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Differences in phosphorylation of phosphoglucomutase 1 in beef steaks from the *longissimus dorsi* with high or low star probe values

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ABSTRACT

The objective of this study was to use proteomics to identify alterations of proteins that are related to tenderness. The *longissimus dorsi* (LD) were removed from ten beef carcasses at 24 h postmortem, and the two with the highest (HSP; average kg of force = 6.57) and lowest star probe values (LSP; average kg of force = 3.75) at 14 days postmortem were identified. Two-dimensional PAGE was used to compare the sarcoplasmic fraction of the LD from HSP and LSP steaks. A series of spots identified as phosphoglucomutase 1 (PGM1) were identified. Only the most alkaline isoform was identified as being unphosphorylated. The least phosphorylated isoform (isoform 5) had a greater density of the total protein ($P < 0.05$) and phosphorylated protein ($P < 0.05$) in the samples from HSP steaks compared to the samples from LSP steaks. This study illustrates the importance of identifying posttranslational modifications of proteins in the search for biomarkers.

1. Introduction

Consumers rate tenderness as the primary sensory trait considered when they are making purchasing decisions (Mennecke, Townsend, Hayes, & Lonergan, 2007), and are willing to pay a premium in order to purchase a consistently tender product (Boleman et al., 1997; Miller, Carr, Ramsey, Crockett, & Hoover, 2001; Platter et al., 2005). During the postmortem storage of meat, there are numerous changes in the myofibrillar and sarcoplasmic protein profile that may play a role in postmortem tenderization and, as a result, have the potential to be indicators of tenderness. It is well documented that some of these changes in the protein profile result from proteolysis (Koohmaraie, 1996; Lonergan, Huff-Lonergan, Wiegand, & Kriese-Anderson, 2001) or solubility changes (Goll, Thompson, Li, Wei, & Cong, 2003; Zeece, Robson, Lusby, & Parrish, 1986) during postmortem aging. These changes can disrupt the myofibril and alter the tenderness of the muscle (Olson & Parrish, 1977). However, in addition to changes in protein proteolysis and solubility, there are posttranslational modifications to proteins that can occur in living muscle tissue. Many proteins are regulated by posttranslational modification and these modifications can include phosphorylation, methylation, acetylation, glycosylation, and lipodation (Hu, Guo, & Li, 2006). Posttranslational modifications can alter the properties of proteins by changing their structural conformation and regulating the function of these proteins in muscle tissue (Hu et al., 2006).

Phosphoglucomutase (PGM1) has been identified as a protein that is potentially associated with differences in tenderness (Anderson, Lonergan, & Huff-Lonergan, 2012; Bouley, Meunier, Culioli, & Picard, 2004), but the role of the enzyme in postmortem tenderization remains unclear. Protein from tender beef samples have been reported to have less PGM1 compared to proteins extracted from tough beef steaks (Picard et al., 2010). Tougher samples of the *longissimus dorsi* and *semitendinosus* have been shown to have a greater abundance of the most alkaline isoform of PGM1 (Anderson, Lonergan, Fedler, et al., 2012; Anderson, Lonergan & Huff-Lonergan, 2012; Bouley et al., 2004). Additionally, it was observed that the more tender samples of the *longissimus dorsi* and *semitendinosus* had a greater abundance of the more acidic isoforms of PGM1 (Anderson, Lonergan, Fedler, et al., 2012; Anderson, Lonergan & Huff-Lonergan, 2012; Bouley et al., 2004). Results from these studies suggest that PGM1 is related to tenderness. Both studies also identified multiple isoforms of PGM1 with similar molecular weights, suggesting that the PGM1 isoforms identified had been posttranslationally modified.

Phosphoglucomutase 1 is a glycolytic enzyme that catalyzes the interconversion of glucose-1-phosphate and glucose-6-phosphate through the intermediate glucose-1,6-bisphosphate and thus plays a pivotal role in the regulation of glycogen metabolism (Cori & Cori, 1936). Phosphoglucomutase 1 can be posttranslationally modified through phosphorylation, acteylation and methylation. A phosphorylated form of phosphoglucomutase has been found to be greater in abundance in diabetic muscle compared to normal muscle (Højlund et al., 2003). Thus noting a change in phosphorylation may result in differences in metabolism and phenotype. There are nine sites on PGM1 that have been identified as phosphorylation sites and these include two serine residues, three tyrosine residues, and four threonine residues (Gururaj,

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Barnes, Vadlamudi, & Kumar, 2004; Han et al., 2010; Li et al., 2009). Of these nine phosphorylation sites, the only well characterized phosphorylation site is the threonine residue at position 466 in humans which activates PGM1 when phosphorylated (Gururaj et al., 2004). Porcine skeletal muscle PGM is phosphorylated and three distinct forms of the phosphorylated protein (based on phosphoprotein staining) are resolved in the first dimension of a pH 4–10 strip (Huang et al., 2011).

Protein phosphorylation can influence metabolism and muscle and meat phenotypes. In fact, D'Allesandro, Marrocco, et al. (2012) proposed that phosphorylation of several proteins, including PGM1, can be linked to differences in tenderness in beef from Chianina cattle.

These studies suggest that the phosphorylation of PGM1 is more closely related to tenderness determination than is simply the overall abundance of PGM1 (Anderson, Lonergan, Fedler, et al., 2012; Anderson, Lonergan & Huff-Lonergan, 2012; Bouley et al., 2004). Because PGM1 has been identified as an indicator of tenderness in beef and its activity is regulated by phosphorylation, understanding the variations in phosphorylation of PGM1 may help to better define its role in postmortem tenderization. The objective of this study was to use proteomics to identify modifications to the phosphorylation of PGM1 to obtain a better understanding of how PGM1 is related to tenderness.

2. Materials and methods

2.1. Sample collection

Ten market weight British crossbred cattle (age 20 months) were slaughtered at the Iowa State University Meat Laboratory using normal, approved humane slaughter procedures. *Longissimus dorsi* (LD) muscles were removed from carcasses at 24 h after slaughter. Muscles were cut into 2.54 cm thick and 0.65 cm thick steaks. Steaks were cut perpendicular to the long axis of the muscle and individually vacuum packaged. All steaks were aged at 4 °C, for a total aging time of either one or 14 days postmortem, and then were frozen at -20 °C for further analysis. The 2.54 cm thick steaks were used for star probe and sensory analysis and the 0.65 cm thick steaks were used for biochemical analysis.

2.2. Star probe measurement

Steaks were cooked to an internal temperature of 71 °C in an electric oven broiler prior to texture analysis. Star probe measurements were taken to obtain an instrumental indication of texture of the cooked LD steaks (Anderson, Lonergan, Fedler, et al., 2012). Star probe analysis uses the measurement of the peak load necessary to puncture and compress the product to 80% of its height. The star probe attachment consists of a circular, five-pointed star shaped probe attached to an Instron Universal Testing Machine (Instron Corporation, Norwood, MA). The star probe attachment measures 9 mm in diameter with 6 mm between each point. The angle from the end of each point up to the center of the attachment is 48°. The star probe attachment punctured and compressed the product at a crosshead speed of 3.3 mm/s. Each steak was punctured and compressed three times and the maximum force was recorded for each puncture. Measurements were then averaged to determine a star probe value for each steak.

2.3. Protein extraction

From the 10 LD samples collected at 24 h postmortem, the two LD samples that had the lowest star probe value at 14 days (average kg of force = 3.75) were designated as the low star probe samples (LSP) and the two LDs that had the highest star probe value at 14 days (average kg of force = 6.57) were designated as the high star probe samples (HSP). Day 1 samples from HSP steaks and LSP steaks were minced and then powdered using liquid nitrogen and a Waring blender (Waring Laboratory; Torrington, CT) prior to biochemical analysis. Samples (200 g) of each LD were homogenized and powdered in liquid nitrogen. Two separate extracts of sarcoplasmic protein were prepared from each sample. To extract the sarcoplasmic fraction, powdered samples (5 g) were homogenized in three volumes of cold sarcoplasmic extraction buffer (50 mM Tris, 1 mM EDTA, pH 8.5) using a Polytron PT 3100 (Lucerne, Switzerland). Samples were then centrifuged at 40,000 ×*g* for 20 min at 4 °C. The supernatant was filtered through cheesecloth and the protein concentration was determined using the method described by Lowry, Rosebrough, Farr, and Randall (1951) using premixed reagents (Bio-Rad Laboratories, Hercules, CA). Protein concentration of sarcoplasmic fraction was adjusted to 6 mg/ml using cold extraction buffer, separated into aliquots, and stored at -80 °C for further analysis.

2.4. Two-Dimensional PAGE

Each gel was loaded with 900 μg of sarcoplasmic protein extracted from either the samples from HSP steaks or samples from LSP steaks. The first dimension separation of proteins on the basis of isoelectric point (pI) was carried out on Immobiline DryStrips (13 cm, pH 4–7, GE Healthcare, Piscataway, NJ) rehydrated with DeStreak Rehydration Solution (GE Healthcare, Piscataway, NJ) containing 2.5 m*M* DL-dithiothreitol (DTT). First dimension focusing was performed on an Ettan IPGphor 3 isoelectric focusing system (GE Healthcare, Piscataway, NJ). In the initial step, a low voltage (500 V) was applied followed by a stepwise increase to 8000 V to reach a total of 18,500 V h. After isoeletric focusing, strips were equilibrated for 15 min in 10 ml of equilibration buffer (50 m*M* Tris-HCl pH 8.8, 6 *M* Urea, 30% Glycerol, 2% SDS, and a trace of Bromophenol Blue) containing 65 m*M* DTT. This was followed by an equilibration for 15 min in 10 ml of equilibration buffer containing 135 m*M* iodoacetamide (Rozanas & Loyland, 2008).

Second dimension electrophoresis (separation by molecular weight using SDS-PAGE) was run on 8% acrylamide gels (16 × 18 cm; acrylamide: *N,N′*-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% SDS [wt/vol], 0.05% *N,N,N′N*-tetramethylethylenediamine (TEMED), 0.05% ammonium persulfate [wt/vol], and 0.5 *M* Tris-HCl, pH 8.8) on a SE 600 vertical slab gel unit (Hoefer Scientific Instruments, Holliston, MA) at a constant 30 V.

2.5. Staining for phosphorylated and total protein

Gels were run in duplicate on each extract prepared. Gels were stained for phosphorylated protein using ProQ Diamond (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. Gels were imaged (excitation: 480 nm; emission: 530 nm) on an Ettan DIGE Imager (GE Healthcare, Piscataway, NJ) and analyzed to determine the relative abundance of phosphorylated PGM1 isoforms using the DeCyder 2D Software (v. 6.5; GE Healthcare, Piscataway, NJ). Gels were rinsed twice with water for 5 min per wash. Gels were stained for total protein using Sypro Ruby (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. Gels were imaged (excitation: 390 nm; emission: 595 nm) on an Ettan DIGE Imager (GE Healthcare, Piscataway, NJ) and analyzed to determine the relative density of individual phosphoglucomutase 1 isoforms using the DeCyder 2D Software (v. 6.5; GE Healthcare, Piscataway, NJ).

2.6. Two-Dimensional Western blotting for phosphoglucomutase 1

Two-dimensional Western blotting was used to identify PGM1 isoforms. Duplicate gels were run for each extract prepared. Gels for two-dimensional Western blotting for PGM1 were loaded with 300 μg of sarcoplasmic protein extracted from either the samples from HSP steaks or samples from LSP steaks. The first and second dimensions were run under the same conditions using the same type of IPG strips (13 cm, pH 4–7) and gels (8% acrylamide gels; 16 X 18 cm) that were previously described for the gels that were stained for phosphorylated