RESEARCHREPORT

pork checkoff

PORK QUALITY

Title: Characterization of Muscle Glycogen Storage and Utilization: Influence on Pork Quality **– NPB# 99-059**

Investigator: Steven M. Lonergan, Ph.D.

Institution: Iowa State University

Co-Investigator: Elisabeth Huff-Lonergan, Ph.D.

Date Received: 3/12/2001

I. ABSTRACT

Production of inferior quality pork has become a significant impediment to the competitiveness of the pork production industry in the United States. For several decades the pork industry has employed genetic selection strategies to make considerable improvements in efficiency of lean meat production. However, it has become increasingly evident that such advances have had costly side effect, especially regarding water holding capacity, color and texture of fresh pork. It is currently understood that variation in these traits can be significantly influenced by the muscle metabolism in the early postmortem period. One of the major changes in postmortem muscle is a mobilization of energy stored within muscle. This energy is mobilized from long chains of glucose molecules (glycogen) and converted to ATP by the glycolytic pathway. In the absence of oxygen, a by-product of the glycolytic pathway – lactic acid – begins to build up in muscle cells and causes a decline in pH. A typical pH decline is from a physiological 7.1 to approximately 5.7. The extent of lactic acid production and concomitant decline in pH depends primarily upon the glycogen content in the muscle at the time of slaughter.

Recently, a protein that serves as a primer and stabilizer of glycogen during glycogen synthesis - glycogenin- has been identified in muscle. The amount of glycogenin has been hypothesized to dictate the amount of glycogen in muscle. Our objective was to determine the existence of glycogenin in porcine muscle and ascertain the influence this protein had on glycogen content and ultimate pH in pork. A polyclonal antibody specific for glycogenin was utilized to identify the existence of this protein in porcine muscle. Pork loin samples varying in ultimate pH were used to determine the influence of the amount of glycogenin on ultimate pH. Our results demonstrate that pork with a greater abundance of glycogenin had a lower ultimate pH. This result is significant because variation in expression of this protein may explain some of variation in ultimate pH in pork. These results suggest that developing a more thorough understanding of glycogenin and glycogen metabolism in living muscle and in early postmortem muscle will aid our efforts to improve overall quality of fresh pork.

II. INTRODUCTION

Variation in fresh pork color, texture, water holding capacity and tenderness continues to be a significant concern to the pork industry. One major process that impacts quality is the conversion of muscle to meat during the early postmortem period. Throughout this process, muscle cells attempt to produce sufficient energy to maintain normal cellular functions. This energy is mobilized from long chains of glucose molecules (glycogen) and converted to ATP by the glycolytic pathway. In the absence of oxygen, a by-product of the glycolytic pathway – lactic acid – begins to build up in muscle cells and causes a decline in pH. A typical pH decline is from a physiological 7.1 to approximately 5.7. The extent of lactic acid production and concomitant decline in pH depends primarily upon the glycogen content in the muscle at the time of slaughter.

Variations in glycolytic process can influence the rate and extent of pH decline. For example, in the well-characterized porcine stress syndrome (associated with the halothane gene), a rapid decline in pH during the first 30 minutes postmortem is extremely detrimental to pork quality. The combination of a low pH and high temperature in the early postmortem period leads to pale, soft, and exudative (PSE) pork (reviewed by Christian, 1997). In cases where very little glycogen is available, the extent of pH decline is minimal (pH 6.5-6.8). The consequence of this limited pH decline is a dark firm and dry (DFD) product. Low ultimate pH (pH < 5.5) can impact texture, water holding capacity and tenderness. In general, low ultimate pH is associated with less firm texture and diminished water holding capacity. This condition has most recently been associated with the RN (Rendement Napole) gene (Le Roy et al., 1990). The effects of dominant allele for the RN gene on pork quality have been well characterized (reviewed by McKeith, 1998). Briefly, these effects include higher glycolytic potential, a lower ultimate pH, lower water holding capacity, and lower processing yields when compared to pork from pigs with normal genotypes.

While there has been significant focus on the factors that impact pH decline in the early postmortem period (halothane genotype, RN genotypes), relatively little attention has been given to the possibility that variations in postmortem glycogen utilization may be due to the state of glycogen itself. Recent advances in glycogen synthesis and storage research offer new avenues through which to study glycogen metabolism in muscle. These advances include the discovery of the enzyme that is essential for initiation of glycogen synthesis – glycogenin, and the identification of a low molecular mass form of glycogen – proglycogen (Alonso et al., 1995a).

Initiation of glycogen synthesis is catalyzed by glycogenin and involves forming a covalent bond between glycogenin and glucose. Glucose bound to glycogenin can then be used to synthesize long strands of glucose molecule to form proglycogen (Alonso et al., 1995b). Proglycogen serves as a stable intermediate on pathways to form macroglycogen. Macroglycogen still contains the glycogenin enzyme. The liberation of energy from glycogen requires the whole system to go in reverse from macroglycogen to glycogenin through proglycogen. Variations in expression of different forms of glycogenin impact the ability of muscle to store energy in the form of glycogen (Lomako et al., 1995). In fact, genetic abnormalities in the glycogenin gene have been associated with symptoms of non-insulin dependent diabetes (Bailey et al., 1995).

Proglycogen and macroglycogen both contain glycogenin, but differ in the amount of associated carbohydrate. Proglycogen has a molecular mass of 400,000 Da, whereas macroglycogen can reach a molecular mass of 10^7 Da. Recent studies have demonstrated that proglycogen and macroglycogen are metabolically unique as they

differ in rates of degradation and synthesis and in their sensitivities to dietary manipulation in humans (Adamo and Graham, 1998; Adamo et al., 1998).

These new developments in glycogen metabolism research raise questions regarding the conversion of muscle to meat. The amount of glycogenin expressed in a cell will influence how much glycogen the cell can synthesize and store. Therefore, it may impact meat quality by influencing postmortem lactic acid production and pH decline. It is not clear if cells preferentially recruit energy from macroglycogen or proglycogen. The disappearance of these two major pools of glycogen has not been characterized during early postmortem period. Proglycogen, which serves as a stable intermediate in pathways to and from macroglycogen, may impact the rate and extent of glycogen utilization postmortem. We hypothesize that variations in glycogenin expression and/or glycogen forms (macroglycogen, proglycogen) may be "molecular clues" to physiological conditions that may cause the occurrence poor quality in pork loins. Definition of factors impacting glycogen synthesis, storage and postmortem utilization will offer opportunities to apply this knowledge to develop strategies to minimize variation in pork water holding capacity, color, and tenderness.

III. OBJECTIVES

OBJECTIVE 1. Characterize molecular properties of the enzyme that initiates glycogen synthesis and stabilizes glycogen in muscle, glycogenin.

OBJECTIVE 2. Define utilization of specific pools of glycogen during postmortem conversion of porcine muscle to pork.

OBJECTIVE 3. Determine the relationship between the amount of glycogenin and ultimate pH in fresh pork.

IV. PROCEDURES

OBJECTIVE 1. Characterize molecular properties of the enzyme that initiates glycogen synthesis and stabilizes glycogen in muscle, glycogenin.

Localization of glycogenin in pre-rigor and post rigor samples was conducted using several extraction buffers of varying ionic strength. Fractionation buffers included a low ionic strength buffer (40 mM Tris, 1 mM EDTA, pH 7.6, 10 mM mercaptoethanol), and a medium ionic strength (Triton-X) buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 10 % glycerol, 0.1 % Triton X-100, 5 mM EDTA, 10 mM mercaptoethanol). The pellets of each of these extractions were solubilized in 10 mM phosphate, 2 % SDS. Glycogenin was detected by Western blotting using an affinity purified goat polyclonal anti-glycogenin antibody. Localization, solubility and molecular weight were determined on partially separated glycogenin fractions.

OBJECTIVE 2. Define utilization of specific pools of glycogen during postmortem conversion of porcine muscle to pork.

This objective centered on changes in the pools of glycogen during the postmortem period. Ten pigs of similar breeding were slaughtered at the ISU Meat Science Research Laboratory. Longissimus muscle samples (last rib region) were collected from both sides of each carcass at 2 hours postmortem and at 24 hours postmortem to monitor the change in glycogen pools over the first day postmortem. Muscle pH was measured at 2 and 24 hours.

One gram of post rigor muscle was extracted in 10 mL ice-cold 1.5 M perchloric acid and homogenized with a motor-driven Potter-Elvehjem tissue grinder (Wheaton Science Products, Millville, N. J.). The homogenate was incubated on ice for 15 minutes and clarified (2000 x g, 4°C, 15 minutes). The acid soluble portion was used for macroglycogen and free glucose determination. Proglycogen is contained in the acid insoluble pellet.

Macroglycogen determination

Ten mL of 1 M HCl was added to one mL of the acid soluble supernatant for acid hydrolysis of macroglycogen (100° C, 2 hours). The hydrolyzed macroglycogen sample was neutralized with 2 M Trizma base, vortexed, and clarified (2000 x g, 4°C, 15 minutes). The supernatant was transferred to an eppendorf tube for determination of total glucosyl units in the acid soluble fraction. Glucose was determined using pre-mixed reagents (Sigma Chemical) as described previously (Lonergan et al., 2001). Total soluble glucose in the initial homogenate subtracted from the total soluble glucosyl units in the hydrolyzed sample to determine glucosyl units (µM) stored in macroglycogen.

Proglycogen determination

Ten mL of 1 M HCl was added to the perchloric acid insoluble pellet and mixed with a glass rod. The acid suspension was incubated at 100° C for 2 hours to facilitate hydrolysis of proglycogen. The samples were neutralized with 2 M Trizma base, vortexed clarified (2000 x g, 4°C, 15 minutes). The supernatant was transferred to an eppendorf tube for analysis of glucosyl units as described above.

OBJECTIVE 3. Determine the relationship between the amount of glycogenin and ultimate pH in fresh pork.

Sixty pork loins were collected at a commercial slaughter plant and transported to the ISU Meat Laboratory. Meat color, pH, purge loss and drip loss were measured after 7 d aging. Loin samples were aged 7 days and were frozen for glycogenin analysis using immunoblots as described below. Loins were classified in high (average pH = 6.04, n=20) and low (average pH=5.47, n=20) pH groups. Macroglycogen, proglycogen, and free glucose were measured as described for objective 2. Glycogenin was measured using immunoblotting techniques described below.

Sample preparation for electrophoresis

A frozen section (1 g) of longissimus muscle was removed from the center of the longissimus, knife minced, added to 5 mL Triton extraction buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 10 % glycerol, 0.1 % Triton X-100, 5 mM EDTA, 10 mM Mercaptoethanol), and homogenized with a motor-driven Potter-Elvehjem tissue grinder. The homogenate was clarified by centrifugation (25,000 x g) for 15 min at 4°C. The solubolized protein content of the supernatant was determined using pre-mixed reagents (Bio-Rad Laboratories, Hercules, CA). Samples were diluted with water to 6.4 mg/mL. One volume of each sample was immediately combined with 0.5 volumes sample/buffer tracking dye solution (3 mM EDTA, 3% [wt/vol] SDS, 20% [vol/vol] glycerol, .003% [wt/vol] pyronin Y, and 30 mM Tris-HCl, pH 8.0) (Wang, 1982) and 0.1 volume of ß-mercaptoethanol. The protein concentration of the gel sample was 4 mg/mL. Gel samples were heated to 50°C for 20 min and then frozen at -30°C for subsequent analysis.

SDS-PAGE Electrophoresis and Western Blotting

Fifteen percent polyacrylamide separating gels (acrylamide:bisacrylamide = 100:1 [wt:wt], pH 8.8) were used with 5% polyacrylamide stacking gels (acrylamide:bisacrylamide = 100:1 [wt:wt] pH 6.8) to fractionate whole-muscle proteins. Protein samples (60 µg, longissimus samples prepared as described above) were loaded onto the gels. Gels (10 cm wide x 12 cm tall x 1.5 mm thick) were run on Hoefer SE280 system (Amersham Pharmacia Biotech, Piscataway, NJ) at a constant voltage (120 V) for approximately 2.75 h. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 2 mM EDTA, 15% [vol/vol] methanol). Samples were blotted onto a PVDF membrane at a constant voltage (90 V) for 1.5 h in a Hoefer TE22 transfer tank (Amersham Pharmacia Biotech, Piscataway, NJ). The temperature of the transfer buffer was maintained between 4°C and 8°C using a refrigerated circulating water bath.

Post transfer membranes were incubated for 1 h at 25°C in PBS-Tween (80 mM disodium hydrogen orthophosphate, anhydrous, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, 0.1 % [vol/vol] polyoxyethylene sorbitan monolaurate [Tween-20]) containing 5% (wt/vol) nonfat dry milk prepared as described by the supplier of the chemiluminescent detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Troponin-T blots were incubated with affinity purified polycolonal goat anti-rabbit glycogenin diluted 1:20,000 (gift of Dr. Joseph Lomako, University of Miami, Miami FL) in PBS-Tween for 1 h at 25°C. Blots were rinsed three times, 10 min per rinse, in PBS-Tween. Bound primary antibody was labeled with a mouse anti-goat peroxidase-conjugated secondary antibody (A2554, Sigma Chemical, St. Louis, MO) diluted 1:25,000 in PBS-Tween, for 1 h at 25°C. Blots were rinsed in PBS-Tween three times, 10 min per rinse, to remove unbound secondary antibody. A chemiluminescent system was used to detect labeled protein bands as described by

the supplier (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). Intensity of glycogenin bands on immunoblots from each pH group was compared.

V. RESULTS

OBJECTIVE 1. Characterize molecular properties of the enzyme that initiates glycogen synthesis and stabilizes glycogen in muscle, glycogenin.

Glycogenin was detected in all samples evaluated using an anti-rabbit glycogenin antibody on immunoblots. There was an apparent increase in the 38 kDa and 44 k Da doublet in soluble fractions with time postmortem. This is most likely explained as the release of glycogenin from the larger proglycogen and macroglycogen molecules during postmortem aging time. Glycogenin band intensity did not decrease with longer postmortem storage times suggesting that it is not degraded during postmortem storage. This is advantageous as post-rigor samples could be used to determine variations in glycogenin. Glycogenin was soluble in the low ionic strength extraction buffer and in the Triton-X extraction buffer. No glycogenin was found in the pellet of either extract. These properties are consistent with the observations of the properties of rabbit skeletal muscle glycogenin. Lomako et al., (1988) observed a isolated an active glycogenin enzyme from rabbit skeletal muscle that was detected as a single band on an immunoblot. This band had a molecular weight of 39 kDa. The presence of two bands (38 kDa and 44 kDa) in samples fractionated from porcine skeletal muscle indicates that some variation in the protein due to expression or post-translational modifications may exist in porcine muscle. The significance of the observation of two bands is not yet determined.

OBJECTIVE 2. Define utilization of specific pools of glycogen during postmortem conversion of porcine muscle to pork.

Glucosyl units were detected in both the macroglycogen and proglycogen fractions. When a greater proportion of the glucosyl units were fractionated with the macroglycogen fraction at 2 hours, the samples had a lower ultimate pH (Figure 1). Conversely, when a greater proportion of the glucosyl units were fractionated with the proglycogen fraction, a higher ultimate pH was attained (Figure 2). This differentiation indicates the rate of conversion of macroglycogen to proglycogen, along with the total glycogen in porcine muscle at 2 hours postmortem can significantly affect the ultimate pH of pork. This association may be useful in developing attempts to measure pork quality. Total glucosyl units in each of the glycogen pools as well as the total glucosyl units present at 2 hours postmortem all had a significant effect on the change in pH from 2-24 hours postmortem (Figure 3).

There was very little detectable macroglycogen at 24 hours postmortem (Figure 4) regardless of ultimate pH. This indicates that the conversion of macroglycogen to proglycogen is not likely to determine the extent of pH decline. Residual glucosyl units in samples at 24 hours postmortem were isolated with the proglycogen fraction and detected as free glucose and glucose-6-phosphate. Total glucosyl units may have a significant influence on ultimate pH when the pH is high. However, it is clear that residual glucose and proglycogen are quite variable at low pH.

OBJECTIVE 3. Determine the relationship between the amount of glycogenin and ultimate pH in fresh pork.

Table 1 summarizes the quality traits measured in the low and high pH groups. Not surprisingly, the loins in the low pH group had poorer water holding capacity and

were lighter in color than the high pH group. Table 2 demonstrates that the low pH group had higher residual glucose and proglycogen levels. Group differences in residual macroglycogen or in the ratios of proglycogen and macroglycogen to the total glucosyl units were not detected. These observations indicate that conversion of macroglycogen to proglycogen is not a limiting step in postmortem muscle glycolysis.

Glycogenin catalyzes the initiation of glycogen synthesis in muscle. Since one glycogenin molecule remains covalently bound to the glycogen molecule, it is reasonable to hypothesize that the amount of glycogenin could be a limiting factor in determination of the total glycogen in muscle and potentially the ultimate pH in pork. Figure 5 demonstrates a more intense band at both 44 and 38 kDa for samples with a pH of 5.5 compared to the others on the blot. This evidence supports the hypothesis that a higher content of glycogenin is associated with lower ultimate pH. This hypothesis is supported by a recent report that glycogenin is proportional glycogen content in human muscle (Shearer et al., 2000). This result is significant because variation in expression of this protein may explain some of variation in ultimate pH in pork. These results suggest that developing a more thorough understanding of glycogenin and glycogen metabolism in living muscle and in early postmortem muscle will aid our efforts to improve overall quality of fresh pork.

Literature Cited

Adamo, K. B., and T. E. Graham. 1998. Comparison of traditional measurements with macroglycogen and proglycogen analysis of muscle glycogen. J. Appl. Phsyiol. 84:908-913.

Adamo, K. B., M. A. Tarnopolsky, and T. E. Graham. 1998. Dietary carbohydrate and the postexercise synthesis of proglycogen and macroglycogen in human skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 275: E229-E234.

Alonso, M. D., J. Lomako, W. M. Lomako and W. J. Whelan. 1995a. A new look at the biogenesis of glycogen. FASEB J. 9: 1126.

Alonso, M. D., J. Lomako, W. M. Lomako and W. J. Whelan. 1995b. Catalytic activities of glycogenin additional to autocatalytic self-glucosylation. J. Biol. Chem. 270:15315.

Bailey, J. M., E. P. Lillehoj, A. N. Sidawy, B. Jones and J. L. Cohen. 1995. Measurement of glycogenin utilization for glycogen synthesis in type II diabetic cells by use of specific immunoassay for apo-glycogenin. Bioch. Soc. Trans. 23:323S.

Christian, L.L. 1997. Effect of the stress gene on quality. Proceedings Pork quality summit. P37-48.

Enfalt, A. C. 1997. Pig meat quality – influence of breed, RN genotype and the environment. Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala.

Keppler, D., and K. Decker. Glycogen. In: Methods of Enzymatic Analysis Third edition Volume 6. (Bergmeyer, ed). Verlag Chemie, Deerfield Beach, FL.

Le Roy, P, J. Naveau, J. M. Elsen and P. Sellier. 1990. Evidence for a new major gene influencing meat quality traits. Genet. Res. Camb. 55:33.

Lomako, J. M. Wieslawa, M. Lomako, and W. J. Whelan. 1988. A self-glucosylating protein is the primer for rabbit muscle glycogen biosynthesis. FASEB J. 2:3097-3103.

Lomako, J., W. M. Lomako and W. J. Whelan. 1995. Glycogen metabolism in quail embryo muscle: the role of glycogenin primer and the intermediate proglycogen. J. Biochem. 234:343.

Lonergan, S. M., E. Huff-Lonergan, L. J. Rowe, D. L. Kuhlers, and S. B. Jungst. 2001. Selection for lean growth efficiency in Duroc pigs: Influence on pork quality. J. Anim. Sci. (In Press).

McKeith, F. L. 1998. The effect of the RN genotype on pork quality. Proc. Recip. Meat Conf. 51:118.

Shearer, J., I. Marchand, P. Sathasivam, M. A. Tarnopolsky, and T. E. Graham. 2000. Glycogenin activity in human skeletal muscle is proportional to muscle glycogen concentration. Am. J. Physiol. Endocrinol. Metab. 278:E177-E180.

Wang, K. 1982. Purification of titin and nebulin. Methods Enzymol. 85:264-274.

Table 1. Summary of glycogen fraction content in samples with low and high ultimate pH (n=40).

Trait	High pH group		Low pH group		P value
	Mean	S.E.	Mean	S.E.	
pH	6.04	.068	5.47	.04	
Glucose	4.7	.53	35.4	3.00	< 0.01
Proglycogen	.957	.24	5.92	.27	< 0.01
Macroglycogen	0.1	.04	0.12	.04	NS
PG:Total	0.14	.03	0.15	.01	NS
Glucose:Total	0.85	.03	0.84	.04	NS

Table 2. Summary of quality traits in pork from low and high ultimate pH (n=40)

Trait	High pH group		Low pH group		P value
	Mean	S.E.	Mean	S.E.	
pH	6.04	.068	5.47	.04	
Purge (% product lost in 7 days)	0.48	.15	1.17	.21	<.05
Drip loss (% product lost in day)	1.2	.14	1.7	.125	<.05
Hunter Color					
L	42.0	.91	49.3	.86	< 0.01
a	4.71	.63	7.14	.56	<.05
b	8.79	.48	11.57	.32	< 0.01

Figure 1.
Relationship between ultimate pH and the ratio of glucosyl units in macroglycogen at 2 hours postmortem

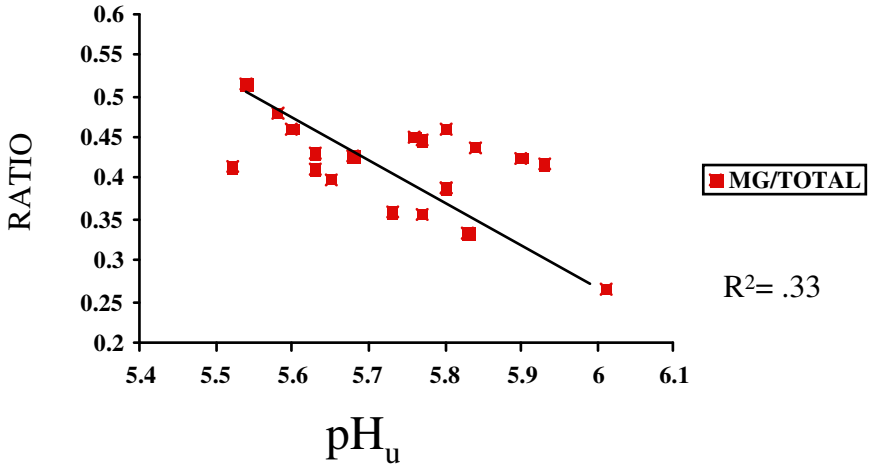

Figure 2.
Relationship between ultimate pH and the ratio of glucosyl units in proglycogen at 2 hours postmortem

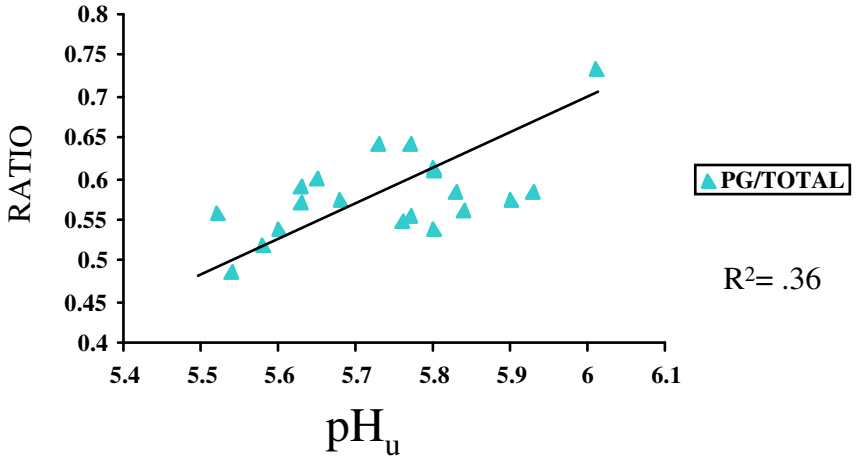

Figure 3.
Relationship between glycogen components at 2 hours postmortem to the change in pH from 2 to 24 hours postmortem

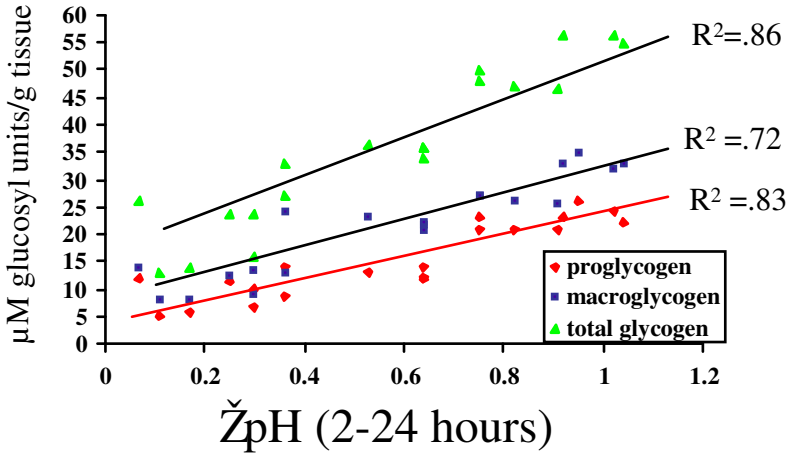

Figure 4.
Residual glycogen components across a range of ultimate pH

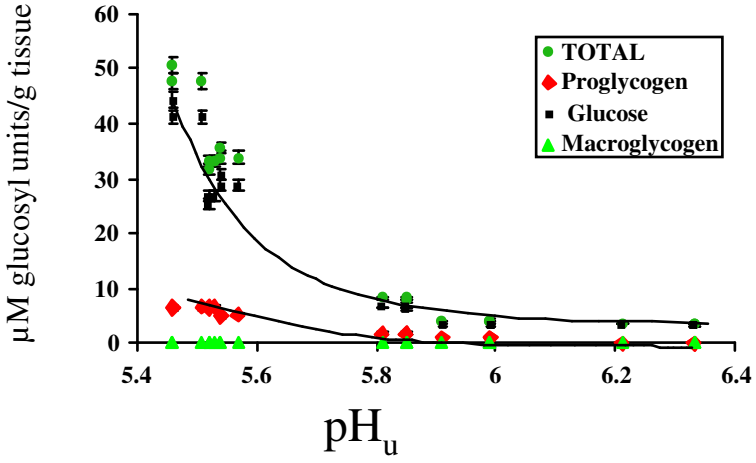

Figure 5.
Relationship between ultimate pH and glycogenin content