RESEARCH REPORT
PORK QUALITY

Title: Relationship between glycolytic oscillations and pork color and water-holding capacity - **NPB#02-082**

Investigator: Matthew E. Doumit

Co-Investigators: Nicholas L. Berry, Emily E. Helman, Chuck P. Allison

Institution: Michigan State University

Date Received: March 2, 2004

Abstract: Our objective was to determine the relationships between pork loin color, water-holding capacity and 1) the pattern of glycolysis dictated by characteristics of soluble muscle proteins, and 2) the influence of glycolytic metabolites on the pattern of glycolysis. Characteristics of superior (n=6) and inferior (n=6) quality loins, respectively, were as follows: 45 min pH (6.40 ± 0.06 vs 5.92 ± 0.10), percent fluid loss after centrifugation (10.22 ± 0.47 vs 20.70 ± 0.32), percent drip-loss by the suspension method (0.66 ± 0.08 vs 3.23 ± 0.45), and day 1 L* (51.37 ± 0.66 vs 56.68 ± 0.86). Longissimus muscle samples were obtained at 20 min postmortem and were immediately frozen in liquid nitrogen and stored at -80° C until analysis. Soluble muscle proteins were extracted, gel-filtered and used to determine *in vitro* patterns of glycolysis. Aliquots of glycolytic reaction mixture were removed every 2 min for 46 min and acidified to halt enzyme activity. Enzymatic assays were used to quantify concentrations of ADP, ATP, and lactate. An oscillatory pattern of glycolysis was observed using extracts from both superior and inferior quality pork. No differences in the average ATP:ADP ratio or the overall mean concentrations of lactate or adenine nucleotides were observed in reactions using extracts from superior and inferior quality samples ($P > 0.05$). Thus, sarcoplasmic protein extracts do not appear to produce distinct patterns of glycolysis that are associated with differences in pork quality. However, the rate of glycolysis in this system increased with addition of myofibrils (ATPase) and decreased with addition of citrate. This system will permit identification of specific biochemicals that cause differences in the pattern of postmortem glycolysis and muscle acidification.

Introduction: The National Pork Benchmarking Audit found that live weight and the percentage of carcass muscle have increased, while backfat thickness has decreased over the last ten years (Stetzer and McKeith, 2003). Unfortunately, the incidence of pale, soft and exudative (PSE) pork has also increased during that time (Stetzer and McKeith, 2003). Despite an abundance of research describing PSE pork characteristics, and a reduction in the frequency of major gene polymorphisms with known deleterious effects on pork quality, Cassens (2000) concluded that little progress has been made in reducing the incidence of PSE pork.

Understanding events that dictate superior and inferior pork quality is essential for development of new strategies to improve the quality and consistency of pork. Briskey et al. (1966) showed that the rate of postmortem glycolysis is variable in pig muscle. Anaerobic glycolysis increases muscle lactate concentration and hydrogen ion concentration (lowers pH). Pale, soft and exudative pork is caused by the denaturation of muscle proteins that result when carcass muscles experience a low pH and high temperature (Briskey and Wismer-Pedersen, 1961). However, low early postmortem pH does not explain all PSE seen in pig muscle. Pork with an apparently normal pH decline may exhibit pale and/or soft and exudative characteristics. Observed inconsistencies in pH decline within a muscle may suggest differences in the pattern of pH decline among muscles or carcasses. Oscillatory behavior of the glycolytic pathway *in vitro* has been well documented (Tornheim and Lowenstein, 1973; 1974; Tornheim et al., 1991).

It is not known if postmortem skeletal muscle exhibits glycolytic oscillations and associated bursts of acidification. Oscillatory glycolysis could account for apparent inconsistencies in pH measurements as well as tissue metabolite data. Bursts of acidification resulting from early postmortem glycolytic oscillations would likely cause more extensive protein denaturation than a gradual pH decline. Thus, glycolytic oscillations could be important determinants of pork color and water-holding capacity. We hypothesize that inferior pork water-holding capacity and color are associated with more pronounced glycolytic oscillations, which exacerbate protein denaturation in pork loin muscle.

Objectives: The objective is to determine the relationships between pork loin color, water-holding capacity and 1) the pattern of glycolysis dictated by characteristics of soluble muscle proteins, and 2) the influence of glycolytic metabolites on the pattern of glycolysis. This study will determine the potential impact that oscillations in glycolysis and pH decline have on pork quality, and will also identify enzyme and metabolite regulators of glycolytic oscillations.

Materials & Methods:

Animal and Meat Quality Data Collection

Sires from Duroc and HAL-1843-free Pietrain lines were mated to Yorkshire and F1 Yorkshire-Landrace gilts. Crossbred progeny were raised in uniform conditions at the Michigan State University (MSU) Swine Teaching and Research Farm. Four gilts from each sire group were harvested on each of four days within a 2-week period at the MSU Meat Laboratory. Muscle tissue and pork quality data were collected as previously described by Allison et al. (2003). Based on differences in 45 min pH, CWHC, DRIP1, and L*, samples from three Duroc- and three Pietrain-sired pigs with superior meat quality traits, and from three Duroc- and three Pietrain-sired pigs with inferior meat quality traits were selected. Pigs that were most extreme in their quality differences were used in the current experiment.

Preparation of muscle protein extracts

Muscle protein extracts were obtained using methods adapted from those described for rat muscle by Tornheim et al. (1991). Frozen LM samples (~7 g) were homogenized in 3 volumes of cold homogenization buffer (90 mM potassium phosphate, pH 6.5, and 180 mM potassium chloride). Homogenization was performed using a Polytron (Brinkman, Westbury, NY) for 3 x 30-sec bursts (setting 4), with 15 sec between bursts. Samples were continuously cooled with ice during homogenization. The homogenate was stirred slowly at 4° C for 1 hour, and then centrifuged at 4° C (SS 34 rotor of a Sorvall RC2-B centrifuge) at 31,000 x g for 10 min to remove insoluble material. A fat cap was carefully removed from the surface with a small spatula and supernatant fluid was decanted and filtered through cheesecloth to remove particulate matter that was not pelleted. The supernatant fluid was then centrifuged at 85,000 x **g** for 30 minutes at 4° C to remove the membrane fraction. The resulting supernatant fluid obtained was adjusted to contain 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM dithiothreitol. Then 7 mL of supernatant were placed on a column (Bio-Rad Column 2.5 cm ID x 14 cm length, cat. No. 738-0017) packed with Sephadex G-25 resin (fine; Amersham Pharmacia). Sephadex beads were prepared by soaking in homogenization buffer. Beads were stirred hourly for 3 h, and allowed to swell for a total of 12 h prior to being transferred to the column. The column was flushed with 0.5 L of homogenization buffer to ensure that all Sephadex beads were properly packed and equilibrated prior to sample loading. Protein was eluted from the column with a buffer containing 15 mM potassium phosphate, pH 6.5, 280 mM potassium chloride, 5 mM EDTA, and 0.1 mM dithiothreitol at a rate of 2 mL per minute and collected in 0.5 mL fractions. A Bio-Rad Biologic LP Chromatography System (Cat. No. 731-8300) and Bio-Rad Model 2128 fraction collector were used for gel filtration and collection of proteins. Protein concentrations of gel-filtered samples were determined using a commercial kit (BioRad Protein Assay Kit, BioRad Laboratories, Hercules, CA) based on the method of Bradford (1976). Bovine serum albumin was used as a protein standard. Samples with the highest protein concentrations were pooled and frozen in 1 mL aliquots and stored at -80° C for later use. Care was taken to remove all filtrate from the column by flushing the system with an additional 0.5 L of elution buffer, as well as 0.5 L of de-ionized distilled H_2O. The column was prepared for the next sample filtration by flushing with 0.5 L of homogenization buffer.

Glycolytic Reaction Conditions

Gel-filtered samples containing muscle protein were exposed to a reaction mixture to initiate glycolysis as described by Tornheim et al. (1991) with minor modifications. The reaction mixture was made in a 10X stock (20 mL), and the final reaction was performed in a volume of 2 mL in a polypropylene microcentrifuge tube that contained 4 mM aspartate, 10 mM glucose, 7.5 mM orthophosphate, 25 mM imidazole HCl, pH 6.9, 8.3 mM magnesium chloride, 0.2 mM calcium chloride, and 1 mM ATP, 0.3 mM GTP, and 0.1 mM NAD, 0.25 mg/mL crude myofibrils, and 1 mg/ml gel-filtered protein. The reaction was started by adding 33.6 µL of a stock yeast hexokinase (5.0 µl of hexokinase in 495 µL of de-ionized distilled H_2O, Sigma cat. No. H-5625) to the 2 mL volume, which provided 0.06 units/mL hexokinase activity in the reaction. Tubes containing reaction mixture were then inverted 3 times to mix the contents. The reaction mixture was maintained in a water bath at 30° C. Samples of 75 µl were removed every 2 minutes from 0 to 46 minutes and placed in 75 µL of 2 N perchloric acid to deproteinize the sample. Upon completion of the experiment, acidified samples were centrifuged at 1,500 x **g** for 10 minutes in an Eppendorf 5415 centrifuge. Following centrifugation, perchloric acid in samples was neutralized with 30 uL of 5.4 N KOH. The samples were then re-centrifuged at 1,500 x **g** for 10 minutes.

Supernatant fluid was kept on ice and assays were performed within 2 hours. Unless otherwise indicated, data represent results of an individual experiment.

ATP, ADP and Lactate Quantification

Adenosine triphosphate (ATP) was quantified using the enzymatic method of Lamprecht and Trautschold (1972), with the exception that the assay was adapted to a 96-well microtiter plate. The ATP assay reagent consisted of 50 mM triethanolamine buffer, 10 mM NAD, 0.1 M magnesium chloride, and 0.5 M glucose. The total reaction volume was 230 µL and the quantity of ATP was determined by following the conversion of NAD^+ to NADH at 340 nm using a VERSAmax plate reader (Molecular Devices, Sunnyvale, CA).

Assays were performed in duplicate with 10 µL of sample from the deproteinized reaction mixture followed by 200 µL of assay reagent. An initial absorbance (A1) at 340 nm was recorded. A stock of glucose-6-phosphate dehydrogenase was prepared in H_2O, and 10 µL (0.46 U/mL in final reaction mixture) was added to each well to start the reaction. The contents of each well were mixed and incubated at 37°C for 15 minutes, and a second A_{340} reading (A2) was taken. This was a modification of the original protocol. It was necessary to run the initial reaction to completion to avoid confounding effects of residual G-6-P produced in the glycolytic reaction. A stock of hexokinase (Sigma cat. No. H-4502) was also prepared and 10 µL (1.8 U/mL in final reaction mixture) was added to each well. The contents of each well were mixed and incubated at 37°C for 40 minutes. Upon completion of the reaction the absorbance at A_{340} (A3) was again recorded and the change in absorbance (A3-A2) was used for ATP determination. Fresh ATP standards (1 mM, 0.5 mM, 0.25 mM, 0.125 mM, and 0 mM) were prepared daily, and ATP concentrations of sample unknowns were calculated based on the standard curve. Final concentrations of ATP were multiplied by a dilution factor of 2.4 to account for dilution of sample during acidification and neutralization.

The enzymatic determination of adenosine diphosphate (ADP) also involved the utilization of methods from Lamprecht and Trautschold (1972). The assay involved an initial creatine kinase reaction to quantitatively convert ADP to ATP. The concentration of ADP was determined as the difference between the ATP concentration measured before and after conversion of ADP to ATP. Final concentrations were derived as previously stated for ATP. Lactate accumulation was measured in samples collected from the glycolytic reaction procedure using methods from Sigma (Procedure No. 826-UV).

Results:

Patterns of glycolysis derived using soluble proteins from superior and inferior quality pork.

An *in vitro* glycolytic system was used to determine if muscle protein extracts from pigs that produced superior and inferior quality pork exhibit different patterns of glycolysis. Loins from pigs designated superior quality had higher 45 min pH, lower fluid loss in samples subjected to centrifugal force, lower drip loss, and lower Day 1 L* values than loins from pigs designated inferior in quality (Table 1).

Supernatant from each longissimus muscle extract was gel-filtered and this extract was used to determine the pattern of *in vitro* glycolysis for each pig. The largest fluctuation in adenine nucleotide concentration occurred from 0 to 20 min following the initiation of the glycolytic reaction in samples of most pigs. Thus, samples were classified as either oscillatory or non-oscillatory based on their pattern of energy generation during the first 20 min of the reaction. Figure 1 depicts a non-oscillatory pattern of glycolysis. The rate of glycolysis in this sample appears to parallel the rate of ATP utilization, such that the ATP concentration deviates little, while lactate steadily

accumulates. This pattern of glycolysis was hypothesized to be associated with superior quality pigs, but was exhibited by both superior and inferior quality pigs. In contrast, figure 2 depicts an oscillatory pattern of glycolysis. This oscillatory pattern was hypothesized to occur in inferior quality pigs and be associated with localized acidification that may exacerbate protein denaturation and increase fluid loss from product. However, the oscillatory pattern was observed in extracts from both inferior and superior quality pigs.

As expected, ATP and ADP concentrations were inversely related to each other in most samples, regardless of quality designation. There were no differences in the average ATP/ADP ratio or the overall mean concentrations of lactate or adenine nucleotides in reactions using extracts from either superior or inferior quality loin samples (Table 2). Additionally, sire breed had no effect on the concentrations of adenine nucleotides or lactate. Our data suggest that protein extracts from muscle yielding superior and inferior quality pork do not produce distinct patterns of glycolysis that explain differences in pork loin quality. Regulation of the glycolytic pattern may be attributed to differences in metabolic effectors or the rate of energy utilization during postmortem glycolysis.

Influence of added myofibrils (ATPase) or metabolites on the rate or pattern of glycolysis.

The rate of ATP utilization is positively associated with the rate of pH decline. Species with a higher proportion of white muscle fibers, such as pigs and poultry, undergo more rapid postmortem ATP utilization and glycolysis than red muscle species (i.e. beef and lamb) due to more active myosin ATPase and more abundant SR calcium ATPase. Increased ATPase activity may reduce the ATP/ADP ratio and induce production of glycolytic "burst" activity. To determine the effect of added ATPase from a myofibrillar source on the pattern of glycolysis, protein extracts from a pig of each quality type (1 mg/mL of gel-filtered protein) were used in combination with 0, 0.25, and 0.50 mg/mL of myofibrils. Although the added ATPase caused increased lactate accumulation over the reaction period of 0 to 46 min (Fig. 3), the pattern of glycolysis was not affected by the addition of myofibrils.

Effects of metabolite concentrations on the pattern of glycolysis were also determined. Muscle extracts were exposed to reaction mixtures that reflect the "at death" profile of glycolytic intermediates of fast- and slow-glycolyzing pork loin muscles reported by Kastenschmidt et al. (1968). These conditions resulted in comparable rates of lactate accumulation. Although citrate is not a glycolytic intermediate, it is known to be a potent physiological inhibitor of phosphofructokinase (PFK), which is generally regarded as the primary regulatory enzyme of glycolysis. Tornheim et al. (1991) showed that addition of citrate to extracts of rat hind limb muscle decreased the frequency of the oscillations and delayed the first burst of PFK activity. When 0.15 mM citrate was included in the reaction mixture containing pig muscle extract, the rate of lactate accumulation was decreased (Figure 4). This demonstrates that the *in vitro* system used is responsive to a known physiological regulator of glycolysis.

Discussion: Pig muscle extracts produce glycolysis with an oscillatory behavior, but the patterns of glycolysis obtained using extracts from superior and inferior pork were similar. Pronounced oscillations occurred in the first 20 min after initiation of glycolysis of some samples. Bursts of glycolysis would be expected to result in a more acidic local environment. If this occurred in muscle, one would expect less desirable pork color and WHC due to increased denaturation of protein at the major sites of energy utilization (myosin ATPase and calcium ATPase), to which glycolytic enzymes are functionally

coupled. However, if patterns of glycolysis differ between postmortem muscles that exhibit variation in meat quality, these patterns were not mimicked by extracts containing glycolytic enzymes from those muscles.

All samples exhibited an oscillatory pattern ($\geq$2 oscillations) of glycolysis from 20 to 46 min, but the amplitude of these oscillations was lower than those observed from 0 to 20 min. It appears that reduced amplitude of oscillations occurring from 20 to 46 min may be a result of the system losing finite control over glycolysis due to low pH. At the onset of the reaction, the pH is near neutral, but as the reaction proceeds, the pH decreases to ~ 5.7, the rate of glycolysis slows, ATP production decreases, and lactate accumulates at a reduced rate. The more subtle oscillations observed at lower pH would be expected to have less adverse effects on pork quality than more pronounced oscillations, which would be predicted to occur earlier postmertem and at higher muscle temperatures.

Although *in vitro* glycolytic patterns observed in this study were not associated the quality of pork from which protein extracts were derived, the system used was responsive to known regulators of glycolysis. This system should provide a useful tool to identify biochemicals that regulate postmortem glycolytic rate and thereby influence pork quality.

References

Allison, C.P., R.O. Bates, A.M. Booren, and M.E. Doumit. 2003. Pork quality variation is not explained by glycolytic enzyme capacity. Meat Sci. 63:7-22.

Briskey, E.J., L.L. Kastenschmidt, J.C. Forrest, G.R. Beecher, M.D. Judge, R.G. Cassens, and W.G. Hoekstra. 1966. Biochemical aspects of post-mortem changes in porcine muscle. J. Agric. Food Chem. 14:201-207.

Briskey, E.J. and J. Wismer-Pedersen. 1961. Biochemistry of pork muscle structure. II. Preliminary observations of biopsy samples versus ultimate muscle structure. J. Food Sci. 26:306-313.

Cassens, R.G. 2000. Historical perspectives and current aspects of pork meat quality in the USA. Food Chem. 69:357-363.

Kastenschmidt, L.L., W.G. Hoekstra, and E.J. Briskey. 1968. Glycolytic intermediates and co-factors in “fast-” and “slow-glycolyzing” muscles of the pig. J. Food Sci. 33:151-158.

Lamprecht, W., P. Stein, F. Heinz, and H. Weisser. 1974. In “Methods of Enzymatic Analysis” Vol. 4, p.1781. Academic Press, Inc., New York, NY.

Stetzer, A.J., F.K. McKeith. Quantitative strategies and opportunities to improve quality. Final Report, Benchmarking Value in the Pork Supply Chain. AMSA, Savoy, IL. 2003. P1-6.

Tornheim, K. and J.M. Lowenstein. 1973. Oscillations in metabolite concentrations during the operation of the cycle in muscle extracts. J. Biol. Chem. 248:2670-2677.

Tornheim, K. and J.M. Lowenstein. 1974. Interactions with oscillations of the glycolytic pathway in muscle extracts. J. Biol. Chem. 249:3241-3247.

Tornheim, K., V. Andrés, and V. Schultz. 1991. Modulation by citrate of glycolytic oscillations in skeletal muscle extracts. J. Biol. Chem. 266(24):15675-15678.

Lay Interpretation: A system was developed to monitor *in vitro* glycolysis catalyzed by pig skeletal muscle proteins. Oscillatory and non-oscillatory patterns of *in vitro* glycolysis were observed using muscle extracts from both superior and inferior quality pork, but no differences between extracts from superior and inferior quality pork were observed. This *in vitro* system was responsive to known regulators of glycolysis and will be a useful tool for identifying specific biochemicals that cause differences in the pattern and rate of glycolysis, and subsequently influence postmortem pH decline and pork quality.

Contact Information:
Dr. Matthew E. Doumit
Associate Professor
Departments of Animal Science and Food Science & Human Nutrition
3385B Anthony Hall
Michigan State University
East Lansing, MI 48824
Phone: (517) 355-8452 ext. 203 Fax: (517) 432-0753
e-mail: doumitm@msu.edu

Table 1. Means and standard deviations for loin muscle quality of superior and inferior pigs[a]

	Superior Quality		Inferior Quality	
	Mean	Stdev	Mean	Stdev
45 min pH	6.40	0.14	5.92	0.24
CWHC, %	10.22	1.15	20.70	0.78
Drip 1, %	0.66	0.19	3.23	1.10
Day 1 L*	51.37	1.61	56.68	2.11

[a] n = 6 pigs in each quality category (superior and inferior).

Table 2. Mean concentrations of adenine nucleotides and lactate observed in glycolytic reactions from 0 to 46 min using protein extracts from either superior or inferior quality pork.[a,b]

	Superior Quality		Inferior Quality	
	Mean	Stdev	Mean	Stdev
ATP, mM	0.539	0.256	0.610	0.268
ADP, mM	0.216	0.137	0.192	0.120
ATP/ADP	4.506	3.390	5.361	3.239
Lactate	1.707	0.790	1.673	0.745

[a] n = 6 pigs in each quality category (superior and inferior).
[b] No differences were observed in reactions using extracts from superior and inferior quality samples ($P > 0.05$).

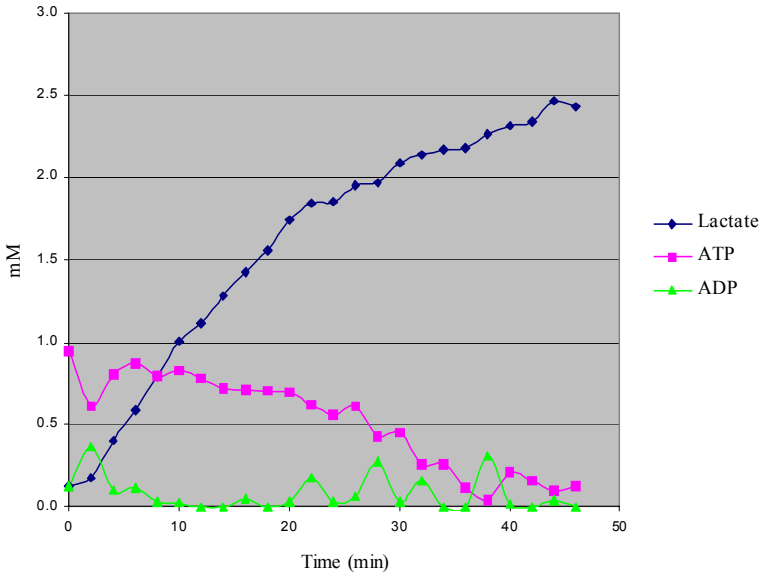

Figure 1. Adenine nucleotide (ADP and ATP) and lactate concentrations of a superior quality Pietrain pig exhibiting non-oscillatory glycolytic behavior from 0 to 20 min after initiation of glycolytic reaction.

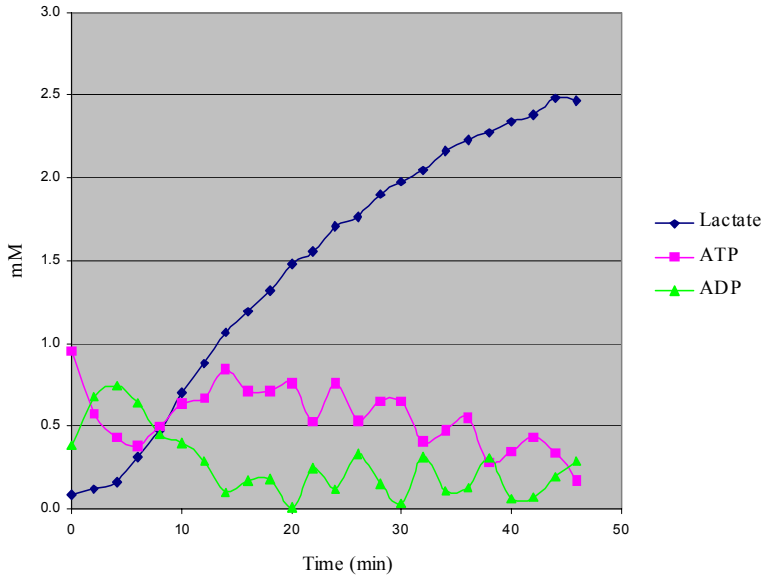

Figure 2. Adenine nucleotide (ADP and ATP) and lactate concentrations of a superior quality Pietrain pig exhibiting an oscillatory pattern of glycolytic behavior from 0 to 46 min after initiation of glycolytic reaction.

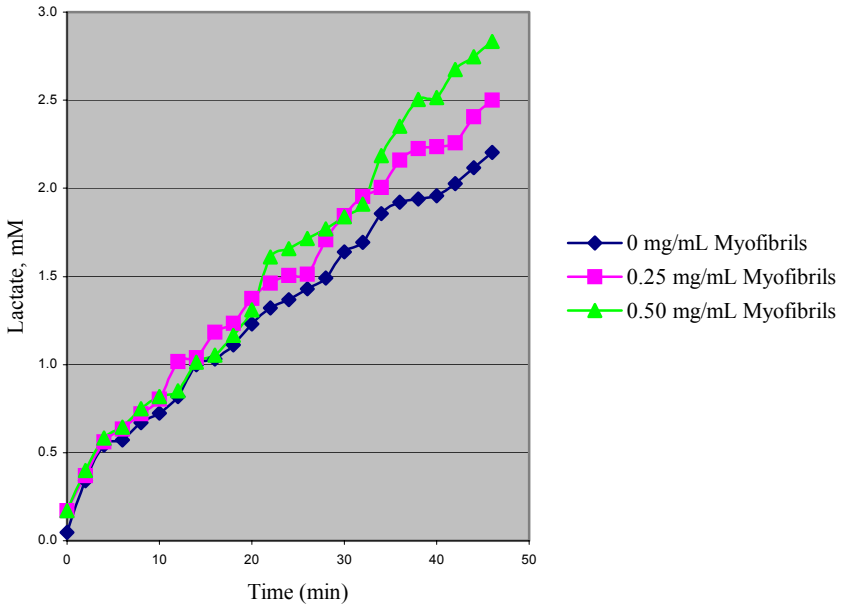

Figure 3. Lactate accumulation in glycolytic reaction mixture is increased with addition of myofibrils.

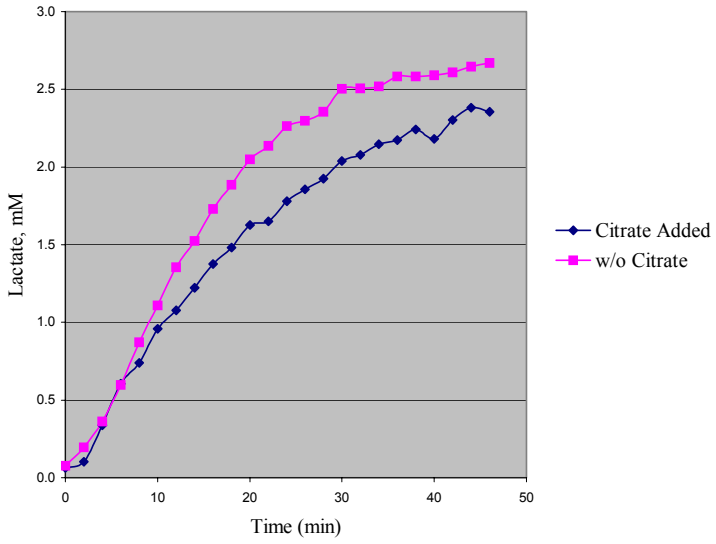

Figure 4. Lactate accumulation in glycolytic reaction mixture is decreased by addition of 0.15 mM citrate.