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Review

Mechanisms of water-holding capacity of meat: The role of postmortem biochemical and structural changes

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Abstract

Unacceptable water-holding capacity costs the meat industry millions of dollars annually. However, limited progress has been made toward understanding the mechanisms that underlie the development of drip or purge. It is clear that early postmortem events including rate and extent of pH decline, proteolysis and even protein oxidation are key in influencing the ability of meat to retain moisture. Much of the water in the muscle is entrapped in structures of the cell, including the intra- and extramyofibrillar spaces; therefore, key changes in the intracellular architecture of the cell influence the ability of muscle cells to retain water. As rigor progresses, the space for water to be held in the myofibrils is reduced and fluid can be forced into the extramyofibrillar spaces where it is more easily lost as drip. Lateral shrinkage of the myofibrils occurring during rigor can be transmitted to the entire cell if proteins that link myofibrils together and myofibrils to the cell membrane (such as desmin) are not degraded. Limited degradation of cytoskeletal proteins may result in increased shrinking of the overall muscle cell, which is ultimately translated into drip loss. Recent evidence suggests that degradation of key cytoskeletal proteins by calpain proteinases has a role to play in determining water-holding capacity. This review will focus on key events in muscle that influence structural changes that are associated with water-holding capacity.
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Keywords: Water-holding capacity; Drip loss; Calpain; Proteolysis; pH

Contents

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1. Introduction

The ability of fresh meat to retain moisture is arguably one of the most important quality characteristics of raw products. It has been estimated that as much as 50% or more of the pork produced has unacceptably high purge or drip loss (Kauffman, Cassens, Scherer, & Meeker, 1992; Stetzer & McKeith, 2003). Product weight losses due to purge can average as much as 1–3% in fresh retail cuts (Offer & Knight, 1988a) and can be as high at 10% in PSE products (Melody et al., 2004). In addition to the loss of salable weight, purge loss also entails the loss of a significant amount of protein (Offer & Knight, 1988a; Offer & Knight, 1988b). On average, purge can contain approximately 112 mg of protein per milliliter of fluid; mostly water-soluble, sarcoplasmic proteins (Savage, Warriss, & Jolley, 1990).

The majority of water in muscle is held either within the myofibrils, between the myofibrils and between the myofibrils and the cell membrane (sarcolemma), between muscle cells and between muscle bundles (groups of muscle cells). Once muscle is harvested the amount of water and location of that water in meat can change depending on numerous factors related to the tissue itself and how the product is handled (Honikel, 2004; Honikel & Kim, 1986). Over the years there have been numerous reviews devoted to factors that influence water-holding capacity (Hamm, 1986; Honikel, 2004; Honikel & Kim, 1986; Offer & Knight, 1988a; Offer & Knight, 1988b). This review will give an overview some of the major factors influencing water-holding capacity and will specifically focus on recent developments in understanding how changes in the structure of postmortem muscle can influence drip loss.

2. Structure of skeletal muscle

Skeletal muscle has a very complex organization, in part to allow muscle to efficiently transmit force originating in the myofibrils to the entire muscle and ultimately to the limb or structure that is moved. A relatively thick sheath of connective tissue, the epimysium, encloses the entire muscle. In most muscles, the epimysium is continuous with tendons that link muscles to bones. The muscle is subdivided into bundles or groupings of muscle cells. These bundles (also known as fasciculi) are surrounded by another sheath of connective tissue, the perimysium. A thin layer of connective tissue, the endomysium, surrounds the muscle cells themselves. The endomysium lies above the muscle cell membrane (sarcolemma) and consists of a basement membrane that is associated with an outer layer (reticular layer) that is surrounded by a layer of fine collagen fibrils imbedded in a matrix (Bailey & Light, 1989).

When muscle cells are viewed under a microscope, very regular transverse striations are seen. These striations are caused by specialized contractile organelles, the myofibrils, found in muscle. The striations arise from alternating, protein dense A-bands and less dense I-bands within the myofibril. Bisecting the I-bands are dark lines known as Z-lines. The area between two Z-lines is a sarcomere. The less dense I-band is made up primarily of thin filaments while the A-band is made up of thick filaments and some overlapping thin filaments (Goll, Robson, & Stromer, 1984). The backbone of the thin filaments is made up primarily of the protein actin while the largest component of the thick filament is the protein myosin. Myosin consists of a tail or rod region that forms the backbone of the thick filament and a globular head region that extends from the thick filament and interacts with actin in the thin filament. The rigor complex formed by the interaction of myosin and actin is often referred to as actomyosin. In electron micrograph images of contracted muscle or of post-rigor muscle the actomyosin looks very much like cross-bridges between the thick and thin filaments, indeed, it is often referred to as such. In postmortem muscle these bonds become irreversible and are also known as rigor bonds. The globular head of myosin also has enzymatic activity; it can hydrolyze ATP and liberate energy. In living muscle during contraction, the ATPase activity of myosin provides energy for myosin bound to actin to swivel and ultimately pull the thin filaments toward the center of the sarcomere. This shortens the myofibril, the muscle cell and eventually the muscle to produce contraction. The myosin and actin can disassociate when a new molecule of ATP is bound to the myosin head (Goll et al., 1984). In post-rigor muscle, the supply of ATP is depleted, resulting in the actomyosin bonds becoming more or less permanent.

Within the structure of the muscle, there are several "compartments" from which drip could originate. These could include the space within the myofibril, the intracellular space outside the myofibril and the extracellular space, including the space between the muscle bundles. Loss of water from each of these compartments may involve slightly different mechanisms. In addition, loss of water from each of these compartments may occur at different times during storage. For example, it would be easy to envision that water found in the extracellular spaces could be lost more easily, with deeper compartments taking more time or force to be released.

3. Location of water in muscle

Lean muscle contains approximately 75% water. The other main components include protein (approximately 20%), lipids or fat (approximately 5%), carbohydrates (approximately 1%) and vitamins and minerals (often

analyzed as ash, approximately 1%). The majority of water in muscle is held within the structure of the muscle and muscle cells. Specifically, within the muscle cell, water is found within the myofibrils, between the myofibrils themselves and between the myofibrils and the cell membrane (sarcolemma), between muscle cells and between muscle bundles (groups of muscle cells) (Offer & Cousins, 1992).

Water is a dipolar molecule and as such is attracted to charged species like proteins. In fact, some of the water in muscle cells is very closely bound to protein. By definition, *bound water* is water that exists in the vicinity of non-aqueous constituents (like proteins) and has reduced mobility, i.e. does not easily move to other compartments. This water is very resistant to freezing and to being driven off by conventional heating (Fennema, 1985). True bound water is a very small fraction of the total water in muscle cells; depending on the measurement system used, approximately 0.5 g of water per gram of protein is estimated to be tightly bound to proteins. Since the total concentration of protein in muscle is approximately 200 mg/g, this bound water only makes up less than a tenth of the total water in muscle. The amount of bound water changes very little if at all in post-rigor muscle (Offer & Knight, 1988b).

Another fraction of water that can be found in muscles and in meat is termed *entrapped* (also referred to as immobilized) water (Fennema, 1985). The water molecules in this fraction may be held either by steric (space) effects and/or by attraction to the bound water. This water is held within the structure of the muscle but is not bound per se to protein. In early postmortem tissue, this water does not flow freely from the tissue, yet it can be removed by drying, and can be easily converted to ice during freezing. Entrapped or immobilized water is most affected by the rigor process and the conversion of muscle to meat. Upon alteration of muscle cell structure and lowering of the pH this water can also eventually escape as purge (Offer & Knight, 1988b).

Free water is water whose flow from the tissue is unimpeded. Weak surface forces mainly hold this fraction of water in meat. Free water is not readily seen in pre-rigor meat, but can develop as conditions change that allow the entrapped water to move from the structures where it is found (Fennema, 1985).

The majority of the water that is affected by the process of converting muscle to meat is the entrapped (immobilized) water. Maintaining as much of this water as possible in meat is the goal of many processors. Some of the factors that can influence the retention of entrapped water include manipulation of the net charge of myofibrillar proteins and the structure of the muscle cell and its components (myofibrils, cytoskeletal linkages and membrane permeability) as well as the amount of extracellular space within the muscle itself.

4. Physical/biochemical factors in muscle that affect water-holding capacity

4.1. Net charge effect

During the conversion of muscle to meat, lactic acid builds up in the tissue leading to a reduction in pH of the meat. Once the pH has reached the isoelectric point (p*I*) of the major proteins, especially myosin (pI = 5.4), the net charge of the protein is zero, meaning the numbers of positive and negative charges on the proteins are essentially equal. These positive and negative groups within the protein are attracted to each other and result in a reduction in the amount of water that can be attracted and held by that protein. Additionally, since like charges repel, as the net charge of the proteins that make up the myofibril approaches zero (diminished net negative or positive charge) repulsion of structures within the myofibril is reduced allowing those structures to pack more closely together. The end result of this is a reduction of space within the myofibril. Partial denaturation of the myosin head at low pH (especially if the temperature is still high) is also thought to be responsible for a large part of the shrinkage in myofibrillar lattice spacing (Offer, 1991).

4.2. Genetic factors

Accelerated pH decline and low ultimate pH are related to the development of low water-holding capacity and unacceptably high purge loss. Rapid pH decline resulting in ultimate or near ultimate pH while the muscle is still warm causes the denaturation (loss of functionality and water binding ability) of many proteins, including those involved in binding water. The most severe purge or drip loss is often found in PSE (Pale, Soft, and Exudative) product from pigs that have inherited a mutation in the ryanodine receptor/calcium release channel (halothane gene) in the sarcoplasmic reticulum (Fujii et al., 1991). This mutation results in impairment of the ability of this channel to control calcium release into the sarcoplasm of the muscle cell, particularly under periods of physical stress. Accelerated release of calcium causes rapid contraction and an increase in the rate of muscle metabolism and in the rate of pH decline (Bendall & Wismer-Pedersen, 1962; Lundstrom, Essen-Gustavsson, Rundgren, Edforslilja, & Malmfors, 1989). This particular mutation in the halothane gene can be identified in parent stock. Because a commercial test for this mutation exists, the United States industry has virtually eliminated this gene in most commercial herds.

The Halothane gene is but one example of a condition that can result in PSE. Other factors can cause PSE meat to occur. Before harvest, short-term stress in normal animals can accelerate their metabolism enough

that the postmortem metabolism in the muscle is accelerated, causing a more rapid pH decline than is seen in non-stressed animals. While the condition may not be as severe as that caused by the Halothane gene, protein denaturation does occur, and drip losses can be greater than in muscle that has a normal, slower rate of pH decline. It should be noted that while the pH of these muscles falls faster than normal, the ultimate pH may not be below normal ranges (Rosenvold & Andersen, 2003).

Other metabolic state and existing conditions of muscle often direct the extent of pH decline in postmortem muscle. Many studies have reported the effect of treatment to decrease glycogen content in muscle to minimize lactate accumulation in postmortem muscle (reviewed by Rosenvold & Andersen, 2003). Genetic factors influencing basal metabolism clearly have the potential to similarly affect lactate accumulation and extent of pH decline. The discovery (Milan et al., 2000) of a non-conserved substitution in protein kinase adenosine monophosphate-activated γ_3-subunit gene (*PRKAG3*) has explained the dominant mutation (denoted RN^-) that accounted for large differences in meat quality and processing yield in the Hampshire pig breed (Monin & Sellier, 1985). The substitution (R200Q) in the PRKAG3 gene causes a 70% increase in muscle glycogen in RN^- homozygous and heterozygous pigs. This increase in glycogen *directly* results in greater production of lactate in postmortem muscle, a lower ultimate pH and poorer water holding capacity in fresh pork.

The *PRKAG3* gene encodes one isoform of one of the regulatory subunits (γ) in mammalian adenosine monophosphate (AMP)-activated protein kinase (AMPK). When subjected to nutritional or environmental stress, the AMP/ATP ratio of eukaryotic cells will rise, triggering the ''AMPK cascade'', stimulating the cells to conserve energy (Thornton, Snowden, & Carling, 1998) and to begin ATP synthesis (Hardie, Carling, & Carlson, 1998). The precise functions of the β and γ regulatory subunits of AMPK are still unknown; however, both are known to be important for kinase activity (Hardie & Carling, 1997). The γ regulatory subunit also may be involved with the AMP-binding site of the AMPK heterotrimeric complex (Cheung, Salt, Davies, Hardie, & Carling, 2000).

While the dominant RN^- mutation that is found in the Hampshire breed of pigs is a nonconservative substitution (R200Q) in the *PRKAG3* gene that causes high glycogen content in skeletal muscle resulting in detrimental effects on processing yield (Milan et al., 2000), other alleles within the same gene are associated with lower muscle glycogen content, and improved meat quality traits (Ciobanu et al., 2001). From three missense mutations identified (*T30N*, *G52R*, *I199V*) in porcine *PRKAG3*, least-squares estimates of genotype means across five commercial pig breeds demonstrate significant effects between the analyzed substitutions and product pH and Minolta L values. In the same study, only four haplotypes were found in the five commercial populations. One of those haplotypes was found to be significantly associated with higher pH and darker meat color (*T30-G52-I199*), while a second had intermediate pH and color and the two others had the lowest pH and the poorest color (Ciobanu et al., 2001). These observations establish a genetic basis for variation in ultimate pH across many breeds and commercial lines, not just Hampshire pigs.

4.3. Steric effects

Myofibrils make up a large proportion of the muscle cell. These organelles constitute as much as 82–87% of the volume of the muscle cell. As mentioned previously, much of the water inside living muscle cells is located within the myofibril. In fact, it is estimated that as much as 85% of the water in a muscle cell is held in the myofibrils. Much of that water is held by capillary forces arising from the arrangement of the thick and thin filaments within the myofibril. In living muscle, it has been shown that sarcomeres remain isovolumetric during contraction and relaxation (Millman, Racey, & Matsubara, 1981; Millman, Wakabayashi, & Racey, 1983). This would indicate that in living muscle the amount of water within the filamentous structure of the cell would not necessarily change. However, the location of this water can be affected by changes in volume as muscle undergoes rigor. As muscle goes into rigor, cross-bridges form between the thick and thin filaments, thus reducing available space for water to reside (Offer & Trinick, 1983). It has been shown that as the pH of porcine muscle is reduced from physiological values to 5.2–5.5 (near the isoelectric point of myosin), the distance between the thick filaments declines an average of 2.5 nm (Diesbourg, Swatland, & Millman, 1988). This decline in filament spacing may force sarcoplasmic fluid from between the myofilaments to the extramyofibrillar space. Indeed, it has been hypothesized that enough fluid may be lost from the intramyofibrillar space to increase the extramyofibrillar volume by as much as 1.6 times more than its pre-rigor volume (Bendall & Swatland, 1988).

During the development of rigor, the diameter of muscle cells has been shown to decrease (Hegarty, 1970; Swatland & Belfry, 1985) and is likely the result of transmittal of the lateral shrinkage of the myofibrils to the entire cell (Diesbourg et al., 1988). Additionally, during rigor development sarcomeres can shorten; this also reduces the space available for water within the myofibril. In fact, it has been shown that drip loss can increase linearly with a decrease in the length of the sarcomeres in muscle cells (Honikel, Kim, Hamm, & Roncales, 1986). More recently, highly sensitive low field nuclear magnetic

resonance (NMR) studies have been used to gain a more complete understanding of the relationship between muscle cell structure and water distribution (Bertram, Purslow, & Andersen, 2002). These studies have suggested that within the myofibril, a higher proportion of water is held in the I-band than in the more protein dense A-band. This observation may help explain why shorter sarcomeres (especially in cold-shortened muscle) are often associated with increased drip losses. As the myofibril shortens and rigor sets in, the shortening of the sarcomere would lead to shortening and subsequent lowering of the volume of the I-band region in myofibrils. Loss of volume in this myofibrillar region (where much water may reside), combined with the pH-induced lateral shrinkage of the myofibril could lead to expulsion of water from the myofibrillar structure into the extramyofibrillar spaces within the muscle cell (Bendall & Swatland, 1988). In fact, recent NMR studies support this hypothesis (Bertram et al., 2002). It is thus likely that the gradual mobilization of water from the intramyofibrillar spaces to the extramyofibrillar spaces may be key in providing a source of drip.

All of the previously mentioned processes influence the amount of water in the myofibril. It is important to note that shrinkage of the myofibrillar lattice alone could not be responsible for the movement of fluid to the extracellular space and ultimately out of the muscle. The myofibrils are linked to each other and to the cell membrane via proteinaceous connections (Wang & Ramirez-Mitchell, 1983). These connections, if they are maintained intact in postmortem muscle, would transfer the reduction in diameter of the myofibrils to the muscle cell (Diesbourg et al., 1988; Kristensen & Purslow, 2001; Melody et al., 2004; Morrison, Mielche, & Purslow, 1998). Myofibril shrinkage can be translated into constriction of the entire muscle cell, thus creating channels between cells and between bundles of cells that can funnel drip out of the product (Offer & Knight, 1988). Extracellular space around muscle fibers continually increases up to 24 h postmortem, but gaps between muscle fiber bundles decrease slightly between 9 and 24 h postmortem, perhaps due to fluid outflow from these major channels (Schafer, Rosenvold, Purslow, Andersen, & Henckel, 2002). These linkages between adjacent myofibrils and myofibrils and the cell membrane are made up of several proteins that are associated with intermediate filament structures and structures known as costameres. Costameres provide the structural framework responsible for attaching the myofibrils to the sarcolemma. Proteins that make up, or are associated with the intermediate filaments and costameres include (among others) desmin, filamin, and synemin. dystrophin, talin and vinculin (Greaser, 1991). If costameric linkages remain intact during the conversion of muscle to meat, shrinkage of the myofibrils as the muscle goes into rigor would be transmitted to the entire cell via these proteinaceous linkages and would ultimately reduce volume of the muscle cell itself (Kristensen & Purslow, 2001; Melody et al., 2004; Offer & Knight, 1988b). Thus, the rigor process could result in mobilization of water out not only out of the myofibril, but also out of the extramyofibril spaces as the overall volume of the cell is constricted (Fig. 1). In fact, reduction in the diameter of muscle cells has been observed in postmortem muscle (Offer & Cousins, 1992). This water that is expelled from the myofibril and ultimately the muscle cell eventually collects in the extracellular space. Several studies have shown that gaps develop between muscle cells and between muscle bundles during the post-rigor period (Offer & Cousins, 1992; Offer et al., 1989). These gaps between muscle bundles are the primary channels by which purge flows from the meat; some investigators have actually termed them "drip channels".

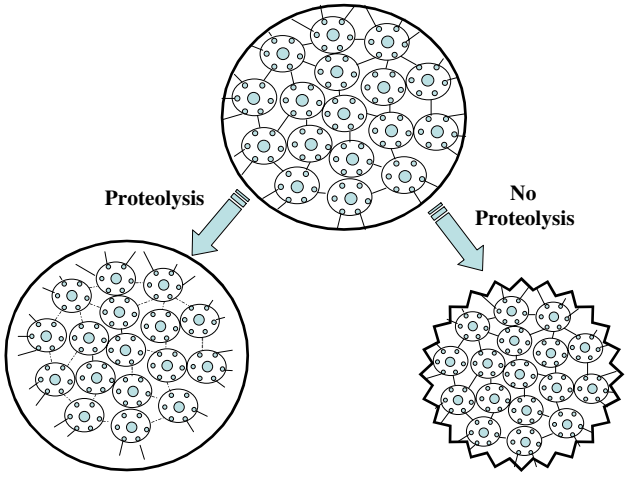

Fig. 1. Schematic over viewing the potential changes in muscle cell diameter during postmortem aging as influenced by proteolysis.

5. Postmortem proteolysis

Since it has been hypothesized that proteinaceous linkages within the cell may influence drip production, it is important to investigate the factors regulating postmortem proteolysis of key proteins.

5.1. Calpain system

The endogenous calpain system plays a major role in regulating proteolysis of muscle proteins under postmortem conditions (Koohmaraie, Schollmeyer, & Dutson, 1986; Lonergan, Huff-Lonergan, Wiegand, & Kriese-Anderson, 2001; Maddock, Huff-Lonergan, Rowe, & Lonergan, 2005). Proteins that are substrates of calpains include proteins like desmin, synemin, talin and vinculin that form the cytoskeletal framework of the muscle cell (Bilak et al., 1998; Evans, Robson, &

Stromer, 1984; O'Shea, Robson, Huiatt, Hartzer, & Stromer, 1979; Schmidt, Zhang, Lee, Stromer, & Robson, 1999).

The calpain system is composed of several isoforms of calcium-dependent cysteine proteases (calpains), and their specific competitive inhibitor, calpastatin (Goll, Thompson, Li, Wei, & Cong, 2003). The two best-characterized isoforms of calpains are μ-calpain and m-calpain, which both degrade the *same* specific set of myofibrillar and cytoskeletal proteins that are degraded as muscle is converted into meat (Geesink & Koohmaraie, 1999; Huff-Lonergan et al., 1996). As the postmortem period progresses, dramatic changes occur within the microenvironment of the muscle cell (e.g., decline in pH, increase in ionic strength) (Winger & Pope, 1981) that can affect calpain activity (Huff-Lonergan et al., 1996; Maddock et al., 2005). As muscle is converted to meat, many changes occur, including: (1) a gradual depletion of available energy, (2) a shift from aerobic to anaerobic metabolism favoring the production of lactic acid, resulting in the pH of the tissue declining from near neutrality to 5.4–5.8, (3) a rise in ionic strength, in part, because of the inability of ATP-dependent calcium, sodium, and potassium pumps to function, and (4) an increasing inability of the cell to maintain reducing conditions. Both μ-calpain and m-calpain have slower rates of activity against myofibrillar protein substrates at pH values and ionic strengths similar to those found in postmortem muscle (Geesink & Koohmaraie, 1999; Huff-Lonergan & Lonergan, 1999; Kendall, Koohmaraie, Arbona, Williams, & Young, 1993). Alterations in pH and/or ionic strengths may cause conformational changes that allow an increase in the hydrophobicity and aggregation of the enzyme. Likewise, pH/ionic strength changes may alter the conformation of substrate proteins and render them less susceptible to cleavage by μ-calpain (Huff-Lonergan & Lonergan, 1999).

Experimental evidence exists that supports the idea that that proteolysis of key cytoskeletal proteins such as the intermediate filament protein, desmin may be related to drip production. These proteins have been shown to be degraded as early as 45 min to 6 h postmortem in some muscles (Melody et al., 2004). Degradation of these proteins at such an early time postmortem would certainly allow water that is expelled from the intramyofibrillar spaces to remain in the cell for a longer period of time. The corollary to this is that *reduced* degradation of proteins that tie the myofibril to the cell membrane (such as desmin) results in *increased* shrinking of the muscle cell, which is ultimately translated into drip loss. Certainly, desmin is not the only candidate protein to be involved. Other proteins associated with intermediate filaments and with the costameres have been implicated. These include the proteins talin and vinculin (Bee, Lonergan, & Huff-Lonergan, 2004; Kristensen & Purslow, 2001; Morrison et al., 1998). These

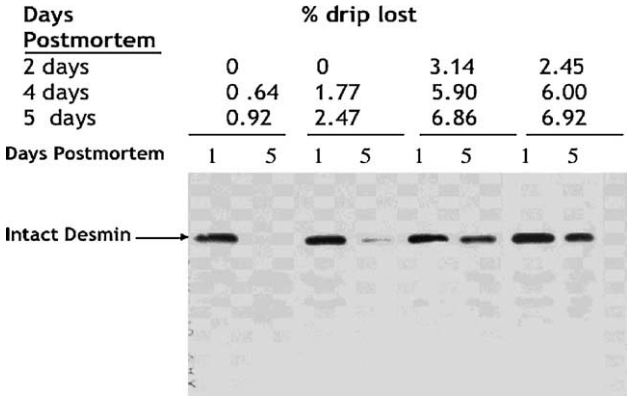

Fig. 2. Relationship between desmin degradation and percentage drip loss in porcine *longissimus dorsi*. Drip loss over the first 5 days postmortem in loins from four different animals. Desmin shown at 1 and 5 days postmortem for each of the animals. Lack of a band indicates desmin has been degraded.

differences in drip loss associated with decreased proteolysis can be seen as early as 24–48 h postmortem (Melody et al., 2004) (Fig. 2). Similar observations have been made in enhanced pork loins (Davis, Sebranek, Huff-Lonergan, & Lonergan, 2004) where reduced degradation of desmin was associated with increased purge loss. Significant correlations between desmin degradation at 1 and 7 d postmortem with pork sirloin purge loss over 7 days in a vacuum package have been reported (Gardner, Huff-Lonergan, & Lonergan, 2005). When desmin degradation was added to the stepwise regression models to predict purge loss, desmin degradation at 1 day postmortem explained 24.1% of the variation in purge loss in the sirloin. In fact, desmin degradation at day 1 postmortem was the first independent variable to enter the model from a list of variables including pH decline, ultimate pH, temperature decline, color and firmness. This observation supports the hypothesis that proteolysis of intermediate filament proteins early postmortem can minimize the flow of water from within the cell to the drip channels.

Because desmin is a known μ-calpain substrate (Huff-Lonergan et al., 1996), it is reasonable to hypothesize that calpain autolysis and activation may explain a portion of the variation of desmin degradation and could subsequently influence drip loss. In an effort to determine what factors influence desmin degradation, μ-calpain autolysis in samples aged one day was determined. The relative intensity of intact desmin at 1 and 5 d postmortem was significantly correlated ($r = .295$, and .270, respectively) with the proportion of μ-calpain large subunit present as the unautolyzed 80 kDa protein at 1 d postmortem. Further, relative intensity of intact desmin at 1, 5 and 7 d postmortem was significantly correlated ($r = -.349$, $-.385$, and $-.378$, respectively) with the proportion of μ-calpain large subunit present as the autolyzed 76 kDa protein

at 1 d postmortem. It has been reported that 52.5% of the variation desmin degradation at 1 d postmortem can be explained in a model that includes loin temperature at 4 h postmortem, loin temperature at 24 h postmortem and percentage of μ-calpain large subunit present as the autolyzed 76 kDa protein at 1 d postmortem (Gardner et al., 2005). A negative correlation between the 76 kDa autolysis product and intact desmin indicates that when very little autolysis occurred within the first day postmortem, a large proportion of desmin remained intact. Autolysis of calpain is the hallmark for activation in postmortem muscle. Thus, it is concluded that conditions favorable for calpain activation are similar to those necessary for desmin degradation.

5.2. Calpastatin

Since rapid proteolysis of intermediate filament proteins (like desmin) in meat has been associated with improved water-holding capacity (Melody et al., 2004; Morrison et al., 1998) and tenderness (Melody et al., 2004) in pork and because there is strong evidence that the calpain enzymes are responsible for postmortem proteolysis observed in pre- and post-rigor muscle (Huff-Lonergan & Lonergan, 1999; Huff-Lonergan et al., 1996; Koohmaraie, 1992) it is important to consider the endogenous inhibitor of μ-and m-calpain, calpastatin. Calpastatin is an effective regulator of calpain activity in postmortem muscle (reviewed by Koohmaraie, 1992). Post-rigor calpastatin activity has explained a high proportion of the variation of meat tenderness (Whipple et al., 1990). There is a documented link between high calpastatin activity, limited postmortem proteolysis of troponin-T (indicated by a limited appearance of a 30 kDa troponin-T degradation product) and high shear force (Koohmaraie, 1992; Lonergan et al., 2001). Melody et al. (2004) documented differences in calpastatin activity between muscles measured 6 and 24 h postmortem in *semimembranosus* (highest), *longissimus dorsi* (intermediate), and *psoas major* (lowest). These differences corresponded to significant differences in desmin degradation between the *psoas major* and *semimembranosus*, providing evidence that variation in calpastatin activity may provide a partial explanation for variation in observed proteolysis. In many cases, the physiological explanation of variation in calpastatin activity has not been explained. It has been hypothesized that there may be a genetic component to variation in calpastatin activity against calpain. Two missense mutations have been identified in the calpastatin gene (*R249K* and *S638R*) to classify four haplotypes, three of which accounted for most of the genetic variation in the US commercial industry populations examined (Ciobanu et al., 2004). Haplotype 1 (*249K-638R*) was found to be the favorable haplotype as it was associated with more juicy and tender pork. These data provide compelling evidence that calpastatin gene variants contribute to differences in fresh pork quality, presumably by regulating calpain activity in meat.

5.3. Role of protein oxidation

Postmortem changes in muscle are also accompanied by a marked increase in indices of oxidation (Harris, Huff-Lonergan, Lonergan, Jones, & Rankins, 2001). Another change that occurs in postmortem muscle during aging of whole muscle products is increased oxidation of myofibrillar proteins (Martinaud et al., 1997). This results in the conversion of some amino acid residues, including histidine, to carbonyl derivatives (Levine, Williams, Stadtman, & Shacter, 1994; Martinaud et al., 1997) and can cause the formation of intra and/or inter protein disulfide cross-links (Martinaud et al., 1997; Stadtman, 1990). In general, both of these changes reduce the functionality of proteins (Xiong & Decker, 1995). Because μ-calpain and m-calpain enzymes contain both histidine and SH-containing cysteine residues at their active sites, they may be particularly susceptible to inactivation by oxidation. Therefore, oxidizing conditions in postmortem muscle may lead to inactivation or modification of calpain activity. In fact, evidence suggests oxidizing conditions inhibits proteolysis by μ-calpain, but might not completely inhibit autolysis (Guttmann, Elce, Bell, Isbell, & Johnson, 1997; Guttmann & Johnson, 1998). In postmortem muscle, there are differences between muscles in the rate that postmortem oxidation processes occur (Martinaud et al., 1997). It has been noted that differences in the rate of oxidation in muscle tissue are seen when comparing the same muscles between animals and/or carcasses that have been handled differently (Juncher et al., 2001). These differences may arise because of differences in diet, breed, antemortem stress, postmortem handling of carcasses, etc. In fact, there have been reports of differences between animals and between muscles in the activity of some enzymes involved in the oxidative defense system of muscle (Daun, Johansson, Onning, & Akesson, 2001). Therefore, there may be genetic differences in susceptibility to oxidation that could be capitalized on to improve meat quality. It is therefore reasonable to hypothesize that differences in the antioxidant defense system between animals and/or muscles would influence calpain activity, proteolysis, and thus quality characteristics influenced by proteolysis such as tenderness and water holding capacity. Experimental evidence exists that indicates high levels of antioxidants in meat can influence proteolysis and early postmortem shear force (Harris et al., 2001; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004b). Rowe et al. (2004b) showed there was a significant increase in proteolysis of troponin-T at 2 days of postmortem aging in steaks from steers fed high levels of 1000 IU α-tocopherol (Fig. 3).

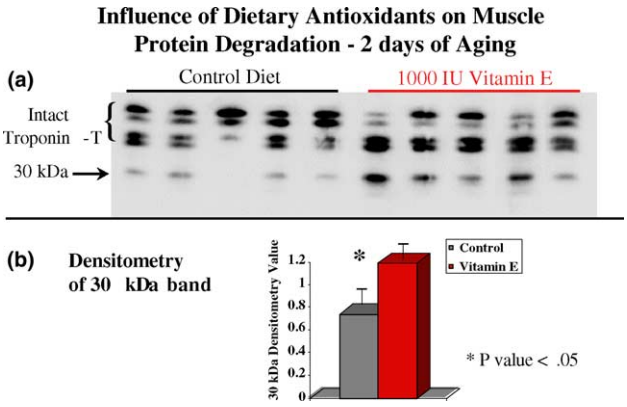

Fig. 3. (a) Western blot of Troponin-T in purified myofibrils from non-irradiated steaks from control diet fed and α-tocopherol (vitamin E) fed steers. (b) Densitometry of the degradation product of Troponin-T (30 kDa band). An increased value indicates more degradation.

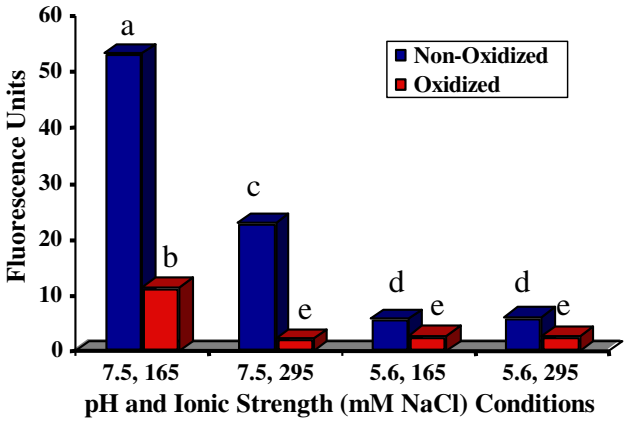

Fig. 4. Effect of oxidizing conditions (100 μM H_2O_2) on the activity of μ-calpain under different pH and ionic strength conditions after 15 min of incubation at 25 °C. The fluorescent peptide Suc-Leu-Leu-Val-Tyr-AMC was used as the substrate. a–e significant differences at $P < 0.01$.

Taken together, these data indicate that very low levels of oxidation *can* influence proteolysis.

The oxidative state of the tissue, which greatly influences the activity of μ-calpain (Guttmann et al., 1997; Guttmann & Johnson, 1998), and the presence of endogenous nitric oxide reaction products (e.g., peroxynitrite, *S*-nitrosothiols) are factors likely influencing calpain that have received little attention (Koh & Tidball, 2000; Maddock et al., 2005; Rowe, Maddock, Trenkle, Lonergan, & Huff-Lonergan, 2003). Because μ-and m-calpain have an oxidizable cysteine residue at their active site, they require reducing conditions to be active (Guttmann et al., 1997). This active site cysteine may also be reversibly *S*-nitrosylated leading to inactivation of the calpain (Koh & Tidball, 2000). Therefore, oxidizing and nitrosylating species in the tissue could inhibit calpains and serve as a regulatory factor.

It has been shown that the presence of oxidizing species does significantly impede the ability of calpains to degrade their substrates. Oxidation with H_2O_2 significantly limits proteolytic activity of μ-and m-calpain against the fluorescent peptide Suc-Leu-Leu-Val-Tyr-AMC, regardless of the pH or ionic strength (Rowe et al., 2003) (Fig. 4). Similar results were seen when using purified myofibrils as the substrate (Maddock, Huff-Lonergan, Rowe, & Lonergan, 2004). Inhibition of calpain with hydrogen peroxide (demonstrated with both the fluorescent substrate and the myofibrils) is reversible as addition of reducing agent (DTT) to the oxidized samples restores activity. In muscle tissue that has been exposed to irradiation to induce oxidation (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004a) during the early postmortem period, degradation of several proteins is arrested, including desmin (Fig. 5) (Rowe et al., 2004b; Rowe et al., 2003). In vitro studies using purified calpain to degrade myofibrils in the presence of hydrogen peroxide (oxidizing agent) have also shown

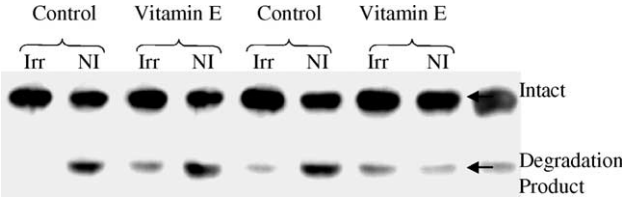

Fig. 5. Western blot of desmin in purified myofibrils from irradiated (Irr) and non-irradiated (NI) beef *longissimus dorsi* aged 7 days postmortem.

that oxidizing conditions may limit the ability of calpain to degrade its substrates including desmin (Maddock et al., 2004). In muscle tissue, oxidizing conditions do appear to reversibly inhibit calpain. Rowe et al. (2004b) used irradiation as a tool to study calpain activity in oxidized early postmortem meat using casein zymography. This activity assay involved running the supernatant fraction extracted from fresh (never frozen) steaks right after irradiation and at each of the days of aging on non-reducing, non-denaturing polyacrylamide gels. These gels contained casein in the separating gel solution. After gels were run, they were incubated at room temperature in a 5 mM calcium chloride solution containing 0.1% 2-mercaptoethanol to reduce reversibly oxidized calpain and allow it to degrade casein. Calpain activity was identified by the appearance of clearing zones (light bands, Fig. 6) after the gel was stained in Coomassie Brilliant Blue (Raser, Posner, & Wang, 1995). Because calpain loses activity after extensive autolysis, *loss of calpain activity during postmortem aging of meat indicates prior activation*. Calpain that is prevented from being active in the tissue will *not* fully autolyze and will thus be able to be activated once the conditions for activity are satisfied (for example, ample calcium and reducing conditions as in the casein gel as-

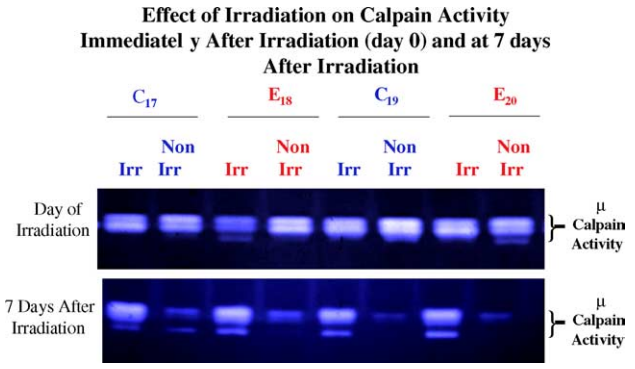

Fig. 6. Casein zymogram of μ-calpain activity in the sarcoplasmic extracts of irradiated (Irr) and non-irradiated (Non Irr) steaks. One steak from each carcass was irradiated and one steak was not. Clear zones (light bands) indicate calpain activity. Extraction was done and gels were run either immediately following irradiation (top panel) or after 7 days of storage following irradiation (bottom panel). C17, E18, C19, E20 indicate samples from different animals.

say). This study showed *consistently* that μ-calpain in non-irradiated samples lost activity faster than in irradiated samples from the same animals. The μ-calpain from the irradiated samples showed little change in activity as aging time progressed. In fact, the irradiated samples had *more* μ-calpain activity at 7 days after irradiation than did the non-irradiated samples from the same carcasses (Fig. 6) potentially indicating μ-calpain was not active in the irradiated muscle. However, once calpain was removed from the tissue and subjected to reducing conditions in the casein zymogram assay, it was active; further evidence that in the tissue, μ-calpain may have been inactivated (but reversibly) by oxidation.

6. Summary

The mechanism of water-holding capacity is centered in the proteins and structures that bind and entrap water, specifically the myofibrillar protein. There is a great body of evidence that demonstrates a direct effect of pH, ionic strength, and oxidation on the ability of myofibrillar protein and myofibrils and muscle cells to entrap water. Independent of these effects, it is clear that the same factors (pH decline, ionic strength, oxidation) also affect proteolysis of key cytoskeletal proteins in postmortem muscle. Variation in water holding capacity at given pH, and temperature of storage is proposed to be at least partially due to variation in proteolysis and the resulting muscle cell shrinkage and mobilization of water to the extracellular space. Because of this, investigations of metabolic, chemical and genetic sources of variation in postmortem proteolysis should provide new insight into mechanisms governing water-holding capacity.

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