Proteomic features associated with tenderness of aged pork loins

Kelsey BreAnn Carlson

Iowa State University

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Proteomic features associated with tenderness of aged pork loins

by

Kelsey BreAnn Carlson

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Meat Science

Program of Study Committee:
Steven Lonergan, Major Professor
Elisabeth Huff-Lonergan
Kenneth Prusa

Iowa State University
Ames, Iowa
2016

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ABSTRACT

Tenderness plays a large role in the definition of meat quality and consumer purchasing decisions. The objectives for this study were to determine differences in proteolysis and sarcoplasmic proteomes that are attributed to variation in tenderness of aged pork loins with similar pH, lipid, and color measurements. Loins (n = 159) were collected one day postmortem from carcasses of Duroc-sired crossbred commercial pigs and aged for approximately 9 – 11 days. Chops (2.54 cm) were collected evaluated for purge, cook loss, pH post-aging, visual color and marbling, Hunter L, a, and b, sensory, star probe measurements (kg), and total lipid. Two libraries of samples with different star probe values were assembled. Final selection limited samples to be within specified ranges for ultimate pH (5.54 – 5.86), marbling score (1.0 – 3.0), and percent total lipid (1.61 – 3.37%). A low star probe group (n = 12, 4.95 kg) and high star probe group (n = 12, 7.75 kg) were utilized for further testing. Data was collected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blots, two dimensional difference in gel electrophoresis (2D-DIGE), and mass spectrometry. The use of SDS-PAGE and Western blots quantified the amount of degradation of the proteins troponin-T, desmin, filamin, and titin in classified sample groups, as well as the rate of autolysis of calpain-1. SDS-PAGE was also used to evaluate predominate myosin heavy chain isoforms to determine fiber type differences in the classification groups. Sarcoplasmic protein abundance and modification of proteins was evaluated by using 2D-DIGE. Two experiments utilizing 2D-DIGE were performed 1) using an 11 cm immobilized pH gradient (IPG) pH 4 – 7 strip and 2) using a 24 cm IPG pH 6 – 9 strip. Protein spots that
differed in abundance were identified from the 2D-DIGE experiments with the use of mass spectrometry.

Calpain-1 was completely autolyzed in both high and low star probe samples, demonstrating calpain-1 was potentially active to some extent in all samples. Extreme differences in proteolysis were exhibited in whole muscle samples where low star probe samples had more troponin-T ($P < 0.01$), desmin ($P < 0.01$), and filamin degradation ($P < 0.01$). Both low and high star probe myofibrillar samples showed degradation of the large protein titin, but select high star probe samples also exhibited intact bands of titin. Results from the 2D-DIGE experiments showed high star probe samples had significantly more metabolic, stress response, and regulatory protein abundance compared to low star probe samples. Specifically, the stress response protein peroxiredoxin-2 was more abundant in high star probe samples in 2D-DIGE experiment one ($P \leq 0.01$, 2 spots) and one-dimensional Western blot confirmations ($P = 0.02$). Low star probe samples showed significantly more degradation of the structural protein desmin as determined in 2D-DIGE experiment one ($P < 0.01$) and one-dimensional Western blot confirmations ($P < 0.01$). These results demonstrate there were extreme proteolytic differences that contributed to measured tenderness of low and high star probe samples. The proteins peroxiredoxin-2 and desmin were found in the soluble protein fraction of muscle as being differentially expressed in classification groups and show potential to be utilized as biomarkers to classify tough and tender aged pork products.
Consumers purchasing decisions for pork products are strongly influenced by eating experience. Pork that is consistently more tender and juicy when evaluated by consumers is more likely to be purchased (Brewer et al., 2001). Pork quality is largely influenced and defined by perceived tenderness (Bray, 1966). During the conversion of muscle to meat, there are a variety of factors that could impact tenderization of meat. Tenderness can be influenced by the amount of lipid in meat (DeVol et al., 1988; Lonergan et al., 2007), collagen content (Nishimura et al., 2009), rate of pH decline (Melody et al., 2004) and ultimate pH of meat (Lonergan et al., 2007). Even if all of these attributes are the same across meat samples, variation in tenderness still may exist. Multiple studies have confirmed that aging meat has beneficial impacts on perceived tenderness (Channon et al., 2004; Ngapo et al., 2013). The proteome of muscle changes during this aging period, impacting muscle structure. These proteomic changes result in degradation of structural proteins that can influence measured tenderness (Huff-Lonergan et al., 1996; Melody et al., 2004). Additional proteomic changes occur postmortem, such as differences in protein abundance. An accumulation of metabolic (Lametsch et al., 2003) and stress related proteins (Jia et al., 2009) are shown in postmortem muscles with differences in tenderness. It is important to understand the changes occurring during the postmortem tenderization process in order to identify biomarkers that could differentiate between tough and tender meat products. Utilization of biomarkers in a production setting could help eliminate inconsistency of products displayed in the retail setting, as
well as the potential for branded products. The current study examined proteomic differences in aged pork *Longissimus dorsi* muscles that had extreme differences in tenderness, but little to no significant influence of pH, lipid, color, and purge. It was hypothesized that variation in fresh pork loin tenderness is attributed to differences in proteolysis and differences in sarcoplasmic proteomes. The objectives for the study were to determine the amount of proteolysis and differences in sarcoplasmic proteomes in samples with extreme star probe values. The results from this study will help pave the way in identifying potential biomarkers to differentiate aged pork loins with varying degrees of tenderness.

**Thesis Formatting**

This thesis was organized into four chapters. Chapter one contains a general introduction explaining the importance of pork tenderness and factors that influence tenderness. Chapter two includes a review of literature on the topic of fresh meat quality and proteomics. Chapter three is titled “Proteomic features associated with tenderness of aged pork loins” and is prepared for submission to the *Journal of Animal Science*. Chapter four contains a general conclusion for the entire thesis.
Literature Cited


CHAPTER 2
REVIEW OF LITERATURE

Introduction

Consumer eating experience of pork is predominately determined by the palatability of the cooked product. Palatability is largely influenced by perceived tenderness. In the retail setting, there is significant variation in fresh pork tenderness presented to consumers (Larsen, 2015). Consumer eating experience can be inconsistent because of the inability to differentiate poor quality products from high quality products. It has been established that consumers are willing to pay a premium of up to $0.37/lb to have guaranteed tender pork products (Sanders et al., 2007). Unfortunately, there are no certified programs that guarantee tenderness to consumers. The lack of differentiation in the quality of pork products coupled with the consumer desire for tender pork demonstrates a need for definition of tenderness from the pork industry.

One way to help define tenderness of pork products is to identify potential biomarkers that could distinguish between tough and tender products. The use of proteomics has aided in the understanding of the tenderization process, and has the potential to further define pork tenderness. In order to identify biomarkers with the use of proteomics, it is fundamental to understand the complexity of muscle and how that complexity plays a role in the conversion of muscle to meat. This review focuses on defining the structure, function, and metabolism of muscle and how these attributes influence meat products.
**Organization of Muscle and Influence on Meat**

Muscle is a complex tissue. Muscle architecture is dependent on organization of proteins that interact with one another to provide form and functions. The three types of muscles found in mammals are skeletal, cardiac, and smooth muscles. These muscle types provide movement, stability, and other vital metabolic and cardiac processes for animals. Skeletal muscle differs from smooth and cardiac muscle because it is under voluntary control. The function and use of skeletal muscle in the live animal plays a major role in the meat product obtained from that specific tissue. The majority of meat products obtained from animals is from skeletal muscle, which will be the focus of this review.

Skeletal muscle is made up of bundles of muscle fibers and individual muscle fibers that are highly organized by collagenous connective tissue layers. The outermost connective tissue layer, termed the epimysium, encapsulates the entire muscle and is the site where intermuscular fat deposits (Goll et al., 1984; Nishimura et al., 1994; Nishimura, 2015). The epimysium is divided into two layers. The thickness of these layers depends largely on muscle function and how much the muscle changes in length and width during use (Nishimura et al., 1994). Muscle is made up of muscle bundles that are surrounded by the connective tissue layer known as the perimysium (Goll et al., 1984; Nishimura et al., 1994; Nishimura, 2015). The perimysium is composed of multiple layers of wavy-patterned sheets that run transversely to the axis of the muscle fiber (Nishimura et al., 1994). Fat deposited around perimysium is termed intramuscular fat (Goll et al., 1984; Nishimura, 2015), also known as marbling. Deposition of intramuscular fat can potentially have an effect on palatability of meat products. Muscle
bundles are further divided into muscle fibers by a thin layer of connective tissue called the endomysium. The endomysium surrounds entire muscle fibers (Rowe, 1978; Nishimura et al., 1994; Nishimura, 2015). The endomysium and the perimysium are connected by loose networks of collagen fibrils that allow for interaction between the two layers (Nishimura et al., 1994). All of these connective tissue layers interact with each other to provide form and function for skeletal muscle. The organizational structure of muscle by these connective tissue layers is fundamentally important for vascular and neural functions, along with fat deposition within and around the muscle (Cassens, 1987). All of these factors can potentially play a role in meat tenderness.

The structure of skeletal muscle fibers, also termed muscle cells, is important for the functionality of the muscle. The size of both muscle fibers and muscle bundles plays a role in the overall texture of meat. Larger muscle bundles and fibers are typically found in muscles used for locomotion (Goll et al., 1984). Large muscle bundles/fibers have a coarser texture in comparison to muscles used for stability, which typically contain smaller muscle bundles and fibers (Goll et al., 1984). Within muscle fibers, proteins are arranged in a manner that facilitates contracting, relaxing, and resting states of muscle. Understanding the diverse network and interactions that occur in muscles is fundamental in understanding tenderness differences in meat.

Muscle fibers are long and threadlike in appearance that can range in diameter from 10 µm to 100 µm or more, extending throughout the muscle (Huxley, 1958). Muscle fibers are multinucleated (Mauro, 1961) and contain an average of 100-200 nuclei per muscle cell (Goll et al., 1984). These fibers are surrounded by a cell membrane known as the sarcolemma, which is found under and immediately adjacent to the
endomysium (Cassens, 1987). The sarcolemma is approximately 70-100 nm thick and is important for transmitting action potentials throughout muscle fibers (Goll et al., 1984). The sarcolemma works in conjunction with transverse tubules. Transverse tubules (T-tubules) are extensions of the sarcolemma that project into the muscle fiber and are responsible for transmitting signals needed for the contraction and relaxation of muscle (Peachey, 1965). The sarcoplasmic reticulum is found directly beneath the sarcolemma and surrounds each myofibril. The sarcoplasmic reticulum also plays a role in contraction and relaxation of the muscle because it acts as a reservoir for calcium that is needed for contraction. The contractile elements of muscle fibers are found within the myofibrils.

Myofibrils are organelles within the sarcoplasm. These myofibrils comprise approximately 80 – 87% of the muscle fiber volume. Inside myofibrils are thick and thin filaments used for contraction and relaxation. Myofibrils are striated in appearance due to alternating light and dark bands in their structure (Cassens, 1987). The light bands are found in the I-band of myofibrils, which contain the thin filaments (Hanson and Huxley, 1953). The thin filaments are primarily composed of the protein actin (Hanson and Huxley, 1953). The dark bands are located in the A-band of the myofibrils. The A-band contains the thick filaments, which are primarily composed of the protein myosin (Hanson and Huxley, 1953). The A-band can also contain portions of the thin filaments, depending on contractile state and it is approximately 1.5 μm in length (Hanson and Huxley, 1953; Huxley and Hanson, 1954). The M-line is found in the center of the A-band, and proteins within the M-line play a role in stabilizing thick filaments.

Myofibrils are made up of repeating structures called sarcomeres. These structures are responsible for muscle contraction. Each sarcomere is bordered by two Z-
lines, which are responsible for holding the thin filaments in the proper position (Goll et al., 1984). The Z-line intersects the I-band of repeating sarcomeres, and each sarcomere contains one A-band and two halves of an I-band. The distance between one Z-line and another in resting skeletal muscle is approximately 2.5 µm (Cross et al., 1981).

**Muscle Contraction and Influence on Muscle Architecture**

The contraction process and apparatus is a highly complex and organized system in living muscle. Therefore, it stands to reason that features of the contractile process and muscle architecture can influence the integrity of meat from skeletal muscle. Muscle contraction involves interaction of the thick and thin filaments in sarcomeres after stimulus from the brain. Stimuli for contraction are transmitted through the muscles fibers via the sarcolemma and transverse tubules. The transverse tubules are responsible for transmitting the signal for contraction to the sarcoplasmic reticulum (Peachey, 1965). One transverse tubule comes together with two sarcoplasmic reticuli to form a triad, which are located throughout the length of a muscle fiber. Depolarization of the transverse tubules (Porter and Palade, 1957) allows for calcium to be released from the sarcoplasmic reticulum into the sarcoplasm of the myofibrils (Spudich and Watt, 1971; Anthony Lai et al., 1988). Calcium is released via ryanodine receptors (Spudich and Watt, 1971; Anthony Lai et al., 1988). The calcium released interacts with regulatory proteins to facilitate contraction within muscles.

The proteins primarily involved with skeletal muscle contraction and relaxation are myosin and actin, along with the regulatory proteins tropomyosin and troponin. Actin is the primary protein comprising the thin filament and can be found in the form of G-actin (globular actin) and F-actin (filamentous actin) (Kabsch and Vandekerckhove,
F-actin is the primary component of the thin filaments and it is arranged in a helical pattern within these filaments (Holmes et al., 1990). Six actin filaments surround one myosin filament to facilitate contraction (Hanson and Huxley, 1953). Actin, the troponin complex, and tropomyosin are highly associated with one another (Hartshorne and Mueller, 1969). The troponin complex aids in the interaction of tropomyosin along F-actin (Lehman et al., 2001; Blumenschein et al., 2005). The regulatory protein troponin is made up of three subunits: troponin-C, troponin-I, and troponin-T, which all play a different role in the contractile process (Greaser and Gergely, 1971; Zot and Potter, 1987). In the presence of low calcium concentration (10^{-7} M/L) (Huxley, 1969), myosin binding sites on actin are blocked by tropomyosin and muscle is in the relaxed state.

Upon the release of calcium from the sarcoplasmic reticulum, the calcium concentration increases to approximately 10^{-5} M/L (Huxley, 1969). At this time, troponin-C binds calcium, ultimately influencing a conformational change in troponin-I (Blumenschein et al., 2005). This conformational change results in movement of troponin-T, the part of the complex associated with actin and tropomyosin (Lehman et al., 2001; Blumenschein et al., 2005). This causes movement of tropomyosin on the actin filament (Lehman et al., 2001; Blumenschein et al., 2005). The movement of tropomyosin exposes myosin binding sites on the thin filament, allowing for interaction between actin and myosin.

The primary protein of the thick filament, myosin, plays a large role in the contractile process. The thick filaments are comprised of approximately 200 – 400 intertwined myosin molecules (Au, 2004). Myosin is a large 520-kDa protein made up of two heavy chains and four light chains that compose the head and tail regions of its structure (Weeds and Lowey, 1971; Rayment et al., 1993; Clark et al., 2002). There are
two heads per myosin molecule. The myosin heads have ATP and actin binding sites that allow myosin to interact with actin in the presence of ATP and elevated calcium levels \((10^{-5}\text{ M/L})\) \cite{Huxley1969,Prochniewicz2004}. Myosin bound ATP is hydrolyzed to ADP and phosphate, which positions myosin in a manner that facilitates actin/myosin (actomyosin) cross-bridge formation between the thick and thin filaments. Myosin exerts a power stroke, which pulls actin towards the center portion of the sarcomere, causing the sarcomere to shorten, and the ADP and phosphate produced from the hydrolysis of ATP are released from myosin \cite{Irving1992,Clark2002}. If ATP is present in the muscle, it will bind to the myosin heads and the myosin will be released from actin, allowing for the muscle to return to its relaxed state when the stimulus for contraction resolves. When ATP stores are depleted within the muscle, the actomyosin cross-bridges will remain, resulting in rigor mortis.

**Contractile and Structural Proteins in Muscle**

*Myosin.* The primary protein of the thick filament is myosin. Myosin is a large 520-kDa protein made up of two 220-kDa heavy chains and four smaller light chains (approximately 20-kDa) that compose the head and tail regions of its structure \cite{Weeds1971,Rayment1993,Clark2002}. Myosin is comprised of two heads that have ATPase activity and actin binding sites to facilitate contraction \cite{Prochniewicz2004}. The N-terminal regions of myosin heavy chain and two light chains comprise the head region of myosin \cite{Clark2002}. The C-terminal regions of the myosin heavy chains makes up the rod portion of myosin \cite{Clark2002}. The rod portion of myosin is involved with myosin polymerization and connecting myosin heads to the thick filament core \cite{Clark2002}. The arrangement of the myosin heads on
the thick filaments allows for connection of the heads to the six surrounding thin filaments (Squire, 1972). Myosin is a dynamic protein that has both catalytic, regulatory, and structural roles within sarcomeres.

*Actin.* The primary protein of the thin filament is actin. The molecular weight of actin is approximately 42-kDa (Potter, 1974). Actin can be found in the globular form (G-actin) or filamentous form (F-actin) (Kabsch and Vandekerckhove, 1992). Two twisted alpha helical strands of F-actin comprise each thin filament (Holmes et al., 1990; Clark et al., 2002). Each alpha-helical turn on the thin filaments is comprised of seven actin monomers (Clark et al., 2002). Thin filaments are found primarily in the I-band in resting muscle and can also be found in portions of the A-band, depending on the contractile state of muscle (Hanson and Huxley, 1953). Actin is anchored at the Z-line by actin-binding proteins, such as filamin and Cap-Z (Clark et al., 2002). Actin is associated closely with tropomyosin and the troponin complex to facilitate contraction (Hartshorne and Mueller, 1969). During muscle contraction, myosin binding sites on actin are exposed, allowing actin and myosin to form cross-bridges with each other in the proper cellular conditions.

*Titin.* One of the largest proteins identified in muscle is titin. Titin has a molecular weight of approximately 3,000-kDa (Wang et al., 1979; Clark et al., 2002). Titin spans half the length of a sarcomere where the N-terminus is anchored at the Z-line and the C-terminus is attached at the M-line (Fürst et al., 1988; Whiting et al., 1989). The major functions of titin are to maintain sarcomere alignment during the contractile process and to help stabilize thick filaments (Horowits et al., 1986). Depending on the location in the sarcomere, titin has different roles. In the I-band, titin is more elastic and aids in
connection between the Z-line and thick filaments (Clark et al., 2002; Au, 2004). The portion of titin in the A-band is directly associated with the thick filaments and plays role in the assembly of these filaments (Labeit et al., 1997; Au, 2004). Titin is associated with a network of proteins located in the M-line that help to stabilize the thick filament within the sarcomere (Au, 2004). Modifications to this protein could be detrimental to the structure of muscle because of the role titin plays within the sarcomere.

**Nebulin.** Nebulin is another large protein (approximately 600 – 900-kDa) that is associated with thin filaments within sarcomeres (Horowits et al., 1986; Clark et al., 2002). Nebulin aids in thin filament assembly, acting as a molecular ruler for the thin filaments (Labeit et al., 1991). Nebulin is closely associated with actin, troponin, and tropomyosin along the thin filament (Labeit et al., 1991). The C-terminal end of nebulin is inserted into the Z-line, whereas the N-terminal end extends to the end of the thin filament where it interacts with the protein tropomodulin (McElhinny et al., 2001; Clark et al., 2002). It is also proposed that nebulin aids in regulation of contraction, signal transduction pathways, and linking myofibrils to intermediate filament proteins (McElhinny et al., 2003).

**Filamin.** Filamin is a large protein (approximately 240 – 300-kDa) that is associated with the Z-line of sarcomeres (Davies et al., 1978; Clark et al., 2002; Huff-Lonergan et al., 2010). Filamin interacts with F-actin at the level of the Z-line (Wang and Singer, 1977; Davies et al., 1978). Filamin also interacts with protein complexes located at the costameres to link the Z-line with the sarcolemma (Peter et al., 2011). The function of filamin in muscle is to aid in stabilization of the cytoskeleton and to help with
Desmin. Desmin is an intermediate filament protein that is essential for the structure of myofibrils. At the level of the Z-line, desmin acts to connect adjacent myofibrils with each other and to connect peripheral myofibrils to proteins assemblies on the sarcolemma, known as costameres (Granger and Lazarides, 1979; Clark et al., 2002). Desmin also functions to align muscle fiber components in myofibrils, such as mitochondria (Clark et al., 2002). The structure of desmin consists of an alpha-helical rod, an N-terminal head, and a C-terminal tail (Geisler and Weber, 1982; Clark et al., 2002; Bär et al., 2004). The head and tail regions of desmin are responsible for proper filament assembly around myofibrils (Bär et al., 2004). In postmortem muscle, intact desmin (55-kDa) is degraded at the head and tail regions of the protein by calpain-1 (Baron et al., 2004). When incubated with the protease cathepsin B, small fragments from the carboxy-terminal end of desmin were cleaved and no clear desmin degradation fragments appeared when analyzed with SDS-PAGE (Baron et al., 2004). Overall, desmin plays an integral role in multiple aspects of muscle organization. Therefore, disruption of myofibrillar organization due to desmin modification or degradation could largely impact muscle fiber structure, potentially influencing tenderness in meat.

Muscle Fiber Types

Muscle is composed of types of red and white fibers that can be classified by contraction speed, glycolytic capacity, and aerobic capacity (Peter et al., 1972). Red muscle fibers contain more myoglobin, mitochondria, and have more oxidative activity than white fibers, whereas white fibers have more glycolytic activity (Close, 1972).
Contraction speed in muscle is dictated by myosin ATPase activity, which provides the energy needed for contraction. Muscles that have fast contraction rates (fast-twitch) have more myosin ATPase activity than slower contracting (slow-twitch) muscles (Bárány, 1967). Red muscle fibers are typically slow-twitch and have the ability to contract over a longer period of time, whereas white muscle fibers are typically fast-twitch fibers that contract in short bursts and are easily fatigued. Electron microscopy images document that Z-lines from white muscle fibers are thicker than red muscle fibers (Eisenberg, 1975). The thicker Z-lines potentially facilitate the strong, short-burst contractions generated from white fibers (Eisenberg, 1975).

Muscle fibers can be classified further based off myosin heavy chain isoforms and rate of ATP breakdown in muscle. Myosin heavy chain is a component of the contractile protein myosin, and is responsible for ATP hydrolysis and actin binding (Reggiani et al., 2000). Myosin heavy chain isoforms play a role in distinguishing muscle fiber types by defining the contractile and metabolic properties of muscle fibers (Choi et al., 2007). Myofibrillar ATPase activity in postmortem muscle is influenced by predominate myosin heavy chain isoform (Bowker et al., 2004). There are eight known myosin heavy chain protein isoforms, and the types primarily found in mammalian skeletal muscle are myosin heavy chain types I, IIa, IIx, and IIb (Reggiani et al., 2000). Red fibers predominately contain types I and IIa isoforms, whereas white fibers contain predominately types IIx and IIb. Myosin heavy chain isoforms can also be grouped based off on contractile speed and metabolism of muscle. Type I muscle fibers are slow-twitch fibers that rely primarily on oxidative metabolism (Reggiani et al., 2000; Gil et al., 2003). Type II fibers rely primarily on glycolytic metabolism and are fast-twitch fibers (Reggiani et al., 2000; Choe
et al., 2008). Type IIx muscle fibers are also known as intermediate fibers. This nomenclature reflects the metabolic activity and intermediate contraction speed of the fibers (Schiaffino et al., 1989; Gorza, 1990).

Muscle fiber type has the ability to influence the postmortem conversion of muscle to meat. The *Psoas major* contains more type I-slow muscle fibers compared to *Longissimus dorsi* and *Semimembranosus* muscles (Melody et al., 2004). In the same study, the *Psoas major* had a more rapid pH decline than the *Longissimus dorsi* and *Semimembranosus* muscles (Melody et al., 2004). Slow muscle fibers have a less developed sarcoplasmic reticulum than fast fibers, so it was presumed that the temperature decline postmortem in combination with more slow muscle fibers resulted in greater calcium concentrations in the muscle, ultimately increasing metabolic activity in the *psoas major* (Melody et al., 2004). Fast fibers (types IIa, IIx, and IIb, classified by predominate myosin heavy chain isoform) have been identified to have greater myofibrillar ATPase capacity and are more susceptible to ATPase denaturation and inactivation from low pH and temperature postmortem than slow fiber types (Bowker et al., 2004). This could ultimately influence energy utilization and cross bridge formation during the conversion of muscle to meat in fast fibers (Bowker et al., 2004).

**Muscle Metabolism**

The primary form of energy currency in the muscle is adenosine triphosphate (ATP), which is important for mechanisms of contraction and relaxation in live muscle and during the conversion of muscle to meat. Muscle stores of ATP are relatively low, approximately 8 – 15 µmol/g of muscle (Goll et al., 1984), so aerobic and anaerobic pathways are essential to produce the amount of ATP needed in living muscles.
Anaerobic metabolism produces ATP for the muscle through the process of glycolysis and by degradation of phosphocreatine (Westerblad et al., 2010). During extended periods of ATP consumption, such as muscle contraction, creatine kinase catalyzes the reaction between phosphocreatine and adenosine diphosphate (ADP) to produce creatine and ATP (Goll et al., 1984; Westerblad et al., 2010). Creatine kinase is also used to replenish the amount of phosphocreatine from this pathway, which is done by rephosphorylating the creatine produced during the formation of ATP (Goll et al., 1984). Another reaction that takes place within the muscle to produce ATP during times of sustained contraction involves the conversion of two ADP molecules to ATP and adenosine monophosphate (AMP), by the enzyme myokinase (Goll et al., 1984; Scheffler and Gerrard, 2007).

Glycolysis produces the high energy electrons needed for the production of ATP via creatine kinase or myokinase pathways. When consumption of ATP exceeds the ability to produce energy for the muscle through these pathways, glycolysis gets stimulated. Anaerobic glycolysis is the breakdown of glucose to pyruvate that can be further broken down into lactate and hydrogen ions, ultimately producing a small amount of ATP for the muscle to be used for cell processes (Scheffler and Gerrard, 2007). Glycogen stores within the muscle can be utilized for energy through the glycolytic pathway if glycogen gets converted to glucose 6-phosphate by the process of glycogenolysis, via the enzyme phosphoglucomutase (Tymoczko et al., 2013). One glucose molecule from glycogen yields a total of three ATP molecules. The steps involved in the glycolytic pathway of muscle are described below:
1. Glycogen in the muscle is broken down to glucose 1-phosphate by the enzyme glycogen phosphorylase.

2. Glucose 1-phosphate is converted to glucose 6-phosphate by the enzyme phosphoglucomutase.

3. Glucose 6-phosphate is isomerized to fructose 6-phosphate by phosphoglucone isomerase.

4. Fructose 6-phosphate is phosphorylated by ATP to fructose 1,6-biphosphate, a reaction catalyzed by phosphofructokinase.

5. Fructose 1,6-biphosphate is split into two triosephosphates that are isomers of each other (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate), a reversible reaction catalyzed by aldolase. Glyceraldehyde 3-phosphate is the product involved with the direct pathway of glycolysis. One molecule of ATP is utilized in this step.

6. Glyceraldehyde 3-phosphate is converted to 1,3-bisphosphoglycerate, a reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase (an oxidation-reduction reaction).

7. 1,3-bisphosphoglycerate transfers a phosphoryl group to ADP, producing one ATP molecule and 3-phosphoglycerate. This reaction is catalyzed by phosphoglycerate kinase.

8. 3-phosphoglycerate is converted to 2-phosphoglycerate via phosphoglycerate mutase, by switching the position of the phosphoryl group.

9. 2-phosphoglycerate is converted to phosphoenolpyruvate, a reaction catalyzed by enolase.
10. Phosphoenolpyruvate is converted to two molecules of pyruvate, via the enzyme pyruvate kinase. During this reaction, a phosphoryl group is transferred to ADP, producing one molecule of ATP.

**Steps 5 through 9 occurs twice, because there are two triosephosphates produced from fructose 1,6-biphosphate that go through the reaction.**

(Tymoczko et al., 2013)

The most efficient means to produce energy in living muscle is through mitochondrial aerobic metabolism (Scheffler and Gerrard, 2007). In aerobic metabolism, pyruvate from the glycolytic pathway is decarboxylated by pyruvate dehydrogenase to form acetyl CoA that can enter the mitochondria, where the tricarboxylic acid (TCA) cycle occurs (Tymoczko et al., 2013). The TCA cycle only functions in aerobic conditions and a net total of 36 ATP molecules can be produced per glucose moiety from glycogen. The steps of the TCA cycle are outlined below:

1. Citrate is formed from oxaloacetate and acetyl CoA, a reaction catalyzed by citrate synthase.
2. Isomerization of citrate to isocitrate, a reaction catalyzed by aconitase.
3. Isocitrate becomes oxidized and decarboxylated by isocitrate dehydrogenase, forming α-ketoglutarate.
4. Carbon dioxide is removed from α-ketoglutarate, and succinyl CoA is formed.
5. Succinyl CoA synthetase catalyzes the reaction of cleavage of the thioester bond of succinyl CoA, forming succinate. This is coupled to the phosphorylation of ADP.
6. Succinate is oxidized to fumarate, a reaction catalyzed by succinate dehydrogenase.

7. Fumarase catalyzes the reaction of fumarate to \( L \)-malate.

8. \( L \)-malate is oxidized to form oxaloacetate, a reaction catalyzed by malate dehydrogenase.

(Tymoczko et al., 2013)

**Conversion of Muscle to Meat**

During the conversion of muscle to meat, muscle undergoes extensive changes both metabolically and structurally. Following exsanguination, there is a loss of homeostasis in the body as a result of blood loss. Blood is the major carrier of oxygen within the body, so during the conversion of muscle to meat, the primary form of muscle metabolism switches from aerobic to anaerobic metabolism due to the loss of oxygen. In summary, skeletal muscle glycogen stores degrade postmortem through glycogenolysis and then enter the anaerobic glycolytic pathway as glucose 6-phosphate to produce ATP for the muscle. Pyruvate builds up from this process and is cleared by the enzyme lactate dehydrogenase (Huckabee, 1958). As a result, lactate and hydrogen ions start to accumulate in muscles. Calcium is also released postmortem from the sarcoplasmic reticulum, causing sustained contraction of muscles, which utilizes energy. ATP stores ultimately get depleted postmortem through degradation (Bate-Smith and Bendall, 1947), so the actin and myosin bonds formed during contraction are unable to release. This process results in rigor mortis.

Lactate and hydrogen ions accumulate from the anaerobic glycolytic process, reducing the pH from approximately 7.4 in living muscle to approximately 5.7, as
exhibited in pork *Longissimus dorsi* muscles (Bate-Smith and Bendall, 1949; Briskey and Wismer-Pedersen, 1961; Greaser, 1986). The rate at which the pH declines in muscles can be influenced by different factors. Temperature influences pH decline in muscles postmortem. Briskey and Wismer-Pedersen (1961) demonstrated that pork muscles chilled at higher temperatures had a steeper pH decline early postmortem. A condition known as PSE (pale, soft, and exudative pork) can result from rapid pH decline or very low ultimate pH levels (Bendall and Swatland, 1988). Stress prior to slaughter can influence pH decline and development of PSE and DFD (dark, firm, and dry) pork. The average pH of pork *Longissimus* muscles measured at 45 minutes after harvest from pigs stressed by exercise prior to slaughter was 6.3 compared to non-stressed pigs that had an average pH of 6.45 (Rosenvold and Andersen, 2003). When stress occurs prior to harvest (high temperatures, transport, mixing of animals, restriction of feed), glycogen reserves in muscles are utilized to respond to the stressor. When glycogen is limited in muscle prior to slaughter, higher ultimate pH can result due to limited glycolysis postmortem. Stressors such as transport (Hambrecht et al., 2005), fasting (Leheska et al., 2002), and movement of animals using loud noises and electrical probes have demonstrated depletion of glycogen in the muscle from pigs (Hambrecht et al., 2005). Muscle fiber type may also influence pH decline and ultimate pH of meat. Oxidative muscle has lower glycolytic potential (measures glycogen, glucose, glucose 6-phosphate, and lactate) and a higher ultimate pH than primarily glycolytic muscle (Hambrecht et al., 2005).

It is shown that pork with high ultimate pH (above 5.80) will be more tender than pork with low ultimate pH (below 5.50) as measured with star probe and sensory analysis (Lonergan et al., 2007). Some of the differences exhibited in tenderness at these extreme
pH ranges can be attributed to the activity of calpain-1, an endogenous protease in muscle. In an experiment examining activity of purified calpain-1 from pork skeletal muscle, calpain-1 had the greatest activity at pH 6.5 compared to pH 7.5 and 6.0 (Carlin et al., 2006). This experiment also demonstrated that at pH 6.5, purified calpain-1 did not lose activity as quickly as the other pH values measured (Carlin et al., 2006). Bee et al. (2007) demonstrated calpain-1 became inactive quicker in pork Longissimus muscles with a faster pH decline. This loss of proteolytic activity early postmortem could explain the lesser extent of desmin and talin degradation in muscles with more rapid pH decline (Bee et al., 2007). These results are consistent with the report of Pomponio et al. (2010), which showed pork loins with a more rapid pH decline lost calpain-1 activity quicker. As noted, a variety of factors can influence both the rate of pH decline and ultimate pH in pork. The role pH plays in the conversion of muscle to meat has the potential to interact with the tenderization process that occurs postmortem.

Factors Influencing Pork Tenderness

Lipid. Producers have been selecting for leaner, more efficient pigs to aid in feeding the growing population and meet consumer demands (Lonergan et al., 2001). The amount of lipid in meat products has the potential to influence measured tenderness. Purchasing decisions by pork consumers can be largely influenced by visual fat content within a product, so understanding consumer desire for marbling attributes is important for the pork industry. The purchasing intent of raw pork chops with different degrees of marbling was evaluated from 142 consumers and the results showed 41.55% of the consumers chose to purchase chops that were lean, 40.14% picked chops that exhibited medium amounts of marbling, and only 18.31% of the consumers selected chops that
were highly marbled (Brewer et al., 2001). However, sensory results from this study demonstrated consumers rated pork that is highly marbled (3.46% intramuscular fat) to be more juicy, tender, and flavorful than leaner chops (1.05% intramuscular fat) after in-home sensory evaluation, showing there are inconsistencies with consumer preference based off of visual examination and eating experiences (Brewer et al., 2001).

Breed type can influence the amount of intramuscular fat deposition in pigs. Duroc pigs have shown an increase in intramuscular fat content compared to other breeds (Channon et al., 2004; Zhang et al., 2007), which could ultimately impact sensory quality. Pigs that were 100% Duroc had increased intramuscular fat content (1.84 %) than pigs that were 100% Large White or 50% Large White/Duroc crosses (Channon et al., 2004). Pigs from that same study that were 100% Duroc were evaluated as being more juicy (Channon et al., 2004). Lipid content in loins from purebred Duroc pigs was significantly more ($P < 0.01$) than purebred Yorkshire, Hampshire, Spotted, Chester White, Poland China, Berkshire, and Landrace pigs (Zhang et al., 2007). Duroc (1769 mg/100 g muscle) and Berkshire (2051 mg/100 g muscle) pigs had increased intramuscular fat in Longissimus dorsi muscles compared to Large White (974 mg/100 g muscle) and Tamworth pigs (1204 mg/100 g muscle) (Wood et al., 2004). This increase in marbling lead to sensory scores that reflected chops to be more tender and juicy from Duroc and Berkshire pigs (Wood et al., 2004). Increased lipid content resulted in decreases in chewiness scores (as determined by a trained panel) and star probe values (kg) in pork loins within an intermediate pH range of 5.50 – 5.80 (Lonergan et al., 2007). Previous research suggests tenderness will only be influenced if intramuscular fat is within the range of 2.0% - 3.5% (DeVol et al., 1988). This demonstrates lipid content
may only be influential to tenderness if it above this specific amount and if percent lipid is lower, the influence may be negligible.

**Collagen Content.** Collagen is found within different the different organizational layers of the muscle (Nishimura et al., 1994; Nishimura, 2015). The epimysium is usually removed from the external layer of the muscle in chops, so it is not usually consumed. The perimysium comprises approximately 90% of intramuscular connective tissue, so it is the main contributor to variations in tenderness exhibited in meat, with regard to collagen content. Collagen can be solubilized in the presence of heat (Hill, 1966), but collagen that is not readily solubilized from the cooking process contributes to the background toughness in meat. In raw pork muscles, the amount of total collagen \( r = 0.857 \) and the thickness of the secondary perimysium \( r = 0.750 \) were significantly correlated with shear-force values (Nishimura et al., 2009). The contribution of collagen to tenderness in cooked meat products is more complex than simply amount of collagen. This is due to collagen cross-linking, which could ultimately affect the heat-solubility of collagen (Tanzer, 1973). In this same study examining raw pork muscles, the heat-solubility of collagen in these pork muscles did not exhibit a significant correlation \( r = -0.077 \) with shear force values (Nishimura et al., 2009). The reason for the low correlation in this study could be due to crosslinking of collagen. Crosslinking of collagen causes collagen to be less heat labile (Lepetit, 2007). These results are consistent with others that found little to no correlation between collagen content of raw meat samples and the texture of cooked meat (Campo et al., 2000; Christensen et al., 2011).

**Sarcomere length.** During the conversion of muscle to meat, muscles contract, causing the sarcomeres within myofibrils to shorten. Temperature has been seen to
influence sarcomere length during the conversion of muscle to meat. If carcasses are chilled too quickly before the onset of rigor mortis, cold shortening can set in (Savell et al., 2005). Feldhusen and Kühne (1992) demonstrated the effects of cold shortening on tenderness of pork *Longissimus* muscles by chilling the muscles to -5°C prior to rigor. This treatment shortened the sarcomere length by 33.5% compared to control muscles (Feldhusen and Kühne, 1992). The shortened sarcomere lengths were significantly correlated with Warner-Bratzler shear force values (*r* = -0.76) early postmortem (Feldhusen and Kühne, 1992), indicating that severely shorter sarcomeres resulted in greater Warner-Bratzler shear force values. Aging the muscles up to 72 hours did resolve some of the differences exhibited in shear force values between cold shortened and control muscle groups (Feldhusen and Kühne, 1992). These results are consistent with Wheeler et al. (2000) showing sarcomere length in unaged pork *Longissimus dorsi*, *Semitendinosus*, *Triceps brachii*, *Semimembranosus*, and *Biceps femoris* muscles explained 40% of the variations seen in tenderness. That study mentioned aging would influence the contribution of sarcomere length to tenderness in these muscles (Wheeler et al., 2000). Although sarcomere length can influence tenderness in early postmortem muscle, there are other processes going on during postmortem aging that can influence the tenderization of muscles.

*Proteolysis.* The process of aging meat in refrigerated conditions is shown to increase tenderness of meat (Channon et al., 2004; Ngapo et al., 2013). During the aging period, muscle proteins are degraded into peptides that can be further degraded into amino acids. This process is defined as proteolysis. Proteolysis during aging occurs from enzymatic action on proteins by endogenous muscle proteases.
The calpain system is a series of proteins that contribute to proteolysis. In muscle, this system is primarily made up of calpain-1, calpain-2, calpain-3, and calpastatin (Goll et al., 1998; Koohmaraie and Geesink, 2006). Calpain-1 and calpain-2 are cysteine proteases primarily associated with postmortem protein degradation of proteins. Calpastatin is the endogenous inhibitor of calpain-1 and calpain-2 (Murachi et al., 1981). Calpain-2 has limited activity postmortem, whereas calpain-1 is largely responsible for proteolysis of muscle proteins postmortem (Geesink et al., 2006; Koohmaraie and Geesink, 2006), so calpain-1 will be the focus of this review. Calpain-1 is made up of two subunits, a large 80-kDa catalytic subunit and a smaller 28-kDa subunit (Dayton et al., 1976; Koohmaraie and Geesink, 2006). The 80-kDa subunit is made up of four domains (I, II, III, and IV) that have different sequences and functions (Huff-Lonergan et al., 2010). Domain I contains the N-terminal of the protein, domain II is the main catalytic unit, and domains III and IV are associated with E-F hand binding sites of calcium (Huff-Lonergan et al., 2010). The 80-kDa subunit is primarily responsible for the catalytic process of protein degradation (Yoshizawa et al., 1995).

The influx of calcium into the sarcoplasm of muscle fibers postmortem and post rigor activates calpain-1 in muscle. Calcium binds to calpain-1, stimulating activity of the protease and degradation of muscle proteins (Goll et al., 1992). Calcium is also needed for the binding of calpain-1 and calpastatin, as well as for calpastatin to inhibit calpain-1 activity (Murachi et al., 1981). Increased calcium levels of 50-150 µM in the muscle fiber also acts to autolyze calpain-1 (Goll et al., 2003). Autolysis lowers the amount of calcium needed for the activation of calpain from 3-50 µM to 0.5-2 µM for half maximal activity (Suzuki et al., 1981; Saido et al., 1994, Goll et al., 2003). Autolysis also leads to
degradation of the 80-kDa subunit to 78-kDa and 76-kDa products by removing the N-terminal of the protein and additional amino acids (Dayton, 1982, Goll et al., 2003, Li et al., 2004). The degradation products (78 and 76-kDa) aggregate to form dimer and trimer molecules that have no proteolytic activity (Li et al., 2004). The formation of these dimers could explain why autolysis of calpain occurs, but no proteolysis. The rate of autolysis can be influenced by cellular conditions such as pH and temperature, which can ultimately influence the rate of calpain-1 activity and autolysis.

Some of the proteins degraded by calpain-1 are important for muscle contraction, structure, and integrity. Huff-Lonergan et al. (1996) examined the extent of degradation of aged bovine myofibrils compared to unaged myofibrils incubated with calpain-1. Results from this study demonstrated degradation of the cytoskeletal proteins titin, nebulin, filamin, desmin, and troponin-T from both the aged myofibrils and myofibrils incubated with calpain-1 (Huff-Lonergan et al., 1996). This is consistent with results from Lametsch et al. (2004), that showed degradation of desmin, troponin-T, myosin heavy chain, myosin light chain I, actin, and tropomyosin isoforms in purified porcine myofibrils incubated with calpain-1 in aging conditions. Taken together, these observations provide convincing evidence that calpain-1 plays a significant role in proteolysis of myofibrillar, cytoskeletal, and intermediate filament proteins during postmortem aging of meat (Huff-Lonergan, 1996; Lametsch et al., 2004).

In pork muscles, degradation of muscle proteins has been associated with increased tenderness. Desmin degradation in pork explained 26% and 38% of the variation in tenderness exhibited between Semimembranosus and Biceps femoris muscles (Wheeler et al., 2000). Melody et al. (2004) examined degradation of titin, desmin, and
nebulin in pork *Longissimus dorsi, Semimembranosus,* and *Psoas major* whole muscle protein samples and discovered that at 48 hours and 120 hours postmortem, *Longissimus dorsi* muscles had greater degradation of these proteins. Protein degradation could help explain why Warner-Bratzler shear force values (kg) measured at 48 hours and 120 hours postmortem were lower in the *Longissimus dorsi* muscles compared to *Semimembranosus* muscles (Melody et al., 2004). The *Psoas major* had the lowest Warner-Bratzler shear force values (kg) at 24 hours postmortem, which could be related to its rapid pH decline, early autolysis of calpain-1, early degradation of titin and desmin, lower amount of collagen, and a faster rate of appearance of calpain-1 bound to myofibrils (Melody et al., 2004).

Muscle pH and rate of pH decline in postmortem muscle can influence calpain-1 activity and autolysis (Carlin et al., 2006; Bee et al., 2007; Pomponio et al., 2010). A more rapid rate of pH decline can result in earlier autolysis and inactivation of calpain-1 (Bee et al., 2007). Melody et al. (2004) demonstrated that the *Psoas major* had the most rapid pH decline when compared to *Longissimus dorsi* and *Semimembranosus* muscles and calpain-1 autolysis occurred in 94% of the samples within 45 minutes postmortem. The *Longissimus dorsi* and *Semimembranosus* muscles showed no evidence of autolysis at 45 minutes postmortem (Melody et al., 2004).

Oxidative conditions can also influence calpain-1 and calpastatin activity. Carlin et al. (2006) examined calpain-1, calpain-2, and calpastatin activity in oxidative conditions using hydrogen peroxide. Both purified calpain-1 and calpain-2 activity decreased in oxidative conditions, and degradation of desmin by calpain-1 in myofibrils was also decreased (Carlin et al., 2006). Oxidation also increased desmin degradation
when a complex was formed between calpain-1 and calpastatin (Carlin et al., 2006). These results indicated the inhibition of calpain-1 by calpastatin is reduced by oxidation (Carlin et al., 2006). Oxidation also impacts protein structure, which can alter susceptibility of calpain-1 degradation of that protein. Chen et al. (2014) demonstrated oxidation altered the secondary structure of desmin, which decreased the rate calpain-1 degradation on this protein. Interestingly, oxidation increased susceptibility of desmin degradation by other proteases, such as caspase-3 and caspase-6 (Chen et al., 2014).

Proteins modified or damaged by oxidation are more susceptible to proteases to prevent accumulation of damaged proteins (Mehlhase and Grune, 2002).

**Use of Proteomics in Evaluating Meat Quality**

Proteomics is the study of proteins and their functions. The utilization of proteomics in meat science has aided in discovering changes that occur during the conversion of muscle to meat. Multiple studies have also examined the tenderization process that occurs postmortem using proteomic techniques (Huff Lonergan et al., 2011; Lametsch, 2011). Understanding the differences in the protein profiles of tough and tender meat products will aid in the identification of a biomarker to be used in the industry to classify pork products. This review will focus on the use of proteomics to understand protein changes during the conversion of muscle to meat and the identification of potential biomarkers for meat quality traits.

Immediately postmortem, there are a variety of changes going on in muscle that could influence the muscle proteome. These changes could impact the tenderization process that occurs during the aging period. Lametsch and Bendixen (2001) used two-dimensional electrophoresis to separate porcine *Longissimus dorsi* muscle proteins by
isoelectric point (pH 4-9) and molecular weight. It was demonstrated that two-dimensional electrophoresis was a suitable method to identify the changes going on in muscle during postmortem storage (0, 4, 8, 24, and 48 hours) (Lametsch and Bendixen, 2001). These results lead to further research in identifying the protein changes postmortem, coupling two-dimensional electrophoresis with the use of mass spectrometry (Lametsch and Bendixen, 2001; Lametsch et al., 2002). Results from these proteomic techniques identified 18 peptides that were associated with nine proteins that changed during postmortem aging (Lametsch et al., 2002). The proteins identified include the structural proteins actin, myosin heavy chain, and troponin-T and the metabolic proteins glycogen phosphorylase, creatine kinase, phosphopyruvate hydratase, myokinase, pyruvate kinase, and dihydrolipoamide succinyltransferase (Lametsch et al., 2002). It is important to note that only fragments of actin (40 and 32-kDa fragments) and myosin heavy chain (56-kDa fragment) were identified, whereas both intact and degradation fragments of troponin-T were identified (Lametsch et al., 2002). All of the fragments of these structural proteins were increased during postmortem storage, whereas intact troponin-T decreased over aging (Lametsch et al., 2002). Some of the metabolic enzymes identified by Lametsch et al. (2002) were also observed to be degraded to an extent during postmortem aging.

The proteomic changes that occur during postmortem aging can influence meat quality attributes, such as water holding capacity. Di Luca et al. (2013) examined proteins from pork Longissimus muscle exudate to identify biomarkers related to water holding capacity. Two-dimensional difference in gel electrophoresis (2D-DIGE) was used to label proteins, separate by isoelectric point (pH 4-7)/molecular weight, and compare spot
abundance to identify proteins/peptides that were differentially expressed between samples with high (approximately 6%), intermediate (approximately 4%), or low (approximately 2.5%) drip loss during aging (Di Luca et al., 2013). The identified proteins from this study consisted of structural, metabolic, stress response, and transport proteins (Di Luca et al., 2013). When samples with high drip loss and low drip loss were compared, high drip loss samples had significantly lower abundance of triosephosphate isomerase, creatine kinase M-type, serum albumin, and transferrin (Di Luca et al., 2013). High drip loss samples exhibited increased abundance of the regulatory protein β tropomyosin (Di Luca et al., 2013). The abundance of the stress related proteins heat shock protein 70, heat shock cognate 71, and stress-induced phosphoprotein were lower in the extreme (high/low) samples compared to the intermediate drip loss samples (Di Luca et al., 2013). The lower abundance could be due to differences in protein localization within the muscle, such as associating more with myofibrillar proteins or the nucleus of cells during times of stress (Beere, 2004; Di Luca et al., 2013). This study proposed that transferrin (an iron transport protein) and triosephosphate isomerase (involved with the glycolytic pathway) are potential biomarkers for increased water holding capacity because these proteins were significantly more expressed in samples with low drip loss (Di Luca et al., 2013).

The use of two-dimensional electrophoresis and 2D-DIGE have also been implemented to try to identify potential biomarkers for tenderness in different species. In beef, Anderson et al. (2012) used 2D-DIGE to compare the sarcoplasmic protein fractions from early postmortem *Longissimus dorsi* samples with different star probe values from aged samples. This study identified proteins using mass spectroscopy and found
tropomyosin alpha 1 chain, actin, and myosin light chain 1 were more abundant in the soluble protein fraction of low star probe steaks (Anderson et al., 2012). The protein identified as a potential biomarker for tenderness from this study was myosin light chain 1 (Anderson et al., 2012). Myosin light chain 1 can be extracted with the less soluble myofibrillar protein fraction (Samarel et al., 1986). It was demonstrated that after myofibrils were incubated with calpain-1 at one and 120 minutes, myosin light chain 1 (21-kDa) was identified in the soluble sarcoplasmic fraction of the proteins using Western blots (Anderson et al., 2012). If myosin light chain 1 was degraded postmortem, this could interfere with the actomyosin complex formed during rigor, potentially impacting the texture of meat (Anderson et al., 2012).

The results from Anderson et al. (2012) are consistent with results from Jia et al. (2009), which found increased myosin light chain 1 in the sarcoplasmic protein fraction of more tender beef *Longissimus thoracis* muscles. Jia et al. (2009) used 2D-DIGE to compare aged *Longissimus thoracis* sarcoplasmic protein samples within pH 5-8 from tender and tough steaks that were classified by Warner-Bratzler shear force. In tough samples, stress response proteins such as DJ-1 and heat shock protein 70 were identified as being more abundant (Jia et al., 2009). These stress proteins may be more expressed in the tough sample group because these proteins have chaperone activity to protect cells during times of stress, possibly protecting proteins from proteolysis (Beere, 2004; Zhou et al., 2006). In addition to myosin light chain 1, Jia et al. (2009) identified peroxiredoxin-6 and malate dehydrogenase as being more abundant in the tender group. This study concluded peroxiredoxin-6, an antioxidant enzyme that involved with reducing reactive oxygen species in cells (Paredi et al., 2012), could be used as a
potential biomarker for tenderness of beef steaks because it was more abundant in tender aged samples (Jia et al., 2009).

Lametsch et al. (2003) examined pork Longissimus dorsi whole muscle (sarcoplasmic and myofibrillar) proteins that were collected immediately after exsanguination and 72 hours postmortem using two-dimensional electrophoresis and mass spectrometry. Throughout the aging period, actin fragments, myosin heavy chain fragments, myosin light chain II, and titin fragments increased in abundance (Lametsch et al., 2003). Interestingly, Lametsch et al. (2003) found that myosin light chain 1 abundance was decreased from 0 to 72 hours of aging, unlike results previously reported by Anderson et al. (2012) and Jia et al. (2009). This study also examined protein abundance and correlation with Warner-Bratzler shear force values and identified some actin (r = -0.47, -0.55, -0.44) and myosin heavy chain II fragments (r = 0.49) were significantly correlated with shear force in aged samples (Lametsch et al., 2003).

Metabolic proteins including enolase 1, enolase 3, phosphoglycerate kinase, pyruvate dehydrogenase, glycogen phosphorylase, triosephosphate isomerase 1, and myokinase were found to be increased in abundance during the aging period (Lametsch et al., 2003). Triosephosphate isomerase 1 was found to be significantly correlated to shear force values (r = -0.46, -0.64), indicating glycolysis may play an important role in meat tenderization (Lametsch et al., 2003).

All of the proteomic studies mentioned demonstrate a variety of proteins involved with muscle and their potential influence on meat products. The use of two-dimensional electrophoresis, 2D-DIGE, mass spectrometry, SDS-PAGE, and Western blots can aid in the determination of biomarkers to be used in the meat industry to define meat quality
traits. It is important to keep in mind that in order for a protein biomarker to be applicable for use in the meat industry, it must be easily solubilized, identified, and measured in a rapid manner that can keep up with production speed.

**Summary**

It is important to understand the role muscle proteins play in structure, metabolism, stress response, and proteolysis in order to understand the complex process of conversion of muscle to meat. During postmortem aging and the conversion of muscle to meat, proteins can be modified and abundance can change, ultimately influencing meat quality attributes. It is known that degradation of proteins involved with muscle structure can result in changes in the texture of meat. Proteomic studies demonstrate that in addition to proteolysis, modification and abundance of metabolic and stress related proteins can also influence the tenderization process. Therefore, the objectives of the study described in this thesis were to determine the amount of proteolysis and differences in sarcoplasmic proteomes that contribute to variation in tenderness of aged pork loins.
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CHAPTER 3

PROTEOMIC FEATURES ASSOCIATED WITH TENDERNESS OF AGED PORK LOINS

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*Iowa State University, Department of Animal Science, Ames, Iowa 50011

†Iowa State University, Department of Food Science and Human Nutrition, Ames, Iowa 50011
Abstract

The objectives for this study were to determine differences in proteolysis and differences in sarcoplasmic proteomes that contribute to tenderness variation in aged pork Longissimus dorsi muscles (LM). Loins (n = 159) were collected one day postmortem from carcasses of Duroc-sired crossbred commercial pigs and aged for 9 – 11 days. Chops (2.54 cm) were collected and evaluated for purge, cook loss, pH post-aging, visual color and marbling, Hunter L, a, and b, sensory, star probe (kg), and total lipid. Samples were selected for proteomic experiments based on star probe values and selected samples were within specified ranges for ultimate pH (5.54 – 5.86), marbling score (1.0 – 3.0), and percent total lipid (1.61 – 3.37%). Samples were classified into either a low star probe group (n = 12, 4.95 kg) or high star probe group (n = 12, 7.75 kg). Proteolytic and muscle fiber type data were collected using SDS-PAGE and Western blot analyses. Two-dimensional difference in gel electrophoresis (2D-DIGE) and mass spectrometry were used to examine sarcoplasmic protein abundance and potential modifications. Calpain-1 was completely autolyzed in both high and low star probe samples, demonstrating calpain-1 potentially had been active in all samples. Low star probe whole muscle samples had more troponin-T (P < 0.01), desmin (P < 0.01), and filamin degradation (P < 0.01) than high star probe samples. Both classification groups showed degradation of titin in myofibrillar samples, but some high star probe samples also exhibited intact bands of titin. Results from 2D-DIGE showed high star probe samples had significantly more abundant metabolic, stress response, and regulatory proteins in the sarcoplasmic fraction compared to low star probe samples. The stress response protein peroxiredoxin-2 was more abundant in high star probe samples as determined by 2D-DIGE (P ≤ 0.01, 2 spots).
and Western blot confirmations ($P = 0.02$). Low star probe samples showed significantly more degradation of the structural protein desmin in 2D-DIGE ($P < 0.01$) and Western blot confirmations ($P < 0.01$). The proteins peroxiredoxin-2 and desmin were found in the soluble protein fraction as being differentially abundant between classification groups. These results demonstrate extreme proteolytic differences influenced measured tenderness of low and high star probe samples and soluble desmin and peroxiredoxin-2 may be utilized as biomarkers to differentiate between tough and tender aged pork products.

**Key words:** desmin, peroxiredoxin-2, pork, proteolysis, tenderness, two-dimensional difference in gel electrophoresis

**Introduction**

It is established that tenderness is a fundamental component related to the quality of meat products (Bray, 1966; Becker, 2000; Grunert et al., 2004; Moeller et al., 2010). In the retail setting, there is variation in the quality of pork products presented to consumers (Larsen, 2015). Consumers value quality and are willing to pay a premium of $0.37 per pound for consistently tender pork products (Sanders et al., 2007). Unfortunately, there are no certified programs that guarantee tenderness of fresh pork to consumers. The lack of differentiation in the quality of pork products coupled with the consumer desire for tenderness demonstrates a need for definition of quality from the pork industry. Identifying biomarkers to classify tough and tender pork products could be a way to distinguish high quality pork products for consumers.
Meat tenderness is influenced by factors such as pH (Melody et al., 2004; Lonergan et al., 2007), lipid (Lonergan et al., 2007), collagen content (Wheeler et al., 2000), and degradation of muscle proteins (Huff-Lonergan et al., 1996a; Wheeler et al., 2000; Melody et al., 2004). Researchers have also identified differences in expression of metabolic and stress response proteins in tough and tender beef (Jia et al., 2009) and pork (Lametsch et al., 2003), demonstrating proteomic differences account for a considerable amount of the variation seen in meat quality. Therefore, it is hypothesized that variation in tenderness of aged pork loins that have similar pH, lipid, and color characteristics is attributed to differences in protein degradation and differences in the sarcoplasmic proteomes. The objectives for this study include identifying differences in proteolysis and sarcoplasmic proteomes of aged pork loin chops that vary greatly in instrumental tenderness. Results from this study will aid in identifying potential biomarkers to differentiate low and high quality pork loins.

Materials and Methods

Collection of Pork Loins and Sensory Data

Loins (0.32 cm trim, strap-off) were collected at one day postmortem from 159 carcasses from Duroc-sired crossbred commercial pigs (market weight approximately 122 kg) that were harvested at a commercial facility. Loins were vacuum packaged and transported to Iowa State University on ice. After aging 9 – 11 days at 4°C, the packaged loins were weighed and then removed from the vacuum bags. Loins were removed from the vacuum bags and weighed. The purge from each loin was then poured out of the vacuum bag and weighed. Loin purge was calculated by using the following equation: 

\[ \text{purge/loin + purge}}] \times 100. \] 

Loins were cut into 2.54 cm thick *Longissimus dorsi* (LM)
chops and were trimmed of external fat. Loin pH was measured from blade, center, and sirloin chops using a Hanna HI9025 pH meter (Hanna Instruments, Woonsocket, RI). The pH meter was calibrated before the first measurement each testing day using pH 4 and pH 7 buffers, and maintenance of calibration was monitored between each sample (within range of pH 6.95 – 7.05). Average pH from the center and sirloin were used to determine final pH of the LM. Chop purge was determined by weighing one chop from the center portion of each loin, placing it in a plastic bag for 24 hours of storage at 4°C, and then re-weighing the chop and purge following the storage period. The following equation was used to determine percent chop purge: \[ \text{percent chop purge} = \left( \frac{\text{purge}}{\text{chop} + \text{purge}} \right) \times 100. \]

Visual color scores were assigned using a 6-point scale (1 = pale pinkish gray/white; 6 = dark purplish red; National Pork Board, 2000) and marbling scores were assigned using a 10-point scale (1 = 1% intramuscular fat; 10 = 10% intramuscular fat; National Pork Board, 2000) to center cut chops from each sample by a trained technician using standard pictures. Hunter L, a, and b were measured on one chop from each loin using a Minolta Chroma Meter with a D65 light source, 50 mm aperture, and 0° observer. Two chops from the center portion of each loin (approximately 5 inches from the sirloin end) were used to collect sensory and chop purge data by cooking loin chops to an internal temperature of 68°C on clamshell grills. A trained panel (n = 4, IRB ID: 14-553) evaluated tenderness, juiciness, chewiness, pork flavor, and off flavor using a 10 point category scale (low values indicate lower degrees of characteristics and high values indicate higher degrees of characteristics). Cook loss was determined with the following equation: \[ \text{cook loss} = \left( \frac{\text{raw weight} - \text{cooked weight}}{\text{raw weight}} \right) \times 100. \]

An Instron (Instron Industrial Products, Grove City, PA) fitted with a five point star probe attachment was used to measure instrumental
tenderness on a cooked chop from the center portion of the loin. Star probe was used in this study because it measures the force (in kilograms) to both puncture, shear, and compress meat, similar to the nature of chewing (Huff-Lonergan et al., 2002). The means, ranges, and standard deviations of the measured attributes from the 159 loins are found in Table 1.

**Total Lipid Extraction**

Total lipid extraction was performed on selected samples (n = 24 high star probe, n = 19 low star probe). Loin chops were minced finely with a knife, individually snap frozen in liquid nitrogen, and powdered using a blender (Waring Commercial, New Hartford, CT). Total lipid in the samples was extracted in triplicate as described by Folch et al. (1957), with modifications. Approximately 2 grams of the frozen powdered samples were weighed into test tubes and 17 mL of a methanol:water (3.5:1) mixture was added to each sample. Samples were vortexed for fifteen seconds and then 6.5 mL of chloroform was added to each sample. Each sample was vortexed for 20 seconds and shaken on a wrist-action shaker for 1 hour. Chloroform and 50 mM KCl (7.5 mL of each) were added to each sample and tubes were inverted gently three times. Samples were centrifuged at room temperature for 20 minutes at 500 x g (Damon/IEC DIV., Needham Heights, MA). The aqueous layer of each sample was aspirated and discarded. Ten mL of 50 mM KCl solution was added to each sample and tubes were inverted three times to mix. Samples were centrifuged again at room temperature for 25 minutes at 500 x g and the top aqueous layer was removed in a similar manner. The samples were then filtered with 4.25 cm Whatman paper into scintillation vials using a Buchner funnel and a suction flask. The test tubes were rinsed in 2 mL of chloroform three times, to ensure all of the samples
were out of the tubes. The scintillation vials were transferred into a concentrator for drying (50°C with steady air flow) with caps removed for approximately 2.5 hours to allow the chloroform to evaporate from the vial. Dried vials were then weighed and percent total lipid was calculated for each sample.

**Sample Selection**

The 159 pork samples were sorted based on aged chop star probe values. Samples that fell within the top and bottom 20% of high and low star probe force were chosen to obtain a sample set with extreme differences in star probe values. Marbling scores and pH criteria were applied for further refinement of the experimental groups. Previous research demonstrates extreme pH influences ultimate quality of pork LM samples (pH >5.80 have superior sensory scores and instrumental tenderness values than samples with a pH <5.50), regardless of lipid content (Lonergan et al., 2007). Results from that same study demonstrated that greater lipid content in pork loins with an intermediate pH range (5.50 – 5.80) tends to result in more tender pork (Lonergan et al., 2007). These results demonstrate that pH and percent lipid play a role in ultimate pork quality (Lonergan et al., 2007). Of the selected extremes, additional criteria were used to define the experimental samples for the current study. Pork loin chops were included in the study if they were in range of pH and marbling scores: 5.54 – 5.86 pH post-aging (center and sirloin) and a 1.0 – 3.0 marbling score. This selection narrowed the sample sets to n = 24 for samples with high star probe and n = 19 for samples with low star probe values. Total lipid content and sensory scores (tenderness, chewiness, and juiciness) were used as criteria for final definition of the experimental groups. Sensory scores were evaluated for consistency with star probe data, and any deviating samples were not
selected for the experimental groups. Samples within the range of 1.61 – 3.37% total lipid and pH range of 5.56 – 5.86 (post-aging center and sirloin portion) were selected to obtain a balanced experimental design of n = 12 for both low and high star probe groups (approximately the top and bottom 7.5% of the original 159 pork loins sorted by star probe values). Figure 1 shows the distribution of star probe values (kg) from individual samples in the low and high star probe groups.

**Sample Preparation**

*Whole Muscle Samples.* Whole muscle samples of the selected pork loins were prepared as described by (Huff-Lonergan et al., 1996b), with slight modifications. Whole muscle solubilizing buffer (10 mL) comprised of 10 mM sodium phosphate, pH 7.0 and 2% wt/vol sodium dodecyl sulfate (SDS) was added to powdered samples (0.4 g). This mixture was homogenized with a Dounce homogenizer and samples were centrifuged at 1,500 x g for 15 minutes at 25˚ C (Sorvall Legend RT, Newton, CT). Protein concentrations were determined using the detergent-compatible (DC) protein assay (Bio-Rad, Hercules, CA) and samples were then diluted to 6.4 mg/mL using whole muscle solubilizing buffer. Samples were diluted to a final protein concentration of 4mg/mL with 0.5 vol of Wang’s tracking dye (3 mM EDTA, 3% [wt/vol] SDS, 30% [vol/vol] glycerol, .001% pyronin Y [wt/vol], 30 mMTris-HCl, pH 8.0) and 0.1 vol of 2-mercaptoethanol. Samples were heated for 15 minutes at approximately 50˚ C and stored at -80 °C.

*Sarcoplasmic and Myofibrillar Samples.* Sarcoplasmic samples were prepared as described by Cruzen et al. (2015), with modifications. Powdered muscle samples (2 g) were mixed with 4.5 mL cold sarcoplasmic extraction buffer (4˚C, 50 mM Tris-HCl,
1mM EDTA, pH 8.0) and homogenized using a Polytron PT 3100 (Polytron, Lucerne, Switzerland) until the solution was mixed thoroughly (approximately 20 – 40 seconds). Each sample was then centrifuged at 40,000 x g for 20 minutes at 4°C (Sorvall Super T21, Newton, CT), and the resulting supernatant was filtered through cheesecloth and collected for sarcoplasmic sample preparation. The remaining pellet from each sample was used for myofibrillar protein extraction. Sarcoplasmic protein concentration was determined using the DC protein assay (Bio-Rad, Hercules, CA). Samples for two-dimensional difference gel electrophoresis (2D-DIGE) were prepared to a final concentration of 10mg/mL using cold sarcoplasmic extraction buffer. Sarcoplasmic samples were diluted to 4 mg/mL as described above for whole muscle samples for use in one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analyses.

Myofibrillar protein samples were prepared from the remaining pellet from the sarcoplasmic extraction (Anderson et al, 2012). The initial weight of each pellet was recorded and pellets were mixed with 20 mL of standard salt solution (100mM KCl, 20 mM Potassium phosphate [pH 6.85 – 6.9], 2 mM MgCl₂, 1 mM EGTA, 1 mM NaN₃) by breaking apart the pellets with a spatula and vortex mixing for 10 seconds. This solution was then centrifuged at 1,000 x g at 4° C for 10 minutes (Sorvall Legend RT, Newton, CT) and the supernatant was discarded. Samples were washed in the standard salt solution and centrifuged three times total. Pellets were washed with 20 mL of Tris wash buffer (5mM Tris-HCl, pH 8.0) and broken apart by vortex mixing for 10 seconds. The sample solution was then centrifuged at 3,020 x g at 4° C for 10 minutes, and the supernatant was discarded. The Tris-HCl wash and centrifugation process was
performed a total of two times. The samples were kept on ice throughout all of the washing steps. Myofibrillar proteins were extracted from the washed pellets with 5 volumes (of the initial weight of the pellet) of myofibrillar extraction buffer (8.3 M urea, 2 M thiourea, 2 % CHAPS, 1 % DTT, pH 8.5 with 2 M Tris). Each pellet was broken apart in the solution with a spatula, vortexed for 15 – 20 seconds, and gently mixed at 4°C for thirty minutes. Samples were centrifuged at 10,000 x g at 20°C for 30 minutes (Sorvall Super T21, Newton, CT) and the protein concentration of the supernatant was determined using premade reagents (Bradford Quickstart, Bio-Rad, Hercules, CA). The protein concentration was adjusted to 6.4 mg/mL with myofibrillar extraction buffer and adjusted to 4mg/mL as described for preparation of whole muscle samples.

**SDS-PAGE and Western Blotting**

*Gel systems.* One-dimensional SDS-PAGE gel electrophoresis was performed to examine the extent of protein degradation in structural proteins and to confirm the identity of protein spots from 2D-DIGE. To quantify the extent of troponin-T degradation, desmin degradation/abundance, and presence of peroxiredoxin-2, 15 % polyacrylamide separating gels (10 cm x 10 cm; acrylamide:N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1 % [wt/vol] SDS, 0.05 % [vol/vol] tetramethylenediamine (TEMED), 0.05 % [wt/vol] ammonium persulfate (AMPER), 0.5 M Tris-HCl pH 8.8) were used. The extent of calpain-1 autolysis was determined using 8 % polyacrylamide separating gels. A 5 % stacking gel (10 cm x 10 cm; acrylamide: N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1 % [wt/vol] SDS, 0.125% TEMED, 0.075 % [wt/vol] AMPER, 0.125 M Tris-HCl, pH 6.8) with 10 lanes was used for all of the gels described. SE 260 Hoefer Mighty Small II electrophoresis units (Hoefer, Inc.,
Holliston, MA) were used to run the 15% and 8% gels. The running buffer was comprised of 25 mM Tris, 192 mM Glycine, 2 mM EDTA, and 0.1% [wt/vol] SDS. Troponin-T degradation was determined by using 20 µg of whole muscle protein samples run for approximately 330 volt-hours. Gels to determine desmin degradation in protein fractions were loaded with 40 µg of whole muscle samples and 50 µg of sarcoplasmic samples. Whole muscle and sarcoplasmic desmin samples were run for approximately 360 volt-hours. Identification and abundance of peroxiredoxin-2 was determined by loading 40 µg of sarcoplasmic protein samples run for approximately 360 volt-hours. Gels for calpain-1 Westerns were loaded with 40 µg of whole muscle protein samples and run for approximately 300 volt-hours.

The identification and quantification of the higher molecular weight proteins titin and filamin were performed on two gel systems. Five percent continuous gels (18cm x 16 cm; acrylamide: N,N′-bis-methylene acrylamide = 100:1 [wt/wt], 0.1 % [wt/vol] SDS, 0.067 % TEMED, 0.1 % [wt/vol] AMPER, 2 mM EDTA, 200 mM Tris-HCl, pH 8.0) loaded with 40 µg of myofibrillar protein samples were used to analyze titin degradation. The same running buffer was used as described above, with the addition of 5 mM dithiothreitol (DTT). Gels were run on SE 600 Hoefer electrophoresis units (Hoefer, Inc., Holliston, MA) at a rate of 10 mA for approximately 1600 total volt-hours. After electrophoresis, gels were fixed and stained using the Silver Stain Plus kit (161-0449, Bio-Rad, Hercules, CA) to determine the amount of titin degradation. Samples were run with two myofibrillar protein references with varying degrees of titin degradation. One myofibrillar protein extract was prepared from a pork chop aged for one day, and one extract was prepared from an aged (7 – 10 day) pork loin chop that was
not part of this study. Samples were evaluated for the presence of T1 (intact) and/or T2 (degraded) bands to determine the extent of titin degradation. Filamin degradation was measured by running 3-12% precast TEA-tricine gradient gels (10 cm x 10 cm; PCG2003, Sigma-Aldrich, St. Louis, MO) at a constant rate of 20 volts for a total of approximately 360 holt-hours. The running buffer used for these gels consisted of 60 mM triethanolamine, 40 mM tricine, and 0.1% [wt/vol] SDS, with the addition of 5 mM DTT. Gels were run on SE 260 Hoefer Mighty Small II electrophoresis units.

Transfer Conditions. Following electrophoresis, gels for troponin-T, desmin, calpain-1, peroxiredoxin-2, and filamin were transferred to polyvinylidene difluoride (PVDF) membranes (0.2 µm pore size). The membranes were soaked in methanol prior to the transfer process for approximately 3 – 5 minutes for activation. Gels were transferred onto membranes using TE-22 Mighty Small Transphor units (Hoefer, Inc., Holliston, MA), running for a constant voltage of 90 volts for 1.5 hours, at approximately 4˚ C. The transfer buffer contained 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 15 % [vol/vol] methanol.

Western Blotting. After transfer, gels were discarded and membranes were blocked in a PBS-Tween solution (80 mM Na₂HPO₄, anhydrous, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% [vol/vol] polyoxyethylene sorbitan monolaurate [Tween-20]) containing 5% non-fat dry milk (NFDM). Gels were blocked for approximately 1 hour at room temperature, and immediately after blocking primary antibodies diluted in PBS-Tween were added to the blots. The primary antibody dilutions contained: troponin-T – 1:80,000 000 using monoclonal mouse anti-troponin-T (T6277, JLT-12, Sigma-Aldrich, St. Louis, MO), desmin – 1:40,000 using polyclonal rabbit anti-desmin antibody
produced at Iowa State University (Huff-Lonergan et al., 1996a), calpain-1 – 1:10,000 using monoclonal mouse anti-calpain-1 (MA3-940, Thermo Scientific, Rockford, IL), peroxiredoxin-2 – 1:20,000 using monoclonal rabbit antibody (ab109367, Abcam, Cambridge, UK), and filamin – 1:1,000 using polyclonal rabbit antibody made at Iowa State University (Huff-Lonergan et al., 1996a). The primary antibody solution for filamin was diluted in a solution of PBS-Tween and 5% NFDM. All blots were incubated in primary antibodies overnight (approximately 16 – 20 hours) at 4˚ C. After primary incubation, blots (with the exception of filamin) were washed in PBS-Tween three times for ten minute intervals. Filamin blots were washed with PBS-Tween containing 5% NFDM for the same period. Blots were then incubated for one hour at room temperature with secondary antibodies diluted in PBS-Tween. The dilutions for the secondary antibodies contained: troponin-T – 1:10,000 goat anti-mouse-HRP (conjugated to horseradish peroxidase) antibody (A2554, Sigma-Aldrich, St. Louis, MO), desmin – 1:20,000 goat anti-rabbit-HRP antibody (31460, Thermo Scientific, Rockford, IL), calpain-1 – 1:10,000 goat anti-mouse-HRP antibody (A2554, Sigma-Aldrich, St. Louis, MO), peroxiredoxin-2 – 1:10,000 goat anti-rabbit-HRP (31460, Thermo Scientific, Rockford, IL), and filamin – 1:20,000 goat-anti-rabbit-HRP (31460, Thermo Scientific, Rockford, IL). The secondary antibody for filamin was diluted in 5% NFDM and PBS-Tween. After secondary incubation, all blots were washed with PBS-Tween three times, for ten minute intervals. Proteins were detected using a chemiluminescent detection kit (ECL Prime, GE Healthcare, Piscataway, NJ) and images of blots were obtained and analyzed using a ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and Alpha Ease FC software (v 3.03 Alpha Innotech).
Densitometry was used to quantify the protein bands and comparisons were made by taking the ratio of the measured protein band to the internal reference used for that protein. The 37-kDa intact band, 30-kDa degradation band, and 27 – 30-kDa degradation product of troponin-T (whole muscle fraction) were all measured and compared to corresponding bands of a 7 day aged LM whole muscle pork sample. The 55-kDa intact band and 38-kDa degradation band of desmin (whole muscle fraction) were measured and compared to the corresponding bands of a day 0/7 day aged mixed pork LM whole muscle sample. The 38-kDa degradation band of desmin (sarcoplasmic fraction) was compared to an aged (9 – 11 days) pork LM whole muscle sample. Calpain-1 autolysis (whole muscle) for the appearance of the intact 80-kDa catalytic subunit compared to a day 0/7 day aged mixed pork LM whole muscle sample. Peroxiredoxin-2 (sarcoplasmic fraction) was measured by examining the 22-kDa protein band compared to the same band in a lane that contained 0 day aged pork LM sarcoplasmic sample. The 290-kDa (approximate molecular weight) of filamin (whole muscle fraction) was compared to a 7 day aged postmortem pork LM whole muscle sample. Samples were run in duplicate for each protein measured. Table 2 displays gel, protein, antibody, and Western blotting details for each protein described. All Western blots were performed in duplicate.

**Muscle Fiber Type Analysis**

To determine predominate fiber type of each sample, myosin heavy-chain isoforms (MHC) were measured (type I, types IIa+IIx, and type IIb). Muscle fiber types were determined as described by (Melody et al., 2004), with slight modifications. Whole muscle gel samples were diluted to 0.256 mg/mL with ddH₂O. Each sample (1 vol) was
combined with 1 vol of tracking dye (50% [vol/vol] glycerol, 2% [wt/vol] SDS, 0.1% [wt/vol] bromophenol blue, and 60 mM Tris-HCl, pH 6.8) and 0.5 vol of 2-mercaptoethanol to obtain a final protein concentration of 0.125 mg/mL. Samples were mixed and then stored at -80°C. Prepared gel samples were run on 6% separating gels (18 cm x 16 cm; acrylamide:N,N′-bis-methylene acrylamide = 50:1 [wt/wt], 0.4% [wt/vol] SDS, 0.05% [vol/vol] TEMED, 0.1% [wt/vol] APS, 30% [vol/vol] glycerol, 100 mM glycine, 200 mM Tris, pH 8.8) with a 4% stacking gel (acrylamide:N,N′-bis-methylene acrylamide = 50:1 [wt/wt], 0.4% [wt/vol] SDS, 0.05% [vol/vol] TEMED, 0.1% [wt/vol] APS, 30% [vol/vol] glycerol, 4 mM EDTA, 200 mM Tris-HCl, pH 6.7). SE 600 Hoefer electrophoresis units were used to run the gels. The running buffer for the upper chamber of the electrophoresis unit consisted of 200 mM Tris, 300 mM glycine, 0.2% [wt/vol] SDS, and 0.1% [vol/vol] 2-mercaptoethanol and the running buffer for the lower chamber of the unit consisted of 100 mM Tris and 150 mM glycine. Each gel was loaded with 2 µg of sample and run at a constant rate of 90-volts at 4°C for 6,500 total volt-hours. After electrophoresis, gels were immediately stained with Colloidal Coomassie Blue Stain (1.7% ammonium sulfate, 30% methanol, 3% phosphoric acid, and 0.1% Coomassie G-250) for 20 – 24 hours. Gels were then destained using ddH₂O, and proteins were detected using a ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and Alpha Ease FC software (v 3.03 Alpha Innotech). Densitometry was used to measure the percentages of myosin heavy chain isoforms (Type I, Types IIa+IIx, and Type IIb) over total myosin heavy chain isoforms. All gels for myosin heavy chain determination were performed in duplicate.
Two-dimensional Difference in Gel Electrophoresis

Two-dimensional difference in gel electrophoresis (2D-DIGE) was used to determine differences in the protein profile of the low and high star probe sample groups (Anderson et al., 2012; Cruzen et al., 2015), with slight modifications. Sarcoplasmic protein samples (10 mg/mL) were used for these experiments. A pooled reference was made by combining equal amounts of all samples selected to be in the experiment (n = 24 total) to be used for identification and pick gels. Fifty µg of each individual sample from the two star probe groups were labeled with CyDyes according to the manufacturer’s directions (GE Healthcare, Piscataway, NJ). Individual samples were labeled alternatingly with CyDye3 and CyDye5. Individual samples were relabeled as needed to complete the experiments. Six aliquots of 100 µg of the pooled reference were labeled with CyDye2. After labeling, the protein concentration of all samples was 7.14 mg/mL and labeled samples were stored at -80°C until further use.

Experiment 1 - 11 cm, pH 4-7

Labeled samples were prepared for running on 11 cm pH 4-7 immobilized pH gradient (IPG) strips (GE Healthcare, Piscataway, NJ) by mixing 15 µg of samples from the low star probe group, high star probe group, and the pooled reference for a total of 45 µg of protein samples for each individual strip. Table 3 demonstrates the labeling procedure for each strip. DeStreak rehydration solution (GE Healthcare, Piscataway, NJ) was prepared as directed by the manufacturer (350 µL per strip) by mixing 1.5% IPG buffer (GE Healthcare, Piscataway, NJ) and 20 mM DTT. This rehydration solution was mixed with the protein solutions and added to strips placed in individual wells of a humidified rehydration chamber. Strips rehydrated overnight (approximately 20 – 24
hours) at room temperature. Strips were then transferred to an Ettan IPGphor isoelectric focusing system (GE Healthcare, Piscataway, NJ) for first dimension separation of the proteins (separating by isoelectric point). Each strip was run for a total of 14,000 volt-hours. Strips were placed in tubes and stored at -80°C until equilibration. Strips were equilibrated for two 20 minute washes using equilibration buffer (50 mM Tris-HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS, and trace amounts of bromophenol blue) with 65 mM DTT for the first wash and equilibration buffer with 135 mM iodoacetamide for the second wash. Equilibrated strips (two 11 cm strips per gel) were then loaded onto 12.5% preparative gels (25.5 x 20.5 cm, 1.5 mm thick; acrylamide: N,N’-bis-methylene acrylamide 100:1, 0.1% SDS, 0.05% TEMED, 0.05% ammonium persulfate, 0.5 M Tris-HCL, pH 8.8) using agarose with trace amounts of bromophenol blue as an overlay for second dimension separation (by molecular weight). Gels were run until the overlay was off the bottom of each gel using an Ettan DALT SIX system (GE Healthcare, Piscataway, NJ) with running buffer prepared as described above. Each sample was run in duplicate. Gels were then imaged with an Ettan DIGE Imager (GE Healthcare, Piscataway, NJ) and analyzed using DeCyder™ 2D software version 6.5 (GE Healthcare, Piscataway, NJ) to identify differences in spot abundance between samples of low and high star probe groups.

Experiment 2 – 24 cm, pH 6-9

Labeled samples were prepared for running on 24 cm pH 6 – 9 IPG strips by mixing 25 µg of samples from the low star probe group, high star probe group, and the pooled reference for a total of 75 µg of protein samples for each individual strip. Table 3 shows the labeling procedure for each strip. DeStreak rehydration solution was prepared
as directed by the manufacturer (500 µL per strip) by mixing 2.5% IPG buffer and 30mM DTT. This rehydration solution was mixed with the protein solutions and added to strips placed in individual wells of a humidified rehydration chamber. Strips rehydrated overnight (approximately 24 hours) at room temperature. Strips were run as described above for a total of 72,500 volt-hours. Strips were stored and equilibrated as described above. Equilibrated strips (one 24cm strip per gel) were then loaded onto 12.5% preparative gels (25.5 x 20.5 cm, 1.5 mm thick; acrylamide: N,N’-bis-methylene acrylamide 100:1, 0.1% SDS, 0.05% TEMED, 0.05% ammonium persulfate, 0.5 M Tris-HCl, pH 8.8) using agarose with trace amounts of bromophenol blue as an overlay. Gels were run, imaged, and analyzed as described in experiment 1.

Spot Identification

After analyzing the protein spots in each experiment, significantly different spots between low and high star probe groups were chosen for identification. The previously defined reference sample representing all samples in the experiment was run for protein spot determination. For experiment 1, two 11 cm, pH 4-7 IPG strips were labeled with 1mg of protein and the rehydration solution. Strips were labeled, rehydrated, and run as described above in experiment 1. Strips were equilibrated and run on 12.5% preparative gels (18cm x 16 cm, 1.5 mm thick; filtered reagents) using the SE 600 Hoefer electrophoresis system until the agarose was off the gel. For experiment 2, two 24 cm, pH 6 – 9 IPG strips were labeled with 1.5 mg of protein and the rehydration solution and strips were rehydrated and run as described above in experiment 2. Strips were loaded onto 12.5% gels (25.5 x 20.5, filtered reagents) and run on an Ettan DALT SIX system. One-dimensional gels SDS-PAGE gels were prepared to confirm the identity of the high
molecular weight protein filamin. Preparative gels (8%, 18cm x 16 cm, 1.5 mm thick; filtered reagents) was loaded with 200 and 225 µg of protein and run for 2,200 volt-hours. Gels were stained using filtered Colloidal Coomassie Blue Stain (1.7% ammonium sulfate, 30% methanol, 3% phosphoric acid, and 0.1% Coomassie G-250) and destained with filtered ddH₂O.

Spots and protein bands picked for identification were excised from the gels and sent to the Iowa State University Protein Facility. Spots were digested with trypsin using Genomic Solutions Investigator ProGest automated digester (Chelmsford, MA), separated by liquid chromatography, and analyzed by tandem mass spectrometry (MS/MS) using a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Rockford, IL). Resulting raw peptide fragment patterns were compared to the database search program MASCOT (MatrixScience, London, UK) to identify proteins.

**Statistical Analysis**

All pH, loin and chop purge, visual color and marbling, sensory, cook loss, total lipid, Western blot, and MHC data were analyzed using the MIXED procedure in SAS (v.9.4, SAS Inst., Cary, NC). The model included the fixed effect of force (high or low star probe force). The analyses for troponin-T, desmin, and peroxiredoxin-2 included a random effect of gel in the model. Least squares means and the standard errors were reported for all measured attributes, and the significance level was determined at $P \leq 0.05$. 
All 2D-DIGE data were analyzed using DeCyder™ 2D software version 6.5 using Student’s paired t-test to determine differences in protein spot relative abundance between high and low star probe groups. The significance level was determined at $P \leq 0.10$.

**Results**

*Fresh Meat Characteristics and Sensory Analysis.* There was no difference in average ultimate pH (center and sirloin portion) of the LM between the low star probe group (5.71 ± 0.03) and the high star probe group (5.66 ± 0.03, $P = 0.19$). The low star probe group had higher marbling scores (2.3 ± 0.14) than the high star probe group (1.9 ± 0.14, $P = 0.03$), as well as higher percent total lipid from extraction (2.61 ± 0.13 low star probe; 2.23 ± 0.13 high star probe, $P = 0.05$). Visual color scores were not different between the two sample groups ($P = 0.52$). Hunter color measurements showed no difference in $L$ and $b$ values, whereas samples in the low star probe group were more red ($a = 14.5 ± 0.19$) than samples in the high star probe group ($a = 13.89 ± 0.19$, $P = 0.04$). The sensory results showed low star probe samples were more tender ($P < 0.01$), less chewy ($P < 0.01$), more juicy ($P < 0.01$), and had more pork flavor ($P < 0.01$) than high star probe samples, while there was no difference in off flavor between the two groups ($P = 0.22$). Percent loin and chop purge were not affected by classification groups ($P > 0.05$). However, samples in the high star probe group did have approximately 4 percentage units more cook loss than samples in the low star probe group ($P = 0.01$). Table 4 contains all fresh meat characteristics and sensory data for the classification groups.
Proteolysis. Protein bands for Troponin-T Western blots were measured using densitometry by analyzing the 37-kDa intact band, the 30-kDa degraded band, and the 27 – 30-kDa degraded product in the whole muscle protein fraction of samples in low and high star probe groups (Figure 2). Intact troponin-T was 26% less abundant in low star probe samples than in the high star probe samples ($P < 0.01$, Table 5). Consequently, abundance of the 30-kDa degradation band and 27 – 30-kDa degradation product of troponin-T were increased by over 100% in the low star probe samples ($P < 0.01$, Table 5). Desmin degradation was analyzed using densitometry to measure the abundance of the 55-kDa intact band and the 38-kDa degradation band in the whole muscle protein fraction of samples (Figure 3). Similar to troponin-T results, abundance of intact desmin was 59% less in the low star probe samples ($P < 0.01$, Table 5). A degradation product corresponding to the 38-kDa band was 255% more abundant in low star probe samples in comparison to high star probe samples ($P < 0.01$, Table 5). The intact band of filamin (290-kDa) was measured using densitometry (Figure 4). Filamin also demonstrated similar proteolytic results where low star probe samples had 33% less abundant intact filamin compared to high star probe samples ($P < 0.01$, Table 5). Intact and degraded protein bands of filamin were successfully identified using mass spectrometry in whole muscle samples. The peptides identified were compared to the peptide sequence of full length filamin (*homo sapiens*, accession # Q14315). Peptides were dispersed evenly throughout the entire protein of intact filamin. Identified peptides from the degraded band included peptides from the entire protein, excluding amino acids at the N-terminal (AA 2616-2725).
Calpain-1 autolysis was examined using Western blots to identify the appearance of an intact 80-kDa band, as well as 78-kDa and 76-kDa autolysis products in whole muscle samples (Figure 5). In all of the samples, the only band identified was the 76-kDa band, indicating calpain-1 was completely autolyzed in both low and high star probe groups. Titin degradation was determined using stained SDS-PAGE gels to identify the presence of intact (TI – approximately 3,000-kDa) and degraded (T2 – approximately 2,400-kDa) titin in myofibrillar samples (Figure 6). Some samples in the high star probe group did exhibit a small amount of T1, indicating some intact titin was still present in samples. Intact titin (T1) was not present in any samples from the low star probe group. Degraded titin (T2) was present in all samples from both classification groups.

*Myosin heavy chain.* Type IIa+IIx and IIb myosin heavy chain isoforms were analyzed using colloidal coomassie blue stained gels to measure the percentage of each isoform in whole muscle protein samples (Figure 7). In both sample groups, there were no differences in the proportion of type IIa+IIx and TypeIIb myosin heavy chain isoform percentages. The proportion of type IIa+IIx in both groups was approximately 22.3% ± 1.75 and the proportion of type IIb was approximately 77.7% ± 1.75 of the total myosin heavy chain measured (Table 6). These results indicate the predominate myosin heavy chain isoform in both classification groups is type IIb.

2D-DIGE Experiment 1 (11 cm, pH 4 – 7). A total of 481 spots were found in the sarcoplastic fraction of the LM from the 24 samples. Of those spots, 102 were found to be significantly different between low and high star probe groups \((P < 0.10)\), and 16 of the most prevalent spots were picked for identification (Figure 8, Table 7). The identified protein spots included groups of structural proteins, metabolic proteins, and stress
response proteins. Desmin (spot 319) was the only protein found more abundant in the low star probe group \( (P < 0.01) \), while all other proteins identified were more abundant in the high star probe group (Table 8). One-dimensional Western blots were performed on the sarcoplasmic fraction of samples to confirm and quantify amounts of desmin and peroxiredoxin-2 in both sample groups.

*Desmin.* Western blots from SDS-PAGE gels did confirm the presence of degraded desmin (approximately 34-kDa) in the sarcoplasmic fraction of low and high star probe sample groups (Figure 9). Densitometry was used to quantify the amount of degraded desmin, and similar to the 2D-DIGE results, there was more degradation in low star probe samples \( (P < 0.01, \text{Table 9}) \). Intact desmin was not detected in the sarcoplasmic fraction of samples from either classification group.

The peptides identified from mass spectrometry of the spot identified as desmin were compared to peptides of full length desmin (*Sus scrofa*, accession # P02540, Figure 10). The identified peptides comprised almost the entire rod portion of desmin (amino acids 110 – 413). The theoretical molecular weight of the identified peptide region was calculated at 34-kDa, and the theoretical isoelectric point was calculated at 4.90, similar to results obtained from the 2D-DIGE experiment (Figure 8). No peptides were identified from the head or tail region of desmin from spot 319 in this experiment.

*Peroxiredoxin-2.* Western blots from SDS-PAGE gels did confirm the presence of peroxiredoxin-2 in sarcoplasmic LM samples (Figure 11). Densitometry was used to quantify the abundance of peroxiredoxin-2 in low and high star probe force samples. Similar to results from the 2D-DIGE experiment, peroxiredoxin-2 was significantly more
abundant in high star probe samples compared to low star probe samples \( (P = 0.02, \text{Table 9}) \).

2D-DIGE Experiment 2 (24 cm, pH 6 – 9). In order to evaluate proteins in the pH range of 6 – 9 between the two star probe groups, a second 2D-DIGE experiment was performed. A total of 179 spots were found in the sarcoplasmic fraction of the LM from the 24 samples. Of those spots, 11 were found to be significantly different between low and high star probe groups \( (P < 0.10) \), and three of the most prevalent spots were identified (Figure 12). All three spots were identified as phosphoglycerate mutase 2 (Table 10). Of the three spots, the more acidic spot (spot 158) was more abundant in low star probe samples, while the other two spots (spots 153 and 179) were more abundant in the high star probe samples (Table 11).

Discussion

Selection of loins that were similar in pH and color, but had extremely different star probe values was a key feature in this experiment. There was a 2.80 kg difference between the average force for the low star probe group (4.95 kg) and the average for the high star probe group (7.75 kg) (Table 4). The sensory analysis revealed the low star probe group was significantly more tender, juicy, and less chewy than the high star probe group. The amount of marbling and total lipid were significantly different between low and high star probe groups, but the difference in percent total lipid between average low and average high star probe samples was only 0.38\% \( (\text{range} = 1.61 – 3.37\%) \). Variation in marbling did not account for the variation exhibited in sensory evaluation for tenderness and juiciness when chops were cooked to different temperatures \( (R^2 = 0.10 \text{ maximum}) \) (Rincker et al., 2008). The small, yet significant difference in the measured a
values between low and high star probe groups indicates the low star probe samples were measured to be more red than high star probe samples. This measured difference in the Hunter a values did not influence the color scores assigned by trained personnel, as there was no difference in visual color scores between the classification groups. There were no significant differences between the low and high star probe groups for pH, color (L and b values), and loin and chop purge. These results show there was little association with these measured attributes to tenderness. This suggests the extreme differences in tenderness in these samples is due to other processes or events that occur postmortem.

Degradation of proteins influences muscle structure and can play a role in meat tenderness (Taylor et al., 1995; Wheeler et al., 2000; Melody et al., 2004). The calpain system, specifically calpain-1, plays a large role in proteolysis of muscle proteins (Huff-Lonergan et al., 1996a; Lametsch et al., 2004; Geesink et al., 2006). In this study, the appearance of the 76-kDa autolysis product of calpain-1 demonstrates calpain-1 was completely autolyzed in samples from both classification groups. Activation of calpain-1 is closely paralleled with autolysis (Andrea et al., 1996). Autolysis lowers the amount of calcium needed for activation of calpain-1 (Suzuki et al., 1981). In pork LM, the 76-kDa autolyzed products of calpain-1 were negatively correlated with the intact proteins desmin \( r = -0.57 \), vinculin \( r = -0.18 \), and talin \( r = -0.66 \), demonstrating autolysis is closely associated with activity of calpain-1 (Bee et al., 2007). Results indicate calpain-1 had been potentially active in all of the samples from the current study. Calpain-1 in muscle degrades the proteins desmin, troponin-T, and titin during postmortem aging in beef (Huff-Lonergan, 1996) and pork (Melody et al., 2004). The rate of activation and autolysis of calpain-1 could impact the amount of protein degradation by calpain-1,
which can be limited by pH decline (Carlin et al., 2006; Bee et al., 2007) or oxidative conditions (Carlin et al., 2006; Chen et al., 2014). Unfortunately, data were not collected on pH decline postmortem from these samples, but differences exhibited in pH decline could potentially explain the differences in proteolysis exhibited between classification groups in this study. The visual color scores and Hunter L and b measurements were not different between classification groups, indicating there were probably no extreme pH decline differences. Moving forward, it would be beneficial to measure the amount of oxidation in these samples to determine if there was a difference in cellular environment between sample groups that could help explain the proteolytic differences.

Degradation of proteins occurs during the postmortem aging period (Huff-Lonergan, 1996b; Lametsch et al., 2004). The samples used in this study were all aged for 9 – 11 days and results showed degradation of the proteins troponin-T, desmin, filamin, and titin in both classification groups. The low star probe group exhibited more degradation products of troponin-T (28 – 30-kDa products) and desmin (38-kDa) than high star probe groups. These results are consistent with observations that showed degradation of these proteins accounts for differences in measured instrumental tenderness values in pork muscles (Melody et al. (2004). For example, it was demonstrated that LM samples had more degradation of troponin-T, less intact desmin, and lower Warner-Bratzler shear force values (kg) than tougher Semimembranosus muscles (Melody et al., 2004).

Troponin-T is a key regulatory protein involved with the actomyosin complex formed during contraction. Degradation of this protein could demonstrate there is a weakening of this actomyosin complex, ultimately influencing tenderness (Huff
Lonergan et al., 2010). Degradation of troponin-T could also just be an indicator of protein degradation in general. Desmin is an intermediate protein that is an integral part of myofibril structure that aids in connecting adjacent myofibrils to each other and with other muscle fiber components (Clark et al., 2002). It stands to reason that degradation of this protein as seen in this study could ultimately alter integrity of the alignment of myofibrils to each other and to the sarcolemma.

Filamin is a large protein located in the Z-line. Filamin interacts with F-actin and functions to aid in stabilization of the muscle cytoskeleton and helps with transmitting chemical signals at the Z-line (Wang and Singer, 1977; Clark et al., 2002). The dimerization of filamin is essential for interaction filamin with F-actin (Pudas et al., 2005). Extreme proteolytic differences in degradation of filamin were observed between classification groups where low star probe samples had significantly less intact filamin. Myofibrils isolated from low and high Warner-Bratzler shear force beef samples had different rates of filamin degradation during aging (Huff-Lonergan et al. (1996a). In that study, low shear force samples degraded earlier postmortem than high shear force samples (Huff-Lonergan et al., 1996a).

Intact (290-kDa) and degraded (278.3-kDa) filamin were successfully identified in high and low star probe samples from the current study. These results demonstrate that removal of the 109 amino acids from the carboxy-terminal end in degraded filamin is likely the first cleavage of intact filamin observed in postmortem muscle. The carboxy-terminal end of filamin is responsible for dimerization and interaction with other cellular components such as β integrins, androgen receptors, and portions of potassium channels (Stossel et al., 2001; Feng and Walsh, 2004; Pudas et al., 2005; Nakamura et al., 2011).
Therefore, the loss of this carboxy-terminal end causes the loss of ability for this protein to form dimers and impairs anchoring to the Z-line.

Degradation products of titin (2,400-kDa) were also found in samples from both classification groups. Select high star probe samples did exhibit presence of intact titin (T1 band, 3,000-kDa). The presence of intact titin in aged samples is a novel result because titin is typically degraded earlier postmortem by calpain-1 (Huff-Lonergan et al., 1995; Taylor et al., 1995; Melody et al., 2004; Rowe et al., 2004). The rate of titin degradation is known to be slower in beef that exhibits high shear force values, regardless of animal age or gender (Huff-Lonergan et al. (1995). Low star probe samples from the current study may have exhibited a faster rate of postmortem titin degradation due to differences in calpain-1 activity. If calpain-1 became inactive at a quicker rate, protein degradation could be limited in samples (Li et al., 2004; Bee et al., 2007). This could potentially explain the proteolytic differences in troponin-T, desmin, filamin, and titin between low and high star probe samples in the current study. In future studies, it would be beneficial to measure calpain-1 activity and protein degradation in early postmortem muscle to evaluate how these attributes relate to tenderness in aged pork. Results from this study demonstrate proteolysis played a major role in the differences in tenderness exhibited between the classification groups. It would be beneficial to understand the rate of proteolysis as well as the oxidative conditions of the muscle to understand why these differences were observed.

There were no differences in proportion of myosin heavy chain isoforms identified from the two classification groups. Myosin heavy chain type IIb was the predominate isoform in samples from both classification groups. These results are
consistent with other observations that showed the predominate myosin heavy chain isoform in pork LM samples 45 minutes postmortem was type IIb (Melody et al., 2004). Muscle fibers that contain predominately type IIb myosin heavy chain isoforms are fast-twitch fibers that rely primarily on glycolytic metabolism (Reggiani et al., 2000). Myosin heavy chain isoforms (I, IIa, IIx, IIb) differ in their ability to migrate on SDS-PAGE gels (Schiaffino et al., 1989). Type Ia/IIx isoforms tend to comigrate together during electrophoresis and migrate the least, type IIb isoforms migrate a little further down the gel, and type I isoforms migrate the most during electrophoretic conditions (Schiaffino et al., 1989). During aging, myosin heavy chain can be degraded in pork LM (Lametsch et al. 2003). A 56-kDa fragment of the globular head domain of myosin heavy chain was identified to increase in abundance during postmortem aging in whole muscle pork samples (Lametsch et al. 2003). Pork LM with low total protein solubility and low pH at 45 minutes postmortem also resulted in degradation of myosin heavy chain (Choi et al., 2010). Degradation of myosin heavy chain during postmortem aging could influence the migration pattern of myosin heavy chain isoforms. This degradation of myosin heavy chain could ultimately influence the results from the present study since samples from both classification groups were aged 9 – 11 days. Examination of fiber types through other methods may be a more precise technique to identify if there are fiber type differences in these aged pork muscles.

The results from both 2D-DIGE experiments in the sarcoplasmic fraction show there are differences in protein abundance and modification between low and high star probe samples. The spots that were identified primarily consisted of metabolic, stress response, regulatory, and structural proteins. Most of the spots that were different in
abundance were identified as being more abundant in high star probe samples, whereas
the spots identified as the metabolic protein phosphoglycerate mutase 2 and the structural
protein desmin were more abundant in low star probe samples.

*Glycolytic Proteins*

The identified protein spots involved with glycolytic metabolism included
pyruvate kinase (Spot 300), triosephosphate isomerase (Spots 402, 407, and 419), and
phosphoglycerate mutase 2 (Spots 155, 158, and 179, 2D-DIGE experiment two).
Pyruvate kinase was 40% more abundant in high star probe samples. Pyruvate kinase
catalyzes the reaction converting phosphoenolpyruvate and ADP to two molecules of
pyruvate and ATP (Tymoczko et al., 2013). Pyruvate kinase is most active in the
phosphorylated form (Cowan and Storey, 1999). The isoelectric point for pyruvate kinase
is approximately 7.53. The spot identified as pyruvate kinase in the current study was
more acidic than 7.53. This could mean the spot identified is most likely the
phosphorylated form of pyruvate kinase since phosphorylation is known to shift the
isolectric point of proteins to be more acidic (Julien and Mushynski, 1982). Various
phosphorylated forms of pyruvate kinase may exist and phosphorylated forms of this
enzyme were identified in postmortem PSE pig muscle (Schwäгеle et al., 1996).
Phosphorylated pyruvate kinase identified in PSE pork has been shown to exhibit greater
activity in low pH conditions (Schwäгеle et al., 1996). Although there was not a
significant difference in ultimate pH between classification groups, the rate of pH decline
could have influenced the abundance of pyruvate kinase. If high star probe samples had
exhibited a faster pH decline, resulting in lower pH early postmortem, this could
potentially increase abundance of this enzyme.
Three spots of triosephosphate isomerase were significantly more abundant in high star probe samples. Triosephosphate isomerase catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Alber et al., 1981). Glyceraldehyde 3-phosphate is the substrate directly involved with the glycolytic pathway (Tymoczko et al., 2013). Triosephosphate isomerase was significantly correlated with Warner-Bratzler shear force values immediately postmortem ($r = -0.46$) and after aging for three days ($r = -0.64$) in pork LM whole muscle proteins (Lametsch et al., 2003). Postmortem muscle is dynamic, so it is difficult to compare early postmortem samples to aged samples. Evidence of increased triosephosphate isomerase in high star probe samples might indicate muscle was more reliant on glycolytic metabolism to produce ATP.

In experiment two of the current study, two spots of phosphoglycerate mutase 2 (Spots 153 and 179) were identified as being more abundant in high star probe samples. These two spots were the more alkaline spots of the three identified phosphoglycerate mutase 2 spots. In addition, spot 158, identified as phosphoglycerate mutase 2 in experiment two, was more abundant in low star probe samples. Spot 158 was identified towards the acidic portion of the gel, indicating potential phosphorylation of this protein. Phosphoglycerate mutase 2 is responsible for catalyzing the transfer of phosphate from 3-phosphoglycerate to produce 2-phosphoglycerate through a 2,3-bisphosphoglycerate intermediate (Kondoh et al., 2005; Tymoczko et al., 2013). Tyrosine kinases are associated with the phosphorylation of phosphoglycerate mutase 2 (Fothergill-Gilmore and Watson, 1989). Phosphorylated phosphoglycerate mutase 2 is the active form of the enzyme (Walter et al., 1999). The phosphorylated form of phosphoglycerate mutase is
less stable than the non-phosphorylated form (Rose and Dube, 1976; Nairn et al., 1995). Phosphorylation could be due to excess of this protein in the cell or because tyrosine resides on the enzyme are more accessible for modification (Fothergill-Gilmore and Watson, 1989). Phosphorylation has also been shown in other metabolic proteins as being related to tenderness. Beef steaks with high star probe values exhibited more abundant phosphoglucomutase 1 in the least phosphorylated form (most alkaline spot), as determined by staining for phosphorylated proteins (Anderson et al., 2014). Results from the current study and Anderson et al. (2014) demonstrate the importance of identifying posttranslational modifications in proteins to be used as biomarkers to in order to understand activity of these proteins and their contribution to meat tenderness.

Other Metabolic Proteins

The identified proteins cytoplasmic malate dehydrogenase (Spot 347), ATP synthase beta subunit (Spot 253), creatine kinase M-type (Spot 301), and glycerol-3-phosphate dehydrogenase (Spots 333 and 336) are associated with other aspects of energy metabolism. Cytoplasmic malate dehydrogenase (Spot 347) is a protein involved with energy metabolism in cells. This protein represented in this spot was 35% more abundant in high star probe samples. Cytoplasmic malate dehydrogenase catalyzes the reaction of oxaloacetate and NADH to malate and NAD$^+$ as part of the malate-aspartate shuttle (Tymoczko et al., 2013). Cytoplasmic malate dehydrogenase more abundant in the sarcoplasmic fraction of beef LM biopsy samples from more tender beef compared to less tender beef (Jia et al., 2009). This protein was also identified to be more abundant in the sarcoplasmic fraction of pork Semitendinosus muscles collected from pigs immediately postmortem that were subjected to acute heat stress (Cruzen et al., 2015). The study by
Jia et al. (2009) used biopsy samples taken from animals four days prior to harvest. These animals were not exposed to stressors that could occur prior to harvest such as transport or exposure to environmental conditions at the time of sample collection. Abundance of cytoplasmic malate dehydrogenase in high star probe samples could potentially demonstrate that cells were trying to produce more ATP to respond to a stressor acting on the cells. Variation in the stress response at the cellular level may have impacted the development of tenderness in postmortem pork.

ATP synthase beta subunit (Spot 253) and creatine kinase M-type (Spot 301) were significantly more abundant ($P < 0.01$) in high star probe samples. ATP synthase beta subunit is an enzyme that uses proton motive force from the electron transport chain to aid in generating ATP for cells (Stock et al., 1999). This protein was identified as being more abundant in the red portion of Semitendinosus sarcoplasmic muscle samples from pigs subjected to acute heat stress, indicating greater potential for ATP production via oxidative phosphorylation (Cruzen et al., 2015).

Creatine kinase M-type is responsible for catalyzing the reaction between phosphocreatine and adenosine diphosphate (ADP) to produce creatine and ATP (Westerblad et al., 2010). Creatine kinase M-type fragments were more abundant in the soluble fraction of beef LM chops stored for 24 hours postmortem (Jia et al., 2007) and pork LM whole muscle protein muscles after 48 hours of storage (Lametsch et al., 2002). Lametsch et al. (2002) demonstrated that even though fragments of creatine kinase were observed, there was no decrease in abundance of the full length protein because it is extremely abundant in muscle.
The two spots of glycerol-3-phosphate dehydrogenase (Spots 333, 336) tended to be significantly more abundant in high star probe samples ($P = 0.08$, $P = 0.06$). Glycerol-3-phosphate dehydrogenase plays a role in the production of NAD$^+$ that can be used in glycolysis or the TCA cycle to produce ATP (Jia et al., 2007). This protein was also identified in postmortem beef LM muscle after 24 hours of storage, demonstrating maintenance of ATP levels in muscle after harvest (Jia et al., 2007). Increased abundance of ATP synthase beta subunit, creatine kinase M-type, and glycerol-3-phosphate dehydrogenase demonstrate potential increase in ATP utilization in postmortem muscle from high star probe samples.

**Stress Response Proteins**

The proteins protein deglycase DJ-1 (Spot 419), peroxiredoxin-6 (Spot 390), and peroxiredoxin-2 (Spots 423 and 428) were identified as being more abundant in the sarcoplasmic fraction from the high star probe samples. These spots are all associated with cellular stress response. Protein deglycase DJ-1 was 54% more abundant in high star probe samples compared to low star probe samples. This protein functions as an antioxidant protein and is known to act as a molecular chaperone or protease to refold or degrade proteins damaged by stress (Moore et al., 2005). Protein deglycase DJ-1 was increased in abundance in beef LM during aging (Jia et al., 2007; Laville et al., 2009). Jia et al. (2009) identified this protein as being more abundant in the sarcoplasmic fraction of aged beef LM samples with higher Warner-Bratzler shear force values.

Peroxiredoxins are a group of proteins (20 – 30 kDa) that function to protect cells from oxidative damage or play a role in controlling hydrogen peroxide concentration in cells (Rhee et al., 2001). There are different isoforms of peroxiredoxin in organisms that
differ in developmental expression patterns, distribution in cells, and reaction intermediates formed (Rhee et al., 2001). The protein spot identified as peroxiredoxin-6 in the present study was 29% more abundant high star probe samples. Peroxiredoxin-6 functions as a peroxidase to protect cells from damage from stress (Wu et al., 2009). Peroxiredoxin-6 was identified as being a potential biomarker for tenderness because it was more abundant in muscle biopsies from live animals and one hour postmortem samples from beef LM that had lower seven day Warner-Bratzler shear force values (Jia et al., 2009). The results from Jia et al. (2009) are contradictory to the typical cellular protective function peroxiredoxin-6 exhibits, potentially indicating peroxiredoxin-6 was not activated at the sample collection times in this experiment. Results from the current study were taken on aged pork muscles, demonstrating peroxiredoxin-6 may have been activated and involved with cellular protection later postmortem.

Two spots identified as peroxiredoxin-2 were 37% and 51% more abundant in high star probe samples. Peroxiredoxin-2 is an antioxidant protein that reduces stress in cells by protecting the cell from damage by hydrogen peroxide molecules, damage from heat, and helps to increase resistance to oxidative stress (Oláhová et al., 2008). Peroxiredoxin-2 functions to protect cells by acting as a molecular chaperone, a peroxidase, and a cellular signaler during times of stress (Oláhová et al., 2008). Peroxiredoxin-2 was identified to increase in abundance in sarcoplasmic beef LM collected immediately after harvest and abundance continued to increase until 24 hours postmortem (Jia et al., 2007). Peroxiredoxin-2 was positively correlated ($r = 0.67$) with Warner-Bratzler shear force values in aged pork *Longissimus* muscles (Hwang et al., 2005).
Because of the large role peroxiredoxin-2 plays in the cellular stress response and previous research suggesting this protein plays a role in the tenderization process, one-dimensional Western blots were run to confirm the identity and abundance of this protein in the current study (Figure 11, Table 9). The results both confirmed the spot identity as peroxiredoxin-2 and the differences in protein abundance between classification groups. High star probe samples exhibited more peroxiredoxin-2 in sarcoplasmic protein samples compared to low star probe samples \((P = 0.02)\). The abundance of these stress response proteins in the high star probe samples from the current study suggest high star probe samples may have had more oxidative stress. The high star probe samples may be producing these stress related proteins in response to stress. Therefore, oxidative stress may be an undefined source of variation in proteolysis and tenderness in aged pork.

**Regulatory Proteins**

The regulatory proteins myosin light chain 1 (Spot 428) and tropomyosin alpha-1 chain (Spot 354) were identified as being more abundant in high star probe samples. Myosin light chain is a regulatory protein that is part of the large protein myosin. Myosin consists of two heavy chains (approximately 220-kDa) and four light chains (approximately 20-kDa) (Weeds and Lowey, 1971; Rayment et al., 1993; Clark et al., 2002). The four light chains include two regulatory chains (20-kDa) and two essential chains (17 – 27-kDa) (Timson, 2003). Myosin light chain 1 is involved with the interaction of actin during the contractile process (Lowey et al., 1993). Myosin light chain 1 as was more abundant in the sarcoplasmic protein fraction of one day postmortem beef LM samples that exhibited low star probe values (taken after 14 days of aging) (Anderson et al., 2012). That same report demonstrated that myosin light chain 1 released
into the soluble protein fraction very rapidly by the protease calpain-1, as determined by digesting myofibrils with calpain-1 (Anderson et al., 2012). The reason for the discrepancy in results between Anderson et al. (2012) and the current study is unknown, but could be due to species differences. Muscles from pork and beef exhibit differences in predominate fiber type and pH decline that could ultimately influence the solubility of myosin light chain and release of this protein into the sarcoplasmic fraction of the muscle.

The spot identified as tropomyosin alpha-1 chain was 47% more abundant in high star probe samples compared to low star probe samples \( (P = 0.03) \). Tropomyosin and the troponin complex work in conjunction with each other to regulate the interaction between actin and myosin during contraction (Oe et al., 2009). Tropomyosin alpha-1 chain was less abundant in the sarcoplasmic fraction of beef LM chops with high star probe force values (Anderson et al., 2012). There are three different isoforms of tropomyosin and tropomyosin alpha-1 chain is the isoform predominately found in fast-twitch muscle fibers, such as the Longissimus muscle (Oe et al., 2009).

The results of identifying tropomyosion alpha-1 chain in the sarcoplastic fraction could indicate that high star probe samples are composed of more fast fibers, demonstrating more release of this protein during the postmortem tenderization process. Tropomyosin alpha-1 chain and myosin light chain 1 are typically associated with the myofibrillar protein fraction, so identification of these proteins in the soluble fraction demonstrates there was release or degradation of these proteins into the sarcoplastic fraction. The results in the current study are different than what has been previously reported, demonstrating there may be a species difference due to fiber type or conversion
of muscle to meat that could be impacting solubility of these proteins. All together when examining protein differences, tropomyosin and myosin light chain 1 are associated with the actomyosin interaction that occurs during contraction. Even though the solubility of these proteins indicates interruption of the actomyosin interaction, it does not seem to play a significant role in measured tenderness between the low and high star probe samples in this study because these fragments were more abundant in the high star probe samples.

*Structural Proteins*

The structural protein desmin (Spot 319) was more abundant in the sarcoplasmic extracts from the low star probe samples. Desmin is an intermediate filament protein that is vital for the structure of muscle cells. At the level of the Z-disk, desmin connects adjacent myofibrils to each other and connects peripheral myofibrils to the sarcolemma (Granger and Lazarides, 1979; Clark et al., 2002). Desmin degradation products were already identified in the current study and were more abundant in low star probe whole muscle protein samples using one-dimensional Western blots. The spot identified as desmin in the 2D-DIGE experiment one was 90% more abundant in the low star probe samples.

In the 2D-DIGE experiment examining the sarcoplasmic protein fraction, desmin migrated to lower molecular weight on the separating gel than intact desmin (55-kDa). The molecular weight of the fragments identified from mass spectrometry were calculated at approximately 34-kDa (Figure 10). These results indicate that a fragment of desmin was identified in the sarcoplasmic fraction of aged samples. Additional analysis of the peptides identified from mass spectrometry showed the rod portion of desmin was
identified (Figure 10). Desmin is comprised of a non-alpha helical amino terminal head, highly conserved alpha-helical rod, and a carboxy-terminal tail (Bär et al., 2004). The head and tail portions of this protein are responsible for proper filament assembly around the myofibrils (Bär et al., 2004). Desmin is a substrate for endogenous proteases. Degradation of desmin by calpain-1 is a result of cleavage of the head and tail regions of this protein (Baron et al., 2004). When incubated with another protease cathepsin B, only small fragments from the carboxy-terminal end of desmin were cleaved and no clear degradation fragments were identified (Baron et al., 2004). The results from Baron et al. (2004) demonstrate calpain-1 was likely the protease responsible for degradation of desmin in the current study. Typically, desmin is associated with the insoluble myofibrillar fraction of the muscle due to its association with the structure of myofibrils, therefore it is novel to find the rod portion of desmin in the soluble fraction of muscle.

In order to confirm the results from the 2D-DIGE experiment, one-dimensional Western blots were performed on sarcoplasmic protein extracts. Results confirmed that only desmin fragments (34-kDa) were identified in the soluble muscle fraction (Figure 8) and these fragments were significantly more abundant in the low star probe samples (100% more abundant, $P < 0.01$). Because desmin is a substrate for calpain-1 and incubation with this protease results in cleavage at the head and tail regions (Baron et al., 2004), it stands to reason that the extreme differences in desmin degradation exhibited in classification groups may be due differences in proteolytic activity by calpain-1. The identification of this desmin fragment in the sarcoplasmic fraction of the muscle demonstrates the potential for this protein to be utilized as a biomarker for tenderness. Sarcoplasmic proteins are easily solubilized and found in the purge of meat. Using
proteins found in meat exudate, such as desmin fragments, can be a rapid way of identifying protein biomarkers for tenderness without further processing or extraction of samples.

Although there was no difference in myosin heavy chain isoforms in the classification groups, there still may be a difference in fiber type that could be measured with other analyses. Desmin degradation occurs at a faster rate and to a greater extent in type IIb fibers compared to type I fibers in pork LM (Muroya et al., 2010). The authors speculated that differences in desmin degradation could be due to fiber type differences influencing calpain activity (Muroya et al., 2010). Cellular environment can also impact the activity of proteases to degrade desmin in muscle. Oxidation of desmin changes the secondary structure of desmin, which increases the susceptibility to proteolysis by caspases (Chen et al., 2014). Oxidation is also known to decrease the activity of calpain-1 and calpain-2, ultimately reducing desmin degradation by calpain-1 (Rowe et al., 2004; Carlin et al., 2006). Measuring both the rate of protease activity and the cellular conditions could be beneficial to understanding why the extreme differences in desmin degradation were observed between classification groups.

Other Proteins

Some other proteins identified in experiment one consist of eukaryotic translation initiation factor 5A-1 (spot 455) and mitochondrial aldehyde dehydrogenase (spot 245). Mitochondrial aldehyde dehydrogenase was 50% more abundant in high star probe samples compared to low star probe samples ($P = 0.02$). The function of mitochondrial aldehyde dehydrogenase is to protect cells from oxidative stress by detoxifying acetylatedalddehydes in cells (Ohsawa et al., 2003). Eukaryotic initiation factor 5A-1 was 30%
more abundant in high star probe samples and only tended to be different between classification groups \((P = 0.07)\). This protein has a variety of functions in cells, but most notably plays a role in translation of proteins (Li et al., 2010). This protein also plays a key role in translation elongation of proteins in cells that have been stressed from oxidation (Li et al., 2010). The increased abundance of both of these proteins demonstrates even further that high star probe samples exhibited some sort of cellular stress that impacted protein expression and possibly activity.

**Conclusions**

Tenderness in commodity pork is influenced by a variety of factors. The sample libraries (high and low star probe) in the current experiment were assembled to be similar in pH, lipid content, and color. Variation in tenderness in aged pork LM from this study was attributed to differences in proteolysis and differences in sarcoplasmic proteomes. Samples with low star probe values exhibited substantially more degradation of the proteins troponin-T, desmin, and titin. Desmin degradation fragments were also found in the soluble fraction of the muscle and desmin was increased in low star probe samples by a substantial amount. Samples with high star probe values had more abundant metabolic, stress response, and regulatory proteins. Peroxiredoxin-2, a key protein in the cellular response to oxidative stress, was substantially more abundant in the soluble fraction of high star probe samples. These results demonstrate desmin and peroxiredoxin-2 may be potential biomarkers to differentiate between tough and tender pork. Both of these proteins were found in the soluble protein fraction of the meat, which allows for the potential of rapid detection of these proteins without further extraction procedures. Further evaluation of muscle fiber type, cellular conditions, and rate of proteolytic
activity needs to be performed in order to understand why these drastic differences in the proteome of aged pork samples exists. Once it is more clearly outlined why these extreme differences exist, procedures to utilize biomarkers to define pork quality could potentially eliminate tenderness inconsistencies in aged pork products.
Literature Cited


Table 1. Summary of the fresh pork loin quality and sensory traits from the initial sample of 159 fresh-never frozen, aged commercial pork loins

<table>
<thead>
<tr>
<th>Item</th>
<th>Range</th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average pH(^1)</td>
<td>5.40 – 6.18</td>
<td>5.64</td>
<td>0.12</td>
</tr>
<tr>
<td>Percent Loin Purge(^2)</td>
<td>0.00 – 4.41</td>
<td>1.37</td>
<td>1.04</td>
</tr>
<tr>
<td>Percent Chop Purge(^3)</td>
<td>0.29 – 3.95</td>
<td>1.18</td>
<td>0.67</td>
</tr>
<tr>
<td>Marbling Score(^4)</td>
<td>1.0 – 4.0</td>
<td>2.2</td>
<td>0.71</td>
</tr>
<tr>
<td>Color Score(^5)</td>
<td>2.0 – 4.5</td>
<td>3.1</td>
<td>0.57</td>
</tr>
<tr>
<td>L value(^6)</td>
<td>42.5 – 55.5</td>
<td>49.7</td>
<td>2.23</td>
</tr>
<tr>
<td>a value(^6)</td>
<td>12.5 – 16.2</td>
<td>14.2</td>
<td>0.69</td>
</tr>
<tr>
<td>b value(^6)</td>
<td>1.91 – 4.23</td>
<td>3.0</td>
<td>0.51</td>
</tr>
<tr>
<td>Tenderness(^7)</td>
<td>1.0 – 10.0</td>
<td>6.6</td>
<td>1.72</td>
</tr>
<tr>
<td>Chewiness(^7)</td>
<td>1.0 – 10.0</td>
<td>4.3</td>
<td>2.04</td>
</tr>
<tr>
<td>Juiciness(^7)</td>
<td>3.0 – 10.0</td>
<td>6.8</td>
<td>1.27</td>
</tr>
<tr>
<td>Pork Flavor(^7)</td>
<td>1.0 – 7.0</td>
<td>3.5</td>
<td>1.05</td>
</tr>
<tr>
<td>Off Flavor(^7)</td>
<td>1.0 – 6.0</td>
<td>1.6</td>
<td>1.02</td>
</tr>
<tr>
<td>Cook Loss(^8)</td>
<td>13.1 – 28.4</td>
<td>21.7</td>
<td>3.23</td>
</tr>
<tr>
<td>Instron – Star Probe(^9)</td>
<td>4.16 – 10.1</td>
<td>6.17</td>
<td>1.08</td>
</tr>
</tbody>
</table>

1 Average pH taken from Center and Sirloin portion of the *Longissimus dorsi*
2 Percent loin purge = [purge/(loin + purge)] x 100
3 Chops were cut 2.5 cm thick and percent chop purge = [purge/(chop + purge)] x 100
4 National Pork Board standards, 10-point scale (1 = 1% intramuscular fat; 10 = 10% intramuscular fat)
5 National Pork Board standards, 6-point scale (1 = pale pinkish gray/white; 6 = dark purplish red)
6 Hunter L, a, and b determined with Minolta Chroma Meter with D65 light source, 50 mm aperture, and 0° observer
7 As determined by a trained panel (n = 4) using a 10-point category scale, low values indicate lower degrees of characteristics and high values indicate higher degrees of characteristics
8 Chops were cooked to an internal temperature of 68°C on clamshell grills. Percent cook loss = [(raw weight – cooked weight)/raw weight] x 100
9 Measured in kg (Huff-Lonergan et al., 2002)
Table 2. Gel loading conditions, protein fractions, and antibody dilutions used to examine troponin-T, desmin, calpain-1, peroxiredoxin-2, filamin, titin, and myosin heavy chain isoforms in aged pork loins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Muscle Fraction</th>
<th>Protein Load</th>
<th>Acrylamide Percentage(^1)</th>
<th>Reference(^2)</th>
<th>(^1^*) Antibody Dilution(^3)</th>
<th>(^2^*) Antibody Dilution(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troponin-T(^6)</td>
<td>Whole muscle</td>
<td>20 µg</td>
<td>15%</td>
<td>7 Day LM (Whole Muscle)</td>
<td>1:80,000</td>
<td>1:10,000 GAM(^4)</td>
</tr>
<tr>
<td>Desmin(^7)</td>
<td>Whole muscle</td>
<td>40 µg</td>
<td>15%</td>
<td>0/7 Day LM (Whole Muscle)</td>
<td>1:40,000</td>
<td>1:20,000 GAR(^5)</td>
</tr>
<tr>
<td>Desmin(^7)</td>
<td>Sarcoplasmic</td>
<td>50 µg</td>
<td>15%</td>
<td>Aged LM (Whole Muscle)(^13)</td>
<td>1:40,000</td>
<td>1:20,000 GAR(^5)</td>
</tr>
<tr>
<td>Calpain-1</td>
<td>Whole muscle</td>
<td>40 µg</td>
<td>8%</td>
<td>0/7 Day LM (Whole Muscle)</td>
<td>1:10,000</td>
<td>1:10,000 GAM(^4)</td>
</tr>
<tr>
<td>Peroxiredoxin – 2(^9)</td>
<td>Sarcoplasmic</td>
<td>40 µg</td>
<td>15%</td>
<td>0 Day LM (Sarcoplasmic)</td>
<td>1:20,000</td>
<td>1:10,000 GAM(^4)</td>
</tr>
<tr>
<td>Filamin(^10)</td>
<td>Whole Muscle</td>
<td>80 µg</td>
<td>3 – 12% (gradient)</td>
<td>7 Day LM (Whole Muscle)</td>
<td>1:1,000</td>
<td>1:20,000 GAR(^5)</td>
</tr>
<tr>
<td>Titin(^11)</td>
<td>Myofibrillar</td>
<td>40 µg</td>
<td>5%</td>
<td>1 Day LM &amp; Aged LM (Myofibrillar)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Myosin heavy chain(^12)</td>
<td>Whole Muscle</td>
<td>2 µg</td>
<td>6%(^14)</td>
<td>Pork Diaphragm</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\) Acrylamide/Bis-Acrylamide ratio 100:1 for separating and stacking (5%) gels for troponin-T, desmin, calpain-1, and peroxiredoxin-2

\(^2\) All reference samples used in this study were obtained from pork *Longissimus dorsi* (LM), unless otherwise specified

\(^3\) Primary and secondary antibody incubations were diluted in PBS-Tween solution (80 mM Na\(_2\)HPO\(_4\), anhydrous, 20 mM NaH\(_2\)PO\(_4\), 100 mM NaCl, 0.1% [vol/vol] polyoxyethylene sorbitan monolaurate [Tween-20])

\(^4\) GAM – goat-anti mouse-HRP antibody (A2554, Sigma-Aldrich)
GAR – goat-anti rabbit- HRP antibody (31460, Thermo Scientific)

Primary antibody – T6277, JLT-12, Sigma-Aldrich

Primary antibody prepared at Iowa State University

Primary antibody – MA3-940, Thermo Scientific

Primary antibody – ab109367, Abcam

Primary antibody prepared at Iowa State University and antibody was diluted in 10 mL PBS-Tween solution mixed with 5% non-fat dry milk

Gels were stained using the Silver Stain Plus kit (161-0449, Bio-Rad) and appearance of T1 (intact) and T2 (degraded) titin bands were analyzed

Gels were stained using Colloidal Coomassie Blue Stain and analyzed for appearance of myosin heavy chain isoforms (type I, types IIa+IIx, and type IIb).

0 Day LM whole muscle sample was also run on desmin sarcoplasmic gels to confirm there was no appearance of intact desmin in sarcoplasmic extract

Acrylamide/Bis-Acrylamide ratio 50:1 for separating and stacking (4%) gel
Table 3. CyDye strip labeling procedure for two-dimensional difference in gel electrophoresis experiments 1 and 2

<table>
<thead>
<tr>
<th>Strip</th>
<th>CY2</th>
<th>CY3</th>
<th>CY 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pooled Reference</td>
<td>Low Star Probe 5</td>
<td>High Star Probe 129</td>
</tr>
<tr>
<td>2</td>
<td>Pooled Reference</td>
<td>High Star Probe 131</td>
<td>Low Star Probe 9</td>
</tr>
<tr>
<td>3</td>
<td>Pooled Reference</td>
<td>Low Star Probe 10</td>
<td>High Star Probe 132</td>
</tr>
<tr>
<td>4</td>
<td>Pooled Reference</td>
<td>High Star Probe 134</td>
<td>Low Star Probe 12</td>
</tr>
<tr>
<td>5</td>
<td>Pooled Reference</td>
<td>Low Star Probe 16</td>
<td>High Star Probe 139</td>
</tr>
<tr>
<td>6</td>
<td>Pooled Reference</td>
<td>High Star Probe 142</td>
<td>Low Star Probe 17</td>
</tr>
<tr>
<td>7</td>
<td>Pooled Reference</td>
<td>Low Star Probe 21</td>
<td>High Star Probe 147</td>
</tr>
<tr>
<td>8</td>
<td>Pooled Reference</td>
<td>High Star Probe 150</td>
<td>Low Star Probe 22</td>
</tr>
<tr>
<td>9</td>
<td>Pooled Reference</td>
<td>Low Star Probe 31</td>
<td>High Star Probe 152</td>
</tr>
<tr>
<td>10</td>
<td>Pooled Reference</td>
<td>High Star Probe 155</td>
<td>Low Star Probe 36</td>
</tr>
<tr>
<td>11</td>
<td>Pooled Reference</td>
<td>Low Star Probe 37</td>
<td>High Star Probe 159</td>
</tr>
<tr>
<td>12</td>
<td>Pooled Reference</td>
<td>High Star Probe 160</td>
<td>Low Star Probe 38</td>
</tr>
</tbody>
</table>
Table 4. Summary of the fresh pork loin quality and sensory characteristics from samples selected for low and high star probe groups.

<table>
<thead>
<tr>
<th>Item</th>
<th>Low Star Probe (n = 12)</th>
<th>High Star Probe (n = 12)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Least Squares Mean</td>
<td>Least Squares Mean</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Standard Error</td>
<td>Standard Error</td>
<td></td>
</tr>
<tr>
<td>Average pH(^1)</td>
<td>5.71 0.03</td>
<td>5.66 0.03</td>
<td>0.19</td>
</tr>
<tr>
<td>Marbling Score(^2)</td>
<td>2.3 0.14</td>
<td>1.9 0.14</td>
<td>0.03</td>
</tr>
<tr>
<td>Total Lipid(^3)</td>
<td>2.61 0.13</td>
<td>2.23 0.13</td>
<td>0.05</td>
</tr>
<tr>
<td>Color Score(^4)</td>
<td>3.3 0.16</td>
<td>3.1 0.17</td>
<td>0.52</td>
</tr>
<tr>
<td>L value(^5)</td>
<td>48.9 0.61</td>
<td>49.6 0.61</td>
<td>0.41</td>
</tr>
<tr>
<td>a value(^5)</td>
<td>14.5 0.19</td>
<td>13.89 0.19</td>
<td>0.04</td>
</tr>
<tr>
<td>b value(^5)</td>
<td>2.91 0.13</td>
<td>2.86 0.13</td>
<td>0.79</td>
</tr>
<tr>
<td>Tenderness(^6)</td>
<td>8.1 0.32</td>
<td>3.9 0.32</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Chewiness(^6)</td>
<td>2.3 0.31</td>
<td>7.9 0.31</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Juiciness(^6)</td>
<td>7.8 0.29</td>
<td>6.3 0.29</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Pork Flavor(^6)</td>
<td>4.3 0.29</td>
<td>2.9 0.29</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Off Flavor(^6)</td>
<td>1.3 0.32</td>
<td>1.9 0.32</td>
<td>0.22</td>
</tr>
<tr>
<td>Percent Loin Purge(^7)</td>
<td>1.01 0.30</td>
<td>1.14 0.30</td>
<td>0.77</td>
</tr>
<tr>
<td>Percent Chop Purge(^8)</td>
<td>0.85 0.21</td>
<td>1.09 0.21</td>
<td>0.42</td>
</tr>
<tr>
<td>Percent Cook Loss(^9)</td>
<td>19.3 0.93</td>
<td>23.0 0.93</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\(^1\) Average pH taken after aging period from the center and sirloin portion of the Longissimus dorsi.
\(^2\) National Pork Board standards, 10-point scale (1 = 1% intramuscular fat; 10 = 10% intramuscular fat).
\(^3\) Total lipid extracted as described by Folch et al. (1957) (with slight modifications).
\(^4\) National Pork Board standards, 6-point scale (1 = pale pinkish gray/white; 6 = dark purplish red).
\(^5\) Hunter L,a,b determined with Minolta Chroma Meter with D65 light source, 50 mm aperture, and 0° observer.
\(^6\) As determined by a trained panel (n = 4) using a 10-point category scale, low values indicate lower degrees of characteristics and high values indicate higher degrees of characteristics.
\(^7\) Percent loin purge = [purge/(loin + purge)] x 100
\(^8\) Chops were cut 2.54 cm thick and percent chop purge = [purge/(chop + purge)] x 100
\(^9\) Chops were cooked to an internal temperature of 68°C on clamshell grills. Percent cook loss = [(raw weight – cooked weight)/raw weight] x 100.
Table 5. Proteolysis of troponin-T and desmin in aged pork *Longissimus dorsi* (LM) whole muscle samples. Samples were analyzed using densitometry, comparing measured protein bands to corresponding bands of an internal reference sample (troponin-T – 7 day aged whole muscle LM sample, desmin – mixed 0/7 day aged whole muscle LM sample, and filamin- 7 day aged whole muscle LM sample).

<table>
<thead>
<tr>
<th>Item</th>
<th>Low Star Probe (n = 12)</th>
<th>High Star Probe (n = 12)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Least Squares Mean</td>
<td>Standard Error</td>
<td>Least Squares Mean</td>
</tr>
<tr>
<td>Troponin-T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37-kDa Intact Band</td>
<td>0.94</td>
<td>0.05</td>
<td>1.27</td>
</tr>
<tr>
<td>30-kDa Degradation Band</td>
<td>1.37</td>
<td>0.07</td>
<td>0.65</td>
</tr>
<tr>
<td>Degradation Products (27 - 30-kDa)</td>
<td>1.25</td>
<td>0.06</td>
<td>0.60</td>
</tr>
<tr>
<td>Desmin - Whole Muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55-kDa Intact Band</td>
<td>0.28</td>
<td>0.06</td>
<td>0.68</td>
</tr>
<tr>
<td>38-kDa Degradation Band</td>
<td>1.10</td>
<td>0.10</td>
<td>0.31</td>
</tr>
<tr>
<td>Filamin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>290-kDa Intact Band</td>
<td>0.92</td>
<td>0.08</td>
<td>1.37</td>
</tr>
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</table>
Table 6. Myosin heavy chain isoform analysis from aged *Longissimus dorsi* muscles from low and high star probe samples.

<table>
<thead>
<tr>
<th>Myosin heavy chain isoform</th>
<th>Low star Probe (n = 12)</th>
<th>Standard Error</th>
<th>High Star Probe (n = 12)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Types IIa+IIX</td>
<td>22.3%</td>
<td>1.74</td>
<td>22.3%</td>
<td>1.75</td>
</tr>
<tr>
<td>Type IIb</td>
<td>77.7%</td>
<td>1.74</td>
<td>77.7%</td>
<td>1.75</td>
</tr>
</tbody>
</table>
Table 7. Proteins identified from aged pork *Longissimus dorsi* muscles with individual peptides and Mowse scores

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein ID</th>
<th>Species</th>
<th>Accession #</th>
<th>pI$^1$</th>
<th>Mass (kDa)$^1$</th>
<th>Sequence Coverage %$^2$</th>
<th>Identified Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>245</td>
<td>Mitochondrial aldehyde dehydrogenase</td>
<td><em>Sus scrofa</em></td>
<td>Q2XQV4</td>
<td>6.87</td>
<td>56.9</td>
<td>23.22</td>
<td>LLCGGGAADDR YGLAAAVFTK VAFTGSTEVGHLIQVAAGK VVGNFDSR VTLELGGK AAFQLGSPWR LGPALATGNVVVMK VSEQTPTLTVANLIK TIEEVIGR TLPIDGDYFSYTR FKTIEEVIGR</td>
</tr>
</tbody>
</table>

Mowse Score$^3$: 1115
<p>| 253 | ATP synthase beta subunit | <em>Bos taurus</em> | P00829 | 5.27 | 56.2 | 55.68 | FTQAGSEVSALLGR VAFTGLTVAEYFR AIAEGLIYPAVDPLDSTSR AHGGYSVFAGVGER VALVYGQMNEPPGAR FLSQPFQVAEVFTGHGK IMDPNIVGSEHYDVAR TIAMDGTGELVR TIVLIMELINNVAK TIVLIMELINNVAK IMNVIGEPIDER VVDLLAPYAK LMNVIGEPIDERGPIK IPVGPETLGR IMNVIGEPIDER EGNLYHMEMIESGVINLK TREGNDLYHMEMIESGVINLK IGLFGGAGVGK TREGNDLYHMEMIESGVINLK IPVGPETLGR IMNVIGEPIDER EGNLYHMEMIESGVINLK TVLIMELINNVAK TREGNDLYHMEMIESGVINLK VLSR LVLEVAQHLGESTVR ADKLAEESH IMNVIGEPIDERGPIK |
| 300 | Pyruvate kinase | <em>Felis catus</em> | P11979 | 7.53 | 58.0 | 6.59 | NTVGICTIGPSAR GDLGIEIPAEEK LDIDSPPITAR |
| 301 | Creatine kinase M-type | <em>Sus scrofa</em> | Q5XLD3 | 7.09 | 43.0 | 62.99 | GTGGVDTAAVGSVFDVSNADR | LGSSEVEQVQLVVDGVK | LSVEALNSLTGEFK | GGDDLDPNYVLSRR | RGTGGVDTAAVGSVFDVSNADR | GQSIDDMIPAQK | DLFDPNQDR | LSVEALNSLTGEFKGK | SMTEQEQQQLIDDHFLFDKPVSPLLASGMAR | TDLNHENLKR | SFLVWVNEEDHLR | FEEILTR | ALTLIEIYKK | ALTLIEIYK | AGHPFMWNEHLGYVLTCPSNLGTGLR | AEEEPDLSK | LNFKAEEEYPDLSK | FCVGLQK | TDLNHENLKGDDLDPNYVLSRR | GYTLPPHCSR | IEEIFKK | LMVEMEK | GIWHNDNK | 5478 |</p>
<table>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>319</td>
<td>Desmin</td>
<td><em>Canis familiaris</em></td>
<td>Q5XFN2</td>
<td>5.27</td>
<td>53.3</td>
<td>33.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1621</td>
</tr>
<tr>
<td>333</td>
<td>Glycerol-3-phosphate dehydrogenase (GPD1)</td>
<td><em>Bos taurus</em></td>
<td>Q5EA88</td>
<td>6.89</td>
<td>37.6</td>
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<td>4.74</td>
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<td><em>Bos taurus</em></td>
<td>O77834</td>
<td>6.38</td>
<td>25.1</td>
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<td>Triosephosphate isomerase</td>
<td><em>Sus scrofa</em></td>
<td>Q29371</td>
<td>7.46</td>
<td>26.7</td>
<td>68.55</td>
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| 407 | Triosephosphate isomerase | *Sus scrofa* | Q29371 | 7.46 | 26.7 | 70.97 | KNNLGELINTLNAAK
LPADTEVVCAAPTAYIDFAR
VHAHALAEGLGVIACIGEK
DLGATWVVLGHSER
IYYGGSVTGATCK
NNLGENIINTLNAAK
VHAHALAEGLGVIACIGEKLDER
VVLAYEPWVAIGTGBK
RHFVGESDELIKQK
HVFGESEDELIKQK
TATPQQAQEVHEK
IAVAAQNCYK
VIADNVKDWNK
TATPQQAQEVHEKLR
DLGATWVVLGHSERR
KNNLGENIINTLNAAK
KFFVGGNWK
EAGITEKVVFQQT
VIADNVKDWNKVVLAYEPWVAIGTGBK
DWNKVVLAYEPWVAIGTGBK
EAGITEK
VIADNVK
FFVGGNWK
VFQQT
MNGRKNNLGENIINTLNAAK
VHAHALAEGLGVIACIGEKLDEREAGITEK
LDEREAGITEK
KNNLGENIINTLNAAK
VHAHALAEGLGVIACIGEKLDER
LPADTEVVCAAPTAYIDFAR
NNLGENIINTLNAAK
VHAHALAEGLGVIACIGEK
HVFGESEDELIKQK
DLGATWVVLGHSER
TATPQQAQEVHEKLR
| 16488 |
### Table 7. Continued

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<th>Mw</th>
<th>PM / MM</th>
<th>Partial amino acid sequence</th>
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<td><em>Bos taurus</em></td>
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<td>428</td>
<td>Peroxiredoxin-2</td>
<td><em>Sus scrofa</em></td>
<td>P52552</td>
<td>4.82</td>
<td>14.2</td>
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<td>455</td>
<td>Eukaryotic translation initiation factor 5A-1</td>
<td><em>Oryctolagus cuniculus</em></td>
<td>P10160</td>
<td>5.24</td>
<td>16.8</td>
<td>35.06</td>
<td>VHLVGIDIFTGK</td>
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</table>

1. Isoelectric point (pI) and molecular weight (MW) are theoretical
2. Percentage of the MASCOT protein sequence covered by identified matching peptides from trypsin digest
3. MOWSE – Molecular weight search, score used to calculate the similarity in molecular weight of the peptides from trypsin digest and the proteins from the MASCOT database
Table 8. Identified proteins from two-dimensional difference in gel experiment 1 (11 cm immobilized pH gradient strip, pH 4-7).

<table>
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<th>Protein</th>
<th>Ratio$^1$</th>
<th>P - value</th>
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</thead>
<tbody>
<tr>
<td>245</td>
<td>Mitochondrial aldehyde dehydrogenase</td>
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<td>253</td>
<td>ATP synthase subunit beta</td>
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<td>300</td>
<td>Pyruvate kinase</td>
<td>-1.40</td>
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<tr>
<td>301</td>
<td>Creatine kinase M-type</td>
<td>-1.32</td>
<td>&lt;0.01</td>
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<td>319</td>
<td>Desmin</td>
<td>1.90</td>
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<td>Glycerol-3-phosphate dehydrogenase (GPD 1)</td>
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<td>0.08</td>
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<td>336</td>
<td>Glycerol-3-phosphate dehydrogenase (GPD 1)</td>
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<td>0.06</td>
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<td>347</td>
<td>Malate dehydrogenase, cytoplasmic</td>
<td>-1.35</td>
<td>0.03</td>
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<tr>
<td>354</td>
<td>Tropomyosin alpha-1 chain</td>
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<td>390</td>
<td>Peroxiredoxin-6</td>
<td>-1.29</td>
<td>0.03</td>
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<tr>
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<td>Triosephosphate isomerase</td>
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<td>0.02</td>
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<td>Triosephosphate isomerase</td>
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<td>Peroxiredoxin-2</td>
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<td>Peroxiredoxin-2</td>
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<td>Myosin light chain 1</td>
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$^1$Ratio indicates spot abundance differences between low and high star probe samples (low star probe/high star probe; negative values = less abundant in low star probe, positive values = more abundant in low star probe)
Table 9. Abundance of desmin and peroxiredoxin-2 in aged pork *Longissimus dorsi* (LM) sarcoplasmic samples run on SDS-PAGE gels. Samples were analyzed using densitometry, comparing measured protein bands to corresponding bands of an internal reference sample (desmin – 9-11 day aged whole muscle LM sample, peroxiredoxin-2 – 0 day aged whole muscle LM sample).

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<td><em>(n = 12)</em></td>
<td><em>(n = 12)</em></td>
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<td>Least Squares Mean</td>
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<td>Standard Error</td>
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<td><strong>Desmin</strong></td>
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<td>34-kDa Degradation Band</td>
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<td>22-kDa Band</td>
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Table 10. Proteins identified from aged pork *Longissimus dorsi* muscles with individual peptides and Mowse scores.

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<th>P-value</th>
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<th>Coverage %</th>
<th>Peptides</th>
<th>Mowse Score</th>
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<td><em>Bos taurus</em></td>
<td>Q32KV0</td>
<td>1.14</td>
<td>0.05</td>
<td>8.88</td>
<td>28.7</td>
<td>47.83</td>
<td>TLWTILDGTDQMWLPVVR HGESTWNQENR MEFDICYTSVLK KAMEAVAAQGK ALPFWNDEIAPIQIK FCGWFDAELSEK FLGDEETVVRK VLAIAHGSNLK HYGGLTGLNK SFDIPPPPMDEK AMEAVAAQGK RSFDIPPPPMDEK HGEEQVK</td>
<td>2942</td>
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<tr>
<td>158A</td>
<td>Phosphoglycerate mutase 2</td>
<td><em>Bos taurus</em></td>
<td>Q32KV0</td>
<td>-1.31</td>
<td>&lt;0.01</td>
<td>8.88</td>
<td>28.7</td>
<td>50.6</td>
<td>MEFDICYTSVLK ALPFWNDEIAPIQIK HGESTWNQENR VLAIAHGSNLK FCGWFDAELSEK HYGGLTGLNK AMEAVAAQGK FLGDEETVVRK SFDIPPPPMDEK KAMEAVAAQGK HGEEQVK RSFDIPPPPMDEK HGEEQVK</td>
<td>2942</td>
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Table 10. Continued

| 179 | Phosphoglycerate mutase 2 | Bos taurus | Q32KV0 | 1.14 | 0.01 | 8.88 | 28.7 | 47.8 | ALPFWNDEIAPOIK TLWTILDGTDQMWLPVVR MEFDICYTSVLK VLIAAHGNSLR FLGDEETVR HYGGTLGLNK HGESTWNQENR FCGWFDAELSEK AMEAVAAQGK SFDIPPPPMDKEK FLGDEETVRK SFDIPPPPMDKEK RSDPFPIMPMDKEK HGEEQVK | 2946 |

1Isoelectric point (pI) and molecular weight (MW) are theoretical
2Percentage of the MASCOT protein sequence covered by identified matching peptides from trypsin digest
3MOWSE – Molecular weight search, score used to calculate the similarity in molecular weight of the peptides from trypsin digest and the proteins from the MASCOT database
Table 11. Identified proteins from two-dimensional difference in gel experiment 2 (24 cm immobilized pH gradient strip, pH 6-9).

<table>
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<td>Phosphoglycerate mutase 2</td>
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<td>0.05</td>
</tr>
<tr>
<td>158</td>
<td>Phosphoglycerate mutase 2</td>
<td>1.31</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>179</td>
<td>Phosphoglycerate mutase 2</td>
<td>-1.14</td>
<td>0.01</td>
</tr>
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</table>

1Ratio indicates spot abundance differences between low and high star probe samples (low star probe/high star probe; negative values = less abundant in low star probe, positive values = more abundant in low star probe).
Figure 1. Average star probe force (kg) from pork Longissimus dorsi chops from individual samples in the low star probe group (n = 12) and the high star probe group (n = 12).
Figure 2. Representative Western blot of intact and degraded troponin-T in aged pork *Longissimus dorsi* (LM) whole muscle samples. Intact bands (37-kDa), degradation bands (30-kDa) and degradation products (27 – 30-kDa) were compared to corresponding bands of a 7 day aged pork LM sample (Ref). Star probe values (kg) are provided for the samples and samples are labeled high/low depending on star probe force.
Figure 3. Representative Western blot of intact and degraded desmin in aged pork *Longissimus dorsi* (LM) whole muscle samples. Intact bands (55-kDa) and degradation bands (38-kDa) were compared to corresponding bands of a mixed 0/7 day aged pork LM sample (Ref). Star probe values (kg) are provided for the samples and samples are labeled high/low depending on star probe force.
Figure 4. Representative Western blots of intact filamin in aged pork LM from low and high star probe samples. A MW Std was used to identify the approximate molecular weight of the protein bands (A). Samples were compared to a 7 day aged LM (Ref) to identify abundance of intact filamin (B).
Figure 5. Representative Western blot of calpain-1 autolysis in aged Longissimus dorsi (LM) whole muscle pork samples. Samples in low and high star probe groups were compared to a day 0/7 mixed LM whole muscle sample (Ref) to identify the presence of intact calpain-1 (80-kDa), and autolyzed (78-kDa and 76-kDa) protein bands.
Figure 6. Representative SDS-PAGE SYPRO Ruby stained gel showing titin degradation in aged *Longissimus dorsi* (LM) whole muscle pork samples. Samples in low and high star probe groups were evaluated for presence of the intact 3,000-kDa titin band (T1) and the degraded 2,400-kDa titin band (T2). A 0 day aged whole muscle LM sample and a 7 day aged LM whole muscle sample (from outside of this experiment) were run on the gel to provide references for T1 and T2 bands.
Figure 7. Representative SDS-PAGE colloidal coomassie blue stained gel used to evaluate myosin heavy chain isoforms. A diaphragm sample was used as a reference (Ref) to identify location of Type IIa and Type I myosin heavy chain isoforms. Samples (Low and High) were analyzed for percentage of abundance of Type IIa+IIx and Type IIb isoforms.
Figure 8. Representative 2D-DIGE gel from the sarcoplasmic fraction of aged pork Longissimus dorsi showing identified proteins. Immobilized pH gradient strips (11cm, pH 4 – 7) were loaded with 45 µg of CyDye labeled protein (15 µg each of CyDye 2, 3, and 5) and strips were run on 12.5% SDS-PAGE gel. Proteins labeled with CyDye2 shown.
Figure 9. Representative Western blots of desmin from the sarcoplasmic fraction of aged pork *Longissimus dorsi* (LM) muscles. A molecular weight standard (MW Std) was run on each SDS-PAGE gel to confirm the approximate molecular weight of the protein bands (A). Samples were compared to the degradation band of an aged whole muscle (WM) LM sample. A 0 day aged LM sample was run on Western blots to provide a reference of intact desmin (B).
Figure 10. Amino acid breakdown of the full length protein of desmin (*sus scrofa* – accession # P02540). The highlighted regions represent peptides of desmin identified from tandem mass spectrometry. The identified peptides comprised almost the entire of the rod region of desmin (indicated with red brackets, amino acid 110 – 413).
Figure 11. Representative Western blots of peroxiredoxin-2 in aged pork *Longissimus dorsi* (LM) muscles from low and high star probe samples. A molecular weight standard (MW Std) was used to identify the approximate molecular weight of the protein bands (A). Samples were compared to a 0 day aged LM muscle (Ref) to identify abundance of peroxiredoxin-2 (B).
Figure 12. Representative 2D-DIGE gel from pork sarcoplasmic *Longissimus dorsi* showing identified proteins. Immobilized pH gradient strips (24 cm, pH 6 – 9) were loaded with 75 µg of CyDye labeled protein (25 µg each of CyDye 2, 3, and 5) and strips were run on 12.5% SDS-PAGE gel. Proteins labeled with CyDye2 shown.
CHAPTER 4

GENERAL CONCLUSIONS

Results from the current study show that even when there are no significant differences in pH, color, and purge in aged pork loins, extreme variations in measured tenderness (star probe and sensory) still exist. This demonstrates that protein degradation and differences in sarcoplasmic proteomes is important in influencing tenderness than the previously mentioned attributes. It is essential to continue researching the mechanisms by which these proteomic differences occurs in order to understand utilization of protein biomarkers in a production setting. Within this sample set, it would have been beneficial to collect pH decline data to understand if the rate of pH decline influenced differences exhibited in proteolysis and protein abundance. More precise methods to determine fiber type could be utilized to identify if the extreme differences in metabolic proteins expressed in low and high star probe samples were influenced by predominate muscle fiber type. It would also be beneficial to measure if there were any oxidation differences between low and high star probe samples to see if oxidative stress influenced the increased abundance of stress response proteins in high star probe samples. Moving forward, the collection of these data would aid in further interpretation of results from this study.

These results demonstrated that low star probe samples exhibited more protein degradation than high star probe samples. The intermediate protein filament desmin was identified as being a potential biomarker to classify tender pork. High star probe samples
exhibited less protein degradation, but significantly more abundant metabolic, stress related, and regulatory proteins. Samples in the high star probe group may have been better equipped to protect the cell from stress that occurs during the conversion of muscle to meat, resulting in more energy allocation for expression of stress response proteins. Stress response proteins, such as peroxiredoxin-2, may act to protect muscle cells from postmortem degradation. Peroxiredoxin-2 was identified as being a potential biomarker to differentiate tough pork. Both of these proteins were identified in the soluble protein fraction of pork, demonstrating no further extraction procedures would be needed to identify these proteins. This is extremely beneficial for the pork industry because the purge from meat can be utilized to identify these biomarkers. Next steps would include identification of rapid tests to measure these proteins in a production setting. Once the technology is available to differentiate between low and high quality pork, the possibility for a grading system or branded products could be utilized by the pork industry to provide consumers with consistently tender products.