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CHARACTERIZATION OF POSTMORTEM FACTORS REGULATING PORK COLOR, WATER HOLDING CAPACITY AND TENDERNESS

By

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ABSTRACT

CHARACTERIZATION OF POSTMORTEM FACTORS REGULATING PORK COLOR, WATER HOLDING CAPACITY AND TENDERNESS

By

Chuck P. Allison

The rate of biochemical reactions which occur in porcine skeletal muscle prior to, and immediately following harvest have a dramatic impact on both quality and quantity of pork produced. The objective of these studies was to quantify biochemical characteristics of porcine *longissimus dorsi* (LM) muscle and determine their relationship to pork color, water holding capacity and tenderness. *Longissimus dorsi* muscle samples were excised from carcasses at 20 min postmortem for quantification of phosphofructokinase and pyruvate kinase capacity. Phosphofructokinase and pyruvate kinase activities were not correlated with LM pH, purge, drip loss, or color ($P > .31$). Tenderness, measured by Warner-Bratzler shear (WBS), was optimized by aging loin chops for 7 d. Western blot analysis of desmin from 4 tender (<4 kg) and 4 less tender (>4.7 kg) samples revealed that desmin degradation paralleled decreases in WBS through postmortem storage. These data indicate that variation in pork color and water holding capacity is not associated with variation in glycolytic enzyme capacity. Additionally, tenderization of most pork loin chops is complete by d 7 and tenderization coincides with desmin degradation.

This thesis is dedicated to my parents who have always believed in me and given me the courage to pursue my dreams. This is also for Melissa, who has sacrificed closeness with her family so that I can further my education, and has been there with love and encouragement every step of the way.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP – Adenosine Diphosphate

AMP – Adensoine Monophosphate

AMPK – AMP-Activated Protein Kinase

ATP – Adenosine Triphosphate

BSA – Bovine Serum Albumin

CWHC – Centrifuge Water Holding Capacity

DFD – Dark, Firm and Dry

DRIP1 – Suspension Drip Measured from 24 to 48 h postmortem

DRIP6 – Drip loss measured under simulated retail display conditions day 6 to day 7 postmortem

F-6-P – Fructose-1,6-phosphate

L6 – Minolta (CIE) L* values taken at d 6 postmortem

L7 – Minolta (CIE) L* values taken at d 7 postmortem

LD – *Longissimus Dorsi*

LDH – Lactate Dehydrogenase

LM – Longissismus Muscle

MCE – Mercaptoethanol

MSU – Michigan State University

MW – Molecular Weight Standards

NADH – Nicotinamide Adenine Dinucleotide, reduced form

NFDM – Non-fat Dry Milk

PEP – Phosphoenol Pyruvate

PK – Pyruvate Kinase

PSE – Pale, Soft and Exudative

RN$^-$ - Rendement Napole

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TBS – Tris Buffered Saline

WBS – Warner Bratzler Shear value

INTRODUCTION

With the production of faster growing pigs that yield leaner, heavier muscled carcasses, pork quality problems have arisen. Selection procedures have been implemented by seedstock swine producers to eliminate sources of inferior pork quality in the last ten years. The removal of the halothane gene from many genetic nucleus herds led scientists to believe that the source of inferior quality pork had been eliminated. However, it has been estimated that 10-20% of pigs harvested today still produce pale, soft and exudative (PSE) pork that has unacceptable meat color, water-holding capacity and/or tenderness.

Much research has focused on the causes of PSE meat. It has been well documented that environmental stressors and high ambient temperatures or fluctuating daily temperatures increase the occurrence of PSE pork. It is generally accepted that a rapid postmortem pH decline, while muscle temperature remains high, is the cause of PSE in pork. However, research to date has been unable to conclusively establish the factors that are responsible for the conversion of porcine muscle to PSE meat.

The most immediate approach to reducing the occurrences of PSE pork is through selection towards halothane resistant breeding stock, careful management throughout the life of the animal and rapid chilling of the carcass. However, the ultimate goal is to establish a better understanding of the biochemical processes that are responsible for the variation observed in ultimate pork quality.

These studies were conducted to: 1) quantify the enzymatic capacity of regulated steps in glycolysis and determine their relationship to porcine *longissimus dorsi* muscle traits (pH decline, color and water-holding capacity); and 2) quantify the optimal tenderization time of porcine *longissimus dorsi* muscle and determine if degradation of desmin is associated with pork muscle tenderization.

Collectively, these data will provide a better understanding of biochemical mechanisms responsible for differences in pork quality. This knowledge could lead to manipulation of biochemical systems that lead to PSE pork muscle. Thus, allowing suppliers to produce and market animals possessing superior color, water holding capacity and tenderness.

LITERATURE REVIEW

Skeletal Muscle

Orcutt et al. (1990) demonstrated that skeletal muscle constitutes 37% of the live weight of pigs. However, skeletal muscle did not evolve for human consumption, but rather to provide a means of locomotion through carefully orchestrated contraction and relaxation cycles. The same processes that make locomotion possible are also responsible for attempting to maintain homeostasis in the muscle after exsanguination. Therefore, a basic understanding of how living tissue functions provides insight into the problems that may arise in the postmortem conversion of muscle to meat.

Skeletal Muscle Contraction

During skeletal muscle contraction, chemical energy, derived from the hydrolysis of ATP, is converted into mechanical force (Regnier et al., 1995). In adult, fast-twitch skeletal muscle, the signal for excitation-contraction coupling is transferred across the triad junction from transverse tubules to the terminal cisternae. This wave of depolarization causes ryanodine receptors to open and results in release of calcium from the sarcoplasmic reticulum (Coronado et al., 1994). Calcium diffuses from the terminal cisternae and binds to troponin molecules. When calcium is bound to troponin, it relieves the inhibition that tropomyosin exerts on crossbridge formation between actin and myosin. The activated troponin interacts with tropomyosin causing it to shift its position along the actin filament. The shift by tropomyosin allows the myosin head to attach to

the actin molecule and initiates a crossbridge cycle (Potter et al., 1995). Pate and Cooke (1989) suggested the following basic cycle occurred during skeletal muscle contraction after the actin-myosin crossbridge had formed. Inorganic phosphate is released from the myosin head and a power stroke results, in which the myosin head "pulls" the actin filament toward the m-line. The z-lines move closer together (sarcomere shortening) resulting in the force needed for locomotion. Simultaneously, ADP is released and ATP attaches to the myosin head causing dissociation of the actin-myosin complex. Subsequently, the hydrolysis of ATP to ADP and inorganic phosphate by myosin ATPase initiates a new cycle. When the stimulus for muscle contraction ceases, the sarcolemma and the transverse tubules become repolarized. The calcium ATPase pumps the calcium back into the sarcoplasmic reticulum. Additionally, tropomyosin regains its original position over the myosin-binding site. In the absence of an attachment between actin and myosin, tension is not generated and the stretching imposed by elastic components in the muscles cause the filaments to slide passively over one another. The muscle returns to the resting state.

White et al. (1964) demonstrated that ATP and its immediate source of replenishment, creatine phosphate, could be maintained by oxidative phosphorylation in slow working muscle. However, in rapidly contracting muscle, the oxygen supply becomes insufficient and ATP must be synthesized anaerobically via glycolysis.

Postmortem Changes in Skeletal Muscle

Once an animal has been exsanguinated muscle tissues are no longer capable of generating energy via aerobic methods. The muscle is therefore forced to switch to anaerobic processes to generate ATP. The biochemical reactions that occur after exsanguination are largely responsible for the ultimate quality of meat. The muscles of living pigs are moderately dark in color, firm in texture and dry in appearance (Briskey, 1964b). The magnitude of change in muscle characteristics from those of living muscle is a direct result of the rate and extent of postmortem anaerobic glycolysis, pH decline and temperature decline.

It is generally accepted that the net product of anaerobic glycolysis from glucose is 2 ATP, 2 lactate and 2 H^+. Thus, the postmortem demands for ATP and increased ATP production will result in more hydrogen ion accumulation and a reduced ultimate pH. The rate of glycolysis is determined by the utilization of energy in the cell. The major sites of ATP utilization are the myofibrillar (myosin) ATPase, calcium ATPase and sodium/potassium ATPase. Myosin ATPase catalyzes the hydrolysis of ATP to ADP and an inorganic phosphate generating the energy to drive the crossbridge cycle (Bechtel and Best, 1985). The amount of ATP hydrolyzed by myosin ATPase during a single muscle twitch is approximately .3 mmoles/l (Infante and Davies, 1962). The calcium ATPase functions to resequester calcium into the SR so that relaxation of skeletal muscle can occur. Bechtel and Best (1985) calculated the amount of ATP to resequester the calcium released during a single muscle twitch to be about .1 mmoles/l. The sodium/potassium pump functions to maintain the ion gradient of the cell or a net

negative charge on the inside of the cell. This is accomplished by pumping 3 sodium ions out of and 2 potassium ions into the cell for every ATP molecule hydrolyzed (Bechtel and Best, 1985).

The extent of glycolysis is related to the amount of glucose or glucose equivalents that are available to be processed through glycolysis and the stability of glycolytic enzymes. Monin et al. (1981) demonstrated that glycolytic potential, a measure of available substrates and the end product in anaerobic glucose metabolism, could be used as an indicator of the potential postmortem lactate formation and consequently H^+ accumulation. When muscle ATP stores become depleted, permanent actin-myosin crossbridges form and the muscle is said to be in rigor.

Forrest et al. (1963) noted that the ultimate gross morphology of porcine muscle ranged from pale, soft and exudative to dark, firm and dry in appearance. The patterns of pH decline (Briskey, 1964a), and/or the pH and temperature relationships in the muscle prior to the onset of rigor mortis are associated with the ultimate muscle classification (Sayre and Briskey, 1963). A brief description of each condition will follow.

Red, Firm and Non-exudative Pork

Many different criteria have been used to predict the conversion of muscle to red, firm and non-exudative pork (normal pork). Wismer-Pedersen and Briskey (1961) demonstrated that when glycolysis proceeds at an intermediate rate, which requires 6-12 hours until lactate production ceases, the muscle exhibits a grayish pink to red color, moderately firm structure and dry

appearance. In 1963, Sayre and Briskey reported that if the pH of the muscle is above 6.0 at 2 hours postmortem and temperature is below 35°C at the onset of rigor, the muscle would exhibit normal properties regardless of the ultimate pH. Additionally, they observed highly significant correlations between muscle protein solubility and water-holding capacity. Scopes and Lawrie (1963) demonstrated that as the normal ultimate pH of 5.5 is attained, certain sarcoplasmic proteins are already denatured. These data demonstrated that even under normal glycolytic conditions, there is a gradual drop in the solubility of sarcoplasmic proteins and a slight decrease in water-holding capacity of the meat.

The National Pork Producers Council's Pork Quality Solutions Team (1998) has developed targets for normal, firm and non-exudative fresh pork quality. These targets are to be used as ranges for pork *longissimus* traits measured at 24 hr postmortem. The solutions team suggests that ultimate pH range from 5.6 to 5.9 and color range from 3 to 5 visually or when measured objectively in the range of 49 to 37 (Minolta CIE L). Most importantly, they suggest that the drip loss not exceed 2.5%.

Pale, Soft and Exudative Pork

Pale, soft and exudative (PSE) pork is often associated with a rapid pH decline that results in low muscle pH in combination with high muscle temperature. Fujii et al. (1991) demonstrated that PSE pork is associated with a mutation in the ryanodine receptor. The mutation appears to result in a hypersensitive gating of the calcium release channel where channel opening is facilitated and closing is inhibited. Although it is currently possible to identify pigs

that are homozygous or heterozygous for this mutation, breeds and lines of pigs that do not possess this genetic defect still produce pork carcasses that exhibit PSE characteristics (Pommier and Houde, 1993). A survey of 14 packing plants by Kauffman et al. (1992), revealed that approximately 16% of pork produced exhibited PSE characteristics. Moreover, the Pork Chain Quality Audit (Cannon et al., 1996) and the National Pork Producers Council (1991), found that >10% of all pork carcasses generated in the United States contained PSE meat.

Pale, watery meat develops as a result of excitement of stress-sensitive pigs before the harvest process, which cause an increase in body temperature and an accelerated rate of postmortem anaerobic metabolism. This increase in glycolytic rate causes an increase in hydrogen ion accumulation in the muscle. The fact that PSE characteristics are hard to induce in pigs that are less susceptible to stress suggests that there is an innate difference among genetic lines that causes some pigs to have a greater predisposition to develop an abnormal pH decline (Gerrard, 1997).

Briskey et al. (1959) demonstrated that, although normal at death, muscles that were ultimately PSE had significantly lower pH values and higher muscle temperature at 40 min postmortem. Moreover, the pH values decreased rapidly to 5.5 or lower, while the temperature remained at 36-41°C. A rapid drop in pH accompanied by a high temperature results in the denaturation of approximately 20% of the sarcoplasmic and myofibrillar proteins (Honikel and Kim, 1986). This helps to explain the undesirable pale color and exudative characteristics associated with PSE muscle after harvest.

As postmortem muscle pH approaches the isoelectric point of 5.1, the net protein charge is reduced, as are repulsive forces between myofilaments (Wismer-Pedersen, 1971). This results in a decrease in the spacing observed between myofilaments. Thus, fresh meat with a lower ultimate pH will have less net protein charge, decreased myofilament spacing (i.e. less space for water to be trapped) and less interaction with water, which is a polar substance.

Honikel and Kim (1986) suggested that the wateriness observed in PSE muscle is determined by breaks in the cell membrane through which fluid can quickly exude from the cell. Additionally, they observed the myofibrillar protein myosin in the drip from PSE muscles and suggest that myosin would not be able to escape the cell if the membranes were intact. According to Offer (1991), denaturation of sarcoplasmic proteins has a major influence on the increase in paleness, while denaturation of the myofibrillar proteins is responsible for the decreases in water-holding capacity. Warriss and Brown (1987) reported a biphasic relationship between 45-minute postmortem pH, reflectance and drip loss. These authors demonstrated that muscles with a lower 45-minute pH had more drip loss and were paler.

Potentially, the most important aspect of PSE pork is the reduced water-holding capacity of the meat. The inability of meat to bind water affects packer profitability, functionality and versatility of the product and most importantly consumer acceptability (Topel et al., 1976). Kauffman et al. (1978) demonstrated that during transit PSE hams lost three times more moisture than normal hams and seven times more moisture than dark, firm and dry hams (DFD).

Additionally, moisture losses during curing, smoking and chilling are higher in PSE hams than those observed in normal or DFD hams. Compared to normal hams and shoulders, hams and shoulders with severe PSE exhibit 33% and 11% lower water-holding capacities, respectively (Cannon et al., 1995).

Dark, Firm and Dry Pork

Briskey et al. (1959) demonstrated that if there was a very limited pH decline and rigor mortis took place at a relatively high pH, the muscle remained dark red in color, firm in texture and dry in appearance. He also noted that if glycolysis proceeded extremely slowly and rigor mortis occurred over a long period of time, the same ultimate muscle quality would occur.

Dark, firm and dry pork is often associated with long-term stress that results in depletion of muscle glycogen prior to harvest. As a result, there is less glycogen to fuel anaerobic glycolysis, less lactate and hydrogen ion accumulation and a higher ultimate pH (>6.0). Because of this higher ultimate pH, proteins in DFD meat have a relatively high net protein charge and undergo minimal protein denaturation. Both of these factors contribute to an increase in myofilament spacing (Wismer-Pedersen, 1971), higher water holding capacity and minimal extracellular fluid accumulation (Kauffman et al., 1994).

Kauffman et al. (1999) subjected eight pork loins representing DFD and PSE conditions to a series of objective and subjective measures to demonstrate extremes in meat quality. When compared with PSE loins, DFD loins averaged 1.5 units higher ultimate pH, 4.7% less drip loss and 136% more bound water. Additionally, DFD loins were darker, firmer and more tender.

With all the positive factors associated with DFD pork (i.e. increased water holding capacity, decreased light reflectance and decreased protein denaturation) it would seem that it would be the target of pork producers. However, there are negatives associated with DFD meat that make it undesirable. Many of the bacterial species that grow on meat survive within a pH range of 5.0-8.0 with optimum growth occurring around pH 7.0 (Cannon et al., 1995). This makes DFD meat a prime target for bacterial growth. Additionally, the dark appearance of DFD meat does not appeal to the average American consumer (Kauffman, 1993).

Control of Glycolytic Metabolism

The rate of postmortem glycolysis plays a major role in the ultimate quality of postmortem tissue. White et al. (1964) and Conn and Stumpf (1966), indicated that the rate of each enzyme-catalyzed reaction is related to the concentration of active enzyme, the availability of substrates, coenzymes and cofactors, the presence of activators or inhibitors, and the temperature and pH conditions. Scrutton and Utter (1968), stated that availability of substrate or regulation of catalytic activities of rate limiting enzymes, or both, are probably the major factors contributing to regulation of glycolytic flux. Two controlled steps in glycolysis, catalyzed by phosphofructokinase and pyruvate kinase, as well as antemortem and postmortem factors will be discussed in terms of their regulation or influence on the ultimate quality of meat.

Phosphofructokinase

Phosphofructokinase (PFK) catalyzes the conversion of fructose-6-phosphate (F-6-P) to fructose-1, 6-phosphate and utilizes one ATP. The implication that phosphorylation of F-6-P was a rate controlling step in glycolysis was first made by Passonneau and Lowry (1962). Mansour and Mansour (1962), demonstrated that PFK limited the rate of glycolysis in liver flukes. These findings led Sayre et al. (1963) to investigate PFK activity of porcine muscle extracts in relation to PSE development of the intact muscle. Activity of PFK was not associated with the rate of postmortem glycolysis or with the physical properties of the muscle. This would seem to exclude PFK as a contributing factor to the development of PSE muscle. However, Crabtree and Newsholme (1972) demonstrated that differences in PFK activity among muscles of different species reflect demonstrable differences in capacities for glycolytic flux. Glycolytic flux refers to the rate of flow of intermediates through a metabolic pathway.

Fiber-type associated differences in glycolytic flux capacity, in addition to differences in ATPase activities among fiber types, have long been associated with species differences in rate of postmortem pH decline. Connett and Sahlin (1996) concluded that type II fibers would be expected to have increased fructose-6-phosphate, which would lead to a slightly greater fraction of PFK being used at any given state of the phosphate energy system. The absolute rate of glycolytic flux will depend on the amount of enzyme present in that fiber. Additionally, PFK activity *in vivo* has been shown to be influenced by a variety of

cellular regulators (Mansour, 1963). Among these are substrate availability, allosteric control, phosphorylation and dephosphorylation and product accumulation.

Pyruvate Kinase

Pyruvate kinase (PK) catalyzes the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP. In a comprehensive study of PK by Schwägele et al. (1996), skeletal muscle PK from halothane sensitive and normal pigs was purified. These researchers found that the enzyme from both sources had an apparent molecular weight of 52 kD and existed as a tetramer in solution. Although no modifications were observed in the subunit size or the structure of the enzyme, differences were found in the activity of the isolated enzyme. Schwägele et al. (1996) found that skeletal muscle PK isolated from halothane sensitive pigs had four-fold more specific activity as compared to the enzyme isolated from normal muscle. More importantly, they found a striking difference in the pH dependency of PK from the two sources of animals. Pyruvate kinase isolated from halothane sensitive animals retained 70% of its activity when assayed at pH 5.5, whereas normal muscle retained less than 10% of its activity. Using isoelectric focusing on polyacrylamide gels, they demonstrated that normal muscle has two isoforms of PK, whereas a third isoform is present in PSE muscle. They further demonstrated that the third isoform is highly phosphorylated and is most likely responsible for the increase observed in specific activity, reduced K_m for phosphoenolpyruvate and a shift in the pH dependency of PK. The later results in PK being active under the acidic

conditions that rapidly occur in PSE muscle. This may allow the continued rapid accumulation of lactate and hydrogen ions in PSE muscle under conditions that would result in slow glycolysis in muscles of normal animals. Existence of this isoform was not determined in living muscle, and it is not known whether this phosphorylated PK isoform is a causative factor or an artifact of the rapid glycolysis in PSE muscle. It is currently unknown if similar regulation of PK occurs in halothane negative animals that subsequently produce PSE pork.

Genetic Predisposition

The genetic makeup of a pig can have a significant influence on the ultimate quality of the meat. Two genetic mutations that have received considerable attention are the halothane gene and the Rendement Napole gene (RN^-). Both of these mutations have resulted in deleterious effects on meat quality.

Halothane Gene

It became clear in the 1960's that predisposition of pigs to produce PSE pork had a marked genetic component. Danish and American scientists discovered that certain breeds contained a large portion of animals that were prone to produce PSE pork (Briskey, 1964b). Moreover, they found a close association between animals that produced PSE pork and animals that had porcine stress syndrome (Briskey, 1964b; Topel et al.,1969; Judge, 1972). Eikelenboom and Minkema (1974) demonstrated that porcine stress could be triggered by the anesthetic gas halothane.

The halothane mutation has been reported to involve an arginine to cysteine transition in the SR Ca^{++} release channel (ryanodine receptor) protein at residue 615 (Fujii et al., 1991). It has been determined that the mutation results in a hypersensitive calcium release channel, which results in elevated sarcoplasmic Ca^{++} and increased energy consumption due to crossbridge cycling (myosin ATPase) and calcium ATPase. The calcium ATPase pump works more chronically to resequester the excess calcium back to the sarcoplasmic reticulum lumen. Thus, increased myosin ATPase and Ca^{++}-ATPase activity leads to elevated muscle temperature and accelerated glycolytic metabolism, which often results in the development of PSE meat.

Pigs that express the halothane gene have been used for their ability to enhance carcass muscling. However, reduced yields associated with increased water loss negate improvements in leanness and mass of carcasses. Fresh and processed meat from halothane-positive animals has been shown to have undesirable cooking and eating characteristics compared to that of halothane negative animals (Boles et al., 1991).

In a study by Pommier and Houde (1993), 913 boneless pork loins were selected in a commercial cutting operation. The loins were selected by a trained grader to obtain a representative sample of PSE meat (CIE $L \geq 53.5$) and a normally colored control group. The genotype with respect to malignant hyperthermia was assessed using a restriction endonuclease assay. These authors conceded that the results from this study clearly illustrate the harmful effects of the halothane gene in a commercial operation, but added that

preharvest management practices may be more important than elimination of animals that posses the halothane gene. Moreover, they suggested that there are other genetic predispositions that ultimately influence meat quality that have not yet been discovered.

Napole Gene

The Hampshire breed has received considerable attention with respect to the pale color of lean tissue and decreased product yield. Some Hampshire pigs possess high antemortem levels of muscle glycogen and their muscle undergoes extended postmortem glycolysis, resulting in a low ultimate pH and a pale color (Sayre et al., 1963). The corresponding muscle was later termed acid meat and the gene was referred to as the Rendement Napole gene (Sellier and Monin, 1994). Le Roy et al. (1990) showed that napole yield was 8% higher in normal pigs than napole gene carriers. Napole yield is measured as the curing and cooking yield of a 100g sample of *semimembranosus* muscle and is used as an indicator of technological yield. The mediocre meat quality observed in the Hampshire breed cannot be attributed to halothane sensitivity. It is well established that the incidence of the halothane gene is essentially zero for the breed (Webb et al., 1982). Sayre et al. (1963) reported two to three times higher glycogen levels at harvest in Hampshire-sired pigs versus Chester White and Poland China breeds. Although the rate of muscle pH decline is similar to that of pigs with normal meat quality, the ultimate pH is lower than normal.

Until recently the identity of the RN^- gene was unknown. Milan et al. (2000) determined that a mutation in AMP-activated protein kinase (AMPK)

resulted in abnormal metabolism of glycogen. When active, AMPK is expected to inhibit glycogen synthesis and stimulate glycogen degradation. Therefore, the AMPK mutation in pigs results in elevated muscle glycogen stores and leads to increased postmortem lactate and hydrogen ion accumulation, resulting in a low ultimate pH. High levels of glycogen alone do not fully explain the phenomenon of an extended glycolysis resulting in meat with a low ultimate pH and PSE conditions (Monin and Sellier, 1985). The Hampshire pigs used by Monin and Sellier (1985) exhibited a lower ultimate pH that was not associated with a higher lactate content nor with differences in buffering capacity. Additionally, other factors, such as enzyme stability at lower pH, must be a key player in the continuation of glycolytic metabolism in these animals. Thus, it is imperative to investigate factors regulating the cessation of glycolysis in systems that glycogen is not limiting.

Harvest Procedures

The stress that is placed on an animal prior to harvest and the events that occur following exsanguination directly influence meat quality. Various production methods have been implemented to either reduce stress or alter metabolism of the animal prior to harvest. Grandin (1994) recommended 2-4 h rest period after arrival at the abattoir to allow the animal's metabolism to return to resting levels. Holding pens, loading chutes, restrainers and alleyways leading to the final drive should enhance the natural movement of pigs to minimize pre-harvest stress.

It is important to remember that the main goal in harvesting pigs is to be as consistent and time efficient as possible. The quicker the heat can be dissipated from the carcass, while preventing cold shortening, the more likely it will result in product of acceptable quality. Heat can be removed faster by minimizing the stun to stick and stun to chill times, and by increasing the intensity and duration of chilling (NPPC, 2000).

Pork Tenderness

Historically, pork *longissimus* muscle has been considered to be relatively tender (DeVol et al., 1988). However, with single trait selection for increased lean, unfavorable changes have occurred in pork color and water holding capacity and there is growing concern adverse effects on tenderness may have also occured. Cameron (1990) found that single trait selection for increased carcass lean resulted in less tasty, less juicy and less tender pork.

Determinants of Meat Tenderness

The relationship between sarcomere shortening and rigor-associated toughening has been reported for lamb muscle by Wheeler and Koohmaraie (1994). As muscle enters rigor, permanent actin-myosin crossbridges form. By 24 h postmortem, sarcomere length will be inversely related shear force values. However, with postmortem storage substantial improvement in tenderness can be observed with minimal increase in sarcomere length. The increase in sarcomere length has been shown to be somewhat of an artifact due to the methods used to measure this length (Gann and Merkel, 1977).

Using electron micrographs, Gann and Merkel demonstrated that the region where there is myosin-actin interaction stays the same through postmortem storage, while there is separation adjacent to z-discs.

Conflicting reports exist with regard to the main determinants of pork tenderness. Wheeler et al. (2000) concluded that the main determinants in *longissimus dorsi* tenderness are connective tissue, sarcomere length and desmin degradation at d 1 postmortem. However, van Laack and coworkers (2001) concluded that connective tissue, intramuscular fat content and myofibrillar structure were the main controllers in tenderness.

Pigs are slaughtered at approximately 6 months of age and the immature connective tissue does not significantly influence pork tenderness (Avery et al., 1996). It is generally accepted that intramuscular fat content is responsible for approximately 10-12% of the variation observed in ultimate tenderness. In contrast, van Laack and coworkers (2001) demonstrated that in Duroc pigs intramuscular fat accounted for 47% of the variation in Warner-Bratzler shear values. Additionally, they found no relationship between intramuscular fat content and Warner-Bratzler shear values for Berkshire and Hampshire pigs.

Extensive research has been conducted to quantify the relationship between sarcomere length and pork tenderness. In a random sample of 120 pork carcasses from a commercial harvest facility, DeVol et al. (1988) reported that in *longissimus dorsi*, sarcomere length averaged 1.83 μm and ranged from 1.66 to 2.00 μm. In this study, sarcomere length accounted for 7% of the variation observed in WBS values. Wheeler et al. (2000), suggested that if

sarcomere length could be extended to at least 2.0 μm, the *longissimus* muscle would be tender regardless of collagen content or proteolysis. The inconsistent effects of increasing sarcomere length on shear force values indicates the high degree of interaction between sarcomere length, proteolysis of specific muscle proteins and connective tissue on tenderness differences (Hostetler et al., 1972). Wheeler and Koohmaraie (1999) demonstrated that sarcomere length does not affect the extent of proteolysis in lamb muscle.

Causes of Postmortem Tenderization

Several studies have focused on the causative factors that influence postmortem tenderization. The improvement in meat tenderness during postmortem storage at refrigerated temperatures has been known since the turn of the 20th century. In 1907 Lehmann reported that there was a 30% increase in tenderness of meat stored for 8 d postmortem (reviewed by Penny, 1980). Although this improvement can be measured both subjectively and objectively, the mechanisms responsible for postmortem tenderization are not completely understood.

There is substantial evidence that suggests proteolysis of key myofibrillar proteins is responsible for meat tenderization and improvements in tenderness are mediated by the calpain system (Koohmaraie, 1988, 1992a, 1994). Guroff (1964) was the first to document the existence of a calcium-and sulfhydryl-dependent proteinase from rat brain. Meyer et al. (1964) reported the existence of a similar proteinase in skeletal muscle and it was later purified from porcine skeletal muscle by Dayton et al. (1976 a,b).

The calpain system consists of μ-calpain (the form of the proteinase active at micromolar calcium concentrations), m-calpain (the form of the proteinase active at millimolar calcium concentrations) and calpastatin (a protein that specifically inhibits both forms of calpain at their respective calcium concentrations required for activation; Koohmaraie, 1992b). Based on the results of numerous experiments, it can be concluded that proteolysis of key myofibrillar proteins by μ-calpain is the underlying mechanism of meat tenderization that occurs during postmortem storage at refrigerated temperatures (Koohmaraie, 1996).

The main proteins that have been implicated in postmortem tenderization include the inter- (e.g., desmin and vinculin) and intra-myofibril (e.g., titin, nebulin and troponin-T) linkages. The function of these proteins is to maintain the structural integrity of myofibrils (Price, 1991). Thus, the degradation of these proteins would cause weakening of myofibrils and result in tenderization. However, phosphorylation of myofibrillar proteins has been shown to reduce degradation of these proteins by calpains (Di Lisa et al., 1995; Zhang et al., 1998). Phosphorylation of calpastatin (endogenous calpain inhibitor) renders it a more effective inhibitor of m-calpain than unphosphorylated calpastatin (Salamino et al., 1994). The relationship between proteolysis of key muscle specific proteins and pork tenderness is unknown. Moreover, the mechanism(s) responsible for controlling the rate of postmortem tenderization is currently unresolved.

CHAPTER 1

PORK QUALITY VARIATION IS NOT EXPLAINED BY GLYCOLYTIC ENZYME CAPACITY

ABSTRACT

My objective was to determine if increased glycolytic enzyme capacity is associated with rapid postmortem pH decline that leads to inferior pork color and water-holding capacity. Duroc (n=16) or HAL-1843 free Pietrain (n=16) sired gilts were harvested within a two-week period. Temperature of the *longissimus* muscle (LM) was logged continuously from 45 min to 22 h postmortem at 5 min intervals and LM pH was measured at 20, 45, 180 min and 22 h postmortem. Temperature of LM at 45 min postmortem was negatively correlated with 45 min pH ($P < .05$). *Longissimus* muscle L* values for chops at 24 h postmortem ranged from 49.6 to 60.2. Fluid loss (Purge) ranged from .79 to 9.91% in vacuum packaged loin sections stored at 4°C from d 1 to d 6 postmortem. After purge determination, two 2.5-cm-thick loin chops were cut and allowed to drip in a simulated retail case at 4°C overnight. Drip loss ranged from .3-1.8%. All measures of fluid loss were correlated to all L* values (d 1, 2, 6 and 7 postmortem; $P < .01$). Phosphofructokinase and pyruvate kinase activities in LM sarcoplasmic fractions were quantified using coupled enzyme assays. Phosphofructokinase and pyruvate kinase activities were not correlated with LM pH, purge, drip loss, or color ($P > .31$). These data indicate that variation in pork color and water holding capacity is not associated with variation in the capacity of enzymes to catalyze regulated steps of glycolysis.

INTRODUCTION

Pigs that are homozygous HAL-1843 positive and heterozygous carriers have been used for their ability to increase the amount of lean tissue in a carcass. However, expression of this mutated gene (ryanodine receptor, Ca^{++} release channel) also increases the risk of developing pale, soft and exudative (PSE) meat (Monin et al., 1981). Although it is currently possible to identify pigs that are HAL-1843 homozygous and carriers, breeds and lines of pigs that do not possess this genetic defect still produce pork carcasses that exhibit PSE characteristics (Pommier and Houde, 1993). The biochemical mechanisms that contribute to PSE pork are not completely understood. However, it is known that the rate and extent of pH decline influence meat color and water-holding capacity. Rate and extent of pH decline in muscle may be determined by the rate of glycolysis, buffering capacity, and/or glycogen storage (Milan et al., 2000).

Bendall (1973) and Warriss et al. (1989) demonstrated that characteristics of postmortem pH decline are determined by the physiological state of muscle at the time of stunning. Antemortem stress and anaerobic postmortem conditions lead to accumulation of muscle H^+ via glycolysis. Rapid postmortem glycolysis causes a rapid pH decline while muscle temperature is relatively high. This leads to denaturation of muscle proteins and a subsequent loss of water-binding capacity (Briskey, 1964b). Meat that has undergone a rapid postmortem pH decline is generally paler in color and more exudative than meat that has undergone a gradual pH decline.

Two major regulatory steps in glycolysis are those catalyzed by the enzymes phosphofructokinase (PFK) and pyruvate kinase (PK). Sayre et al. (1963) reported that PFK and phosphorylase activity *in vitro* were not associated with rate of pH decline in *longissismus dorsi* muscles of Hampshire, Poland China and Chester White pigs. However, Schwägele et al. (1996) demonstrated that muscle from halothane sensitive pigs had four times more total PK activity than control pigs. Additionally, Schwägele et al. (1996) showed that PK purified from muscle of halothane sensitive pigs lost only 30% of its PK activity, compared to a loss of >90% activity of PK purified from control pig muscle, when PK was assayed at pH 5.5 rather than pH 7.0. These authors demonstrated that PK in LM of halothane sensitive pigs was more active and less pH labile due to phosphorylation of the enzyme. It is unknown if similar regulation of glycolysis contributes to PSE meat in HAL-1843 free pigs. Thus, my hypothesis is that increased capacity of rate-limiting glycolytic enzymes contributes to rapid glycolysis and leads to inferior pork color and water-holding capacity.

MATERIALS AND METHODS

Animals and meat quality data collection

Sires from Duroc and HAL-1843 free Pietrain lines were used to inseminate Yorkshire and F_1 Yorkshire-Landrace gilts. Progeny (n=32) were raised in uniform conditions at the Michigan State University (MSU) Swine Teaching and Research Farm. Four Duroc and four HAL-1843 free Pietrain sired gilts were harvested on each of four days within a two-week period at the MSU Meat Laboratory. All procedures were performed in accordance with guidelines

Table 1.1. Timeline for meat quality measurements.

	TIME POSTMORTEM						
MEASUREMENTS	**20MIN**	**45MIN**	**180MIN**	**DAY 1**	**DAY 2**	**DAY 6**	**DAY 7**
pH	pH20min	pH45min	pH180min	pH22h			
TEMP	20 min	45min	——//	——22h			
COLOR				L1	L2	L6	L7
DRIP LOSS				DRIP1		DRIP6	
CWHC				XX			
PURGE				X	——//		——X

set forth by the MSU Animal Use and Care Committee.

A time line for data collection is shown in Table 1.1. Loin muscle area, 10th and last rib backfat thickness, and subjective color, firmness and marbling were determined according to current NPPC guidelines (NPPC, 2000) after chilling the carcasses for 22 h at ~2°C. Temperature of the *longissimus* muscle (LM) adjacent to the last rib was measured at 20, 45, 180 min and 22 h postmortem using a hand held temperature probe. From 45 min to 22 h postmortem, temperature was logged every five min using DeltaTRAK FlashLink Dataloggers (Pleasanton, CA). *Longissimus* muscle pH was measured at 20, 45, 180 min and 22 h postmortem with a portable pH meter equipped with a puncture-type combination pH electrode (Model 1140, Mettler-Toledo, Woburn, MA). At these same times postmortem, samples were also obtained for subsequent measurement of pH using the iodoacetate method (Bendall, 1973). Initial samples (20 min postmortem) were taken midway between the last rib and the cranial edge of the ilium on the left side of the carcass. Subsequent LM samples (45 min, 180 min and 22 h) were taken approximately one inch cranial to the previous sampling site. *Longissimus* muscle samples were cut into 0.5 cm^3 pieces, frozen in liquid nitrogen, and stored at -80°C. At 24 h postmortem, a section of loin was removed from the right side of the carcass between the 11th rib and the last lumbar vertebra. From the cranial edge of the loin section, duplicate 10 g samples were obtained and used to determine water-holding capacity by high-speed centrifugation (CWHC; 40,000 x **g** for 30 min) at 24 h postmortem (Honikel and Hamm, 1994). Two 2.5 cm thick loin chops were

removed from between the 12th and last rib of the section. These chops were used to determine color (15 min bloom time; CIELab L*, a*, b*, D65, 2° standard observer and 50 mm orifice) at 24 (L1) and 48 h (L2) postmortem with a Minolta chromameter (CR-310; Ramsey, NJ). Drip loss was determined by suspending chops from 24 to 48 h postmortem at 4°C (DRIP1; Honikel and Hamm, 1994). The remaining sections were vacuum packaged at 24 h postmortem in Cryovac shrink bags using a Multivac machine (Koch, Type AG 800) and stored at 4°C until d 6 postmortem. The difference between d 6 and 24 h loin weight was divided by 24 h weight and expressed as purge. After determination of purge, two 2.5-cm-thick loin chops were cut from the cranial end of the loin section and allowed to bloom for 15 min before color determination (L6). Chops were allowed to drip for 24 h at 4°C on styrofoam trays with a moisture-impermeable overwrap to simulate retail conditions. Drip loss (DRIP6) and color (L7) were recorded on d 7 postmortem.

Sample Preparation for Enzyme Assay

Longissimus muscle samples used to determine glycolytic enzyme capacity were obtained at 20 min postmortem as described above. Chemicals used in muscle extraction and enzyme assays were purchased from Sigma Chemical Company (St. Louis, MO). Frozen LM samples (1 g) were homogenized in 10 volumes of ice-cold extraction buffer (75 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EGTA, pH 7.0, containing 50 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, and 6 mg/l of leupeptin). Homogenization was performed on ice using a Polytron (Brinkman, Westbury, NY) for 2 x 20 sec

bursts (setting 4). The homogenate was fractionated into sarcoplasmic and myofibrillar components by centrifugation at 10,000 x **g** for 15 minutes. The supernatant fluid was saved and the pellet was resuspended in 10 ml of extraction buffer and centrifuged again at 10,000 x **g** for 15 minutes. The two supernatant fluids were combined. Protein concentration of supernatant fluid was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). In preliminary experiments, I found that the sarcoplasmic fraction (supernatant) contained > 95 % of the PK and PFK activity (Table 1.2). Thus, I used the sarcoplasmic fraction for quantification of PK and PFK activity.

Enzyme Assay

Pyruvate kinase activity was measured using a coupled enzyme assay described by Schwägele et al. (1996). The assay was adapted so that it could be performed in a 96-well microtiter plate. The total reaction volume was 210 µl, which consisted of 3 mM magnesium acetate, 50 mM KCl, 0.2 mM EDTA, 0.2 mg/ml bovine serum albumin (BSA), 30 mM imidazole (pH 7.0), 1mM adenosine diphosphate (ADP), 0.7 mM phosphoenolpyruvate (PEP), 0.1 mM nicotinamide adenine dinucleotide, reduced form (NADH), 2 U/ml lactate dehydrogenase (LDH) and 10 µl of a 1:10 dilution of sarcoplasmic fraction. Activity was measured by following the oxidation of NADH to NAD^+ at 340 nm on a VERSAmax plate reader (Molecular Devices, Sunnyvale, CA) at 22° C. The assay was linear from 0 to 50 s. Assays performed in the absence of substrate (PEP) contained less than 1% of sample activity, demonstrating that conversion of NADH to NAD^+ was coupled to PK activity. Pyruvate kinase activity was

Table 1.2. V_{max} values (μmol/min/ml), standard deviations and ranges for enzyme capacity by fraction (n=6). All fractions are diluted to be comparable to the crude homogenate.

TRAIT	MEAN	SD	RANGE
Pyruvate Kinase			
Crude homogenate	252.97	13.11	233.80-274.4
Supernatant	289.98	15.45	271.20-310.95
Washed Pellet	11.62	4.36	5.88-16.56
Phosphofructokinase			
Crude homogenate	147.25	22.46	115.45-179.80
Supernatant	142.92	9.00	134.60-159.05
Washed Pellet	8.92	5.03	3.16-17.46

calculated from the V_{max} that was determined from the SOFTmax PRO software (Molecular Devices, Sunnyvale, CA) and expressed as μmole/min·mg^{-1} protein. Samples were simultaneously assayed in triplicate and the intra-assay coefficient of variation was <4%. To approximate activity at ultimate postmortem pH this assay was also repeated with the pH of the buffer lowered to 5.5 with HCl.

Phosphofructokinase was quantified using a coupled enzyme assay described by Scopes (1977). This reaction was performed in a 96-well microtiter plate that contained a total reaction volume of 210 μl of 3 mM magnesium acetate, 50 mM KCl, 0.2 mM EDTA, 0.2 mg/ml BSA, 30 mM Tris base (pH 8.0), 1 mM fructose-6-phosphate (F-6-P), 0.2 mM NADH, 1 mM adenosine triphosphate (ATP), 0.2 mM PEP, 2 units each of PK and LDH/ml and 10 μl of a 1:4 dilution of sarcoplasmic fraction. The assay was performed at 22°C and was linear from 0 to 180 s. Assays performed in the absence of substrate (F-6-P) contained approximately 27% of the total activity. This could be attributable to F-6-P, ADP or ATPases present in the sample. Therefore, negative control assays with no F-6-P were performed for each sample. The V_{max} from control assays were subtracted from the V_{max} for assays performed with F-6-P. The total activity is expressed as μmole/min·mg^{-1} protein. Samples were simultaneously assayed in triplicate and the intra-assay coefficient of variation was <2%.

In vitro Phosphorylation of Pyruvate Kinase

An attempt was made to phosphorylate PK in crude supernatant fluid using the procedure outlined by Schwägele et al. (1996) for purified PK. Briefly, the phosphorylation reaction was performed in 200 μl of 5 mM Bis-Tris, pH 6.5,

50 μM ATP, 5mM magnesium acetate, 20 mM KCl containing 200 μg equivalent of protein and 4 units of protein kinase. Prior to use, 1 mg of cAMP-dependent protein kinase A was reconstituted in 200 μl of dithiothreitol (50 mg/ml) and allowed to incubate at room temperature for 10 minutes. The samples were incubated overnight at 30° C in a water bath. The enzymatic activity was quantified by assaying the original sample and the phosphorylated sample simultaneously as described above. I was unable to show an increase in the enzyme capacity of PK after *in vitro* phosphorylation. V_{max} values measured at pH 5.5, following phosphorylation showed a >88% reduction in specific activity when compared to its non-phosphorylated counterpart (data not shown). No attempt was made to verify if PK was phosphorylated with this method. It is possible that other sarcoplasmic proteins were preferentially phosphorylated, instead of PK, under the conditions described.

Data Analysis

Data were analyzed using a mixed model that contained the random main effect of harvest date and the fixed main effect of genetic line. Least squares means by sire line of meat quality traits were compared using a protected least significant difference test (Freud and Wilson, 1997). Pooled within-class correlation coefficients were calculated for meat quality data with enzyme capacity by ordinary least squares analysis of variance for a randomized complete block design.

RESULTS AND DISCUSSION

The lines for this study were selected based on previous research demonstrating a predisposition to produce progeny with differences in postmortem metabolism, pork color and water holding capacity. It has long been known that HAL-1843 positive pigs are prone to produce PSE meat. Schwägele et al. (1996) demonstrated that PK activity is higher in halothane sensitive pigs and that increased PK activity is due to enzyme phosphorylation. These authors suggested that elevated PK activity and greater enzyme stability under acidic conditions contributed to the incidence of PSE in these animals. The current study was designed to determine if capacity of PK and PFK explains variation in meat quality of pigs that are HAL-1843 free.

Pietrain-sired hogs produced lighter weight carcasses with larger loin muscle areas than Duroc-sired hogs at a similar age (Table 1.3). No differences in pH, color, water-holding capacity and glycolytic enzyme activity were found between sire groups ($P > 0.06$). Therefore, meat quality data and their relationship to glycolytic enzyme activity are presented and discussed collectively for all pigs.

The portable pH meter and the iodoacetate method of measuring pH produced similar results ($r = 0.83$). Additionally, similar correlations existed between meat quality traits and pH values obtained by either method (data not shown). Therefore, only pH values obtained using the portable pH meter are reported. The iodoacetate method has been shown to produce unavoidable alkalinization that leads to pH values approximately 0.1 pH unit higher than the

Table 1.3. Least square means and ranges for carcass measurements

				BACKFAT			
SIRE LINE	**n**	**LIVE WT (kg)****	**HCW (kg)****	**10TH RIB (mm)**	**LAST RIB (mm)**	**LEA (cm^2)*****	**LENGTH (cm)****
Duroc	16	134	103	19.8	30.7	51.5	86.2
	RANGE	*122-142*	*91-108*	*12.7-25.4*	*17.8-40.6*	*41.5-60.6*	*83.2-91.2*
Pietrain	16	128	98	16.5	27.4	61.5	84.0
	RANGE	*115-141*	*86-101*	*10.2-25.4*	*15.2-38.1*	*52.9-73.2*	*79.2-86.9*

** Least square means differ, P<.01

*** Least square means differ, P<.001

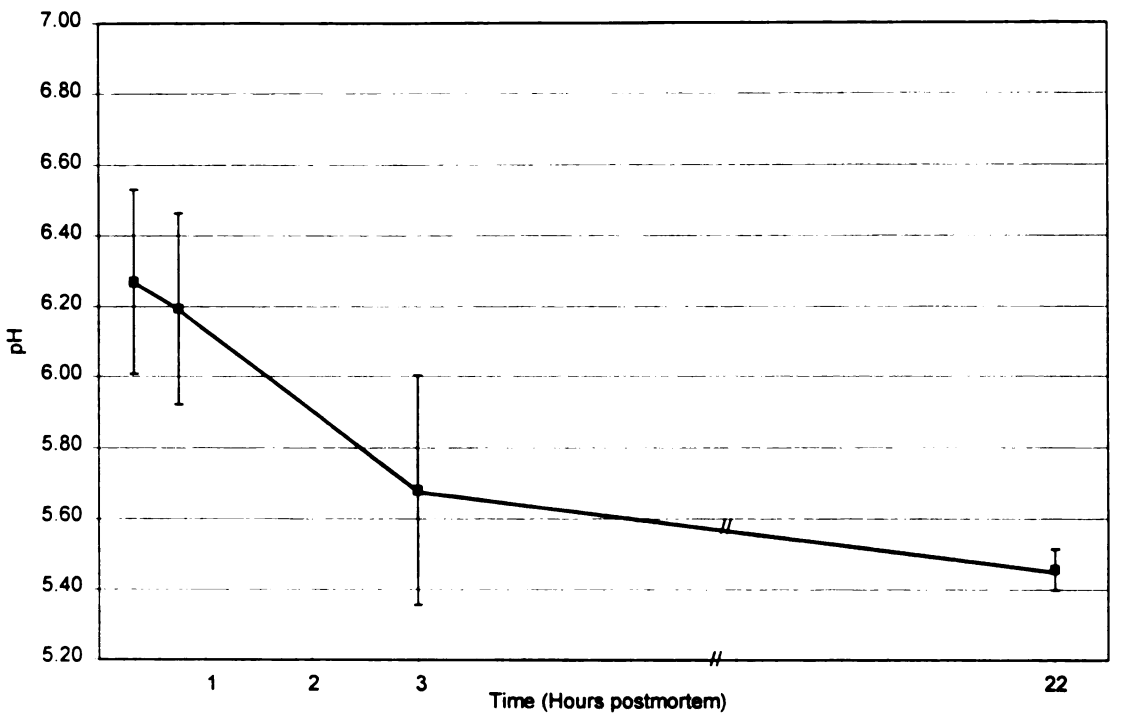


Figure 1.1: *Longissimus* muscle pH decline. Measurements were taken with a portable pH meter and puncture type probe adjacent to the last rib. Vertical lines at data points represent means ± S.D.

actual pH (Bendall, 1973; Dutson, 1983). I also found that pH values obtained using the iodoacetate method were approximately 0.1pH unit higher than those obtained with a portable pH meter.

Figure 1.1 shows the rate and extent of postmortem pH decline. Relatively large standard deviations associated with mean pH values within the first 3 h postmortem reflect differences in the rate of pH decline among *longissimus* muscles. The range in LM pH was 5.8 to 6.7, 5.6 to 6.6 and 5.3 to 6.4 at 20 min, 45 min and 3 h postmortem, respectively. In this study, differences in ultimate pH (22 h postmortem) were relatively small (Figure 1.1; range 5.4 to 5.6).

Figure 1.2 depicts the temperature decline in LM measured with a portable temperature probe at 20 min, 45 min, 3 h and 22 h postmortem, and measured continuously from 45 min postmortem to 22 h postmortem. Although the temperature decline curves among carcasses are generally parallel, differences in initial muscle temperature exist. Differences in muscle temperature at 45 min postmortem may result from variation in initial body temperature, heat produced during the conversion of glycogen to lactic acid and/or the hydrolysis of creatine phosphate and ATP (Bendall, 1973). I observed an inverse relationship between temperature at 45 min postmortem and pH at 45 min postmortem (Table 1.4). This relationship has also been reported by McCaw et al. (1997).

Several measures of color and water-holding capacity were made. Day 1 and d 2 measures were performed on different chops than d 6 and d 7. However, the four chops were within approximately a 13 cm region of the same

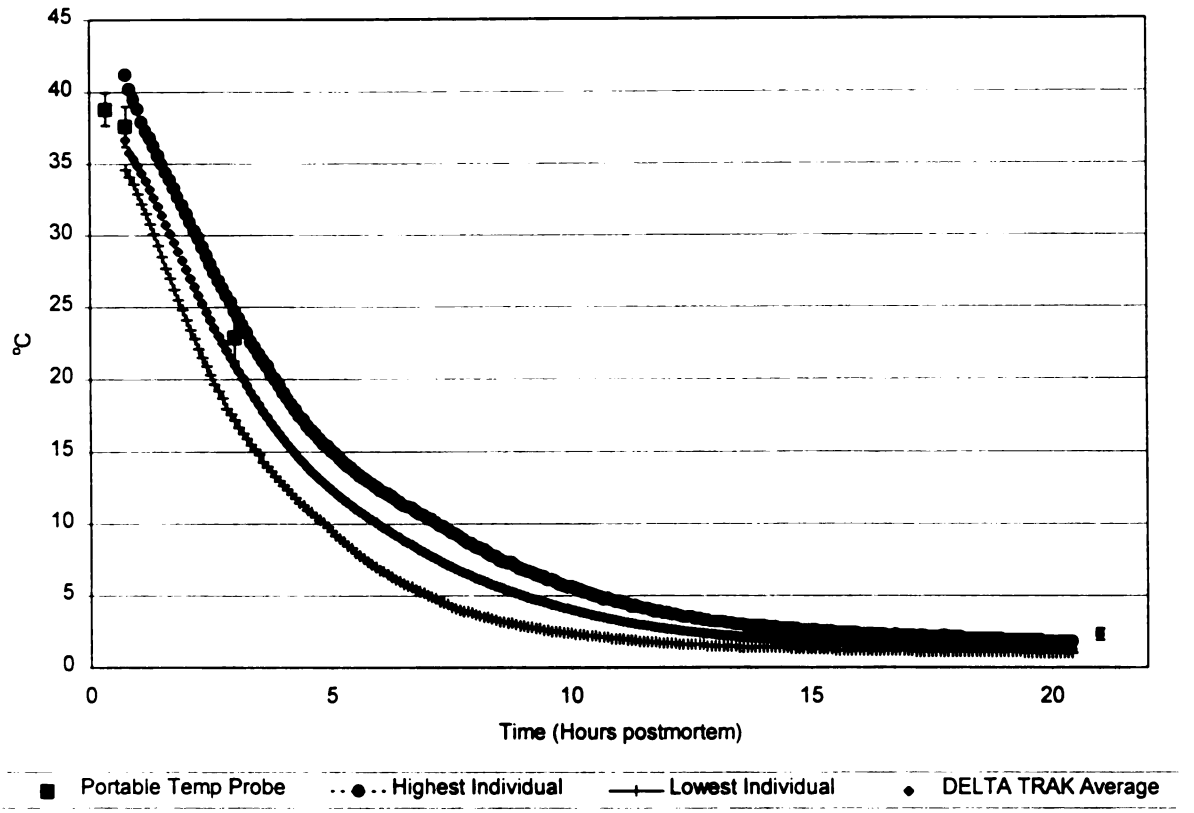


Figure 1.2. Postmortem *logissimus muscle* temperature decline. Means ± standard deviations of temperature measured using a hand-held temperature probe are represented by (■). Average temperature decline measured using the dataloggers is indicated by (◆). The individual carcass that had the highest temperature (●) and the carcass with the lowest temperature (+) are illustrated to show the range in temperature observed.

Table 1.4: Correlation coefficients between temperature, pH, color and water holding capacity

	temp20m	temp45m	temp180m	temp22h	pH20m	pH45m	pH180m	pH22h	L1	L2	L6	L7	DRIP1	CWHC	PURGE
temp45m	.522**														
temp180m	.237	.546**													
temp22h	-.418**	-.294	-.363*												
pH20m	-.270	-.325	.319	.049											
pH45m	-.187	-.375*	.325	.011	.781**										
pH180m	-.161	-.356	-.362*	-.077	.731**	.814**									
pH22h	-.178	-.236	-.420*	-.111	.354	.412*	.534**								
L1	.119	.444*	.359	-.019	-.312	-.470**	-.226	-.175							
L2	.161	.516*	.370*	-.046	-.425*	-.576**	-.292	-.145	.921**						
L6	.210	.407*	.331	-.018	-.549**	-.694**	-.483**	-.404*	.755**	.837**					
L7	.231	.472*	.385*	-.049	-.533**	-.706**	-.528**	-.450**	.769**	.858**	.965**				
DRIP1	.172	.461*	.528**	-.086	-.625**	-.633**	-.482**	-.313	.733**	.828**	.783**	.772**			
CWHC	.167	.334	.429*	.046	-.559**	-.614**	-.428*	-.348	.672**	.739**	.720**	.748**	.874**		
PURGE	.256	.167	.183	-.045	-.619**	-.757**	-.496**	-.265	.556**	.702**	.789**	.752**	.724**	.652**	
DRIP6	-.049	.230	.255	.108	-.534**	-.542**	-.406*	-.173	.694**	.713**	.729**	.693**	.731**	.633**	.626**

* Correlation are different from zero P < .05

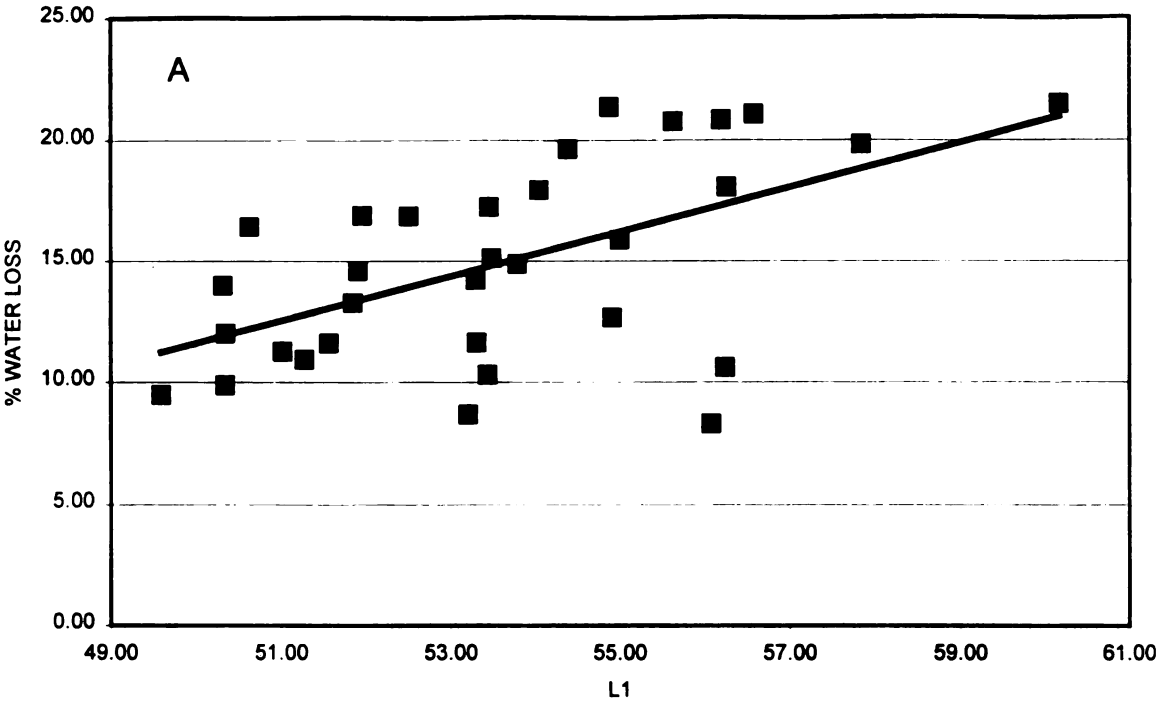
** Correlations are different from zero P < .01

loin section. Different measures of water-holding capacity (DRIP1, CWHC, PURGE and DRIP6) and color (L1, L2, L3 and L6) were highly correlated to each other (Table 1.4). *Longissimus* muscle L* values were positively correlated with water loss (Figure 1.3). Both water loss and L* value were inversely related to LM pH from 20 to 180 min postmortem (Table 1.4). Of the pH measures, 45 min pH was most highly correlated with water loss and color (Table 1.4). This supports conclusions made by Sayre and Briskey (1963) and Sellier and Monin (1994), that the rate of pH decline, as measured by 45 min pH, influences the degree of protein denaturation during rigor onset. The relationships between LM water loss and 45 min pH are graphically depicted in Figure 1.4. Although different measures of water loss did not give the same absolute number, samples with superior or inferior water-holding properties were identified by all methods of measuring water-holding capacity or water loss used in this study (Figure 1.3 and 1.4). Although no severe PSE pork was observed among the samples in the current study, the variation in pork color and water-holding capacity was of practical importance to packer profitability and consumer acceptance.

Twenty-two-hour pH was not related to early postmortem measures of color or water-holding capacity (Table 1.4). Warriss and Brown (1987), demonstrated that ultimate pH was related to reflectance and exudate, but only accounts for about 15% of the variation in these traits. In this study, the lack of a relationship between 22 h pH and water-holding capacity or L* values at d 1 and 2 may be attributed to the lack of variation in ultimate pH.

A modest correlation (r~-.4) between 22 h pH and L* at d 6 and 7 was

Figure 1.3. Relationship between L1 and measures of water loss in *longissimus* muscle. Panel A: Water-holding capacity (■) was measured by high-speed centrifugation (CWHC). Panel B: Purge (▲) was measured on boneless loin section from d 1 to d 6 postmortem stored at 4°C. Drip 1 (*) was assessed using the suspension method from d 1 to d 2. Drip 6 (♦) was measured on duplicate chops under simulated retail display case conditions from d 6 to d 7.


A
25.00
20.00
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% WATER LOSS
49.00
51.00
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57.00
59.00
61.00
L1

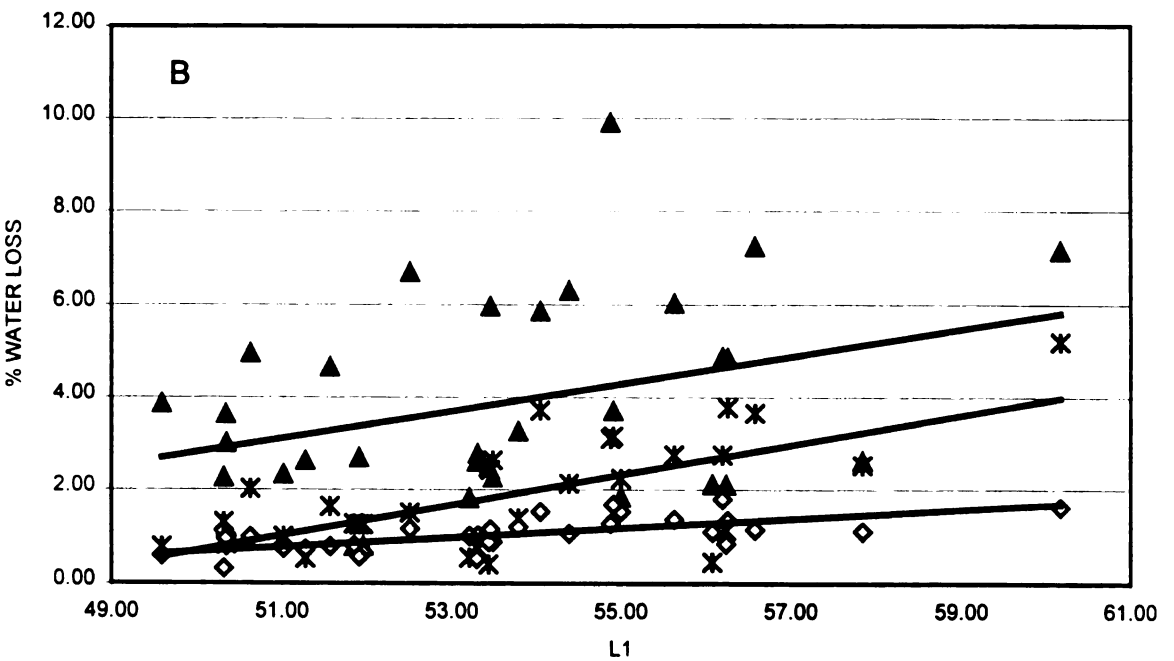
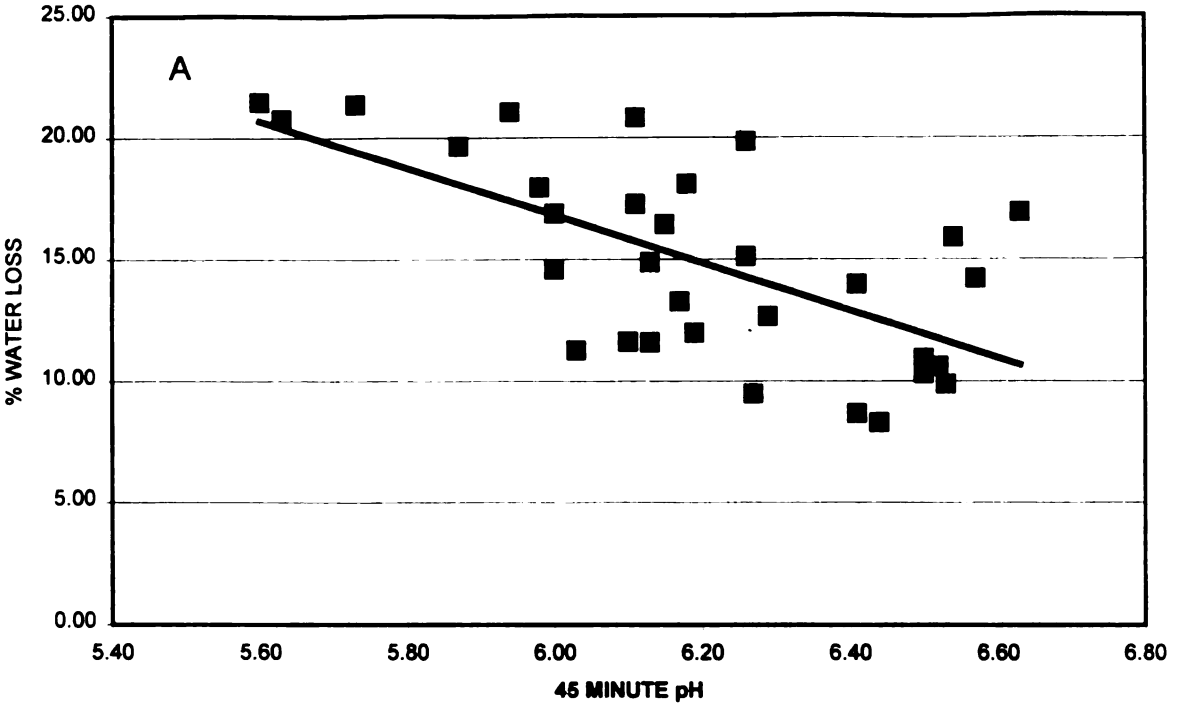

B
12.00
10.00
8.00
6.00
4.00
2.00
0.00
% WATER LOSS
49.00
51.00
53.00
55.00
57.00
59.00
61.00
L1

Figure 1.4. Relationship between 45 min pH and measures of water loss in *longissimus* muscle. Panel A: Water-holding capacity (■) was measured by high-speed centrifugation (CWHC). Panel B: Purge (▲) was measured on boneless loin section stored at 4°C from d 1 to d 6 postmortem. Drip 1 (*) was assessed using the suspension method from d 1 to d 2. Drip 6 (♦) was measured on duplicate chops under simulated retail display case conditions from d 6 to d 7.


A
25.00
20.00
15.00
10.00
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0.00
% WATER LOSS
5.40
5.60
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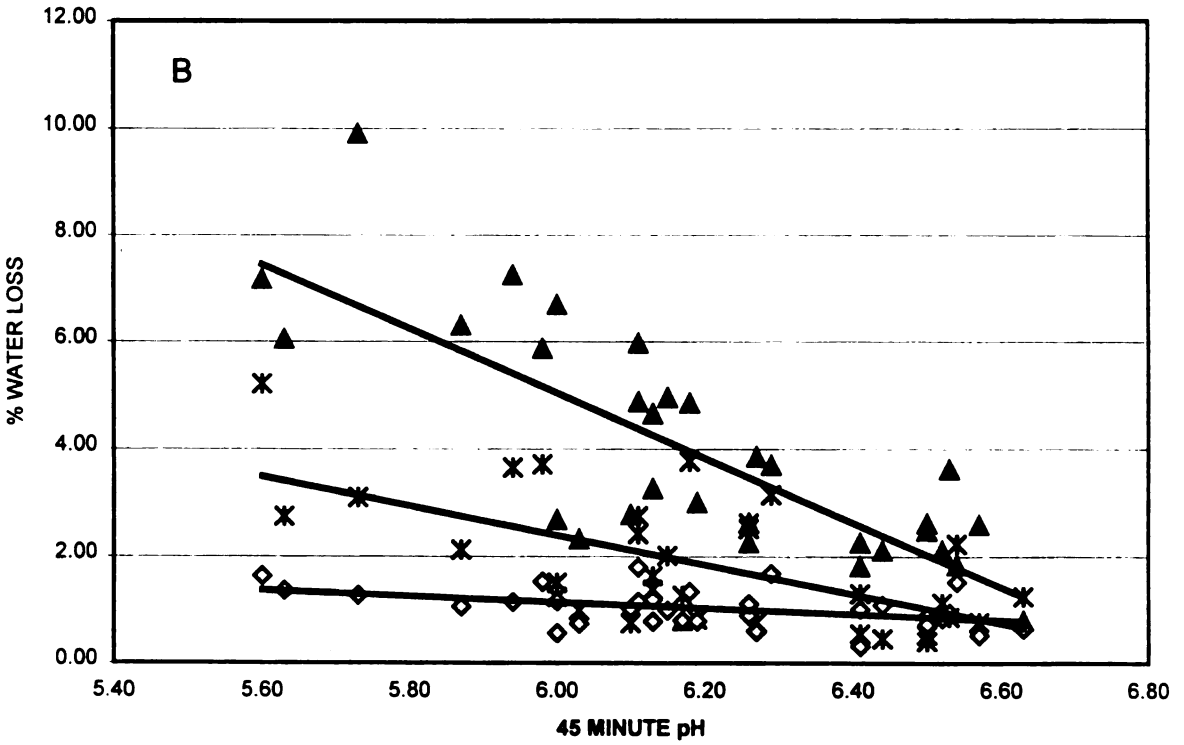
6.20
6.40
6.60
6.80
45 MINUTE pH


B
12.00
10.00
8.00
6.00
4.00
2.00
0.00
% WATER LOSS
5.40
5.60
5.80
6.00
6.20
6.40
6.60
6.80
45 MINUTE pH

observed (Table 1.4). It is possible that slight differences in ultimate pH may impact long term color stability. Indeed, myoglobin stability is reduced at lower pH and higher temperatures. During normal rigor development, reflectance begins to increase rapidly below pH 6.2 (Warris and Brown, 1987). The negative correlation observed between 22 h pH and L* at day 6 and 7 indicates that pork with a lower ultimate pH becomes lighter during postmortem storage.

Coupled enzyme assays were used to quantify total activity of PFK and PK in the sarcoplasmic fraction of *longissimus* muscles. Since these assays were conducted under controlled conditions *in vitro*, it should be recognized that activity data reflect the *in vitro* capacity of these enzymes to catalyze their respective reactions, rather than *in vivo* activity. It is also worth noting that the practice of measuring V_{max} values under optimal conditions *in vitro* and relating them to *in vivo* flux rates is based on sound theoretical framework (Newsholme and Crabtree, 1986). The *in vitro* activity of PFK and PK ranged from .32 to .67 and 3.8 to 6.4 μmole/min·mg^{-1} protein, respectively. These activities are similar to those previously reported for porcine LM (Schwägele et al., 1996; Rosochacki et al., 2000). I observed no correlation between glycolytic enzyme capacity and LM pH, color or water-loss (Table 1.5). In contrast, Schwägele et al. (1996) demonstrated that PK in LM of halothane sensitive pigs, which are prone to producing PSE meat, was more active and less pH labile due to phosphorylation of the enzyme. My findings indicate that a similar regulation of glycolysis does not contribute to the variation observed in pork quality of HAL-1843 free pigs. I also measured activity of PK at pH 5.5 and observed a loss of >88% PK activity

Table 1.5: Correlation coefficients between glycolytic enzyme capacity and pork quality traits*

	pH45	L1	L2	L6	L7	DRIP1	CWHC	PURGE	DRIP6
PFK	.183	-.177	-.085	-.012	-.09	-.152	-.181	.012	-.056
PK	-.082	.198	.117	-.039	.024	-.038	-.053	.085	.057

*Correlations are not significantly different from zero at the P > .1 level.

in all LM samples at this pH compared to activity measured at pH 7.0. These observations are similar to the reduction in activity seen by Schwägele et al. (1996) for control pigs, but much greater than the 30% loss in PK activity observed for halothane sensitive pigs in that study. This provides additional evidence that phosphorylation-dependent stabilization of enzyme activity, as observed by Schwägele et al. (1996) for halothane sensitive pigs, is not a key factor regulating variation in pork quality of HAL-1843 free pigs. My findings support data previously reported by Sayre et al. (1963) who demonstrated that differences in specific activity of glycolytic enzymes was not related to ultimate meat quality. My findings are also consistent with those of Scopes et al. (1974), who showed that different concentrations of PFK and PK did not affect the rate at which glycolysis proceeded *in vitro*. I conclude that in HAL-1843 free pigs, LM color and water-holding capacity are not associated with the capacity of rate-limiting glycolytic enzymes.

IMPLICATIONS

In this study, L* value was positively correlated to all measures of water loss. Forty-five min postmortem pH was most highly correlated to water loss. Differences in glycolytic enzyme capacity do not appear to be responsible for the variation observed in pork quality of HAL-1843 free pigs. Additional research is necessary to identify other biochemical factors, such as allosteric enzyme regulators, physiological buffering compounds, or mechanisms regulating ATP utilization in muscle, that influence the rate of pH decline and consequently govern pork quality.

CHAPTER 2

ANALYSIS OF POSTMORTEM TENDERIZATION IN PORCINE *LONGISSIMUS DORSI* MUSCLE

ABSTRACT

My objective was to quantify the rate and extent of postmortem tenderization in porcine *longissimus dorsi* (LD) muscle and determine if proteolysis of desmin corresponds to mechanical measures of tenderness. Berkshire (n=32) and Yorkshire (n=16) sired pigs were harvested on two days at a commercial abattoir. Four 5.72-cm sections of the LD were removed at d 1 from the 11th rib to the 3rd lumbar vertebrae. Loin sections were randomly assigned to aging treatments of 1, 3, 7 and 14 d, vacuum packaged and stored at 4°C. After storage, two 2.5-cm thick chops were cooked to an internal temperature of 71°C on Farberware Open Hearth™ broilers. Chops were cooled overnight at 4°C and three 1.27-cm diameter cores per chop were sheared with a Warner-Bratzler Shear (WBS) machine. No differences in WBS values were observed between breed or loin location ($P>.05$). Shear values decreased ($P<.0001$) from 4.1 kg at d 1 to 3.6 kg and 3.2 kg at d 3 and d 7, respectively. Chops aged for 7 and 14 d had similar WBS values ($P>.05$). Western blot analysis of desmin from 4 tender ($\leq$4 kg) and 4 less tender ($\geq$4.7 kg) samples at d 1 revealed that desmin degradation paralleled decreases in WBS. Intact desmin was typically undetectable in tender samples by d 7 and in less tender samples by d 14. Tenderization of most pork loin chops was completed by d 7, however some chops exhibited additional tenderization and desmin degradation between 7 and 14 d postmortem.

INTRODUCTION

The overall eating quality of a product will influence repeat purchase decisions of today's consumer. Of these quality traits, tenderness is usually accepted as the most crucial (Koohmaraie, 1996). Several studies have shown that various factors in production and processing up to the point of retail display affect tenderness (Wood et al., 1994). Single trait selection for increased carcass lean has resulted in less tasty, less juicy and less tender pork (Cameron, 1990). Additionally, it has been known for almost a century that postmortem storage of product at refrigerated temperatures will improve tenderness (reviewed by Penny, 1980). Although these improvements in tenderness can be measured both subjectively and objectively, the mechanisms that control these improvements are not clearly defined (Koohmaraie, 1991).

During postmortem storage of meat, numerous changes occur in skeletal muscle, which result in loss of structural integrity of the tissue. This loss of structural integrity is thought to be responsible for meat tenderization and the principal mechanism being limited to the proteolysis of myofibrillar proteins (Koohmaraie, 1992b). It has also been documented that meat tenderization results from the postmortem degradation of myofibrillar proteins by calcium-dependent proteases, or calpains (Goll et al., 1992). Increased levels of calpastatin have been shown to inhibit degradation of myofibrillar proteins by calpains (Koohmaraie et al., 1995).

Desmin is an intermediate filament protein that is part of the cytoskeleton of nearly all animal cells (Robson, 1989;1995). It has been demonstrated to

have a subunit molecular weight of 52 kD (Ip et al., 1985). Immunoelectron microscope localization studies indicated that desmin encircles the Z-line and radiates out perpendicularly to the myofibril axis to ensnare and connect adjacent myofibrils (Richardson et al., 1981). Desmin has also been shown to link myofibrillar Z-lines to the cell membrane skeleton (Robson, 1995). Additionally, desmin "knockout" mice have been shown to exhibit severe disruption of muscle cellular organization (Milner et al., 1996).

In preliminary experiments, degradation of desmin, but not degradation of calpastatin or filamin was associated with improved tenderness at d 7 postmortem. The objective of this study was to quantify the rate and extent of postmortem tenderization in porcine *longissimus dorsi* (LD) muscle and determine if proteolysis of desmin corresponds to mechanical measures of tenderness at 1, 3, 7 and 14 d postmortem

MATERIAL AND METHODS

Animals and Meat Quality Data Collection

Sires from Berkshire and Yorkshire lines were used to inseminate F_1 Yorkshire-Landrace gilts. Progeny were raised in uniform conditions at the MSU Swine Teaching and Research Farm. Berkshire (n=32) and Yorkshire (n=16) sired pigs were harvested on two days at a commercial abattoir. Bone-in loin sections were removed from the carcass at d 1 and transported to the MSU Meat Laboratory. Internal temperature of the meat was monitored during transport, as was the temperature of the ice chest. Internal temperature of loin sections never exceeded 4°C and the ice chests never exceeded 5°C. Sections were trimmed

of external fat and removed from the bone. Four 5.72-cm sections of the LD were cut from the 11th rib to the 3rd lumbar vertebrae. The sections were randomly assigned to aging treatments of 1, 3, 7 and 14 d, vacuum packaged and stored at 4°C. After respective storage times, two 2.5-cm-thick chops were cooked to an internal temperature of 71°C on Farberware Open Hearth™ broilers. Temperature was monitored continuously using copper-constantan thermocouples (10.2 cm in length, .081 cm in diameter and accuracy ± 0.1°C; Omega, Stamford, CT), and chops were turned once when the internal temperature reached 40°C. Chops were cooled overnight at 4°C and three 1.27-cm diameter cores per chop were sheared with a Warner-Bratzler shear (WBS) machine.

Sample Preparation for Analysis of Protein Degradation

Frozen *longissimus* muscle samples (1g) were homogenized using a Polytron (Brinkman, Westbury, NY) for 1 x 20 sec burst (setting 4) in 10 volumes of ice cold 50 mM tris, 10 mM EDTA, pH 8.3, containing 2 mM phenylmethylsulfonyl fluoride, and 6 mg/l of leupeptin. Five hundred μl of the crude homogenate were mixed with 500 μl of 2x treatment buffer without MCE (125mM Tris pH 6.8, 4% SDS, 20% glycerol) by repeated pipeting. Samples were heated at 50°C for 20 min, then mixed again by repeated pipeting, heated for 5 min and centrifuged at 16,000 x **g** for 20 min. The supernatant fluid was removed and protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Samples were then mixed with an equal volume of 2x treatment buffer with 10% MCE and heated at 95°C for 3 min.

SDS-PAGE and Immunoblotting

Desmin was resolved by SDS-PAGE (Laemmli, 1970) on .75 mm-thick 10% (37.5:1 acrylamide:bisacrylamide) separating gels with 4% (37.5:1) stacking gels. Electrophoresis of proteins was performed at 200 V for approximately 45 min at room temperature in BIO-RAD Mini Protean II gel assemblies. Proteins were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA) in buffer containing 25 mM Tris, 193 mM glycine, and 15% methanol (Towbin et al., 1974). Lanes containing molecular weight markers were stained with amido black. To prevent non-specific antibody binding, membranes were incubated in blocking buffer (10% non-fat dry milk (NFDM) in Tris-buffered saline [TBS] containing 20 mM Tris pH 7.4, 137mM sodium chloride, 5 mM potassium chloride and .05% Tween-20) for 1 h. Membranes were incubated in primary D76 hybridoma supernatant followed by an alkaline phosphatase conjugated anti-mouse IgG diluted 1:1000 (Sigma) for 45 min. The D76 hybridoma, developed by D.A. Fischman, was obtained from the Developmental Studies Hybridoma Bank and developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. All antibodies were diluted in blocking buffer. Membranes were washed three times with blocking buffer after each incubation. Antibody binding was visualized by exposure to BCIP/NBT (Bio-Rad).

Data Analysis

Data were analyzed using a mixed model that contained the random main effect of the individual pig and the fixed main effects of breed, day (1, 3, 7 and 14

postmortem), location, sex and harvest date along with the two way interactions of breed*day, breed*location, breed*sex, day*location, day*sex and location*sex Least square means by sire line were compared using a protected least significant difference test (Freud and Wilson, 1997). None of the interaction terms were found to be important (P>.5). Therefore, only the main effect of day was evaluated.

RESULTS AND DISCUSSION

Warner-Bratzler Shear

No differences were observed between the two genetic lines with respect to tenderness for any of the aging treatments (P>.2) and data were pooled by day. Shear values decreased (P<.0001) from 4.1 kg at d 1 to 3.6 kg and 3.2 kg at d 3 and d 7, respectively (Figure 2.1). Chops aged for 7 and 14 d had similar WBS values (P>.05; Figure 2.1). In contrast, van Laack et al. (2001) demonstrated that there was a significant improvement in tenderness from d 7 to 14 postmortem. My data are consistent with those reported by Koohmaraie et al. (1991), who demonstrated that maximum tenderness in pork is achieved by d 5 postmortem. Although I did not measure tenderness at d 5, maximum tenderness is achieved between 3 and 7 d postmortem in this study. Collectively, these data show that storage beyond 7 d postmortem does not improve tenderness of pork. A slight numerical decrease in WBS was observed from d 7 to 14 postmortem. This appears to result from continued tenderization of some samples (change in WBS from d 7 to 14 ranged from 0 – 1.65 kg). It is

unclear why tenderization occurs more slowly in some samples or why proteolysis continues to result in additional tenderization in these samples.

Analysis of Desmin Degradation

To quantify the relationship between proteolysis and WBS values, a subset of samples were chosen. Western blot analysis of desmin was performed on samples from 4 tender ($\leq$ 4.0 kg) and 4 less tender (> 4.7 kg) chops at d1 postmortem. Figure 2.2 depicts the Western blot analysis of desmin and the corresponding WBS values. The disappearance of intact desmin corresponds to decreases in WBS values through d 7 postmortem. At d 1 postmortem intact desmin was observed in all samples regardless of WBS value (Figure 2.2 panel A). This helps to explain the low correlation reported by Wheeler et al. (2000) between tenderness and percent desmin degraded by d 1 postmortem. At d 3 postmortem the divergence of desmin degradation between the tender and less tender samples can begin to be observed (Figure 2.2 panel B). By d 7 postmortem, further degradation of desmin can be observed in more tender samples and more intact desmin is present in the samples that measure less tender (Figure 2.2 panel C). However, by d 14 there is very little intact desmin observed in any of the samples and a range in WBS values of 2.3 – 3.5 kg (Figure 2.2 panel D). It is unclear why there is continued degradation of desmin after d 7 with only minor improvements in WBS values. These data provide evidence that there are other factors that need to be elucidated to more fully understand postmortem tenderization.

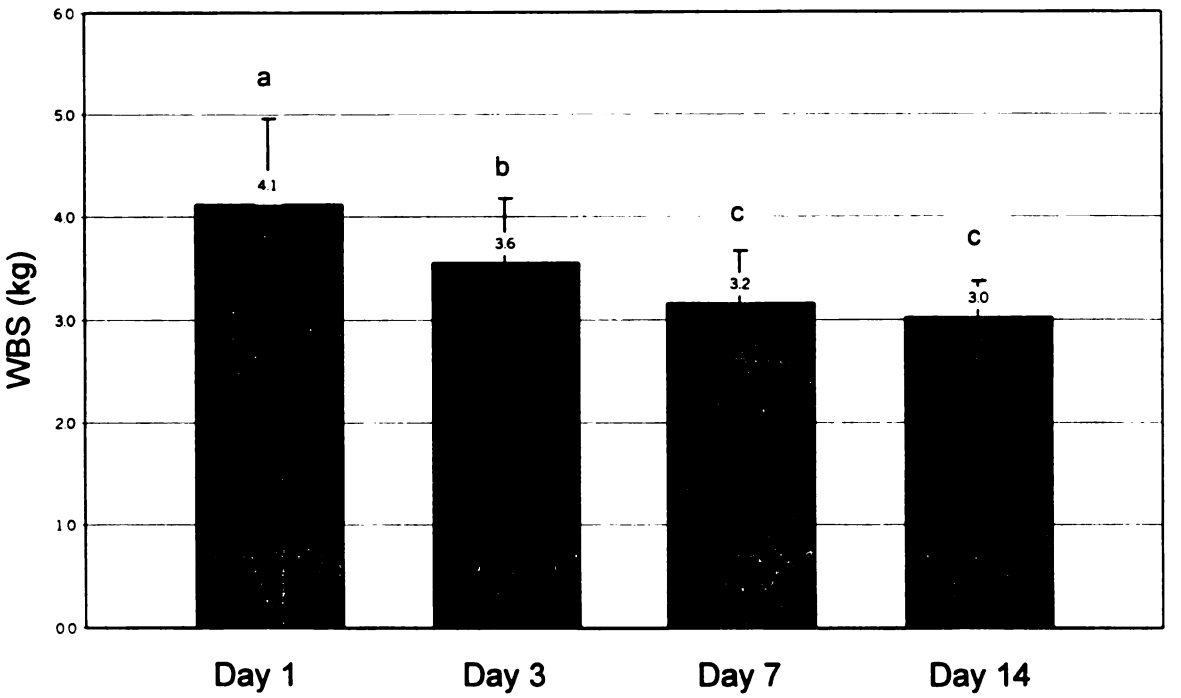
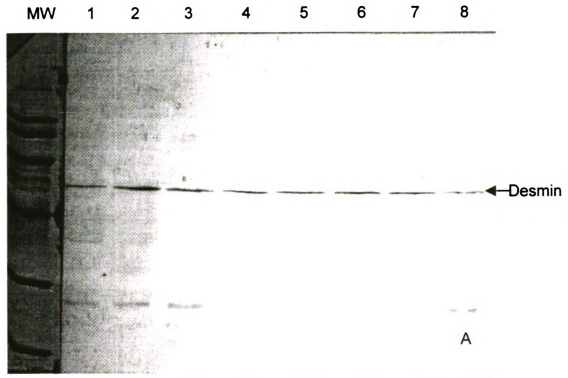


Figure 2.1. Means and standard deviations for WBS values measured at d 1, 3, 7 and 14 days postmortem. Means with different letters differ ($P < .0001$).

Figure 2.2. Western blot detection of desmin degradation using a monoclonal "D76" antibody. The corresponding WBS values are shown at the bottom of each western blot. Molecular weight standards (MW) are, from top to bottom, as follows: myosin, 200 kD; β-galactosidase, 116 kD; phosphorylase b, 97 kD; bovine serum albumin, 66 kD; hen egg-white ovalbumin, 45 kD; bovine carbonic anhydrase, 31 kD; soybean trypsin inhibitor, 21.5 kD. *Panel* A: desmin measured at d 1 postmortem. *Panel* B: desmin measured at d 3 postmortem. The bottom arrow indicates a putative degradation product of desmin.


MW 1 2 3 4 5 6 7 8
Desmin
A
WBS (kg) 3.9 7.0 3.9 3.0 6.0 4.7 5.4 2.9

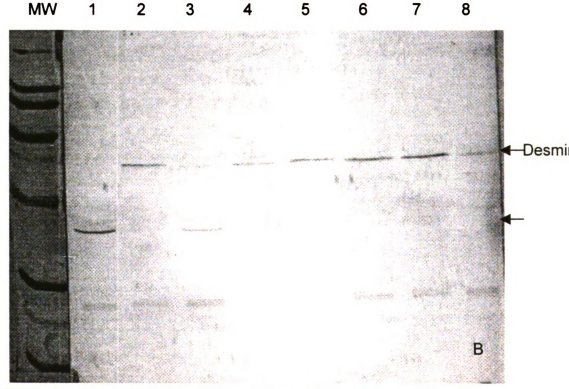
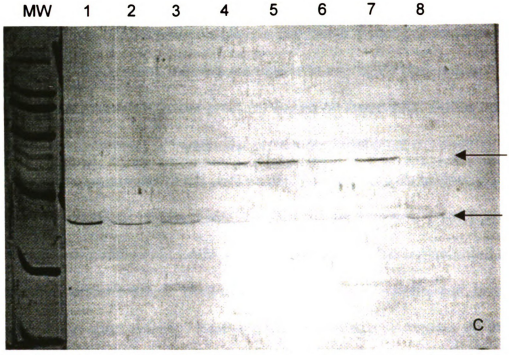
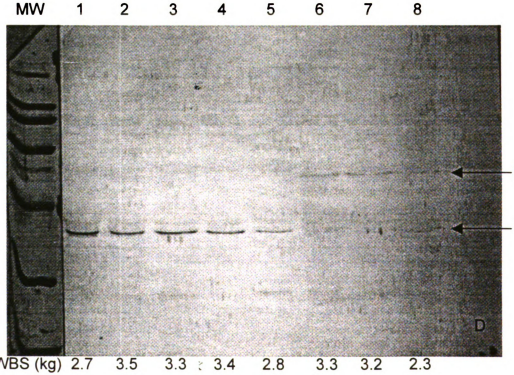

MW 1 2 3 4 5 6 7 8
Desmin
B
WBS (kg) 2.7 5.0 3.0 3.7 4.0 4.4 4.7 3.1

Figure 2.2. Western blot detection of desmin degradation using a monoclonal "D76" antibody. The corresponding WBS values are shown at the bottom of each western blot. Molecular weight standards (MW) are, from top to bottom, as follows: myosin, 200 kD; β-galactosidase, 116 kD; phosphorylase b, 97 kD; bovine serum albumin, 66 kD; hen egg-white ovalbumin, 45 kD; bovine carbonic anhydrase, 31 kD; soybean trypsin inhibitor, 21.5 kD. *Panel* C: desmin measured at d 7 postmortem. The bottom arrow indicates an putative degradation product of desmin. *Panel* D: desmin measured at d 14 postmortem. The bottom arrow indicates a putative degradation product of desmin.


MW 1 2 3 4 5 6 7 8
Desmin
C
WBS (kg) 2.7 4.4 3.0 3.3 4.4 2.9 4.3 2.6


MW 1 2 3 4 5 6 7 8
Desmin
D
WBS (kg) 2.7 3.5 3.3 3.4 2.8 3.3 3.2 2.3

IMPLICATIONS

Tenderness of pork products plays a key role in repeat purchase decisions of consumers. At this point, retailers are unable to segregate pork products and guarantee the consumer a tender product. Based on these data, it does not appear that measurement of myofibrillar protein degradation at d 1 postmortem reflects ultimate tenderness of the pork product. These data do however, suggest that proteolysis can be measured after d 3 postmortem. Detection of tenderness early postmortem would allow for strategies to be implemented to "guarantee" a tender product. Until the strategies can be implemented, pork loin chops should be aged at least 7 d postmortem to optimize tenderness for the consumer.

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