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Excess glycogen does not resolve high ultimate pH of oxidative muscle

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ABSTRACT

Skeletal muscle glycogen content can impact the extent of postmortem pH decline. Compared to glycolytic muscles, oxidative muscles contain lower glycogen levels antemortem which may contribute to the higher ultimate pH. In an effort to explore further the participation of glycogen in postmortem metabolism, we postulated that increasing the availability of glycogen would drive additional pH decline in oxidative muscles to equivalent pH values similar to the ultimate pH of glycolytic muscles. Glycolysis and pH declines were compared in porcine *longissimus lumborum* (glycolytic) and *masseter* (oxidative) muscles using an in vitro system in the presence of excess glycogen. The ultimate pH of the system containing *longissimus lumborum* reached a value similar to that observed in intact muscle. The pH decline of the system containing *masseter* samples stopped prematurely resulting in a higher ultimate pH which was similar to that of intact *masseter* muscle. To investigate further, we titrated powdered *longissimus lumborum* and *masseter* samples in the reaction buffer. As the percentage of glycolytic sample increased, the ultimate pH decreased. These data show that oxidative muscle produces meat with a high ultimate pH regardless of glycogen content and suggest that inherent muscle factors associated with glycolytic muscle control the extent of pH decline in pig muscles.

1. Introduction

Two classical fresh meat quality problems prevail in the meat industry: pale, soft and exudative (PSE) meat and dark, firm and dry (DFD) meat. DFD meat displays a dark red or purple color with a high ultimate pH. The ultimate pH cutoff for classifying meat as DFD is traditionally thought to be above pH 6.0 (Briskey, 1964), yet some argue as low as 5.87 (Page, Wulf, & Schwotzer, 2001). While this condition is more prevalent in meat from ruminants, it also occurs in non-ruminants (McVeigh & Tarrant, 1982; Warriss, 1982; Warriss, Bevis, & Ekins, 1989; Warriss, Kestin, Brown, & Wilkins, 1984). Historically, DFD has been attributed to low glycogen content at death (usually due to stress). Stress prior to slaughter often results in a negative linear relationship between ultimate pH and pre-slaughter muscle glycogen, especially when muscle glycogen content is between 0 and 53 μmol/g glycogen in pigs and cattle (Henckel, Karlsson, Jensen, Oksbjerg, & Petersen, 2002; Immonen & Puolanne, 2000). Therefore, muscle glycogen content early postmortem is useful in predicting postmortem pH decline (Warriss, Bevis, & Ekins, 1989).

Data describing this linear relationship between ultimate pH and glycogen content are collected from studies using the *longissimus lumborum*, after all this muscle is the most economically relevant to consumers interested in fresh meat quality. In pigs, this muscle consists largely of the fast-contracting fibers that are glycolytic in nature. As a result, the ultimate pH of meat produced by this muscle is often near pH 5.5–5.6. A strong relationship between glycogen and ultimate pH suggests that glycogen, or more specifically the lack thereof, is responsible for arresting postmortem glycolysis and leading to pork with an elevated ultimate pH. In pigs, especially, a number of muscles produce meat with an ultimate pH at or above pH 5.9, including the adductor, semitendinosus (red portion), gracilis, semimembranosus, gastrocnemius, and masseter (Huff-Lonergan, Baas, Malek, Dekkers, Prusa, & Rothschild, 2002; Porcine Myology, 2005; Realini, Vénien, Gou, Gatellier, Pérez-Juan, Danon, & Astruc, 2013). These muscles, however, contain lower antemortem glycogen and a higher proportion of slow-contracting fibers than in the *longissimus lumborum*. Therefore, if a linear relationship exists between antemortem muscle glycogen and ultimate pH in all muscles, we hypothesized that providing glycogen in excess will produce meat with an ultimate pH near 5.5–5.6. Testing this hypothesis in vivo is difficult. Increasing glycogen content of muscle through high starch diets has proven unreliable (reviewed by Fernandez and Tornberg (1991); Rosenvold, Essén-Gustavsson, and Andersen (2002); Rosenvold et al. (2001, 2002)). Therefore, we tested the aforementioned hypothesis using our in vitro system designed to recapitulate postmortem glycolysis (England, Matarneh, Scheffler,

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Wachet, & Gerrard, 2014; England, Matarneh, Scheffler, Wachet, & Gerrard, 2015).

To conduct this study, a muscle was needed that met two criteria. First, it must be a predominantly oxidative muscle with high ultimate pH. Second, it must be readily available for easy sampling postmortem. The potential options were the *masseter*, the *diaphragm* or red portion of the *semitendinosus*. All three muscles are predominantly slow-contracting and oxidative, but the *masseter* alone exhibits exclusively type I and type IIA fibers (Johnson, White, & Lawrie, 1986; Realini, Vénien, Gou, Gatellier, Pérez-Juan, Danon, & Astruc, 2013; Toniolo, Patruno, Maccatrozzo, Pellegrino, Canepari, Rossi, D'Antona, Bottinelli, Reggiani, & Mascarello, 2004; Tuxen & Kirkeby, 1990). Therefore, the *masseter* was selected over the *diaphragm* or red *semitendinosus*. While the porcine *masseter* does not contain nearly as high a proportion of type I fibers as cattle (~100%) (Johnson, White, & Lawrie, 1986), it met both criteria for this study.

2. Materials and methods

2.1. Sample collection

Market-weight pigs (100–125 kg) were harvested in the Virginia Tech Meat Center using accepted commercial processing procedures. Following exsanguination, porcine *longissimus lumborum* and *masseter* samples were excised at 5 and 1440 min (24 h) postmortem. Samples were used for meat quality characterization or snap frozen in liquid nitrogen and stored at −80 °C.

2.2. Meat quality characteristics and muscle metabolites

The frozen intact 24 h samples of the *longissimus lumborum* and *masseter* were analyzed for meat quality and muscle metabolites. Specifically glycogen, glucose 6-phosphate, glucose, and lactate were measured according to Bergmeyer (1984) using a method modified for microplate design (Hammelman, Bowker, Grant, Forrest, Schinckel, & Gerrard, 2003). Muscle pH was measured by powdering the tissue and homogenizing it using a solution containing 150 mM KCl and 5 mM sodium iodoacetate at a 1:8 ratio (wt/vol) (Bendall, 1973). Samples were equilibrated to 25 °C, centrifuged at 13,000 ×*g* for 5 min at room temperature and measured immediately thereafter using a Thermo Scientific Orion Ross Ultra Semi-Micro glass electrode (Thermo Scientific, Pittsburgh, PA). Both the unfrozen *longissimus lumborum* and *masseter* were measured for objective color (L^*, a^*, and b^*) using a Minolta colorimeter CR-300 (Konica Minolta Inc., Osaka, Japan) with 8 mm aperture, C illuminant, and 0 observer. The mean value of three separate measurements was used for comparisons between muscles.

2.3. In vitro buffer system

The 5 min porcine *longissimus lumborum* and *masseter* muscles were powdered in liquid nitrogen and homogenized at a 100 mg/mL (1:10 ratio) into an anaerobic glycolysis buffer containing 10 mM Na_2HPO_4, 5 mM $MgCl_2$, 60 mM KCl, 5 mM ATP, 0.5 mM ADP, 0.5 mM NAD+, 30 mM glycogen, 25 mM carnosine, 30 mM creatine and 10 mM sodium acetate (pH 7.4) (England, Matarneh, Scheffler, Wachet, & Gerrard, 2014; England, Matarneh, Scheffler, Wachet, & Gerrard, 2015). Reaction vessels were stored at 25 °C for the duration of the trial. Aliquots were removed from the muscle and reaction buffer homogenate at 0, 60, 120, 240 and 1440 min for further analysis. In this study, one *masseter* and one *longissimus lumborum* sample was used from six separate pigs.

In a separate study, the ratio of total muscle mass to buffer volume was maintained, but the percent composition of *masseter* and *longissimus lumborum* (n = 4 per treatment) was varied in order to titrate ultimate pH of the system. In this study, one *masseter* and one *longissimus lumborum* sample were used from four separate pigs.

In another study, 24 h *masseter* (n = 4 per treatment) was added to the in vitro buffer system with changes. The pH was adjusted pH 6.1 to match the in vivo pH of the 24 h *masseter* and ATP was included at 3 mM or omitted (control). Samples were removed at 0 and 1440-min for pH and lactate. In this study, four separate *masseter* muscles from four pigs were used, but powdered muscle from each masseter was split between treatments.

In the final study, the extent of pH decline for the *masseter* (n = 6 per treatment) was compared with and without a cocktail of mitochondria electron transport chain inhibitors (2 μM rotenone (Complex I), 1 mM potassium cyanide (Complex IV), and 2 μM oligomycin (Complex V)). In this study, six separate *masseter* muscles from six pigs were used, but powdered muscle from each masseter was split between treatments.

2.4. Metabolite analysis

Aliquots were removed from the homogenate for glycogen analysis and mixed with an equal volume of 2.5 M HCl, heated at 90 °C for 2 h, centrifuged at 13,000 ×*g* for 5 min at room temperature, and the resulting supernatant was neutralized with 1.25 M KOH (Bergmeyer, 1984). Samples were removed from the homogenate for lactate, glucose 6-phosphate, ATP analysis and treated with an equal volume of ice-cold 1 M perchloric acid, centrifuged at 13,000 ×*g* for 5 min at room temperature, and the resulting supernatant was neutralized with 2 M KOH (Bergmeyer, 1984).

Glycogen, lactate, glucose, and glucose 6-phosphate were measured from muscle homogenates using previous methods (Bergmeyer, 1984) modified for microplate volumes (Hammelman, Bowker, Grant, Forrest, Schinckel, & Gerrard, 2003). All reactions occurred in 5 mL borosilicate glass tubes and were analyzed spectrophotometrically at 340 nm in triplicate using 96-well microplates. ATP was separated with an HP Agilent 1100 (Agilent Technologies, Santa Clara, CA) using an Accucore C18 2.6 μm 50 mm × 4.6 mm column (Thermo Scientific, Pittsburgh, PA), detected at 254 nm with gradient separation (Bernocchi, Ceconi, Cargnoni, Pedersini, Curello, & Ferrari, 1994; Williams, Vidt, & Rinehart, 2008) and quantified using commercially available ATP (Sigma-Aldrich, St. Louis, MO) as a standard.

2.5. pH analysis

Measurement of muscle pH was conducted according to (Bendall, 1973). The muscle homogenate pH was measured similarly with a slight modification. Four volumes of homogenate were transferred to a new tube and one volume of 25 mM sodium iodoacetate and 750 mM KCl (pH 7.0) was added. Samples were equilibrated to 25 °C, centrifuged at 13,000 ×*g* for 5 min at room temperature and measured immediately thereafter using a Thermo Scientific Orion Ross Ultra Semi-Micro glass electrode (Thermo Scientific, Pittsburgh, PA).

2.6. Acid phosphatase activity assay

Acid phosphatase activity was measure according to a modified procedure by Bergmeyer (1974). Briefly, muscle samples were homogenized in 100 mM K_2HPO_4 (pH 7.4) and centrifuged at 13,000 ×*g* for 5 min at room temperature. Aliquots of the supernatant were added to the reaction buffer containing 41 mM sodium citrate (pH 4.8) and 6.9 mM p-nitrophenylphosphate heated to 37 °C. Following a 10 min incubation, 4 volumes of 100 mM NaOH were added to stop the reaction. The activity was measured in triplicate using 96-well microplates spectrophotometrically at 410 nm and reported as units of activity defined as 1.0 μmol of p-nitrophenylphosphate per minute per g whole tissue at pH 4.8 and 37 °C. In this study, one *masseter* and one *longissimus lumborum* sample was used from eight separate pigs.