RESEARCHREPORT
ANIMAL SCIENCE

Title: **Novel Approach to Controlling Water-Holding Capacity and pH Decline in Pork: The Role of Dietary Lipoic Acid in Calcium Regulation and Glycogen Storage. (#01-095)**

Investigator: Elisabeth Huff-Lonergan

Institution: Iowa State University

Co-Investigators: Steven Lonergan – Iowa State University
Eric Berg - University of Missouri-Columbia

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I. Project title and NPB project identification number

Novel Approach to Controlling Water-Holding Capacity and pH Decline in Pork: The Role of Dietary Lipoic Acid in Calcium Regulation and Glycogen Storage. (#01-095)

Principal Investigators: Elisabeth Huff-Lonergan[1]
Co-Investigators: Steven Lonergan[1], Eric Berg[2]
Institutions: [1]Iowa State University
[2]University of Missouri-Columbia

II. Abstract

One of the most significant quality problems facing the industry is a lack of consistency of important quality attributes including water-holding capacity and product tenderness. One mechanism that may affect pork quality is oxidation of proteins that maintain calcium balance in muscle cells. Increasing the level of an antioxidant like lipoic acid could aid in preventing oxidation of these proteins and maintaining relatively low levels of free calcium for a longer period of time postmortem thus slowing pH decline and improving water-holding capacity. Therefore, it was the objective of this project to establish the role of oxidation in regulating the development of water holding capacity and tenderness in pork. The antioxidant compound lipoic acid was fed to market pigs in an attempt to create differences in oxidation. In this study the doses and delivery system used did not show a difference in quality that could be attributed to lipoic acid treatment. However, this study did indicate the extent of oxidation occurring in meat early postmortem might be related to ultimate pH values and tenderness of the product. Products that had a relatively high level of oxidation had lower ultimate pH values. This study indicates that oxidation that occurs in early postmortem meat can have a significant effect on quality attributes that are influenced by pH. Besides providing this basic information, this study points to the continued need to examine antioxidant compounds that lead to novel solutions (such as modifying finishing diet rations) that can be easily implemented by the producer to improve pork quality.

III. Introduction

A lack of consistency in water-holding capacity remains one of the most significant quality problems facing the pork industry today. Unacceptably high moisture loss from fresh product as purge or drip occurs in as much as 50% of the pork produced (Kauffman et al., 1992). Valuable water-soluble protein, vitamins, and product weight are lost with this moisture loss. It is clear that early postmortem biochemical and biophysical processes contribute to the development of water-holding capacity. The rate and extent of pH decline have an influence on the development of moisture loss. Rapid pH decline resulting in ultimate or near ultimate pH while the muscle is still warm causes the denaturation of many proteins, including those involved in binding water. On the other hand, a normal rate of pH decline that results in a lower than normal ultimate pH also negatively influences the water-holding capacity of the product. Once the pH has reached the isoelectric point (pI) of the major proteins, especially myosin (pI = 5.4) the net charge of the protein is zero, resulting in a diminished capacity to attract and bind water. Factors controlling the rate and the extent of pH decline need to be fully explored if researchers are to ever identify ways to predict and control water-holding capacity. The rate of pH decline is closely linked to the rate of metabolism in muscle immediately prior to slaughter. The most severe PSE product can be caused by a mutation in the ryanodine receptor/calcium release channel in the sarcoplasmic reticulum. 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. This rapid release of calcium causes rapid contraction and an increase in the rate of metabolism and pH decline. This mechanism is but one example that points out the need to understand the regulation of calcium in early postmortem muscle. It is highly likely that perturbations in calcium regulation may be at the root of other heretofore-unexplained variations in the rate of pH decline. One aspect that has not yet been examined is the rate of protein oxidation that occurs in muscle tissue. The role of oxidant signaling either by the generation of reactive oxygen species is a very active area of research in muscle biology. Oxidative modification of the ryanodine receptor (calcium release channels) increases their rate of calcium release into the sarcoplasm, in fact, oxidation of these

channels can override some of the naturally occurring inhibitory factors (i.e. Mg^{2+}) (Donoso et al., 2000). Another important calcium regulatory factor that is inherent in muscle is the sarcoplasmic reticulum Ca^{2+}-ATPase pump (SERCA). This pump removes calcium from the sarcoplasm and returns it to the sarcoplasmic recticulum were it is not available to participate in metabolic processes until released again by the ryanodine receptor. This pump functions as long as ATP is available during the early postmortem period, a point in time when it is critical to maintain low levels of calcium in the cell. This pump, like the ryanodine receptor, can also be oxidized. Oxidation of SERCA pumps greatly diminishes their activity (Klebl et al., 1998), thereby making calcium removal from the sarcoplasm a much slower process. Therefore, it can clearly be seen that tissue oxidation could allow a rapid and early build-up of calcium in the sarcoplasm by impairing the ability of the ryanodine receptor to maintain calcium in the sarcoplasmic reticulum while at the same time reducing the ability of the SERCA pumps to remove that calcium from the sarcoplasm.

Relationship between desmin degradation and percentage drip loss in porcine longissimus dorsi

Days Postmortem	% drip lost			
2 days	0	0	3.14	2.45
4 days	0 .64	1.77	5.90	6.00
5 days	0.92	2.47	6.86	6.92

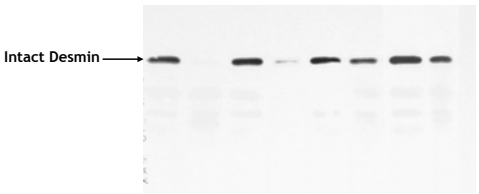


Figure 1. Drip loss over the first 5 days postmortem in loins from 4 different animals. Desmin shown at 1 and 5 days postmortem for each of the animals. Lack of a band indicates desmin has been degraded.

Tissue oxidation could also affect water-holding capacity of pork by inactivating the proteolytic enzyme calpain. When oxidized, calpain loses much of its activity. This is significant because calpain may aid in improving water-holding capacity of meat by degrading proteins that form lateral linkages within the muscle cell. As the pH of the muscle declines during the conversion of muscle to meat, the intricate latticework of the myofibril within the muscle cell shrinks, reducing the space within the cell where water can reside. If the proteinacous linkages between the myofibril and the muscle cell membrane are intact, this 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. It has been suggested that *reduced* degradation of proteins that tie the myofibril to the cell membrane (such as desmin) results in *increased* shrinking of the muscle cell that is ultimately translated into drip loss (Morrison et al., 1998). In fact, as a result of research that was funded last year by NPPC, we have shown that reduced degradation of the protein desmin is related to higher amounts of drip loss (Figure 1). Therefore, since calpain can degrade desmin and oxidation may limit calpain activity, lessening of oxidative processes in muscle may improve water-holding capacity by allowing more degradation of proteins like desmin by calpain.

As can be seen from the discussion above, limiting the amount of oxidation in early postmortem tissue could be very beneficial in controlling water-holding capacity of meat by slowing Ca^{2+} accumulation and thus slowing pH decline. In previous work by our collaborator, Eric Berg, loin muscles from pigs supplemented with lipoic acid were shown to have significantly slower pH declines (Berg et al., 2000). The compound lipoic acid is a very potent antioxidant. It has been called a universal antioxidant because unlike vitamin E (lipid soluble) and ascorbic acid (water-soluble), lipoic acid is both lipid and water-soluble. This characteristic could allow it to slow oxidation of both membrane proteins (like the ryanodine receptor and sarcoplasmic reticulum Ca^{2+}-ATPase pump) and water-soluble sarcoplasmic proteins (like calpain). Additionally lipoic acid is capable of regenerating other antioxidants in the cell such as vitamin E and ascorbic acid (Denke, 2000). Because lipoic acid is a potent antioxidant, investigation of its involvement in postmortem calcium regulation is warranted.

Another factor that can influence the extent of pH decline is the amount of readily available glycogen/glucose. High levels of utilizable glycogen can translate into more lactic acid production and a lower ultimate pH. Glycogen is known to exist as two different "pools" in muscle, macroglycogen and proglycogen. Proglycogen is a stable intermediate in pathways that form macroglycogen. The liberation of energy from glycogen first requires macroglycogen to be re-converted back to proglycogen. Therefore, it is possible that storage of more glycogen as macroglycogen instead of proglycogen may slow or limit the rate of pH decline by altering the immediate availability of glycogen. Many factors may affect this process. It has been shown infusion of muscle with high levels of insulin may favor the formation of macroglycogen (Huang et al., 1997). Lipoic acid can stimulate insulin mediated glucose transport (Perth et al., 2000). Through this mechanism, lipoic acid could potentially favor formation of the less readily available macroglycogen and thus slow or limit pH decline. Studies funded by NPPC at Iowa State

University (S. Lonergan) have shown that the majority of glycogen in post-rigor muscle is macroglycogen, suggesting that it might be less easily used by the postmortem muscle.

IV. Objectives

The ***central hypothesis*** for the proposed research was: *Lipoic acid can slow early postmortem pH decline and improve water holding capacity by slowing oxidative processes that may affect Ca^{2+} regulation and proteolysis. Lipoic acid may also affect water-holding capacity by altering glycogen storage.*

We tested the central hypothesis and accomplished the overall objective of this application by pursuing the following research objectives:

Objective 1. Determine the extent of the impact of tissue oxidation and subsequent postmortem modifications of specific proteins involved in regulating intracellular calcium levels (ryanodine receptor/calcium release channel and the sarcoplasmic reticulum Ca^{2+}-ATPase pump) on drip loss.

Objective 2. Ascertain the relationship between tissue oxidation, desmin degradation and water-holding capacity.

Objective 3. Determine the relationship between lipoic acid supplementation, the storage form of glycogen and drip loss.

V. Procedures

This project was designed as a collaborative effort between Iowa State University (Dr. Elisabeth Huff-Lonergan and Dr. Steven Lonergan) and the University of Missouri/Columbia (Dr. Eric Berg). The pigs used in this project were housed, fed at the Minnesota Agricultural Experiment Station in New Ulm MN. This facility was leased by Triumph L.L.C.; a component of Farmland Industries. Sixty ~ 95 kg (210 lb.) pigs were grouped in pens with equal numbers of barrows and gilts represented in treatment.. Each pen was fed one of three diets: TRT-0 = a traditional finishing diet; TRT-1 = TRT-0 plus 8 mg of lipoic acid per kg of live body weight per pig; TRT-2 = TRT-0 plus 16 mg of lipoic acid per kg of live body weight per pig. Pigs remained on their respective diets until reaching ~118 kg (260 lb.). Pigs were slaughtered at the Farmland facility in Crete, Nebraska. Harvest was done under humane industry standards. The pigs were harvested in two groups on January 8, 2002 (25 pigs) and on January 10, 2002 (29 pigs). The product from these pigs was used for all 3 objectives. The following measurements were made in the packing plant used to characterize the longissimus dorsi from the loin. The pH was measured at 1 hour and 24 hours (longissimus dorsi) postmortem. In addition, at the plant, an approximately 20 gram sample was taken from the longissimus dorsi and at 1 hour and at 24 hours postmortem, frozen in liquid nitrogen and taken to Iowa State University for the analyses listed in the specific objectives below. The carcasses were fabricated at approximately 24 hours postmortem and the loins and hams were transported to the University of Missouri/Columbia. Hunter L*, a* and b* values were recorded on the cut surfaces of the longissimus dorsi. In addition, an experienced panel recorded NPPC scores for loin color, marbling and firmness. Chops were removed for Warner-Bratzler shear-force evaluation. Chops for shear-force evaluation were aged for 2 and 21 days postmortem. One-inch boneless, closely trimmed chops from the longissimus dorsi were removed, weighed and over wrapped in oxygen-permeable over wrap and stored under simulated retail display for 24 hours, 96 hours and 7 days. Drip-loss and Hunter color scores were evaluated at each of the aforementioned time points. The summary of the quality data appears in NPB Final report #01-045 (Supplementation of swine finishing rations with a unique antioxidant (alpha-lipoic acid) to improve fresh pork quality and shelf stability – Eric Berg – Principal Investigator). After the prescribed storage period, the chops were vacuum-packaged, frozen and sent to Iowa State University for the analyses necessary to accomplish the three objectives outlined in this proposal.

Objective 1. **Determine the extent of the impact of tissue oxidation and subsequent postmortem modifications of specific proteins involved in regulating intracellular calcium levels (ryanodine receptor/calcium release channel and the sarcoplasmic reticulum Ca^{2+}-ATPase pump) on drip loss.**

Experimental Methods: The relative amount of oxidation that had occurred in the tissues was evaluated by quantifying the amount of the lipid oxidation products malondialdehyde(MDA) and 4-hydroxy-2 (E)-noneal (HNE) in samples taken at 1 hour postmortem (Lipid Peroxidation Assay Kit., Calbiochem, San Diego, CA)

Objective 2. Ascertain the relationship between tissue oxidation, desmin degradation and water-holding capacity.

Tissue oxidation evaluation was done as outlined in objective 1. Because μ-calpain has been shown to degrade desmin, autolysis of μ-calpain was evaluated by using immunoblotting techniques (Boehm et al., 1998) in all samples. In addition degradation of desmin, was determined in all samples. Protein degradation was determined using SDS-PAGE and immunoblotting techniques (Huff-Lonergan et al., 1996). These results were compared against the pH decline and drip loss and shear force values.

Objective 3. Determine the relationship between lipoic acid supplementation, the storage form of glycogen and drip loss.

Relative amounts of macroglycogen and proglycogen were determined on 2-hour and at 24-hour longissimus (loin) samples from all dietary treatment groups (Huang et al., 1997). These results were compared to the pH/temperature decline data and the drip loss data.

VI. Results

Objective 1. **Determine the extent of the impact of tissue oxidation and subsequent postmortem modifications of specific proteins involved in regulating intracellular calcium levels (ryanodine receptor/calcium release channel and the sarcoplasmic reticulum Ca^{2+}-ATPase pump) on drip loss.**

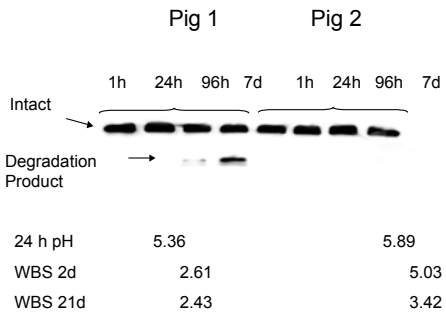
Objective 2. Ascertain the relationship between tissue oxidation, desmin degradation and water-holding capacity.

The results of both objectives 1 and 2 will be discussed together in this section.

In this study we examined the relationship between the degradation of the proteins involved in controlling calcium concentration (sarcoplasmic reticulum Ca^{2+}- ATPase pump (SERCA-1) and the ryanodine receptor, RYR-1), an important structural protein (desmin) and a key protease (calpain), and the meat quality attributes of ultimate pH, the shear force and the amount of early postmortem oxidation.

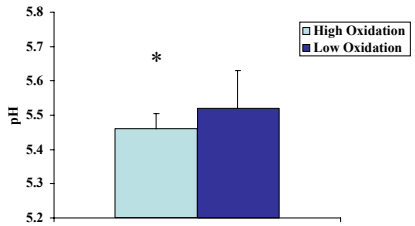
SERCA –1 Results involving proteins that in regulate calcium concentration were particularly interesting. The two proteins (RYR-1 and SERCA), work in concert to play a major role in regulating contraction and maybe more importantly, may play a role in regulating early postmortem metabolism. Impaired function of one or both of these proteins immediately prior to slaughter or early after slaughter could allow calcium levels to rise more rapidly, thus increasing the rate of metabolism and ultimately the rate of pH decline. Both of these proteins (SERCA-1 and RYR-1) are susceptible to being oxidized and are both calpain substrates. Each

Figure 1. Relationship between SERCA-1 Degradation and pH and Shear Force at 2 and 21 Days Postmortem



of these features is relevant to this study. Interestingly, products that had more degradation of SERCA-1 at 96 hours and 7 days postmortem were also from carcasses that had lower ultimate pH values (Figure 1). This is opposite of the expected trend if degradation of this protein is only indicative of overall protein degradation that occurs in the tissue. In general, products with high ultimate pH values tend to show greater protein degradation. This increase in degradation of the SERCA noted in the low ultimate pH samples was unexpected and could indicate a structural/functional difference in this protein from low pH loins compared to SERCA-1 proteins from higher pH loins. In addition to allowing it to be more susceptible to degradation, this "difference" could be related to the greater pH decline. Also, it has been noted that oxidation can inhibit the activity of SERCA pump proteins (Klebl et al., 1998). Inhibition of SERCA activity very early postmortem could potentially allow calcium concentrations to rise more rapidly and thus could cause attainment of ultimate pH more quickly. Indeed, in this study, loins with high levels of oxidation had lower pH values (24 hours) (Figure 2). Another factor to consider is that oxidation of proteins often renders them more susceptible to degradation by endogenous enzymes. If the SERCA-1 proteins were more oxidized this could potentially result in both the lower pH values and greater degree of degradation that was seen in this study. These results are significant because they indicate a protein and/or pathway that could be a potential target to elucidate the mechanism underlying the development of low pH product and may indicate that inclusion of endogenous antioxidants may aid in protecting pork quality.

Figure 2. Relationship Between Level of Oxidation and Ultimate pH



*P < 0.05

RYR-1 In general, there was less degradation of the ryanodine receptor at 24 hours in those samples that had low ultimate pH and high shear force compared to those samples that had a moderate ultimate pH (5.5-5.6) and lower shear force (Figure 3). However, at later times postmortem (96 hours) the degradation was more extensive in the samples from loins with lower pH values (pig 3892, 96 hours, Figure 3) than in the loins with moderate ultimate pH values (pig 3814, 96 hours Figure 3). Again, as was the case with the SERCA, this could possibly indicate some structural or post-translational modification of the protein. In addition, RYR-1 is a calpain substrate and it is known that oxidation of the calpains can inhibit their activity (Rowe et al., 2003). One of the primary RYR-1 degradation products produced by calpain migrates at approximately 150 kDa(Gilchrist et al., 1992). Indeed, when specifically examining the presence of the 150 kDa degradation product of RYR-1 at 96 hours postmortem it was seen that samples from loins that had low levels of oxidation had greater amounts of the 150 kDa degradation product of RYR-1 (Figure 4), possibly indicating greater degradation by calpain. When the autolysis of calpain was examined it was noted that there was more rapid autolysis of μ-calpain in product from samples that had a moderate ultimate pH and less oxidation (Figure 5). This corresponded to those samples that had more RYR-1 degradation at 96 hours. Again, this follows previous research from our lab and others that indicated greater levels of oxidation are associated not only with less activity of μ-calpain, but also with slower rates of calpain autolysis (Guttmann et al., 1997; Rowe et al., 2003).

Figure 3. Relationship Between Ryanodine Receptor Degradation, μ-Calpain Autolysis , Shear Force and pH

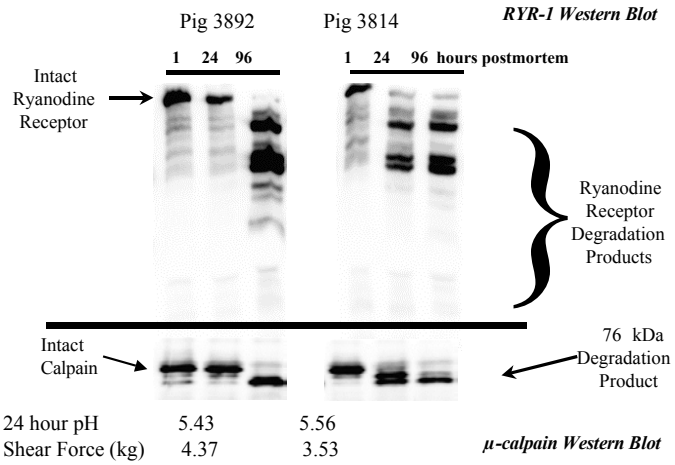


Figure 4. Relationship Between Degradation of the Ryanodine Receptor and Measures of Oxidation (Malondialdehyde = MDA)

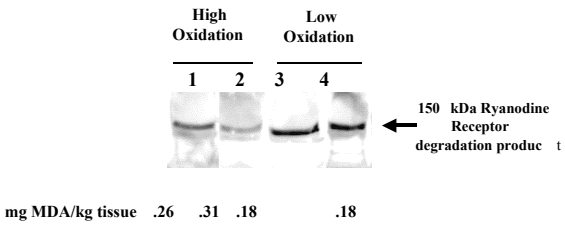
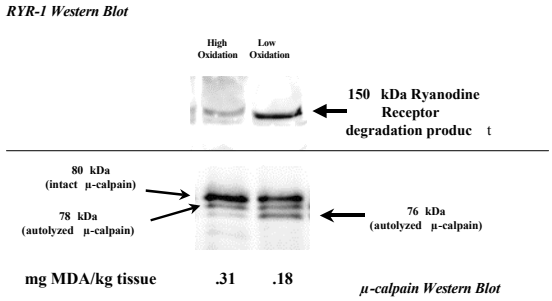


Figure 5. Autolysis of μ-calpain is related to lipid oxidation and RYR-1 degradation



Desmin Degradation of the intermediate filament protein desmin was correlated to lower shear force values. This is in agreement with our previous work and the work of others (Dodge et al., 2001).

Objective 3. Determine the relationship between lipoic acid supplementation, the storage form of glycogen and drip loss.

In this study, the mode of delivery of lipoic acid did not seem to be adequate to elicit a response in the tissue. There were no significant differences between any of the lipoic acid treatment groups for the amount of glycogen in the muscle at 2 hours or at 24 hours postmortem ($P > 0.05$). In addition, because there was little variation in the amount of glycogen at either time point, there were no significant correlations between glycogen levels and pH/temperature decline and/or drip loss in these samples.

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VII. Discussion

Oxidation of key proteins in the muscle cell during the early postmortem period does appear to play a key role in the quality of the product. Therefore, preventing or delaying oxidation in muscle tissue is important in maintaining the quality of the product. One compound that has potential to prevent oxidation in the tissue is lipoic acid. This compound is very interesting as it may have the ability to prevent oxidation in both the water-soluble fraction of the muscle as well as in the cell membrane. In this study, with the doses and means of delivery used we did not see an effect of lipoic acid on meat quality. However, we did note normally occurring differences in levels of oxidation (unrelated to lipoic acid treatment) that were related to differences in some quality attributes as well as the degradation of key proteins. Products that had lower levels of oxidation also had significantly higher ultimate pH values. The level of oxidation was also related to the degradation of proteins that regulate calcium levels in muscle. Since free calcium level in muscle is a driver of muscle metabolism and oxidation can cause deregulation of these proteins, oxidation of these proteins may influence pH decline in early postmortem meat. Therefore, it is possible that by reducing the level of postmortem oxidation in muscle and early postmortem meat, producers and processors may be able to improve some aspects of pork quality. This study points to the need to continue to investigate the use of antioxidant compounds in pig diets that will slow the oxidation that occurs in early postmortem muscle.

VII. Lay Interpretation

In general, products that had a high level of oxidation had lower ultimate pH values than did products with relatively lower levels of oxidation There appeared to be an interaction between the level of early postmortem meat oxidation measurements and the degradation of key proteins involved in calcium regulation. Together these data indicate that oxidation that occurs in fresh meat early postmortem can have a significant effect on quality attributes. Therefore, it is reasonable to continue to pursue research on antioxidants that may be fed to pigs as an avenue to improve pork quality.

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