicker than the lipid, therefore the polyunsaturated fatty acid is protected from oxidation. The α-tocopherol then transfers a hydrogen atom to the lipid peroxy radicals and scavenges the peroxy radicals, which results in the formulation of the tocopheroxy radical (Choe & Min, 2006). Also, lipid-soluble vitamins such as carotenoids protect lipids from oxidation via scavenging peroxy radicals. The water-soluble vitamin, ascorbic acid acts as an endogenous antioxidant by restoring α-tocopherol from the tocopheroxy radical. Additionally ascorbic acid can scavenge oxygen and various radicals (Erickson, 2002).

Meat peptides can also be considered antioxidant compounds, as they increase the oxidative stability of meat (Sohaib et al., 2017). Majority of peptides that have been found to show antioxidant activity, have contained leucine and valine in the amino-terminal position. Additionally, these peptides contain histidine, proline, cysteine, tyrosine, tryptophan, and/or methionine in their amino acid sequence. Peptides containing tyrosine or tryptophan in the carbonyl-terminal position can scavenge radicals and some ROS (Lorenzo et al., 2017). Additionally, anserine and carnosine are found in high concentrations in meat and have been found to be abundant antioxidant endogenous dipeptides. These peptides have been found to chelate transition metals and scavenge peroxy radicals (Erickson, 2002). Additionally, anserine

and carnosine, among additional compounds such as lysine and histidine, sulphur/amine compounds, can trap aldehydes, reduce rancidity and act as catalysis of the heme-protein oxidation (Lorenzo et al., 2017). Glutathione is also another antioxidant peptide, which is capable of reducing reactive ferryl heme-protein to met-heme protein (Galaris et al., 1989)

Lastly, there are endogenous enzymes with antioxidant activity. Superoxide dismutase can remove $O_2^{\bullet -}$ and produce oxygen and H_2O_2, which can be eliminated by catalase (Richards, 2005). Additionally, the glutathione peroxide also prevents lipid oxidation by removing H_2O_2, resulting in water and oxidized glutathione (Chaijan & Panpipat, 2017).

2.2.3 Extrinsic Factors

There are various extrinsic factors that can influence the extent of lipid oxidation in muscle tissues. These extrinsic factors can be dependent on the amount of damage to the muscle tissues before slaughtering, such as stress, as well as post-slaughtering events such as pH, temperature, tenderizing techniques, and processing factors. One of most important factors that influences oxidation in raw and cooked meat is oxygen. Processing techniques such as grinding, mincing, and cooking can result in exposure of phospholipids to oxygen and other contaminants, as well as accelerate the development of rancidity (Min & Ahn, 2005). In cooked meats, it is essential to eliminate oxygen exposure post-cooking to minimize oxidation. Although, when cooked meat is stored in oxygen-depleted environments, lipid oxidation by-products may still be detected. The influence of modified atmospheric packaging (MAP) on lipid oxidation is later discussed in this chapter.

Furthermore, the interactions between substrates and oxidation catalysts are favored by temperature. The decomposition of hydroperoxides increases with an increase in temperature, therefore initiating the propagation phase of oxidation. Frozen meats can also experience

oxidation due to temperature fluctuations, which can result in the formation of ice crystals, increase cell disruption, and release prooxidant compounds (Erickson, 2002). Storage time also influences oxidation, as radicals can cause damage to lipids over long periods. The long storage time releases iron from hemeproteins, which catalyzes multiple reactions within the initiation and propagation phase of oxidation, as previously discussed. The rate at which the meat is thawed is important when it comes to influencing meat quality. Freezing at low temperatures causes less damage because the freezing process is faster, therefore the ice crystals formed are smaller. Also, a reduction in the thawing time results in less exudate and water holding capacity, decreasing the disruption of the muscle fiber structure (Leygonie et al., 2012).

Cooking can promote lipid oxidation in muscle food. Cooking-induced oxidation can be explained by heat-induced changes in muscle components, including the disruption of cellular compartmentalization, the deactivation of antioxidant enzymes, and the exposure of membranal lipids to a prooxidant environment (Schricker & Miller, 1983). Higher temperatures promote Maillard reaction products, which can inhibit oxidation due to their antioxidant activity (Zipser & Watts, 1961). After cooking, meat is stored at low temperatures (4 to -18°C) to minimize further oxidation from occurring. However, even at freezing temperatures, the oxidation rate of meat is reduced but not eliminated (Pikul & Bechtel, 1984).

2.3 Protein Oxidation

Protein oxidation is defined as the covalent modification of a protein native structure induced by reactive oxygen species (ROS) or through indirect reactions with secondary oxidative stress (Zhang et al., 2013). In protein oxidation, ROS reacts with the backbone and side chains of the protein, and causes modifications of the amino acid side chains, and fragmentation, polymerization, and aggregation (Soladoye et al., 2015). ROS are generated during normal

metabolic processes, such as mitochondrial electron transportation reactions, cytochrome P-450 reactions, and peroxisomal fatty acid metabolism. Other external factors can generate ROS such as, photosensitizers, ultraviolet, ozone, air pollution, and ionizing radiation, see Figure 3 (Xiong, 2000). In the muscle food system, myoglobin and hemoglobin act as pro-oxidant proteins and initiate oxidative reactions (Baron & Andersen, 2002).

The oxidation of proteins is known to play an important role in the pathogenesis of degenerative diseases; however, the mechanism of protein oxidation and its effects are largely unknown due to its complexity. The mechanism behind protein oxidation is believed to be similar to that of lipid oxidation, however, the oxidation of proteins involves a higher complexity of pathways and a larger variety of oxidation products (Davies, 1997). Due to the earlier onset of lipid oxidation, it is believed that lipid oxidation products promote protein oxidation. The oxidants directly attack the backbone of the protein, resulting in changes in the protein's secondary and tertiary conformations. For example, the lipid peroxidation by-products, malondialdehyde, and 4-HNE have been found to covalently bind to residues of proteins, resulting in protein oxidation (Requena et al., 1997).

One of the major effects of protein oxidation is the loss of essential amino acids. Protein carbonylation results in the irreversible modification of essential amino acids, like arginine, threonine, and lysine (Estévez et al., 2011; Stadtman & Levine, 2003). These changes lead to chemical changes that may compromise the availability and the metabolic activities of the affected amino acids. Depending on the oxidizing agent, protein oxidation can lead to the loss of sulfhydryl groups, the formation of protein carbonyls, modification of aromatic amino acids, formation of cross-links, among additional changes (Lund et al., 2011). The presence of carbonyl groups is often used as an indicator of ROS-mediated protein oxidation (Whittaker et al., 1997).

Protein carbonyl groups can form as a by-product of oxidation via peptide bond cleavage, ROS modification, or through the adduction of non-protein carbonyl units (Zhang et al., 2013). Carbonyl content is considered a standard marker of protein oxidation, as carbonylation occurs in most oxidized proteins. Carbonyl assays are used as a common method for estimating the extent of oxidative modification. There are additional methods to determine oxidation levels in foods such as HPLC-MS, which is used to quantify concentrations of specific oxidative by-products. Additionally, polymerization and/or fragmentation of oxidized proteins can be measured by SDS-PAGE analysis.

2.3.1 Protein oxidation in meat

The lipid composition of meat can influence the rate of protein oxidation in the muscle system. Skeletal muscle tissue contains high concentrations of endogenous catalysts such as, oxidizable lipids, oxidative enzymes, ferric heme pigments, and transition metal ions. Oxidizing agents that are present in meat can act as precursors or as catalysts for the production of ROS, as well as nonoxygen free radicals (Decker et al., 2000). Also, myosin is highly susceptible to free radical attack, as a brief exposure of myosin to $OH^{\bullet}$ results in a significant reduction in the thermal stability (temperature maxima and enthalpy) of myosin (Liu & Xiong, 2000).

Furthermore, several postmortem processing methods, such as grinding and cooking, have been studied in a variety of meat products and have been shown to contribute to the formation of oxidative species, promoting protein oxidation in muscle systems. Alterations in meat protein's structure can influence the physical and chemical properties of proteins including water-holding capacity, meat tenderness, hydrophobicity, solubility, and cellular functions. Calpain has been found to be the major enzyme involved in the degradation of muscle proteins, contributing to the decrease in water-holding capacity during postmortem aging and the

development of meat tenderness (Koohmaraie, 1992). Postmortem oxidation of calpain has been found to negatively impact fresh meat quality.

Meat products that are ground or minced are more prone to oxidation due to the breakdown of the meat proteins. The process of grinding increases the surface area of the protein and disrupts the cellular compartmentalization and antioxidative system. This process allows pro-oxidants to be in direct contact with proteins, making them more vulnerable to the attack of the ROS (Xiong, 2000). Additionally, temperature level and duration of heat treatment of the meat have been shown to affect oxidation. Heating meat above 60°C can trigger oxidative cleavage of the porphyrin ring, which results in the release of heme iron, leading to the promotion of protein and lipid oxidation (Miller et al., 1994). Protein oxidation in meat has been observed through various changes such as increased surface hydrophobicity and aggregation of the proteins, as well as increased carbonylation, carboxylation, and Schiff base formation (Sante-Lhoutellier et al., 2008). Garcia *et al.* (1996) reported the depletion of heme iron from 100% to 10% has been reported to occur during cooking, in addition to an increase in the non-heme iron (Garcia et al., 1996). Non-heme iron ions are critical for protein oxidation initiation in meat systems. Likewise, higher amounts of carbonyls were produced at high temperatures and increased cooking time; this may be caused by the release of iron and time/temperature's impact on the antioxidant defense mechanism in the meat systems. Food irradiation is the process by which food is exposed to ionizing radiation to reduce the number of harmful bacteria and parasites. Irradiation dose and rate can also affect the extent of protein oxidation in meat systems (Giroux & Lacroix, 1998). A 10kGy of irradiation in food has been found to result in biochemical changes, including the modification of protein structure, cross-linking, fragmentation, and aggregation of protein polypeptide chains. Irradiation also can trigger

radiolysis of water, initiating free radicals in the meat system (Giroux & Lacroix, 1998). Packaging strategies have been utilized in order to limit the effects of irradiation on meat protein oxidation (Henry 2009).

Studies have also reported an increase in protein oxidation with prolonged frozen storage of meat. The extent of protein oxidation in meat during frozen storage can be attributed to the postmortem processing methods applied to the meat, type of animal species, the metabolic profile of the muscle, length and temperature of storage, and the fat content of the animal (Bao et al., 2021). Qian *et al* (2021) found that myofibrillar protein oxidation decreased when storage temperature decreased from -1°C to -12°C. Also, protein denaturation was found to be greater at higher temperatures (Qian et al., 2021). Besides the effects of physical damage of beef during freezing/thawing, oxidative damage to myofibrillar proteins can also impact the water-holding capacity, as well as other desirable meat characteristics (Estévez et al., 2011).

2.3.2 Digestion of oxidized meat proteins

The nutritional quality of protein can be described by its metabolic function, which is mainly dependent on its constituent amino acids, the peptide sequence, spatial arrangement in its native structure, and its bioavailability and concentration (Evenepoel et al., 1998). For proteins to fulfill their required activities in the human body, it is important that they are in their proper structural and chemical state. If there are structural and conformational impairments of the proteins caused by oxidative damage, there may be scissions of the peptide sequence and amino acid side chain modification. These oxidative modifications have been found to alter the protein's structure and function, leading to protein conformational diseases (Xiong, 2000). Among the chemical modifications caused by protein oxidation, carbonylation and the formation

of protein cross-linking has been found to be the most influential on the loss of muscle protein functionality (Xiong, 2000).

During digestion, partially oxidized meat is further exposed to oxidation catalysts in the gastrointestinal tract (Rysman et al., 2016). The oxidative damages of meat can lead to the accumulation of harmful by-products, such as carbonyls. These by-products will be exposed to the gastrointestinal tract, increasing the concentration of oxidation products in the lumen. Research has found that diseases like Alzheimer's, chronic renal failure, and diabetes, among other diseases, are caused by the accumulation of protein carbonyls (Dalle-Donne et al., 2003). Research supports the impact of dietary oxidized proteins on the redox state of intestinal tissues, intestinal flora disturbance, and the onset of local pathological conditions (Keshavarzian et al., 2003). Investigating the impact of oxidized meat proteins during gastric digestion may allow us to understand the impact of oxidized meat proteins on nutrition and human health. Furthermore, the digestive breakdown of dietary oxidized meat proteins may be able to further induce or contribute to the generation and accumulation of reactive chemical species in contact with intestinal cells. Pierre *et al.* (2004) found evidence to support the impact of luminal oxidative stress on genotoxicity and cytotoxicity from the colonic mucosa (Pierre et al., 2004). Van Hecke *et al.* (2014) recently reported that an oxidized protein during digestion of red meat led to the formation of a genotoxic DNA adduct in a simulated colonic environment.

2.4 Methods for preventing oxidation in muscle foods

Various research studies have investigated different methods to prevent oxidation in muscle foods. Modified atmospheric packaging (MAP) is a widely used method for effective preservation of muscle foods. Furthermore, researchers have evaluated the impact of altering the animals' diet on the antioxidative status of the food. The addition of novel food ingredients

containing antioxidant properties have also been well-studied. This section will explore the various methods for preventing oxidation in muscle foods.

2.4.1 Packaging

The purpose of meat packaging is to extend the shelf-life and preserve consumer acceptability. Although there are packaging systems that have been effective at slowing oxidative reactions, some techniques still allow or enhance oxidative modification in the meat system. Modified atmospheric packaging (MAP) is commonly used for extending shelf-life of fresh meat. Consumers typically select meat based on appearance and choose meat that is bright red, as it indicates freshness (Berruga et al., 2005). MAP packaging, usually comprised of 70-80% O_2 and 20-30% CO_2, has provided new opportunities for extending the shelf-life of fresh beef. The purpose of adding oxygen is to maintain the desirable red color of the fresh meat. However, oxygen also is known to promote oxidation in fatty acids and cholesterol and can have detrimental effects on the meat quality (Church, 1994). Several studies have found that beef stored in high oxygen packaging develops premature browning at temperatures lower than expected (Warren et al., 1996). Additionally, MAP packaging has been shown to mitigate the protective effects of antioxidant components, as well as induce the loss of thiol groups and increase protein polymerization (Lund et al., 2007). Clausen *et al. (*2009) found that a packaging system containing high O_2 has a marked negative influence on various eating quality parameters such as meat flavor, juiciness, tenderness, cooking loss, α- tocopherol content, as well as cause an increase in protein and lipid oxidation by-products. The results indicated that tenderness and juiciness in the cooked meat were reduced, and the flavor appeared to be more intense with a "warmed-over/ oxidized taint" note (Clausen et al., 2009) .

In contrast, vacuum or 100% nitrogen packaging has been found to limit protein oxidation in meats. Antioxidant active packaging has been developed in order to mitigate the effects of lipid oxidation (Lund et al., 2007). Avoiding the use of oxygen in packaging, as well as the addition of ingredients with antioxidant properties could limit the detrimental effects of protein oxidation. The inclusion of natural antioxidants within packaging material can protect food from oxidation, resulting in an increased shelf-life. Contini *et al.* (2014) investigated the effects of packaging with a citrus extract coating on cooked turkey meat. The antioxidant active packaging led to a significant reduction in oxidation levels, as well as maintained the turkey's sensory properties, particularly tenderness and overall acceptability, during its storage period. Contini *et al.* (2014) findings reveal that packaging made with an antioxidant coating has the potential to reduce oxidation (Contini et al., 2014).

2.4.2 Modifying the animal's diet

Research has proven that the feeding regime of the animal and its antioxidant status play a large role in the oxidative stability of the food consumed. The muscle tissues of monogastric meat animals are representative of the fatty acid composition of the feed and can incorporate into the cell membranes specific substances that are found in the feeds, such as tocopherol. Muscle food oxidation is typically prevented by reducing the ratio of polyunsaturated fatty acids and fatty acids in animal feed and strategically supplementing the animal feeds with tocopherol or carotenoids. The dietary supplementation of tocopherols and carotenoids has been found to be an effective inhibitor of protein oxidation in muscle foods (Toldrá, 2002). Santé-Lhoutellier *et al.* (2004) found that the animal's diet had a significant effect on myofibrillar protein carbonylation. There was a lower level of protein carbonyls when lambs were fed a pasture diet, compared to being fed a concentrated diet. Additionally, a study on beef feed evaluated the effects of

tocopherol (vitamin E) supplementation in the animal diet. According to the study carried out by Rowe *et al.* (2004) tocopherol supplementation was shown to decrease the development of protein carbonyls in beef (Rowe et al., 2004).

Furthermore, Mercier *et al.* (2004) evaluated the effects of finishing diet on lipid and protein oxidation in beef homogenates. One group of cattle was fed exclusively grass (pasture group) and the other group was fed a diet containing cereals, silage, cattle-cake (mixed diet group). Overall, the study found that the finishing diet of cows had an important effect on the antioxidant status of meat, such as antioxidant enzyme activity and vitamin E content. According to the Thiobarbituric Acid Reactive Substance (TBA-RS) analysis, a method for detecting lipid oxidation, the homogenates of pasture-fed cows had a significantly lower TBA-RS value than mixed diet-fed cows. This indicates that there were lower levels of lipid oxidation in the pasture-fed animals. Similar results were found when they observed the effect diet plays on lipid oxidation in refrigerated meat storage in air. Overall, the pasture-finishing diet presented more advantages over the mixed diet-finishing in terms of lipid oxidation, due to the lower values of TBA-RS (Mercier et al., 2004).

2.4.3 Antioxidants

Natural antioxidants are added to the muscle in order to improve oxidative stability of meat products (Falowo et al., 2014). Food manufacturers use antioxidants to prevent the quality deterioration of their products. Antioxidants are substances that can delay or prevent oxidation in food systems when present at low concentrations. Antioxidants are widely distributed in animal tissue, plant material, and microorganisms; they protect organisms from oxidative stress. Examples of antioxidants are free radical scavengers, singlet oxygen, metal iron chelators, inactivators of reactive oxygen species (ROS), among others. There are various mechanisms

describing how antioxidants work against reactive oxygen species. Primary antioxidants, such as phenolic compounds, break the chain reaction of oxidation by scavenging free radical intermediates. These compounds are able to neutralize the free radicals by donating a hydrogen atom to one radical and receiving another radical to form stable non-radical products (Is & Woodside, 2001).

Secondary antioxidants prevent or slow the rate of oxidation by suppressing the oxidation promotors, including singlet oxygen, pro-oxidative enzymes, metal ions, and additional oxidants. Metal chelators such as ethylenediaminetetraacetic acid (EDTA), citric acid, and phosphoric acid can decrease the pro-oxidant effect of metal ions by forming a stable complex and reducing their redox potential. Some secondary antioxidants have the capability to regenerate primary antioxidants by replenishing the hydrogen atom. The effectiveness of antioxidants is influenced by various factors such as the concentration, temperature, oxidation substrate, structural features, the presence of pro-oxidants, etc. It is important that the antioxidant is used at the optimal temperature, otherwise, it may exhibit pro-oxidant effects when at higher temperatures (Cillard & Cillard, 1986). Also, the activity of the antioxidants is dependent on the reaction kinetics, which is the rate at which an antioxidant reacts with a specific radical.

A variety of plants that are rich in antioxidants have been previously used in frozen muscle foods as an antioxidants source. Turgut *et al.* (2017) evaluated the antioxidant effects of pomegranate peel extract on meatballs that were stored in a freezer. This study expands on a previous study where the antioxidant effects were evaluated during refrigerated storage. Both studies confirmed that pomegranate extract was effective at delaying protein oxidation by inhibiting the accumulation of carbonyls, as well as decelerating the adverse changes that occurred in sulfhydryl and the solubility of proteins. The pomegranate extract did not cause any

unfavorable changes to the meatballs, additionally, the color stability was found to have been improved through the addition of the extract. Overall, the addition of the pomegranate extract is an effective solution to preventing oxidation in refrigerated and frozen meat products (Turgut et al., 2017).

Furthermore, carnosine, a β-alanyl-L-histidine dipeptide with antioxidant properties, is found in high concentrations in skeletal muscle tissue. Nagasawa *et al.* (2001) evaluated the antioxidant activity of carnosine's stability on muscle protein and lipid oxidation using *in vivo* and *in vitro* model experiments. The results indicated that the concentration of muscle carnosine plays a protective role in the mitigation of muscle lipid and protein oxidation by-products under both *in vivo* and *in vitro* studies (Nagasawa et al., 2001). These findings are consistent with other research conducted by Kohen *et al.* (1988). This study revealed that carnosine scavenges peroxyl radicals and is an efficient chelating agent for copper and other transitional metals. Additionally, Kohen *et al.* (1988) found high concentrations of carnosine in skeletal muscle and the brain, which are the two tissues with the most active oxidative metabolism (Kohen et al., 1988). These findings suggest that carnosine may be a biologically significant antioxidant. Additionally, Decker *et al.* (1992) confirmed that carnosine successfully inhibited iron-catalyzed lipid oxidation without chelation, as well as inhibited nonmetal lipid oxidation catalysts (Decker et al., 1992). Overall, the addition of antioxidants to muscle foods has been found to reduce oxidation levels.

2.5 The lipid oxidation by-product (4-ONE)

Cellular oxidative stress on lipids can yield reactive electrophilic aldehydes that are capable of covalently modifying biological macromolecules. The formation of lipid hydroperoxides is a complex process, as it involves various radical intermediates. 4-hydroxy-2-

nonenal (4-HNE) is one of the most studied aldehydes generated by lipid oxidation. 4-HNE has been found to be cytotoxic, facilitate redox instability with myoglobin, as well as modify and inhibit glycolytic enzymes by covalent modification. However, 4-HNE is not the only reactive aldehydic product. Recent studies have reported that the lipid oxidative secondary product, 4-oxo-2-nonenal (4-ONE) is the more reactive cousin of 4-HNE (Lin et al., 2005). Structurally 4-HNE and 4-ONE are similar molecules, but 4-ONE contains a different functional group at the C4 position that has been predicted to make the compound more reactive towards protein nucleophiles compared to 4-HNE The ketone at the C4 position on 4-ONE acts as an additional target for nucleophiles. Also, 4-ONE does not contain a hydroxyl group so the 4-ONE adduct cannot cyclize and may contain two free carbonyl groups, which are reactive towards the primary amines (Feeney et al., 1975).

Lee *et al.* (2000) confirmed for the first time that 4-ONE is a major product of iron (II)-mediated breakdown of lipid hydroperoxides. The study provided evidence that 13-HPODE, which is a prototypic *w*-6 lipid hydroperoxide, undergoes iron-mediated decomposition and forms 4-ONE, see Figure 4 (Lee & Blair, 2000). Furthermore, reactions of 4-ONE with the side chains of Histidine, Lysine, and Cysteine were investigated in the absence of oxygen. The results indicated that there was very little cross-linking for 4-ONE in the absence of oxygen however in the presence of oxygen, cross-linking was enhanced. These findings confirm that 4-ONE has biological properties of modification of proteins (Zhang et al., 2003). Zhu *et al*. (2009) further studied 4-ONE induced cross-linking by focusing on specifically covalent cross-linking of glutathione and carnosine to proteins. 4-ONE was found to form Cystine-Lysine and Histidine-Lysine pyrrole cross-links. Additionally, 4-ONE reacted rapidly with glutathione and carnosine which inhibited 4-ONE-based protein-protein crosslinking. However, unlike 4-HNE, the reaction

of 4-ONE with antioxidants lead to the formation of 4-ketoaldehyde, which is reactive toward primary amines through Paal-Knorr condensation. As a result, glutathione and carnosine can be cross-linked to proteins by 4-ONE (Zhu et al., 2009).

Doorn *et al.* (2006) continued to investigate 4-ONE and 4-HNE and their reactivity towards proteins. The research study identified amino acids that were reactive with 4-HNE and 4-ONE, characterized the chemical structure and determined the preference for amino acid modification. The results indicated that cysteine, histidine, and lysine are reactive with 4-HNE and 4-ONE. Additionally, arginine was also adducted by 4-ONE. This study indicated that both 4-ONE and 4-HNE modify amino acid nucleophiles, however, 4-ONE was found to be more reactive than 4-HNE in regards to the identity and the number of peptide residues modified, as well as the kinetics of the amino acid adduction (Doorn et al., 2006).

There has been a tremendous research effort focusing on understanding the lipid peroxidation product, 4-HNE. Studies have found that 4-HNE is a reactive substance towards proteins that affect the quality of meat, and that 4-HNE causes extensive cellular damage and oxidative pathology in meat. Recent research has evaluated the impact of the oxidative by-product 4-ONE, which is generated from the oxidation of polyunsaturated fatty acyl chains under conditions of oxidative stress. The 4-ONE was recently identified as a toxic and highly reactive secondary product of lipid oxidation in meat that causes severe damage to meat quality and impairment of nutritional profile (Gonzales et al., 2021). Research has shown 4-ONE to be highly cytotoxic and protein reactive (Lin et al., 2005) and more reactive towards thiols and amine groups (Doorn et al., 2006). The 4-ONE is a more potent neurotoxic than HNE, and its rapid reaction with protein is characterized by the formation of a Schiff base with Lysine (Lin et al., 2005). It has been reported to cause cross-linking of the α-synuclein molecule making it a

more stable oligomer, which is an important compound of Parkinson's disease (Näsström et al., 2009). Most of the earlier works related to 4-ONE were emphasized in understanding its role as a mediator in human pathophysiology. Lee and Blair *et al*. (2000) showed that 4-ONE is a significant product of the breakdown of lipid hydroperoxides. During the condition of oxidative stress, polyunsaturated fatty acids are oxidized to generate electrophilic aldehydes, which bind to proteins and polynucleotides, leading to cell death (Lee & Blair, 2000).

Recent studies in our laboratory have shown that 4-ONE is a potent and highly toxic reactive chemical species formed during lipid oxidation in meat. Our results revealed evidence of induced severe vacuolization and rupturing of mitochondrial membranes following incubation with 4-ONE at varying pH levels. These findings supported the claim that 4-ONE inhibits LDH activity and causes extensive damage to mitochondrial function and induced Mb redox instability (Gonzales et al., 2021).

There is a large research gap on the influence of the 4-ONE on myoglobin redox stability, mitochondrial function, and the overall quality of meat products. The identification of 4-ONE mediated protein oxidation in meat is critical in order to understand the potential health implication of dietary oxidized meat proteins. There is currently no research focusing on the role of 4-ONE in mediated protein degradation as a major cause of food deterioration although proteins play such a major role in food systems. Researching the molecular interaction between dietary meat proteins and 4-ONE is a critical topic of study in meat science. The 4-ONE induced degraded meat proteins may have pathological consequences in the gut and on internal organs upon intestinal uptake. There is a considerable knowledge gap on the impact of formed 4-ONE in meats during gastric digestion. Additionally, the detrimental effects of the 4-ONE induced

oxidation of meat proteins may further implicate aggressive oxidative reactions and a potential rise in the human health disorder (Van Hecke et al., 2014).

CHAPTER 3

MATERIALS AND METHODS

3. Material and methods

3.1 Chemicals

4-oxo-2-nonenal, 4-oxo-2-nonenal-d3, 4-hydroxy-2-nonenal, and 4-hydroxy-2-nonenal-d3 were obtained from Cayman Chemical Co (Ann Arbor, MI, USA). Sodium chloride (NaCl), glutaraldehyde, sodium cacodylate, ethanol, propylene dioxide, sodium dodecyl sulfate (SDS), tris-HCl, ammonium bicarbonate, acetonitrile, and formic acid were obtained from Sigma Chemical Company (St. Louis, MO). Pre-cast gels were purchased from Bio-Rad (Hercules, CA). All chemicals and reagents were of analytical grade. All reagents used for TEM imaging were obtained from Electron Microscopy Sciences, Hatfield PA.

3.2 Raw materials

Bovine *Psoas major* (PM) muscle (n=6) representing different animals (USDA Choice, A-level maturity, normal color, and absence of quality defects) were procured two days postmortem from a local beef purveyor, stored in vacuum packaging at ±2°C for up to 24 hours before analyses. To avoid variability in fiber types and fiber orientation, the tissue sample was collected from the whole cross-sectional portion of the PM.

3.3 Proximate composition

Samples (10g; n=3) were cut from the whole cross-sectional portion of PM and trimmed of visible fat and connective tissue. Samples were placed in a muffle furnace at 600°C for two hours to analyze total ash (AOAC Method 942.05). Samples were analyzed for moisture content

by oven drying at 105°C for three hours (AOAC Method 930.05). Protein was analyzed by the Kjeldahl method (AOAC Methods 4500-Norg C and 4500-HN3 C). The fat content was determined using ANKOM^{XT15} Extractor (PVM 1:2003 and AOCS Procedure Am 5-04). Three samples of each muscle tissue were analyzed. All experiments were replicated a minimum of three times on three independent occasions.

3.4. Experiment 1: Assessment of 4-ONE induced bovine skeletal muscle protein degradation.

3.4.1. *Transmission electron microscopy (TEM) imaging*

Fresh PM muscle (FPM), 0.35 grams, was carefully minced with sterilized scissors, mixed thoroughly with 5μmol/L of pure 4-ONE, and incubated in an Eppendorf tube at 37 °C for 72 hours. A 5μmol/L of 4-ONE was used to spike the PM proteins in this study, as previous research has detected levels of 4-HNE in meat to be >5μmol/L (Steppeler et al., 2016). Assumed that 4-ONE and 4-HNE are produced at the same rate, we wanted to evaluate if protein degradation occurs when PM is spiked with low levels 4-ONE. An additional PM sample was prepared without 4-ONE and stored in an Eppendorf tube at 4 °C for 72 hours. After 72 hours, samples were removed and prepared for TEM processing by mixing in 2% Glutaraldehyde / 0.1M NaCacodylate buffer, pH 7.2 for one hour at 21°C. Samples were washed and rinsed a minimum of three times with buffer and placed in 1% osmium tetraoxide / 0.1M NaCacodylate buffer for one hour. The samples were again washed with distilled water three times for ten minutes to remove excess fixative and dehydrated in progressively increasing concentrations of ethanol (25, 50, 75, 100%). Following fixation, samples were again washed twice in 100% ethanol before transitioning into propylene oxide (PO). Samples were then infiltrated with increasing concentrations of Embed 812 resin/PO (25, 50, 75, 100%) with two final changes of

100% resin. The samples were then placed in molds and polymerized at 60°C for 24 hours. Approximately, 70nm thick sections were cut on a Leica UM6 ultramicrotome, and the cut samples were collected on 200 mesh copper grids, and post-stained with 2% uranyl acetate before imaging. Images were taken on a JEOL JEM1011 TEM operating at 80kV.

3.4.2. *Meat protein fraction preparation*

The meat protein fraction was prepared using PM meat to assess 4-ONE induced bovine skeletal muscle protein degradation. Briefly, 2.5g of FPM muscle was minced and homogenized with 25mL of buffer solution (20 mM sodium phosphate buffer, 25mM NaCl, and 5.0mM EDTA- pH 7.0) using the Tissue Tearor ™ at 30,000 rpm for one minute. The slurry was filtered using Whatman filter paper (Whatman® qualitative filter paper, Grade 113, creped, circle, diam. 150 mm) and centrifuged at 10,000*g* for 5 minutes at 20°C. The supernatant was separated and a 1.5mL aliquot (labeled as control) was stored in an Eppendorf tube at -40°C. The remaining supernatant was spiked with 5μmol/L of pure 4-ONE and incubated for different time intervals (24, 48, 72, and 120 hours) in an incubator (Thermo Scientific, Massachusetts, USA) at 37°C for an accelerated storage test. Following each treatment group's incubation period, the samples were removed and carefully shaken, and stored frozen at -40°C until further analysis.

Additional protein samples were separated via SDS-PAGE as negative controls. The negative controls gels included FPM incubated with 1% ethanol (the carrier solution of 4-ONE) for 24, 72, and 120 hours, FPM incubated at 4°C for 3, 5, and 7 days, and FPM incubated at 37°C for 24, 72, and 120 hours.

3.4.3. *Beef patty preparation and cooking*

The FPM was trimmed of visible fat and connective tissue and ground twice using a Hobart grinder (Model, 310, Hobart Inc., Troy, Ohio, US) with a 3.2-mm plate. The ground FPM

was formed into an 80g patty and cooked on a preheated fry pan for about 4-7 minutes until it reached medium temperature (71°C). The cooking temperature was monitored at the geometric center of the patty using a calibrated thermometer.

3.4.3.1 *In-vitro gastric digestion*

Simulated gastric digestion is generally used in food and nutritional sciences in place of conducting human trials that are costly, resource-intensive, and sometimes disputable. Consequently, *in-vitro* alternatives determine endpoints such as the bioavailability of nutrients and the digestibility of macronutrients (e.g., lipids, proteins, and carbohydrates) are used for screening and building new hypotheses. To mimic gastric digestion an i*n-vitro* method was used by preparing the gastric juice with appropriate consideration of the presence of digestive juice elements and their respective concentrations, including digestive pH, time, and other factors. The cooked PM was mixed with simulated gastric juice according to the methods prepared by Peng *et al*. (2020) with slight modifications.

Briefly, 10g of the cooked PM muscle was removed from the patty and homogenized with 36mL of gastric juice, see Table 1 for gastric juice recipe (Peng et al., 2020). The pH of the slurry was adjusted to 2.5 using 12.1M HCl or 10M NaOH and then incubated at 37°C for two hours. The slurry was filtered using filter paper (Whatman® qualitative filter paper, Grade 113, creped, circle, diam. 150 mm). The filtered gastric juice was discarded, and the digested meat was used for further analysis.

A 2.5g portion of the cooked and digested PM (CDPM) muscle was homogenized with 2.5mL of buffer solution (20 mM sodium phosphate buffer, 25mM NaCl, and 5.0mM EDTA- pH 7.0) using the Tissue Tearor ™ at 30,000rpm for one minute. The homogenized meat slurry was filtered using a cheesecloth and the decanted liquid was centrifuged at 10,000*g* for five minutes

at 20°C. The supernatant was separated and 1.5mL of the supernatant (Control) was stored in an Eppendorf tube (1.5mL capacity) at -40°C. Similar to the previous protocol, 5μmol/L of 4-ONE was added to the supernatant and incubated for 24, 48, 72, and 120 hours at 37°C to perform an accelerated storage test. Following the treatment for each group's incubation period, samples were shaken and kept frozen at -40°C until analysis, see Figure 5.

The sarcoplasmic protein fractions were spiked with 4-ONE following cooking and digestion, because we do not yet know how 4-ONE reacts with constituents of the gastric digestion juice, such as urea and Bovine serum albumin (BSA). Therefore, to eliminate variability, we decided to spike 4-ONE after digestion to allow us to understand the interactions of 4-ONE with the cooked and digested protein fractions.

3.4.4. ***SDS-PAGE and protein identification.***

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for the protein fraction separation. A 950μL of the protein supernatant obtained from the homogenized meat model was further combined with 950μL of loading buffer (Tris-HCl, glycerol, SDS, bromophenol blue, dithiothreitol; Fisher Scientific, Suwanee, GA, USA) and 50μL of 2-Mercaptoethanol (Sigma-Aldrich, St. Louis, MO), vortexed, and placed in a boiling water bath for seven minutes. Samples were cooled and stored frozen (-40°C) until further analysis. The gel was loaded with a 10μL of Precision Plus Protein™ dual color standards (Bio-Rad Laboratories, Hercules, CA, USA), and 10μL of the denatured sample was loaded for each additional lane in 12% Criterion TGX Midi Protein Gels (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were separated using the Tris-Glycine-SDS running buffer at 150V for 50 minutes (Fisher Scientific, Suwanee, GA, USA) and the gel was stained with Coomassie brilliant

blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA) and destained using Bio-Rad destain (Hercules, CA, USA). Samples and gels were run in duplicates.

Protein identification was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the University of Georgia Core Proteomic Facility. A 10μL aliquot of each FPM and CDPM protein sample was loaded onto the pre-cast gel and allowed band separation for five minutes. The gel bands were carefully sliced with a sterile surgical blade and rinsed twice with 50% acetonitrile/20mM ammonium bicarbonate (~pH 7.5 – 8.0). The sliced gel piece was dehydrated by adding 100% of acetonitrile and dried using a SpeedVac. Various amounts of Trypsin solution (0.01μg/μL in 20mM ammonium bicarbonate) were added until the gel piece completely absorbed the trypsin solution. The sample tubes were stored in an incubator at 37°C temperature for twelve hours. The tryptic peptides were extracted from the gel piece using 50:50 acetonitrile/H_2O both with 0.1% formic acid. The extracts were dried using a SpeedVac.

The mass spectrometry analyses were performed on a Thermo-Fisher LTQ Orbitrap Elite Mass Spectrometer coupled with a Proxeon Easy NanoLC system (Waltham, MA) located at the Proteomics and Mass Spectrometry Facility, University of Georgia. The peptides were separated by HPLC on a reversed-phase self-packed column/emitter using 200Å, 5μM, Bruker MagicAQ C18 resin. The eluent went directly into the mass spectrometer. Briefly, the two-buffer gradient elution (0.1% formic acid as buffer A and 99.9% acetonitrile with 0.1% formic acid as buffer B) starts with 0% B, holds at 0% B for two minutes, then increases to 30% B in 50 minutes, to 50% B in ten minutes, and 95% B in ten minutes using a flow rate of 0.5μl per minute.

The data-dependent acquisition (DDA) method was used to acquire MS data for protein identification. A survey MS scan was acquired first, and then the top eight ions in the MS scan

were selected following CID MS/MS analysis. Both MS and MS/MS scans were acquired by Orbitrap at the resolutions of 120,000 and 15,000, respectively. The data was acquired using Xcalibur software (version 2.2, Thermo Fisher Scientific).

3.4.7. *Protein band density analysis*

Gel samples were imaged with Bio-Rad Chemidoc XRS+, which uses a mercury UVB lamp with a wavelength of 302nm. The device was computer-controlled using the Bio-Rad Image Lab software (Hercules, CA, USA). After loading the gel into the Chemidoc device, the Image Lab software was loaded, and appropriate parameters were selected for the image capturing and collection. The gel was manually aligned using a digital alignment grid and the image was captured. The software program measured the protein band's molecular weight and density. The band densities were calculated as individual band density relative to total band density in the prospective lane.

3.5. Experiment 2: Detection and quantification of 4-ONE in postmortem bovine skeletal muscle proteins under different conditions.

3.5.1. *Beef patty storage and cooking*

The FPM was trimmed for visible fat and connective tissue and ground twice using a Hobart grinder (Model, 310, Hobart Inc., Troy, Ohio, US) with a 3.2-mm plate. The ground PM was formed into 80g patties, placed on absorbent pads in foam trays, and overwrapped with polyvinyl chloride film (Koch Supplies, Inc., Kansas City, Mo., US). Two groups of patties were stored at 4°C for 0, 3, 5, and 7 days. Following the storage period, the first group of patties (FPM) was used for further analysis of LC-MS/MS. The second group of PM patties was cooked after 0, 3, 5, and 7 days on a preheated fry pan for about 4-7 minutes until they reached medium

temperature (71°C). The cooking temperature was monitored at the geometric center of the patty using a calibrated thermometer.

3.5.2. *Patty digestion*

To mimic gastric digestion, the PM patties were thoroughly mixed with simulated gastric juice according to the methods prepared by Peng *et al*. (2020) with slight modifications. Briefly, 10g of the cooked PM patty was homogenized with 36mL of gastric juice, see Table 1. The pH of the slurry was adjusted to 2.5 ± 0.5 using 12.1M HCl or 10M NaOH and then incubated at 37°C for two hours. The slurry was filtered using filter paper (Whatman® qualitative filter paper, Grade 113, creped, circle, diam. 150 mm). The filtered gastric juice was discarded, and the cooked and digested PM (CDPM) was used for further LC-MS/MS analysis.

3.5.3. *LC-MS/MS*

A 100mg of PM was derivatized by homogenizing 50mM sodium acetate buffer containing 5mM amino-oxyacetic acid, 250μM BHT and 500μM DPTA at pH 5.0. The slurry was spiked with 50μL of 2x deuterated 4-ONE, vortexed, and incubated for one hour on ice. After the derivatization, the samples were briefly vortexed and centrifuged at 10,000g for 10 minutes. Strata-X-AW columns- 331um Polymeric Weak Anion- 60mg/L (Phenomenex, Torrance, CA, USA) were conditioned with 1mL of methanol and equilibrated with 1mL of 100mM sodium acetate (pH 5.3). The samples' supernatant was loaded on the preconditioned columns, and the columns were washed with 1ml of 100mM sodium acetate buffer (pH 5.3) followed by 1mL of methanol. The derivatized aldehydes were eluted with 1mL of 5:95 NH_4OH: MeOH. The samples were stored at -80°C before analysis by liquid-chromatography-tandem mass spectrometry (LC-MS/MS). The LC-MS/MS analysis was performed by the Proteomic and Mass Spectrometry (PAMS) laboratory at the University of Georgia.

The samples were placed in the SpeedVac (Thermo Fisher Scientific, Rockford, IL) until fully dried or until a gel was formed, then resuspended in 125μl of methanol. The samples were sonicated for 5 minutes and then put in an Eppendorf 5415C centrifuge at 6000 rpm/min for at least five minutes. The supernatants from the same sample were taken and combined. The samples were placed in the SpeedVac to dry. Then the samples were resuspended in 120μl of methanol and stored in a 5°C freezer to chill. Samples were centrifuged for at least 10 minutes. The supernatant was placed in an autoinjector vial for analysis. 15μl of the sample was injected onto a Zorbax Eclipse Plus C18, 2.1x100mm, 3.5μm (Agilent Technologies, Inc., Santa Clara, CA) using the following gradient: Solvent A was 0.1% formic acid (FA) and Solvent B was 0.1% FA in acetonitrile. Samples were injected at 20% B and ramped to 50% B over 13 minutes at a flow rate of 0.3 ml/min. Then in 5 minutes ramped to 100 B and held for three minutes. B was ramped back to 20% B where the column equilibrates for 5 minutes before the next run. The mass spectrometer (Bruker, Impact II, Q-TOF) was run using multichannel reaction monitoring (MRM) of 302 m/z with an 8m/z isolation window to bring in both ONE and ONE-d3. Nitrogen was used as the collision gas and Collison Induced Dissociation (CID) was performed at 15eV. Three independent replications were conducted for each 0, 3, 5, and 7-day stored FPM and CDPM samples, at three different times.

3.6. *Data analysis*

Protein identification and modification characterization were performed using Thermo Proteome Discoverer (version 2.2) with Mascot (Matrix Science) and Uniprot database.

CHAPTER 4

RESULTS

4.1. Proximate composition

Proximate composition analysis of wet basis postmortem *Psoas major* (PM) muscle revealed that it contained protein (20.2%), fat (1.6%), and ash (1.3%). Total nitrogen and moisture content were determined to be 3.23% and 75.5%, respectively. The dry basis composition of the PM revealed that it contained 82.5% protein, 6.6% fat, 5.3% ash, and 12.7% total nitrogen, see Table 2.

4.2. Experiment 1: Assessment of 4-ONE induced bovine skeletal muscle protein degradation.

4.2.1. Transmission Electron Microscopy (TEM) imaging

The high-resolution TEM images of postmortem PM muscle incubated with 4-ONE are shown in Figure 6. The fresh PM showed a distinct light and dark band pattern. The TEM image of the PM muscle spiked with 4-ONE shows a pattern of myofibrillar damage and disorientation of the sarcomere arrangement. The TEM image shows considerable damage to the myofibrillar protein structure indicating that 4-ONE induced significant deterioration and disruption to the light and dark protein bands.

4.2.2. 4-ONE interactions and protein analysis

This experiment was designed to explore possible interactions of 4-ONE with postmortem PM muscle proteins. The SDS-PAGE separations of bovine skeletal muscle protein fractions of fresh *Psoas major* (FPM) incubated with 5μmol/L of 4-ONE are shown in Figure 7.

The band patterns resulting from the separations revealed that incubation of FPM with 4-ONE for 120 hours degraded the sarcoplasmic proteins. The non-degraded FPM (Control) contained many proteins ranging in molecular weight from approximately 10kD to 250kD. The bands labeled #1, #3, #4, #5, #6, #7, and #8 are most prominently observed in the control lane. During the initial 24-hour incubation period with 4-ONE, Band #1 was completely degraded. The additional protein bands density faded throughout the incubation time, and after 120 hours they began to completely disappear. Band #8 remains prominent throughout the 72-hour mark of incubation; however, it also began to fade after 120 hours of incubation. The negative control gels, see Figures 8-10, showed minimal protein degradation in the FPM incubated with ethanol for 120 hours, as well as in the FPM proteins incubated at 4°C for 7 days and at 37°C for 120 hours. The lanes on the negative control protein gels contained prominent protein bands compared to the faded protein bands shown in Figure 7. These results confirm that 4-ONE is responsible for the degradation of the fresh meat proteins and other factors such as time, temperature, and ethanol have little to no impact on the 4-ONE induced protein degradation.

Furthermore, the SDS-PAGE separations of the sarcoplasmic protein fractions of cooked and digested *Psoas major* (CDPM) incubated with 1% 4-ONE are shown in Figure 11. The control lane (CDPM without 4-ONE) contained two small molecular weight protein bands. The cooking and *in-vitro* digestion process significantly degraded most of the protein bands that were found in the FPM, shown in the control lane of Figure 7. Throughout the incubation period, Band #1 faded and completely disappeared after 72 hours of incubation with 4-ONE. Band #2 disappeared within 24 hours of incubation. After 120 hours of continued incubation, no protein bands were detected on the SDS-PAGE gel.

A protein identification analysis was conducted with LC-MS/MS for the total fraction of the postmortem PM incubated with 4-ONE. The analysis identified 65 proteins in the FPM and determined their abundance in each fraction by peak area. Several parameters were modified such as scaling and normalization of each run and the removal of keratins. The analysis showed that the peak area of the proteins decreased as the incubation time was increased throughout the incubation period. The identified proteins with the largest change in peak area are shown in Figure 12-A. The peak area of S-formylglutathione hydrolase sharply declined after the initial 24 hours and continued to decline until 120 hours when no protein was detected. Electron transfer flavoprotein's peak area declined rapidly after 24 hours, then slightly increased after 48 hours, and further declined until no peak area was detected after 120 hours. The initial peak area of creatine kinase S-type, $1.0x10^7$, decreased to $5.5x10^6$ after 24 hours and then gradually decreased throughout the incubation period. Adenylosuccinate synthetase isozyme's peak area slightly increased after 24 hours, then decreased until the peak area was no longer detected after 120 hours. Furthermore, the peak area of Enoyl-CoA hydratase gradually decreased to $3.6x10^5$ within 48 hours, no further peak area was detected at hour 72. There was also no peak area of 3-hydroxy acyl-CoA dehydrogenase detected after 120 hours of incubation with 4-ONE.

Fewer proteins were detected in the LC/MS-MS analysis of the CDPM incubated with 4-ONE. Figure 12-B showed a large decrease in the CDPM proteins peak areas after incubation with 4-ONE. The Collagen alpha-2 (I) chain's peak area sharply decreased from $9.0x10^6$ to $3.0x10^5$ after 24 hours, and no peak area was further detected after 48 hours. No peak areas were detected for ATP synthase subunit alpha and Myosin light chain kinase following 48 hours of incubation. MYBPC1 protein's peak area gradually decreased from $1.0x10^6$ to $3.0x10^5$ within 72 hours, and no protein was detected after 120 hours. Additionally, glucose-6-phosphate isomerase

peak area decreased from $2.0x10^6$ to $3.0x10^5$ within 72 hours and was not detected after 120 hours.

4.2.3. Analysis of SDS-PAGE protein band density

Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA) was used to identify the protein bands and calculate their band density. Overall, 18 protein bands were identified in the control sample (FPM, no 4-ONE). The density of bands decreased to 10 over the 120-hour incubation period of the meat sample with 4-ONE. Figure 13-A shows a significant decrease in the Adjustment Volume (Intensity) of Band #4, #5, and #7 over the incubation time. Band #8 had a slight increase after 72 hours of incubation, and then decrease in intensity after 120 hours. Additionally, the Image Lab software detected two total protein bands in the CDPM. After 72 hours of incubation, no proteins bands were detected. As shown in Figure 13-B, there was a significant decrease in Intensity in the two detected cooked and digested protein bands. After 72 hours with 4-ONE, no intensity was detected in the CDPM. This confirms that in both FPM and CDPM, the muscle protein's band volume decreased over time.

4.3. Experiment 2: Detection and quantification of 4-ONE in postmortem bovine skeletal muscle proteins under different conditions.

4.3.1. Detection and quantification of 4-ONE in meat

The purpose of this experiment was to develop an LC-MS/MS method for the detection and quantification of 4-ONE in meat samples. The ratios of 4-ONE/4-ONE-d3 in the FPM and CDPM are shown in Tables 3 and 4. The average ratio of 4-ONE to 4-ONE-d3 in FPM on Day 0 was 0.12 ± 0.03. On Day 3, the average ratio was 0.30 ± 0.31, indicating a large variability in this data set. The variability of the sample sets remained higher for Days 5 and 7 as the average ratios were 0.35 ± 0.26 and 0.40 ± 0.28, respectively. Furthermore, the average ratio of 4-ONE to

4-ONE-d3 in CDPM on Day 0 was 0.29 ± 0.12. On Days 3, 5, 7 the average ratios were 0.80 ± 0.69, 0.38 ± 0.07, and 0.81 ± 0.68, respectively. Although the data substantiates the presence of 4-ONE, the high standard deviations obscure any trends if present.

CHAPTER 5

DISCUSSION

The main objective of this study was to investigate the effects of the lipid oxidation by-product, 4-oxo-2-nonenal (4-ONE) on skeletal muscle proteins in beef. To determine whether 4-ONE induces degradation of muscle proteins, fresh *Psoas major* (FPM) was inoculated with 2% 4-ONE, incubated for 72 hours at 37°C, and transmission electron microscopy (TEM) images were taken. PM was selected based on its oxidative properties. Compared to other types of beef skeletal muscles, PM contains a higher concentration of myoglobin, mitochondria, and other oxidative enzymes resulting in high levels of oxygen consumption rate (OCR). The OCR decreases as the storage time of the PM postmortem increases, this is due to the depletion of coenzymes and substrates in addition to the degradation of enzymes that are involved in the mitochondrial respiration (O'keeffe & Hood, 1982). Canto *et al.* (2016) observed lower color and oxidative stability in beef PM steaks compared to *Longissimus lumborum* (Canto et al., 2016). Additionally, Utrera *et al.* (2014) evaluated the effects of protein oxidation during frozen storage and subsequent processing. Their findings indicated that the PM patties had the highest concentration of carbonyls as well as a decrease in antioxidant protection against the pro-oxidant action of heme-iron (Utrera et al., 2014). The high-resolution images showed significant damage to the myofibrillar protein structure and disorientation of the sarcomere arrangement compared to the image of FPM without 4-ONE, shown in Figure 6. These images provide evidence that 4-ONE induces considerable damage to light and dark protein bands.

Furthermore, FPM and cooked and digested *Psoas major* (CDPM) were spiked with 5μmol/L 4-ONE over four different storage periods (i.e., 24, 48, 72, and 120 hours). The proteins were separated using SDS-PAGE gel electrophoresis to evaluate if 4-ONE induced protein degradation over time, see Figures 7 and 11. Throughout the incubation period with FPM, the bands gradually attenuated. We presume that the decrease of the bands' densities in both gels indicates that 4-ONE degrades smaller molecular weight proteins faster than the larger molecular weight proteins. These findings show that the lipid oxidation by-product, 4-ONE induces muscle protein degradation and may further modify proteins via protein oxidation.

The data from LC-MS/MS proteomic analysis provides further indication that the muscle proteins degraded over the incubation period, see Figure 12. There was a higher abundance of proteins in the controls than in the FPM samples that were incubated with 4-ONE. These results also indicated that 4-ONE caused complete degradation of some proteins. The density of the protein bands provided additional evidence that the Adjustment Volume (Intensity) significantly decreased over the incubation time with 4-ONE. Additionally, some of the proteins completely degraded after 24 hours in the CDPM samples that were incubated with 4-ONE. Overall, the proteomic analysis showed that the ability of ROS-generating systems in *Psoas major* to facilitate protein degradation was enhanced with 4-ONE.

Our second objective was to quantify 4-ONE in FPM and CDPM over 7 days. We successfully detected 4-ONE in all meat samples, however, there was large variability in the ratios of 4-ONE to the standard 4-ONE-d3, see Tables 3 and 4. We believe that this variability is due to the extraction methodology. We do not believe this to be a sampling error because of the large variability in 4-ONE-d3 detection. 4-ONE-d3 detection should remain relatively constant throughout the experiments because the meat is spiked at a standard quantity. We do not foresee

this to be an instrumental fouling problem because there was not a steady decay of the 4-ONE-d3 signal. Therefore, it is believed that 4-ONE and 4-ONE-d3 are not being fully extracted. To prevent this error from occurring in the future, an alternate extraction method needs to be developed. In addition, it is proposed that the meat sample is spiked with 4-ONE-d3 before homogenization. Although the LC-MS/MS readings did not show consistent results, we successfully detected 4-ONE in meat samples indicating that this toxic compound is naturally occurring in meat.

In this study, the levels of spiked 4-ONE-d3 were targeted to simulate the actual amount of 4-ONE found in meat samples. Because there are no previous studies quantifying 4-ONE in food, these levels were adjusted within a reasonable range for the purpose of our study. To understand the precise interactions of 4-ONE with bovine skeletal muscle proteins, it is suggested to repeat these experiments after the further development of the 4-ONE quantification method.

According to Estevez *et al.* (2011), lipid-derived radicals and hydroperoxides promote protein oxidation in muscle food systems (Estévez et al., 2011). Studies have reported that reactive aldehydes formed during lipid oxidation can act as oxidants and attack the backbone of a protein to cause fragmentation, as well as conformational changes in the secondary and tertiary structure of a protein. These changes in the protein structure can cause changes in proteolytic properties due to the oxidation-induced unfolding process. This process increases the surface hydrophobicity of the oxidized protein, causing unfolding to occur (Martinaud et al., 1997). Protein unfolding can influence the physical and chemical properties of proteins, as well as modify the digestibility of proteins, which decreases the nutritional quality of meat (Davies et al.,

1987). We propose that the lipid oxidation by-product, 4-ONE covalently binds to the residues of muscle proteins and promotes protein degradation, see Figure 14.

Furthermore, there is extensive research focusing on 4-hydroxy-2-nonenal's (4-HNE) influence on protein modification. Studies have shown that 4-HNE-induced modification of proteins has been linked with the onset and/or progression of multiple neurogenerative diseases. 4-HNE can form covalent, irreversible bonds with protein adducts that cause degradation of cellular protein functions (Rauniyar et al., 2010). Because 4-ONE is relatively structurally similar to 4-HNE, 4-ONE also can bind and modify peptides. These modifications occur on a variety of nucleophilic residues including the histidine imidazole group, sulfhydryl groups of cysteines, and ε-amino group of lysine via Michael addition. Protein cross-linking is also likely to occur. According to Park and Xiong *et al.* (2007), oxidized myofibrillar proteins contain high amounts of protein-bound malondialdehyde (MDA), which is a lipid oxidation by-product. It is believed that MDA, as well as other carbonyls such as 4-HNE and 4-ONE, contributes to the cross-linking of myofibrillar proteins. One of the major consequences of myofibrillar protein cross-linking is meat toughening due to water loss (Park & Xiong, 2007).

The data collected in this study provided evidence that 4-ONE induces the degradation of bovine skeletal muscle proteins. There was a significant decrease in the protein bands' intensity and the proteins' peak area over time, indicating that the sarcomere protein has been modified by 4-ONE. We propose that 4-ONE covalently binds to sarcomere protein residues and induces protein degradation. Possible effects of 4-ONE-induced degradation include changes in the protein's structure and function, as well as a decrease in the nutritional quality of meat and an increase in meat toughness. The lack of research focusing on 4-ONE induced protein degradation provides us with the opportunity to further study the interactions between meat proteins and 4-

ONE. Future studies should assess the bioprotective role of novel food ingredients to prevent the formation and/or detoxification of 4-ONE in meat.

CHAPTER 6

SUMMARY

Meat has been widely consumed throughout history for its nutritional benefits. Although meat contains high levels of protein and essential vitamins, it also contains lipids that are highly susceptible to oxidation. Our findings indicate that the lipid oxidation by-product, 4-oxo-2-nonenal (4-ONE) is naturally found in *Psoas major* (PM) and induces bovine skeletal muscle protein degradation. These conclusions provide reason that 4-ONE can degrade bovine skeletal proteins, resulting in changes to the protein's structure and function. It appears that 4-ONE covalently binds to the protein residue and induces bovine skeletal muscle protein degradation.

The high-resolution images of fresh *Psoas major* (FPM) muscle incubated with 4-ONE showed myofibrillar damage and disorientation of sarcomere arrangement, indicating that 4-ONE induced significant deterioration and disruption to the protein bands. Additionally, the protein fractions of PM incubated with 4-ONE, performed by SDS-PAGE gel electrophoresis, showed severe degradation of the sarcoplasmic proteins. We found the untreated PM (without 4-ONE) contained intact protein bands that remained visible throughout the 120-hour incubation time, although they attenuated significantly. Similar results were reported when evaluating cooked and digested *Psoas major* (CDPM) incubated with 4-ONE. After 72 hours of incubation with 4-ONE, little to no protein bands were observed in the CDPM. The results of the protein identification analysis conducted by LC-MS/MS showed an overall decrease in the FPM and CDPM protein's peak area over the 120-hour incubation with 4-ONE.

We developed a methodology for 4-ONE detection and successfully detected naturally formed 4-ONE in meat. Although we were able to detect levels of formed 4-ONE, the standard deviations in the data set were too large to conclude possible trends, if any were present. Future studies should develop a better extraction method to decrease the standard error. Additionally, it is proposed that future meat samples are spiked with 4-ONE-d3 before homogenization so that 4-ONE-d3 better reflects the endogenous conditions 4-ONE experiences during extraction. Although there was a large variability within the LC-MS/MS readings, we were able to successfully detect formed 4-ONE in all meat samples.

This study determined that the lipid oxidation by-product, 4-ONE induces degradation of bovine skeletal muscle proteins and is naturally formed in meat. The next steps should include the continuation of method development for the quantification of 4-ONE in meat samples, as well as other food commodities containing high levels of lipids. Following the quantification method development, this study should be repeated with accurate levels of 4-ONE found in meat to fully understand the extent of protein degradation by 4-ONE.

CHAPTER 7

CONCLUSION

This study was conducted to provide the food science community with evidence that the lipid oxidation by-product, 4-oxo-2-nonenal (4-ONE) is an aggressive reactive species responsible for causing skeletal muscle protein degradation. The transmission electron microscopy images showed evidence that 4-ONE when present can cause extensive degradation of muscle proteins. The SDS-PAGE and protein analysis data support these findings that 4-ONE will deteriorate skeletal protein fractions during meat storage and display. The detection and quantification of 4-ONE through LC-MS/MS showed that 4-ONE naturally forms in meat and has the potential to degrade bovine skeletal muscle proteins. Future studies should focus on developing the method for quantifying 4-ONE in meat samples, as well as exploring novel food ingredients to prevent and/or detoxify 4-ONE in food systems.

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Table 1. Chemical composition of simulated gastric digestion juice.

Chemicals	Weight*	Concentration
Sodium chloride	2756 mg	0.27%
Sodium phosphate	308 mg	0.03%
Potassium chloride	827 mg	0.08%
Calcium chloride	304 mg	0.03%
Ammonium chloride	307 mg	0.03%
Hydrochloric acid (37%)	6.5 mL	0.65%
Glucose	653 mg	0.06%
Glucuronic acid	21 mg	0.00%
Urea	87 mg	0.01%
Glucosamine hydrochloride	330 mg	0.03%
Bovine Serum Albumin (BSA)	1.0 g	0.10%
Pepsin	2.5 g	0.25%
Mucin	3.0 g	0.30%

* Gastric juice was prepared by adding chemicals to one liter of ultrapure water

Table 2. Proximate composition of postmortem bovine *Psoas major* muscle.

	Wet basis	Dry basis
Macro/Micronutrients	**%**	**%**
Protein	20.2 ± 0.9	82.5 ± 4.7
Fat	1.6 ± 0.1	6.6 ± 0.2
Ash	1.3 ± 0.1	5.3 ± 0.4
Total N	3.23 ± 0.0	12.7 ± 0.7
Moisture	75.5 ± 0.2	--

Table 3. Ratios of 4-ONE/4-ONE-d3 in fresh *Psoas major* stored for 0, 3, 5, 7 days by LC-MS/MS.

	Area of 4-ONE/4-ONE-d3			
	Day 0	**Day 3**	**Day 5**	**Day 7**
Sample #1	0.15	0.01	0.05	0.11
Sample #2	0.10	0.27	0.49	0.68
Sample #3	0.13	0.63	0.50	0.41
Average	0.12	0.30	0.35	0.40
SD	0.03	0.31	0.26	0.28

*Samples #1, #2, and #3 represent the three samples of *Psoas major* used in this experiment, replicated three times. SD represents the standard deviation.

Table 4. Ratios of 4-ONE/4-ONE-d3 in cooked and digested *Psoas major* stored for 0, 3, 5, 7 days by LC-MS/MS.

	Area of 4-ONE/4-ONE-d3			
	Day 0	**Day 3**	**Day 5**	**Day 7**
Sample #1	0.41	0.58	0.43	1.53
Sample #2	0.29	0.25	0.30	0.73
Sample #3	0.17	1.58	0.41	0.19
Average	0.29	0.80	0.38	0.81
SD	0.12	0.69	0.07	0.68

*Samples #1, #2, and #3 represent the three samples of *Psoas major* used in this experiment, replicated three times. SD represents the standard deviation.

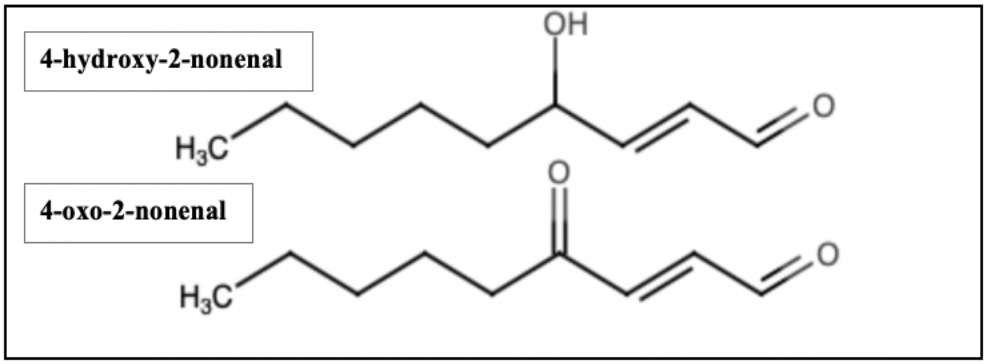


Figure 1. Molecular structure of 4-hydroxy-2-nonenal and 4-oxo-2-nonenal.

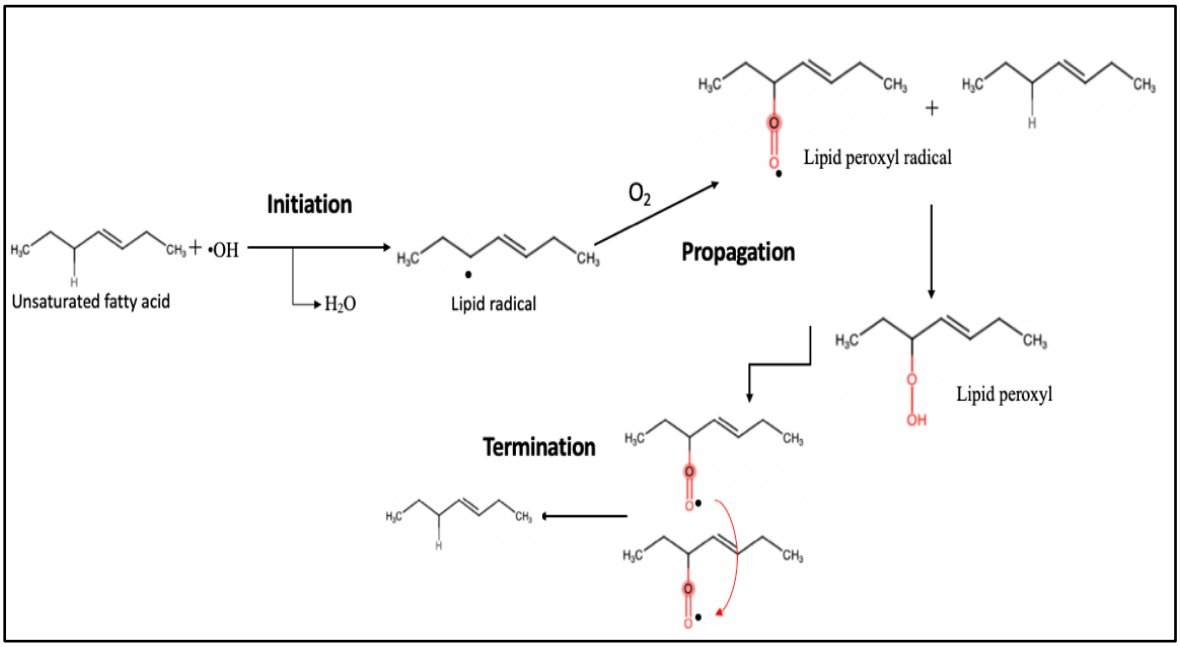


Figure 2. Classical lipid oxidation mechanism.

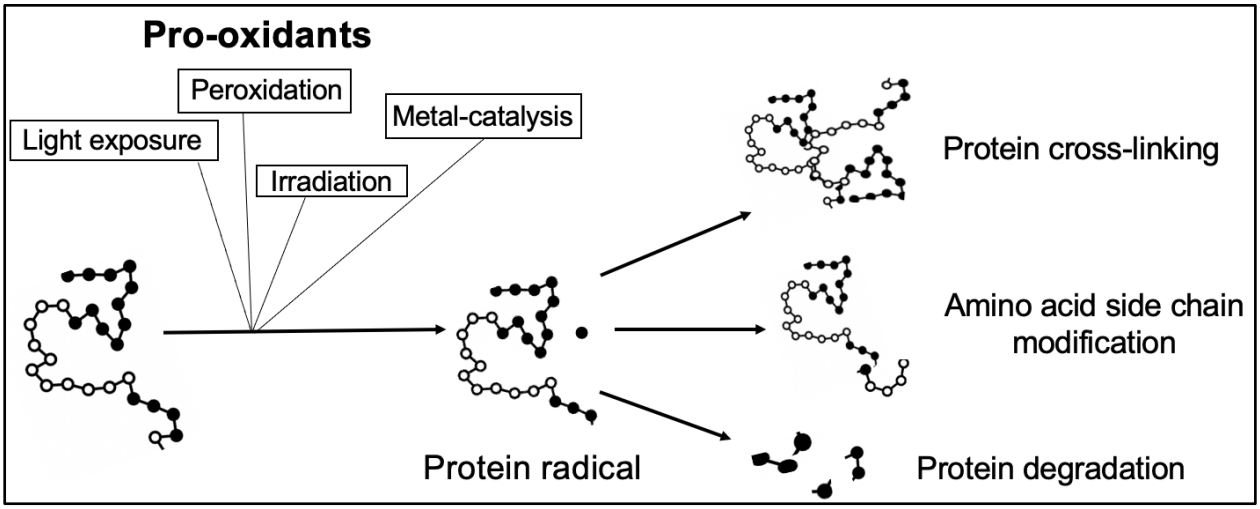


Figure 3. Protein modification via protein oxidation.

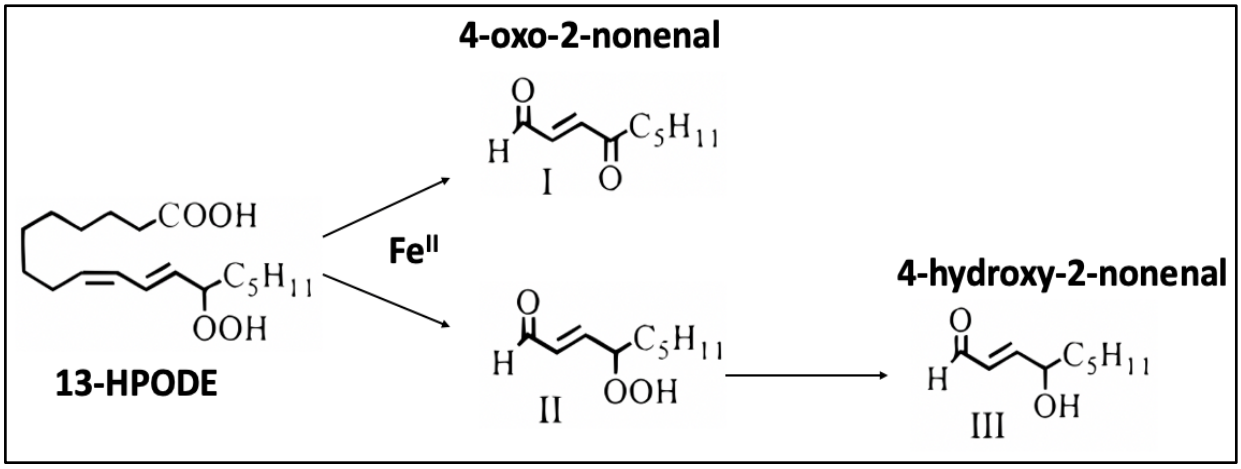


Figure 4. Mechanism of 4-ONE and 4-HNE formation from 13-HPODE.

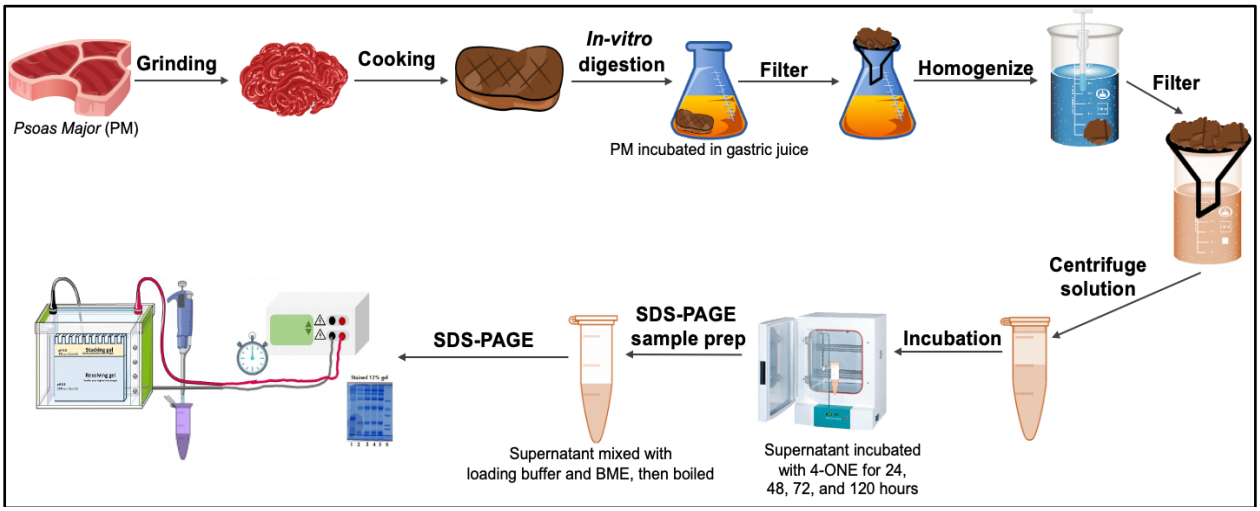
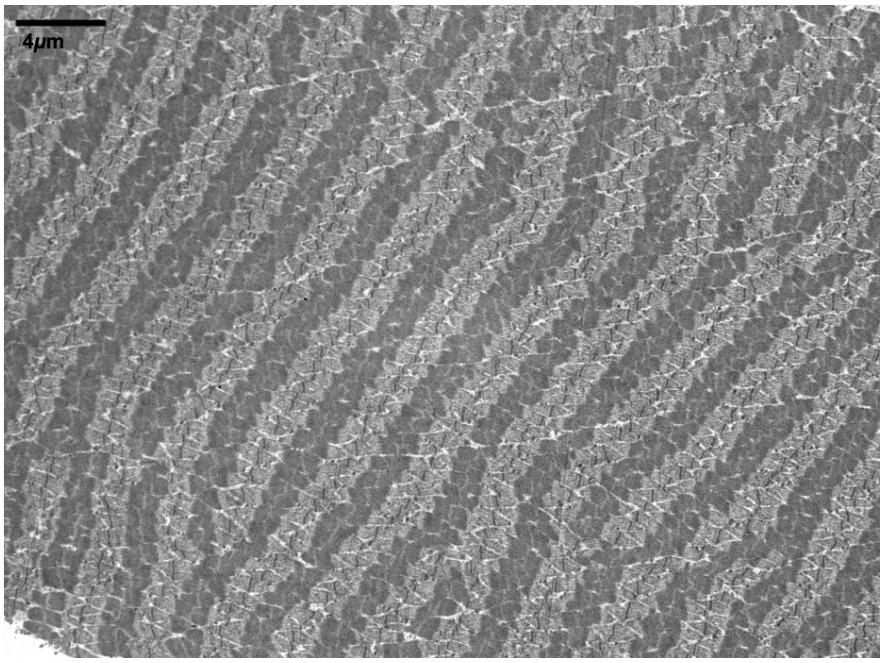
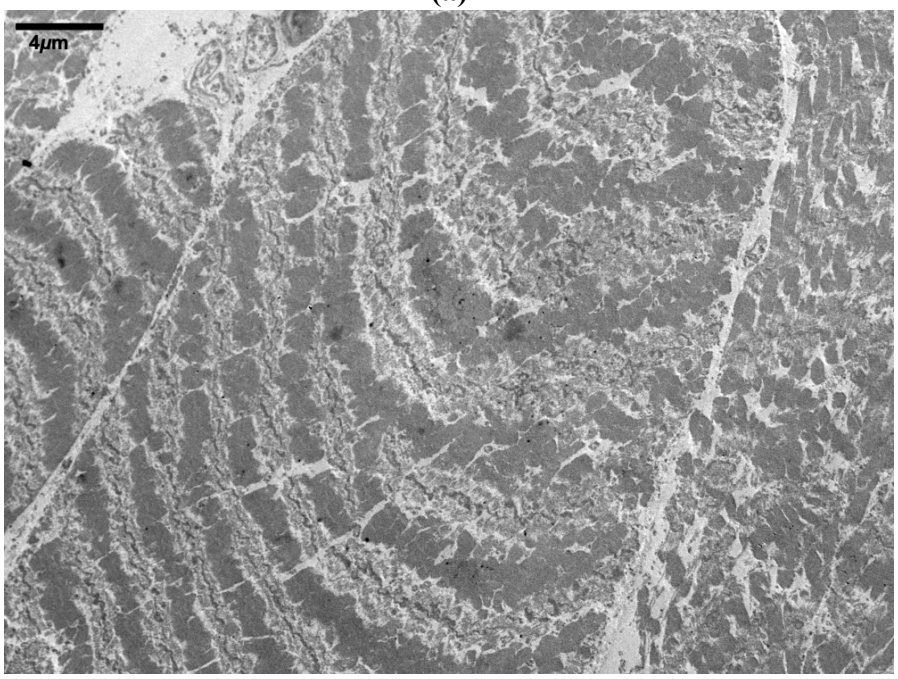


Figure 5. Flow diagram of the cooking, digestion, incubation, and preparation process of the *Psoas major* for SDS-PAGE analysis. Negative control samples followed the same processes except for the addition of 4-ONE. An additional negative control was made by incubating the supernatant with ethanol.



(a)



(b)

Figure 6. Transmission Electron Microscopy images of fresh *Psoas major* incubated at 4°C for 72 hours (a) and *Psoas major* spiked with 1% 4-ONE and incubated at 37°C for 72 hours (b).

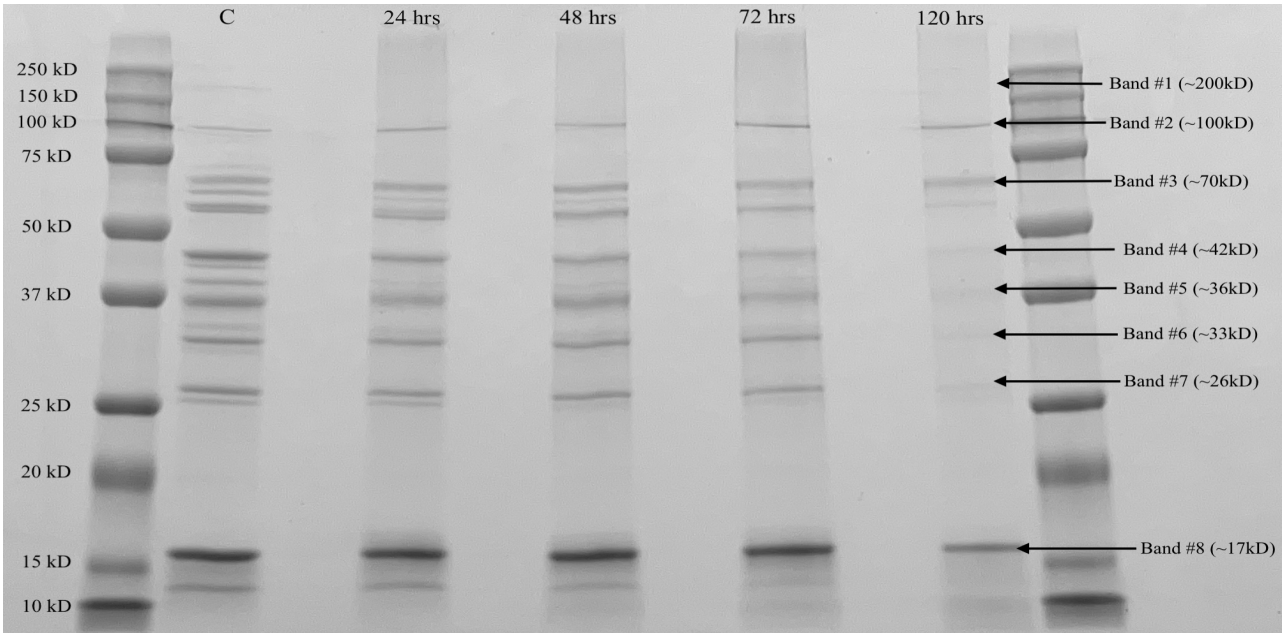


Figure 7. SDS-PAGE gel of isolated protein fractions of *Psoas major* incubated with 4-ONE for 24, 48, 72, and 120 hours at 37°C stained with Coomassie brilliant blue. The control lane is fresh *Psoas major* without 4-ONE.

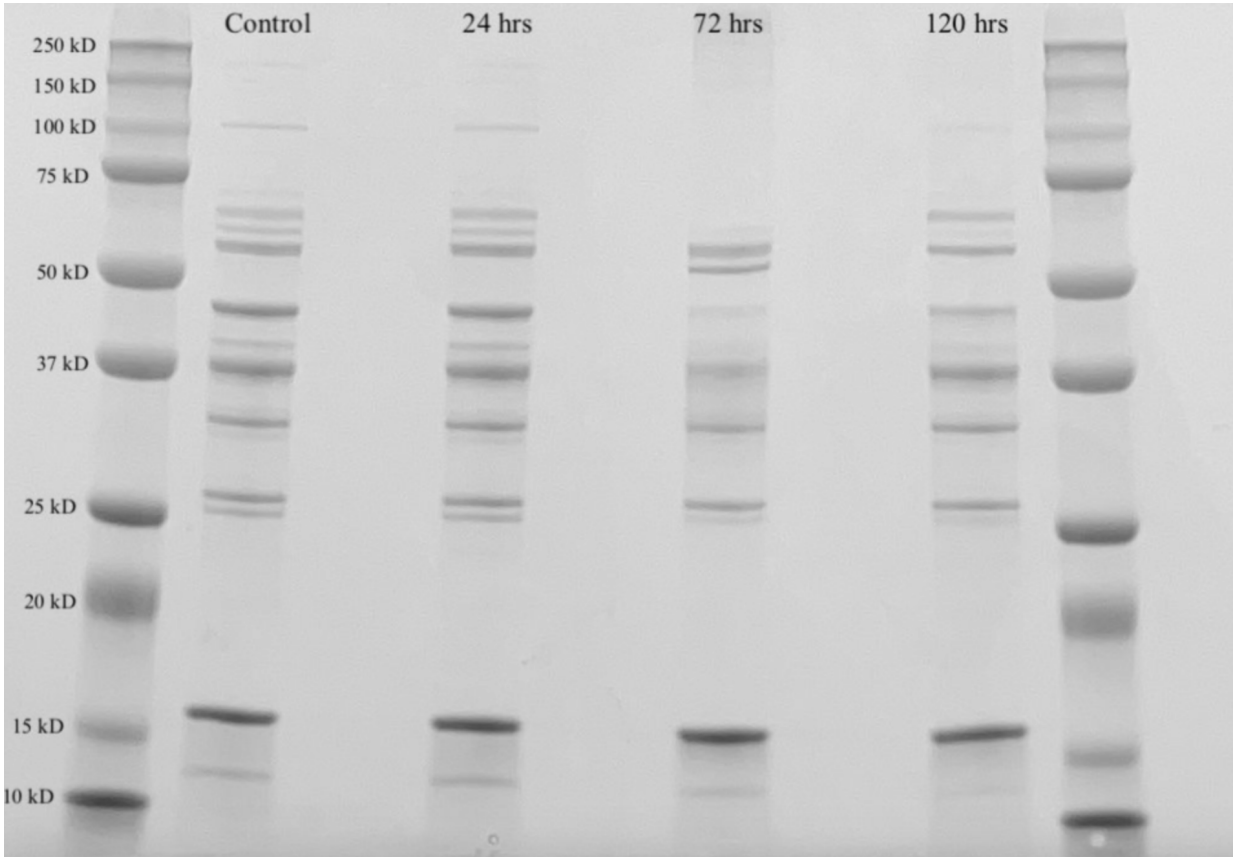


Figure 8. Negative control SDS-PAGE gel of isolated protein fractions of fresh *Psoas major* incubated with ethanol for 24, 72, and 120 hours at 37°C stained with Coomassie brilliant blue. The control lane is fresh *Psoas major* without ethanol.

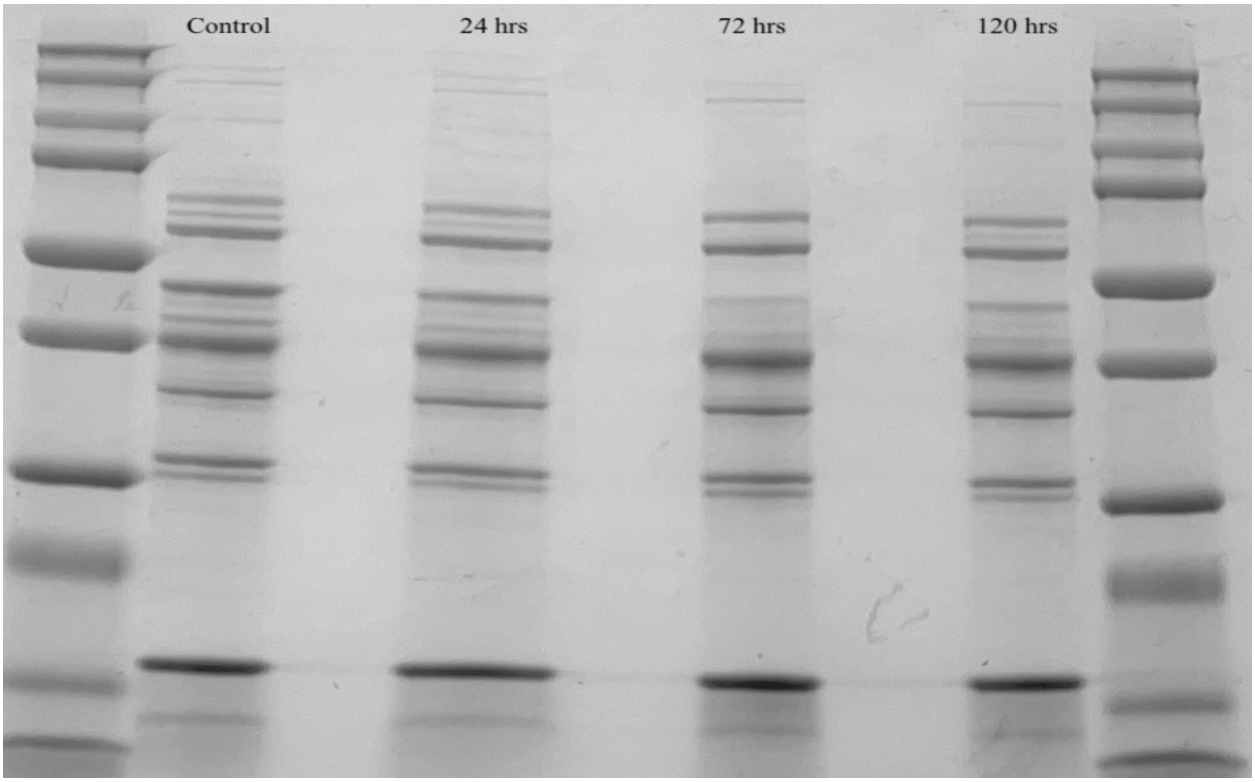


Figure 9. Negative control SDS-PAGE gel of isolated protein fractions of fresh *Psoas major* incubated for 24, 72, and 120 hours at 37°C stained with Coomassie brilliant blue. The control lane is fresh *Psoas major* at time zero.

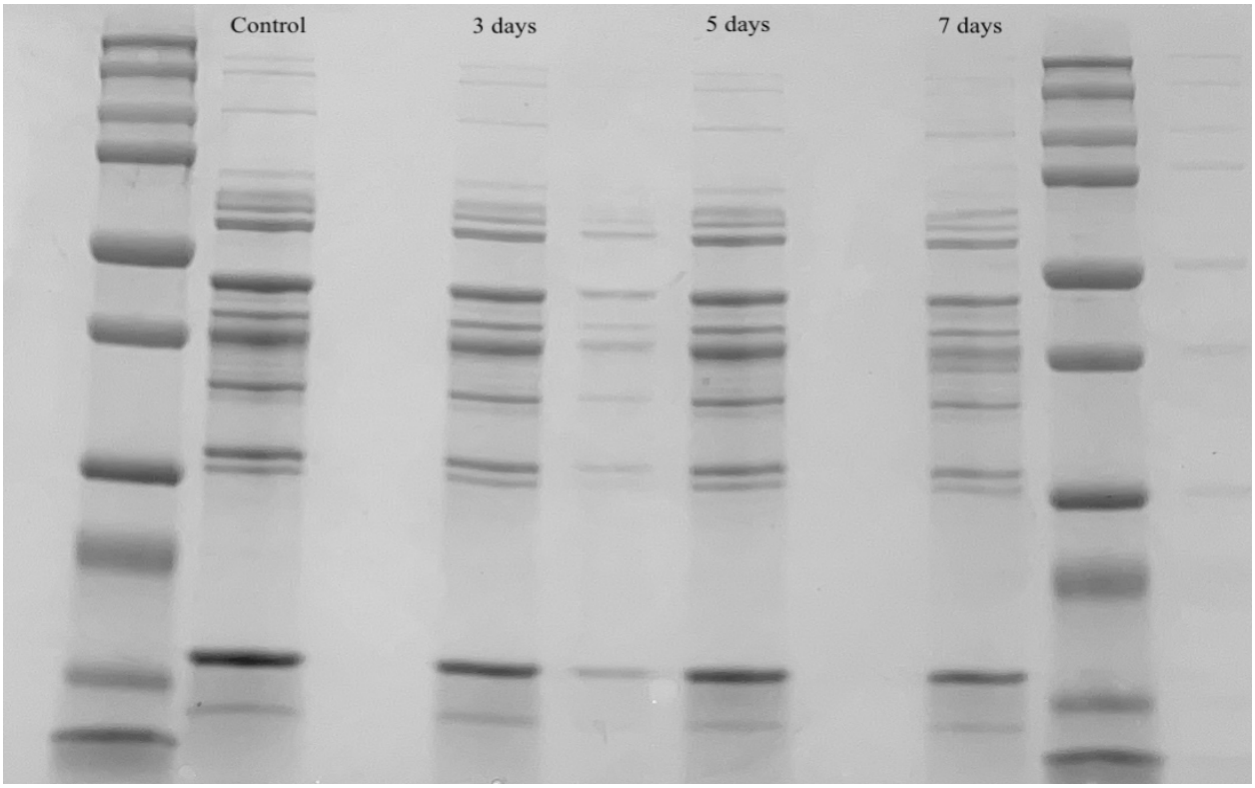


Figure 10. Negative control SDS-PAGE gel of isolated protein fractions of fresh *Psoas major* stored for 3, 5, and 7 days at 4°C stained with Coomassie brilliant blue. The control lane is fresh *Psoas major* at time zero.

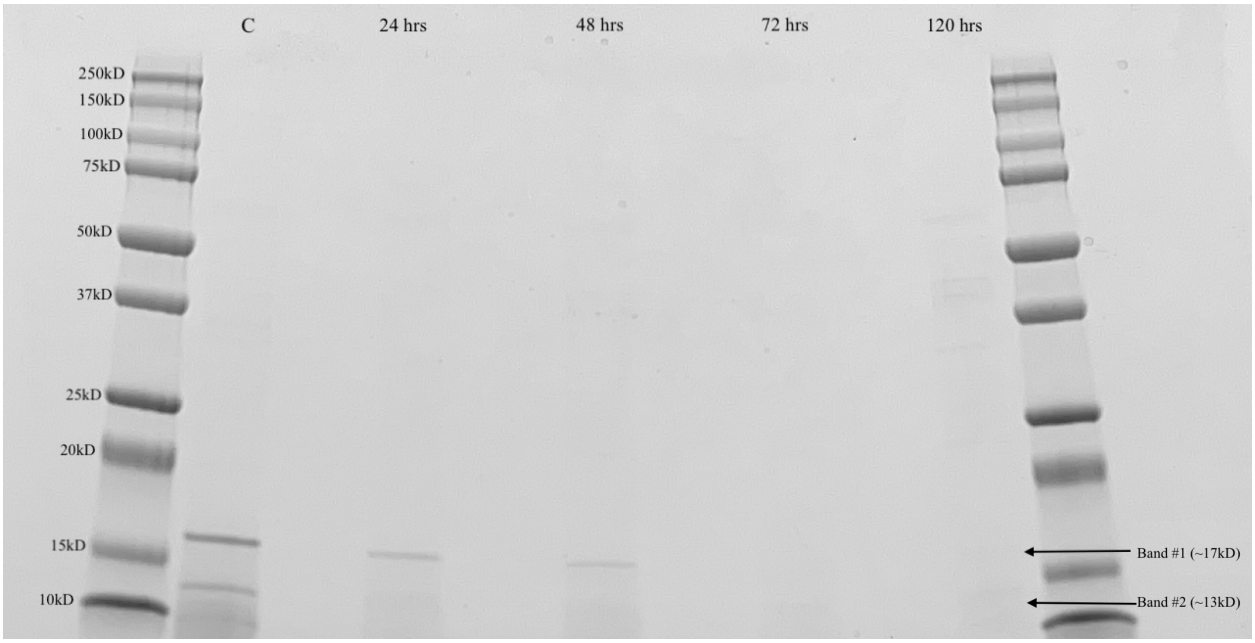
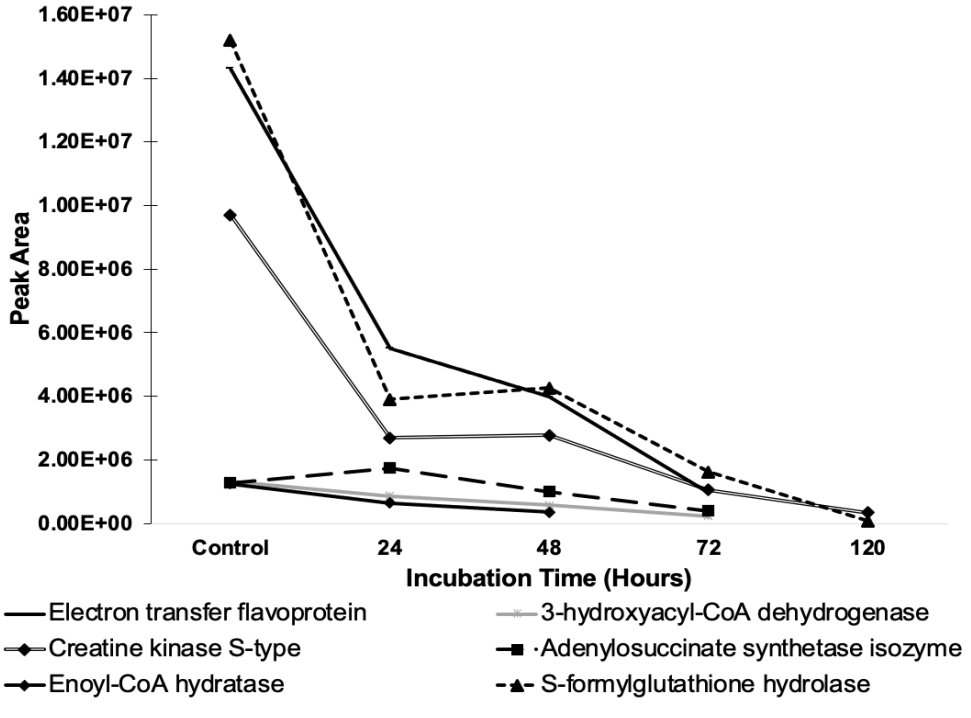
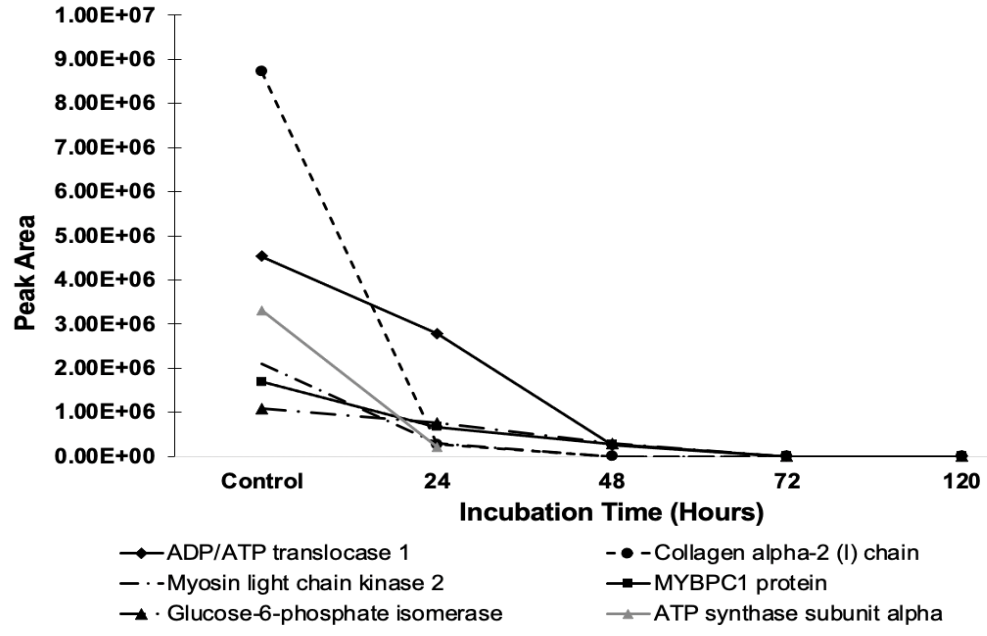


Figure 11. SDS-PAGE gel of isolated protein fractions of cooked, digested *Psoas major* incubated with 4-ONE for 24, 48, 72, and 120 hours at 37°C stained with Coomassie brilliant blue. The control lane is cooked, digested *Psoas major* without 4-ONE.

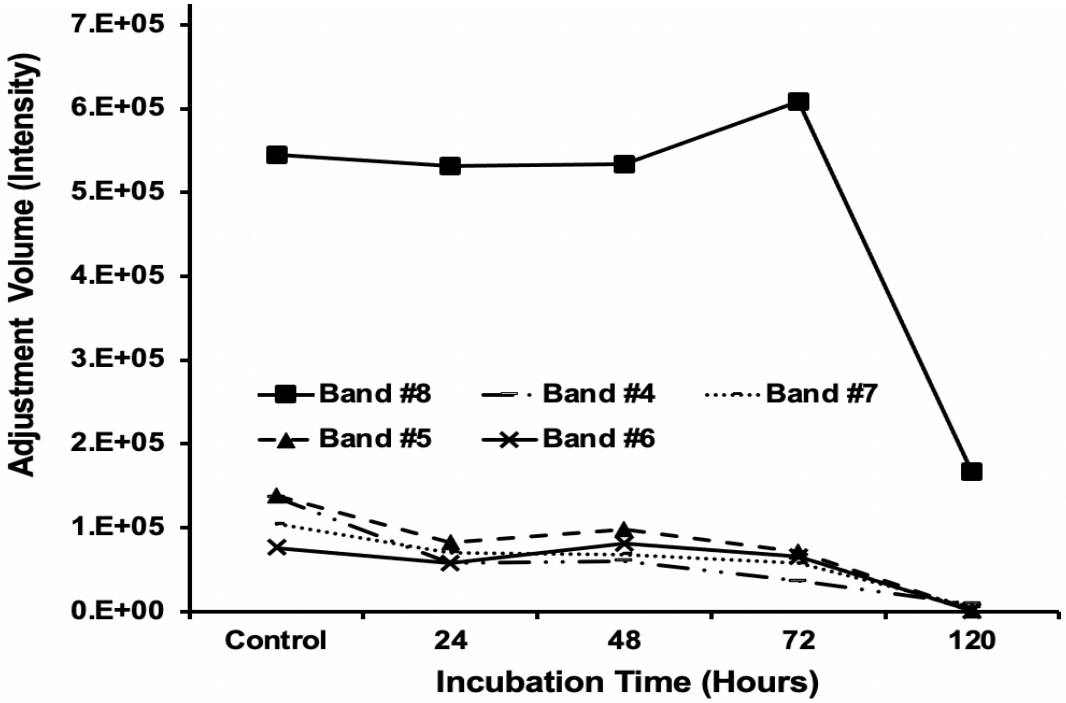


(a)

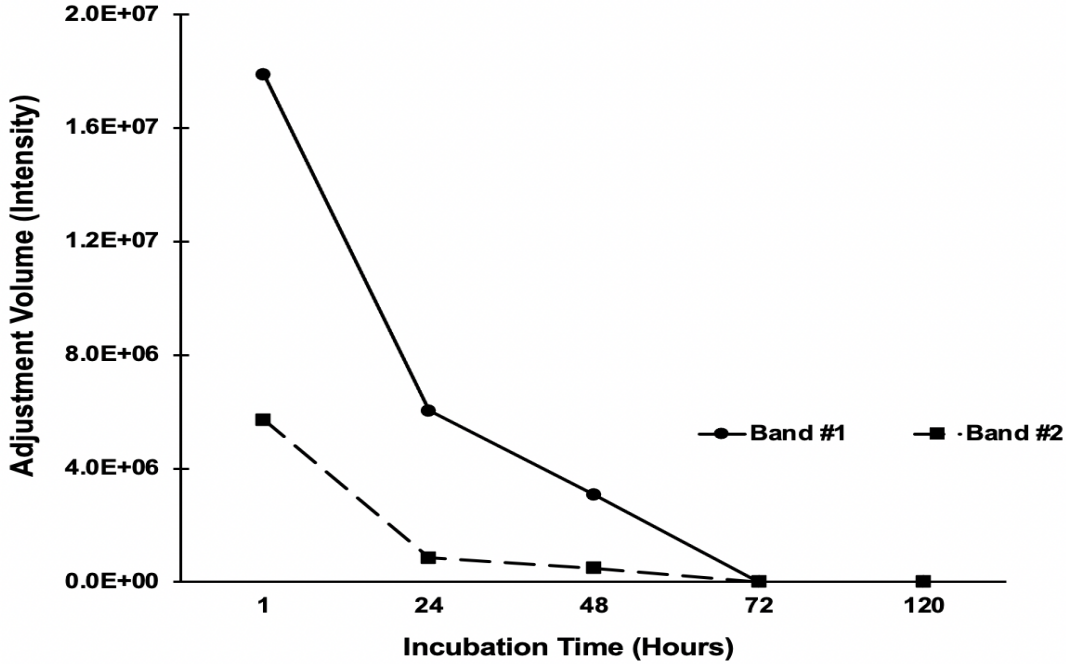


(b)

Figure 12. Peak areas of proteins tentatively identified in fresh *Psoas major* incubated with 4-ONE over 120 hours (a). Peak areas of proteins identified in cooked, digested *Psoas Major* incubated with 4-ONE over 120 hours (b).



(a)



(b)

Figure 13. Adjustment Volume (Intensity) of *Psoas major* protein fractions incubated with 4-ONE over 120 hours (a). Adjustment Volume (Intensity) of cooked, digested *Psoas major* protein fractions incubated with 4-ONE over 120 hours (b).

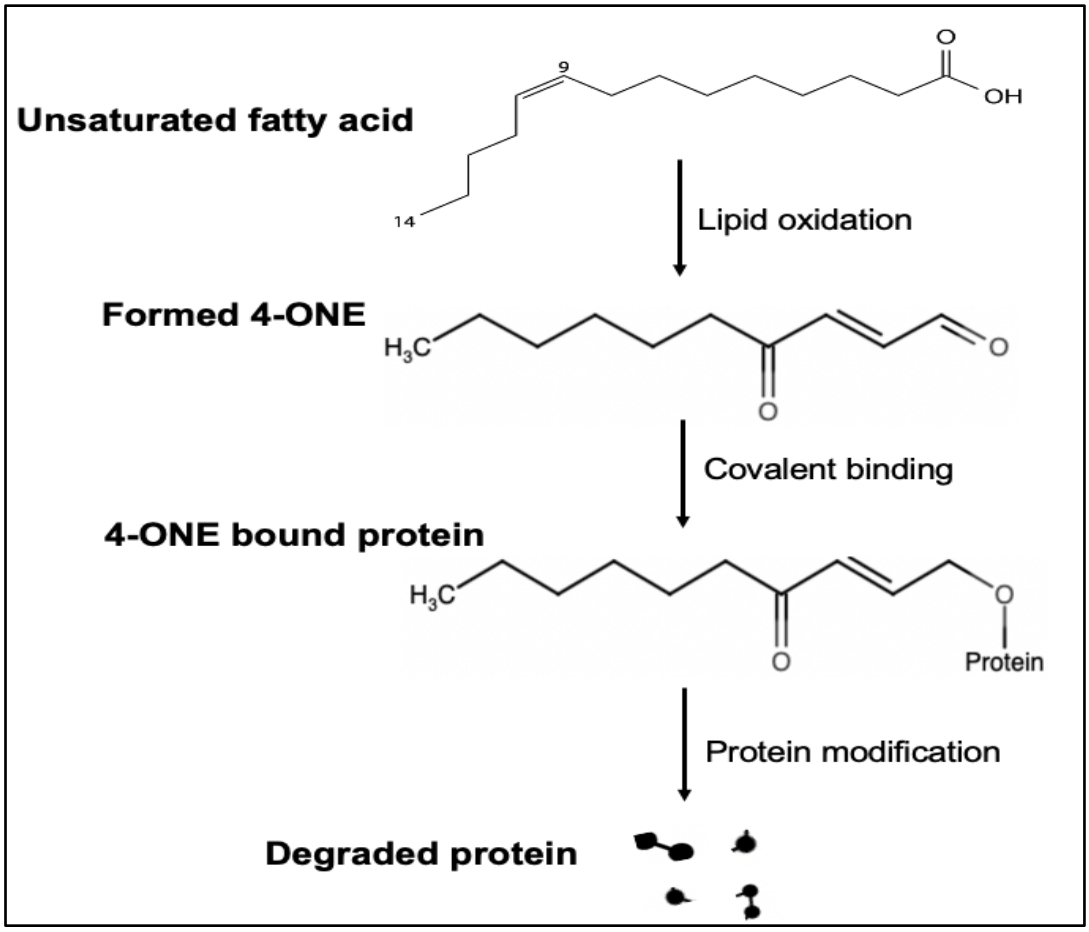


Figure 14. Proposed formation of 4-ONE modified proteins.

APPENDIX A

RELATIVE ABUNDANCE OF FRESH *PSOAS MAJOR* PROTEINS WITH 4-ONE

	Peak Area				
Protein	**Control**	**24 hrs**	**48 hrs**	**72 hrs**	**120 hrs**
Beta-enolase OS=Bos taurus OX=9913 GN=ENO3 PE=2 SV=1	6.55E+09	6.34E+09	4.19E+09	3.22E+09	4.16E+08
Creatine kinase M-type OS=Bos taurus OX=9913 GN=CKM PE=1 SV=2	2.59E+09	1.84E+09	1.20E+09	6.74E+08	8.73E+07
Phosphoglucomutase-1 OS=Bos taurus OX=9913 GN=PGM1 PE=2 SV=1	1.37E+09	2.47E+09	1.83E+09	1.31E+09	1.63E+08
Triosephosphate isomerase OS=Bos taurus OX=9913 GN=TPI1 PE=2 SV=3	1.86E+09	1.37E+09	3.82E+08	4.42E+08	7.43E+07
Hemoglobin subunit beta OS=Bos taurus OX=9913 GN=HBB PE=1 SV=1	5.25E+08	3.99E+08	1.83E+08	1.79E+08	4.09E+07
Phosphoglycerate mutase 2 OS=Bos taurus OX=9913 GN=PGAM2 PE=2 SV=1	4.81E+09	3.16E+09	1.87E+09	1.99E+09	2.40E+08
L-lactate dehydrogenase B chain OS=Bos taurus OX=9913 GN=LDHB PE=2 SV=4	7.71E+08	5.91E+08	4.19E+08	3.35E+08	4.40E+07
Phosphoglycerate kinase 1 OS=Bos taurus OX=9913 GN=PGK1 PE=2 SV=3	1.36E+09	6.82E+08	4.46E+08	3.30E+08	4.66E+07
Aspartate aminotransferase, cytoplasmic OS=Bos taurus OX=9913 GN=GOT1 PE=1 SV=3	5.87E+08	6.03E+08	4.28E+08	4.16E+08	7.23E+07
Glucose-6-phosphate isomerase OS=Bos taurus OX=9913 GN=GPI PE=2 SV=4	7.07E+08	1.42E+09	7.64E+08	7.15E+08	7.28E+07
Albumin OS=Bos taurus OX=9913 GN=ALB PE=1 SV=4	3.33E+08	1.37E+09	9.28E+08	6.33E+08	2.48E+08

Phosphatidylethanolamine-binding protein 1 OS=Bos taurus OX=9913 GN=PEBP1 PE=1 SV=2	3.21E+08	2.36E+08	1.34E+08	1.78E+08	6.12E+07
Aspartate aminotransferase, mitochondrial OS=Bos taurus OX=9913 GN=GOT2 PE=1 SV=2	2.42E+08	2.72E+08	1.83E+08	1.98E+08	2.34E+07
Carbonic anhydrase 3 OS=Bos taurus OX=9913 GN=CA3 PE=2 SV=3	3.99E+09	1.89E+09	1.74E+09	1.13E+09	1.96E+08
Glutathione S-transferase Mu 1 OS=Bos taurus OX=9913 GN=GSTM1 PE=1 SV=3	1.91E+08	1.33E+08	7.77E+07	9.36E+07	1.74E+07
Malate dehydrogenase, mitochondrial OS=Bos taurus OX=9913 GN=MDH2 PE=1 SV=1	1.06E+09	7.38E+07	6.27E+07	3.01E+07	5.53E+06
Malate dehydrogenase, cytoplasmic OS=Bos taurus OX=9913 GN=MDH1 PE=2 SV=3	8.64E+08	6.39E+08	4.35E+08	3.42E+08	5.25E+07
Fructose-1,6-bisphosphatase isozyme 2 OS=Bos taurus OX=9913 GN=FBP2 PE=2 SV=1	1.30E+08	9.16E+07	5.45E+07	5.01E+07	3.71E+06
Hemoglobin subunit alpha OS=Bos taurus OX=9913 GN=HBA PE=1 SV=2	1.15E+08	1.06E+08	3.22E+07	3.64E+07	3.09E+06
Heat shock 70 kDa protein 1B OS=Bos taurus OX=9913 GN=HSPA1B PE=2 SV=1	6.81E+06	1.08E+07	8.46E+06	4.16E+06	2.68E+05
Actin, aortic smooth muscle OS=Bos taurus OX=9913 GN=ACTA2 PE=1 SV=1	7.26E+07	1.25E+08	1.30E+08	1.69E+07	4.39E+07
Glutathione S-transferase P OS=Bos taurus OX=9913 GN=GSTP1 PE=1 SV=2	1.33E+07	1.66E+07	5.13E+06	4.89E+06	4.94E+05
Heat shock cognate 71 kDa protein OS=Bos taurus OX=9913 GN=HSPA8 PE=1 SV=2	9.75E+06	3.56E+07	1.91E+07	9.37E+06	9.57E+05
Glyceraldehyde-3-phosphate dehydrogenase OS=Bos taurus OX=9913 GN=GAPDH PE=1 SV=4	5.84E+08	2.34E+08	2.16E+08	1.15E+08	2.22E+07

Adenosylhomocysteinase OS=Bos taurus OX=9913 GN=AHCY PE=2 SV=3	6.10E+07	6.87E+07	5.18E+07	3.92E+07	3.34E+06
Peroxiredoxin-6 OS=Bos taurus OX=9913 GN=PRDX6 PE=1 SV=3	4.23E+07	2.98E+07	1.62E+07	1.63E+07	2.03E+06
Purine nucleoside phosphorylase OS=Bos taurus OX=9913 GN=PNP PE=1 SV=3	5.52E+07	3.90E+07	2.28E+07	2.50E+07	2.64E+06
Peroxiredoxin-1 OS=Bos taurus OX=9913 GN=PRDX1 PE=2 SV=1	1.15E+08	4.34E+07	2.67E+07	2.73E+07	6.85E+06
Parkinson disease protein 7 homolog OS=Bos taurus OX=9913 GN=PARK7 PE=2 SV=1	1.61E+08	9.74E+07	6.03E+07	7.01E+07	1.94E+07
Citrate synthase, mitochondrial OS=Bos taurus OX=9913 GN=CS PE=1 SV=1	1.03E+08	7.92E+07	6.68E+07	3.74E+07	5.38E+06
Superoxide dismutase [Mn], mitochondrial OS=Bos taurus OX=9913 GN=SOD2 PE=2 SV=1	4.90E+07	3.84E+07	2.36E+07	3.06E+07	3.28E+06
Acetyl-CoA acetyltransferase, mitochondrial OS=Bos taurus OX=9913 GN=ACAT1 PE=2 SV=1	2.78E+07	1.23E+07	1.26E+07	6.38E+06	7.59E+05
Aldehyde dehydrogenase, mitochondrial OS=Bos taurus OX=9913 GN=ALDH2 PE=1 SV=2	2.09E+07	3.53E+07	3.02E+07	1.92E+07	3.45E+06
Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic OS=Bos taurus OX=9913 GN=GPD1 PE=2 SV=3	6.53E+08	8.92E+07	6.78E+07	3.47E+07	5.56E+06
UTP--glucose-1-phosphate uridylyltransferase OS=Bos taurus OX=9913 GN=UGP2 PE=1 SV=2	2.50E+07	3.35E+07	2.14E+07	1.56E+07	2.54E+06
Carbonic anhydrase 2 OS=Bos taurus OX=9913 GN=CA2 PE=1 SV=3	7.52E+07	4.31E+07	3.17E+07	2.55E+07	5.05E+06
Serotransferrin OS=Bos taurus OX=9913 GN=TF PE=2 SV=1	8.45E+06	3.64E+07	2.60E+07	1.45E+07	4.24E+06

Zeta-crystallin OS=Bos taurus OX=9913 GN=CRYZ PE=2 SV=2	5.92E+07	4.13E+07	3.36E+07	2.53E+07	4.21E+06
Retinal dehydrogenase 1 OS=Bos taurus OX=9913 GN=ALDH1A1 PE=1 SV=3	1.16E+07	2.06E+07	1.35E+07	1.22E+07	7.89E+05
Electron transfer flavoprotein subunit beta OS=Bos taurus OX=9913 GN=ETFB PE=1 SV=3	5.19E+07	1.84E+07	1.49E+07	1.09E+07	1.85E+06
Alanine aminotransferase 1 OS=Bos taurus OX=9913 GN=GPT PE=2 SV=1	3.89E+06	6.01E+06	5.05E+06	2.38E+06	1.71E+05
GTP:AMP phosphotransferase AK3, mitochondrial OS=Bos taurus OX=9913 GN=AK3 PE=1 SV=3	6.13E+06	4.27E+06	2.42E+06	1.59E+06	7.07E+04
Alpha-aminoadipic semialdehyde dehydrogenase OS=Bos taurus OX=9913 GN=ALDH7A1 PE=2 SV=4	4.07E+06	7.22E+06	7.58E+06	2.88E+06	8.22E+05
Acylphosphatase-2 OS=Bos taurus OX=9913 GN=ACYP2 PE=1 SV=2	5.40E+07	4.97E+07	3.39E+07	3.90E+07	1.40E+07
Dihydropteridine reductase OS=Bos taurus OX=9913 GN=QDPR PE=2 SV=1	1.09E+07	2.77E+06	3.48E+06	1.86E+06	2.11E+04
Peroxiredoxin-2 OS=Bos taurus OX=9913 GN=PRDX2 PE=2 SV=1	2.05E+07	1.85E+07	1.02E+07	1.04E+07	2.96E+06
Electron transfer flavoprotein subunit alpha, mitochondrial OS=Bos taurus OX=9913 GN=ETFA PE=2 SV=1	1.43E+07	5.51E+06	3.98E+06	9.71E+05	--
Aconitate hydratase, mitochondrial OS=Bos taurus OX=9913 GN=ACO2 PE=1 SV=4	1.89E+06	4.12E+06	4.61E+06	1.37E+06	--
Creatine kinase S-type, mitochondrial OS=Bos taurus OX=9913 GN=CKMT2 PE=2 SV=1	9.71E+06	2.69E+06	2.78E+06	1.05E+06	3.35E+05
Glycerol-3-phosphate phosphatase OS=Bos	1.05E+07	4.75E+06	4.45E+06	2.24E+06	2.70E+05

taurus OX=9913 GN=PGP PE=2 SV=1					
S-formylglutathione hydrolase OS=Bos taurus OX=9913 GN=ESD PE=2 SV=1	1.52E+07	3.90E+06	4.26E+06	1.62E+06	8.27E+04
Annexin A6 OS=Bos taurus OX=9913 GN=ANXA6 PE=1 SV=2	3.56E+06	8.14E+06	7.76E+06	3.89E+06	5.75E+05
Adenylosuccinate synthetase isozyme 1 OS=Bos taurus OX=9913 GN=ADSS1 PE=2 SV=1	1.28E+06	1.74E+06	9.98E+05	3.89E+05	--
3-ketoacyl-CoA thiolase, mitochondrial OS=Bos taurus OX=9913 GN=ACAA2 PE=2 SV=1	6.41E+06	1.85E+06	2.71E+06	1.02E+06	5.52E+04
Mth938 domain-containing protein OS=Bos taurus OX=9913 GN=AAMDC PE=2 SV=1	4.67E+06	5.03E+06	3.89E+06	4.15E+06	8.44E+05
Thioredoxin OS=Bos taurus OX=9913 GN=TXN PE=3 SV=3	4.31E+06	7.32E+06	5.36E+06	5.77E+06	1.08E+06
Thioredoxin-dependent peroxide reductase, mitochondrial OS=Bos taurus OX=9913 GN=PRDX3 PE=1 SV=2	4.89E+06	3.95E+06	1.80E+06	2.78E+06	4.11E+05
Mannose-6-phosphate isomerase OS=Bos taurus OX=9913 GN=MPI PE=2 SV=3	6.67E+05	--	--	1.84E+05	--
Annexin A7 OS=Bos taurus OX=9913 GN=ANXA7 PE=1 SV=2	4.28E+06	2.69E+06	3.14E+06	1.36E+06	5.74E+05

-- Indicates no peak area detected

APPENDIX B

RELATIVE ABUNDANCE OF COOKED AND DIGESTED *PSOAS MAJOR* PROTEINS WITH 4-ONE

	Peak area				
Protein	**Control**	**24 hrs**	**48 hrs**	**72 hrs**	**120 hrs**
Aspartate aminotransferase, cytoplasmic OS=Bos taurus OX=9913 GN=GOT1 PE=1 SV=3	3E+08	3E+08	7.3E+08	5.5E+08	6.9E+08
Myoglobin OS=Bos taurus OX=9913 GN=MB PE=1 SV=3	1E+09	9E+08	6E+08	1.1E+08	1.3E+08
Albumin OS=Bos taurus OX=9913 GN=ALB PE=4 SV=1	3E+08	3E+08	3.9E+08	4.5E+08	4.1E+08
Hemoglobin subunit beta OS=Bos taurus OX=9913 GN=HBB PE=1 SV=1	7E+08	2E+08	1E+08	6.8E+07	5.3E+07
Myosin-7 OS=Bos taurus OX=9913 GN=MYH7 PE=3 SV=3	3E+07	1E+08	2.1E+07	1E+07	8E+06
L-lactate dehydrogenase OS=Bos taurus OX=9913 PE=3 SV=1	1E+08	1E+08	1.2E+08	7.8E+07	8.3E+07
Actin, alpha 1, skeletal muscle OS=Bos taurus OX=9913 GN=ACTA1 PE=2 SV=1	2E+08	3E+08	9E+07	5.9E+07	5.2E+07
Myosin-1 OS=Bos taurus OX=9913 GN=MYH1 PE=2 SV=2	5E+06	3E+06	3E+06	9E+05	2E+05
Myosin-2 OS=Bos taurus OX=9913 GN=MYH2 PE=3 SV=1	1E+06	7E+05	7E+05	7E+05	--
Keratin, type I cytoskeletal 14 OS=Bos taurus OX=9913 GN=KRT14 PE=1 SV=3	7E+06	7E+07	5.3E+07	3.7E+07	2.7E+07
Phosphoglucomutase-1 OS=Bos taurus OX=9913 GN=PGM1 PE=3 SV=1	1E+08	2E+07	2.3E+07	2.2E+07	1.8E+07

Superoxide dismutase [Mn], mitochondrial OS=Bos taurus OX=9913 GN=SOD2 PE=3 SV=3	2E+07	2E+07	4.2E+07	4.7E+07	4.9E+07
Tropomyosin beta chain OS=Bos taurus OX=9913 GN=TPM2 PE=2 SV=1	7E+07	3E+07	2.1E+07	1.6E+07	6E+06
Pyruvate kinase (Fragment) OS=Bos taurus OX=9913 GN=PKM2 PE=2 SV=1	2E+07	2E+07	1.6E+07	8.3E+06	6E+05
Hemoglobin subunit alpha OS=Bos taurus OX=9913 GN=HBA PE=1 SV=2	1E+08	1E+08	4E+07	2.2E+07	1E+07
2-phospho-D-glycerate hydro-lyase OS=Bos taurus OX=9913 GN=ENO3 PE=1 SV=1	6E+07	1E+07	8.6E+06	5.1+E05	7.3+E06
Fructose-bisphosphate aldolase OS=Bos taurus OX=9913 GN=ALDOA PE=1 SV=1	1E+08	4E+07	3.2E+07	2.3E+07	2.3E+07
Filamin C OS=Bos taurus OX=9913 GN=FLNC PE=1 SV=1	2E+07	3E+07	3E+07	3.5E+07	2.6E+07
Cytokeratin-1 OS=Bos taurus OX=9913 GN=KRT1 PE=1 SV=2	5E+06	2E+07	3.7E+07	1.5E+07	2.3E+07
Inositol-1-monophosphatase OS=Bos taurus OX=9913 GN=IMPA1 PE=2 SV=1	2E+06	3E+06	4E+06	4E+06	3.8E+06
Malate dehydrogenase, cytoplasmic OS=Bos taurus OX=9913 GN=MDH1 PE=2 SV=3	3E+07	1E+07	2.1E+07	1.5E+07	1.7E+07
Keratin, type I cytoskeletal 27 OS=Bos taurus OX=9913 GN=KRT27 PE=2 SV=1	1E+07	4E+07	4.7E+07	2.7E+07	1.3E+07
L-lactate dehydrogenase OS=Bos taurus OX=9913 GN=LDHB PE=1 SV=1	5E+06	3E+06	3.4E+06	1.9E+06	2E+0.6
Pyruvate kinase (Fragment) OS=Bos taurus OX=9913 GN=PKM PE=2 SV=1	3E+06	2E+06	2E+06	6.4E06	7.5E0.5
Serpin B6 OS=Bos taurus OX=9913 GN=SERPINB6 PE=3 SV=1	5E+06	5E+06	9E+06	9E+06	1.1E+07

Creatine kinase OS=Bos taurus OX=9913 GN=CKMT2 PE=3 SV=1	1E+07	8E+06	1.6E+07	7362620	1.2E+07
Tropomyosin alpha-3 chain OS=Bos taurus OX=9913 GN=TPM3 PE=2 SV=1	--	--	--	--	--
Uncharacterized protein OS=Bos taurus OX=9913 PE=3 SV=1	--	--	--	--	2E+06
Alanine aminotransferase 1 OS=Bos taurus OX=9913 GN=GPT PE=4 SV=1	--	--	--	--	--
Keratin, type II cytoskeletal 5 OS=Bos taurus OX=9913 GN=KRT5 PE=1 SV=1	--	1E+06	5E+06	2.7E+06	6E+06
Keratin 3 OS=Bos taurus OX=9913 GN=KRT3 PE=1 SV=1	2E+06	--	--	--	--
KRT6A protein OS=Bos taurus OX=9913 GN=KRT6A PE=2 SV=1	--	--	--	--	--
Antithrombin-III OS=Bos taurus OX=9913 GN=SERPINC1 PE=3 SV=1	--	1E+06	6E+06	1E+06	2E+07
Alpha-1,4 glucan phosphorylase OS=Bos taurus OX=9913 GN=PYGM PE=2 SV=1	2E+06	4.6E+05	--	--	--
Glyceraldehyde-3-phosphate dehydrogenase OS=Bos taurus OX=9913 GN=GAPDH PE=1 SV=1	--	--	--	--	--
Aspartate aminotransferase, mitochondrial OS=Bos taurus OX=9913 GN=GOT2 PE=1 SV=2	--	--	--	--	--
Uncharacterized protein OS=Bos taurus OX=9913 PE=4 SV=1	--	--	5.4E+05	--	2.7E+06
Phosphatidylethanolamine-binding protein 1 OS=Bos taurus OX=9913 GN=PEBP1 PE=1 SV=2	--	--	3.4E+05	7.6E+05	1.6E+06
ATP synthase subunit beta OS=Bos taurus OX=9913 GN=ATP5F1B PE=3 SV=1	--	--	--	--	3.8E+06

IF rod domain-containing protein OS=Bos taurus OX=9913 GN=KRT76 PE=3 SV=1	--	--	2.57E+05	--	6.63E+06
Myosin regulatory light chain 2, ventricular/cardiac muscle isoform OS=Bos taurus OX=9913 GN=MYL2 PE=4 SV=1	--	--	--	--	2.05E+06
2-phospho-D-glycerate hydro-lyase OS=Bos taurus OX=9913 GN=ENO1 PE=3 SV=2	--	--	--	--	---
Collagen alpha-1(I) chain OS=Bos taurus OX=9913 GN=COL1A1 PE=1 SV=3	--	1.00E+06	5.02E+06	2.75E+06	6.13E+06
Serpin A3-6 OS=Bos taurus OX=9913 GN=SERPINA3-6 PE=3 SV=1	2.00E+06	--	--	--	--
Glyceraldehyde-3-phosphate dehydrogenase, testis-specific OS=Bos taurus OX=9913 GN=GAPDHS PE=2 SV=1	--	--	--	--	--
Fumarylacetoacetase OS=Bos taurus OX=9913 GN=FAH PE=3 SV=1	--	1.00E+06	6.80E+06	1.44E+06	2.00E+07
ADP/ATP translocase 1 OS=Bos taurus OX=9913 GN=SLC25A4 PE=1 SV=3	2.00E+06	4.66E+05	--	--	--
Cytochrome c OS=Bos taurus OX=9913 GN=CYCS PE=1 SV=2	--	--	--	--	--
LIM domain binding 3 OS=Bos taurus OX=9913 GN=LDB3 PE=4 SV=1	--	--	--	--	--
Collagen alpha-2(I) chain OS=Bos taurus OX=9913 GN=COL1A2 PE=1 SV=2	--	--	5.42E+05	--	2.72E+06
Myosin light chain 1/3, skeletal muscle isoform OS=Bos taurus OX=9913 GN=MYL1 PE=2 SV=1	--	--	3.46E+05	7.65E+05	1.60E+06
Slow skeletal troponin I OS=Bos taurus OX=9913 PE=2 SV=1	--	--	--	--	3.87E+06

Uncharacterized protein OS=Bos taurus OX=9913 GN=ANKHD1 PE=4 SV=3	--	--	2.57E+05	--	6.63E+06
Transforming acidic coiled-coil containing protein 1 OS=Bos taurus OX=9913 GN=TACC1 PE=3 SV=1	--	--	--	--	2.05E+06
Na-K-Cl cotransporter OS=Bos taurus OX=9913 PE=2 SV=1	--	--	--	--	--
Transcription factor AP-2 beta OS=Bos taurus OX=9913 GN=TFAP2B PE=3 SV=1	--	1.00E+06	5.02E+06	2.75E+06	6.13E+06
NOVA alternative splicing regulator 1 OS=Bos taurus OX=9913 GN=NOVA1 PE=4 SV=1	2.00E+06	--	--	--	--
Histone domain-containing protein OS=Bos taurus OX=9913 GN=LOC523458 PE=3 SV=1	--	--	--	--	--
Parkinson disease protein 7 homolog OS=Bos taurus OX=9913 GN=PARK7 PE=2 SV=1	--	1.00E+06	6.80E+06	1.44E+06	2.00E+07
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial OS=Bos taurus OX=9913 GN=DLST PE=3 SV=1	2.00E+06	4.66E+05	--	--	--
Polybromo 1 OS=Bos taurus OX=9913 GN=PBRM1 PE=4 SV=1	--	--	--	--	--
Mitotic spindle organizing protein 2 OS=Bos taurus OX=9913 GN=MZT2 PE=2 SV=1	--	--	--	--	--
Indoleamine 2,3-dioxygenase 2 OS=Bos taurus OX=9913 GN=IDO2 PE=3 SV=2	--	--	5.42E+05	--	2.72E+06
Synaptojanin-1 (Fragment) OS=Bos taurus OX=9913 GN=SYNJ1 PE=1 SV=2	--	--	3.46E+05	7.65E+05	1.60E+06

Endoplasmin OS=Bos taurus OX=9913 GN=HSP90B1 PE=2 SV=1	--	--	--	--	3.87E+06

-- Indicates no peak area detected

APPENDIX C

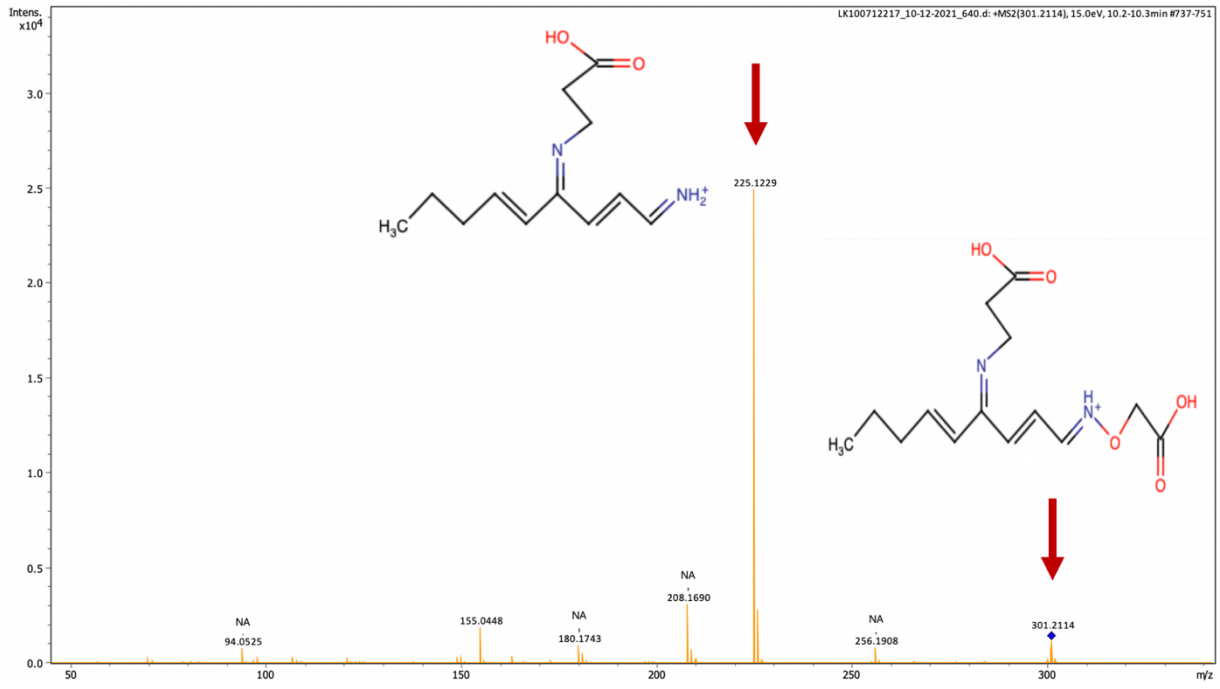


Figure C. CID MASS SPECTRUM AT 15EV OF 4-ONE WITH POSSIBLE ION STRUCTURES.

APPENDIX D

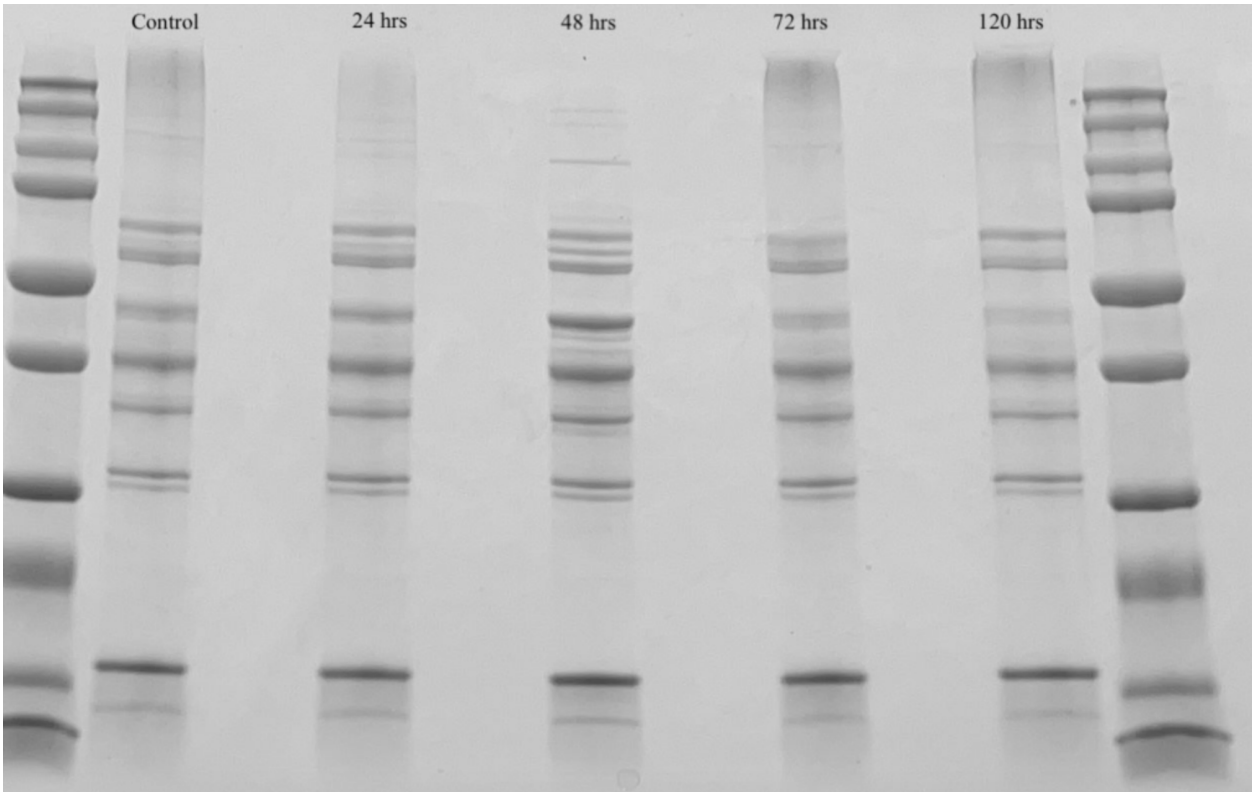


Figure D. SDS-PAGE OF *PSOAS MAJOR* INCUBATED WITH 4-HNE AT 37°C.