Technical note: Protocol for electrophoretic separation of bovine myosin heavy chain isoforms and comparison to immunohistochemistry analysis¹

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ABSTRACT: Myosin heavy chain (MyHC) isoform composition is a primary determinant of contractile speed of muscle fibers. Currently, bovine MyHC isoforms are evaluated using time-consuming histochemical analysis by immunofluorescence or ATPase activity. Electrophoretic separation of MyHC isoforms is more rapid; however, a reliable procedure without use of gradients has not been validated for cattle. Therefore, our objectives were to develop and validate a procedure for separating bovine MyHC isoforms (I, IIa, and IIx) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and compare results to immunohistochemistry (IHC) analysis. Muscle

samples were collected from masseter, sternomandibularis, diaphragm, longissimus lumborum, and cutaneous trunci within 1.5 h postmortem. To determine appropriate conditions for electrophoretic separation, several parameters of gel composition were varied. Bovine MyHC isoforms were clearly separated by increasing glycerol content of polyacrylamide gels to 37%. Identity of MyHC isoforms was confirmed using western blotting. Percent MyHC composition evaluated by gel electrophoresis was consistent with IHC ($P > 0.2$). Thus, SDS–PAGE produces clear separation of MyHC isoforms, and is a viable alternative to IHC-based methods.

Key words: bovine, fiber type, myosin heavy chain, skeletal muscle

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INTRODUCTION

Myosin heavy chain (MyHC) is the primary component of the thick filament in skeletal muscle. The MyHC protein includes a rod portion and a globular head, which has ATPase activity. Hydrolysis of ATP repositions the myosin head during the cross-bridge cycle of muscle contraction, and thus, ATPase activity largely governs the speed of contraction (Barany, 1967). In turn, functional diversity in ATPase activity and contractile speed is largely ascribed to MyHC isoform content (Weiss et al., 1999). Fibers may be “pure” and contain only 1 MyHC isoform;

however, relatively high proportions of fibers may express more than 1 MyHC isoform, particularly during adaptive or transition periods (Schiaffino and Reggiani, 2011). Three MyHC isoforms are expressed in adult bovine muscle (I, IIa, and IIx), whereas the presence of a fourth isoform (IIb) is questionable. Cattle possess the gene for type IIb, and while some have shown evidence that IIb protein is expressed in skeletal muscle (Picard and Cassar-Malek, 2009), this does not appear to be the case for most breeds (Toniolo, 2005).

Muscle properties, including MyHC expression and contractile speed, are dynamic during an animal's lifetime. Evaluating changes in muscle characteristics is important for understanding the impact of experimental manipulations on muscle growth and physiology, as well as meat quality. Classification schemes for describing muscles and muscle fiber types typically incorporate contractile speed based on MyHC properties; specifically, histological cross-sections

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of muscle fibers are analyzed for ATPase activity or MyHC isoform expression.

Current methods for determining bovine MyHC isoform composition are technical and time-consuming; the process includes cutting muscle histology sections, staining using immunofluorescent or immunohistochemistry (IHC) approaches, imaging, and assignment of MyHC type to hundreds of fibers per muscle per animal. In comparison, electrophoretic separation of MyHC isoforms is more rapid and is more practical for analyzing larger sets of muscles or animals. This technique has been used to separate MyHC isoforms in mice, rats, and other species, but a reliable protocol does not exist for bovine isoforms. Therefore, our objectives were to establish a procedure for separating bovine MyHC isoforms (I, IIa, IIx) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), validate this approach using western blotting, and compare results to IHC analyses.

MATERIALS AND METHODS

Sample Collection

Muscles (longissimus lumborum, $n = 36$; cutaneous trunci, $n = 3$; masseter, $n = 3$; diaphragm, $n = 2$; sternomandibularis, $n = 2$) were obtained within 1.5 h postmortem from steers harvested using standard procedures under USDA-FSIS inspection at the University of Florida Meat Laboratory. Muscle samples for electrophoresis were frozen in liquid nitrogen, while muscles used for histology were mounted on a cork using OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA) and frozen in isopentane cooled by liquid nitrogen. Diaphragm and sternomandibularis were not used for histology. All samples were stored at -80°C until subsequent analysis.

Protein Extraction

Muscle (roughly 500 mg) was powdered in liquid nitrogen, and 50 to 100 mg was weighed and diluted in 1 mL of extraction buffer containing 10 mM sodium phosphate (pH 7.0) with 10% (w/v) SDS. Samples were homogenized with a Precellys 24 Homogenizer (Bertin Instruments, Rockville, MD) at 5,000 rpm for 3×10 s. Then, samples were centrifuged at $10,000 \times g$ for 10 min. Supernatants were transferred to a new tube, and protein concentration was determined using a Pierce BCA assay (Thermo Fisher Scientific, Waltham, MA).

Aliquots of supernatant were diluted to obtain the desired protein concentration, which was further diluted 1:3 with a high glycerol Laemmli buffer (sample:Laemmli). The stock high glycerol Laemmli consisted of 83.5 mM Tris (pH 6.8), 2.67% SDS, 6.7% β -mercaptoethanol, 50% glycerol, and 0.01% bromophenol blue. Glycerol concentration of Laemmli was increased in order to facilitate the sample loading, but it was not necessary for successful separation of isoforms. Samples were heated at 95°C for 5 min.

Electrophoresis

Myosin heavy chain isoforms were separated using modifications from SDS-glycerol gel electrophoresis procedures outlined by [Talmadge and Roy \(1993\)](#). Procedures were conducted using a double-wide vertical mini system (CBS Scientific, Del Mar, CA; $20\text{ cm} \times 10\text{ cm}$, $w \times h$) with a 0.75-mm-thick gel with 32-well combs. Initially, several parameters of gel composition were tested, including: percent acrylamide of stacking gel (range: 4% to 5%), percent acrylamide of separating gel (8% or 9%), percent glycerol of stacking gel (range: 30% to 45%), percent glycerol of separating gel (range: 30% to 45%), and acrylamide:bis-acrylamide (37.5:1 or 50:1). Once clear separation of MyHC isoforms was established, those parameters were maintained for further testing.

The resolving gel consisted of 37% glycerol, 8% acrylamide-bis (50:1), 0.2 M Tris (pH 8.8), 0.1 M glycine, and 0.4% SDS. This solution was degassed, and polymerization was initiated by addition of 0.05% ammonium persulfate and 0.025% TEMED (N,N,N',N' -tetramethylethylenediamine). The stacking gel consisted of 37% glycerol, 4% acrylamide-bis (50:1), 70 mM Tris, 4 mM EDTA, and 0.4% SDS. After degassing, polymerization was initiated with 0.1% ammonium persulfate and 0.05% TEMED. The upper running buffer consisted of $2\times$ buffer (0.1 M Tris, 150 mM glycine, and 0.1% SDS); β -mercaptoethanol (0.07%, w/v) was added immediately prior to the run. The $2\times$ running buffer (without β -mercaptoethanol) was diluted 1:1 with water to obtain $1\times$ running buffer used in the lower chamber. Buffers were chilled at 4°C prior to use. Samples were loaded into wells to obtain 0.5 to 2.0 μg total protein per lane. The gel unit was placed in a 4°C room, and electrophoresis was performed at constant voltage: 40 V for approximately 2 h, followed by 80 V for 36 to 40 h. During the last 36 to 40 h, the current was roughly 5 to 6 mA. At completion of the run, gels were washed in ultrapure water 3×5 min. Gels were

then incubated with agitation in a coomassie R250 dye-based reagent (Imperial Protein Stain, Thermo Scientific) for 2 h. The gel was destained overnight with several changes of ultrapure water. The gel was scanned using the Odyssey CLx imaging system (LI-COR Biosciences, Lincoln, NE) and quantified using the manufacturer's Image Studio software.

Western Blotting

Western blotting was used to confirm the identity of MyHC isoforms separated by SDS-PAGE. Gels were prepared and run as described above, and 1 to 2 μ g protein was loaded per lane. Proteins were transferred to nitrocellulose membranes using a wet (tank) transfer system (CBS Scientific, Del Mar, CA). Transfer buffer consisted of 50 mM Tris, 0.38 M glycine, 0.01% SDS, and 10% methanol, and electrophoretic transfer was performed at 40 mA for 7 h at 4 °C. Membranes were stored dry. Rehydrated membranes were then blocked for 1 h at room temperature in Tris-buffered saline (TBS) blocking buffer (StartingBlock, Thermo Scientific). Next, membranes were incubated with primary antibodies (diluted to 0.5 μ g/mL in blocking buffer with 0.1% Tween 20) for 2 h at room temperature. Primary antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA) were of unique isotypes to detect a combination of MyHC using 2-color detection. A primary antibody for all MyHC types (sarcomeric, MF-20; IgG2b) was used in conjunction with BF-32 (MyHC I and IIa; IgM), A4.840 (MyHC I; IgM), SC-71 (MyHC IIa; IgG1), or 6H1 (MyHC IIx; IgM). Primary antibody BA-F8 (MyHC I; IgG2b) was also used. After incubation with the primary antibody, membranes were washed in TBS with 0.1% Tween 20 (TBS-T) 3 $\times$ 5 min. Membranes were then incubated with goat anti-mouse secondary antibodies (LI-COR Biosciences, Lincoln, NE) diluted in blocking buffer with 0.1% Tween 20. IRDye anti-mouse IgG2b 680 (1:15,000) was combined with either anti-mouse IgM 800 (1:10,000) or anti-mouse IgG1 800 (1:5,000); only anti-mouse IgG2b 680 was included for detecting MyHC slow with BA-F8 as the primary. Membranes were incubated with secondary antibody for 1 h at room temperature, and followed by 3 $\times$ 5 min washes in TBS-T. Bands were detected using the Odyssey CLx.

Immunohistochemistry

Immunohistochemistry was utilized to detect MyHC types I, IIa, and IIx, and establish relative

area of fiber types. Procedures were conducted according to Wright et al. (2018). Briefly, muscle cross-sections (10 μ m) were cut on a cryostat (Microm HM 525, Tritech Inc., Edgewater, MD), placed on silanized slides, and stored at -80 °C until subsequent analysis. Primary antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA) of unique isotypes were used to detect MyHC I (BA-F8; IgG2b) and MyHC I and IIa (BF-32; IgM). Then, sections were incubated with appropriate Alexa Fluor dye-conjugated secondary antibodies (Thermo Scientific, Rockford, IL) and Alexa Fluor dye 488 conjugate to wheat germ agglutinin was included to detect cell membranes. Sections were imaged using an Evos FL fluorescent microscope (Thermo Scientific, Rockford, IL). Fibers staining purple were considered type I (positive for both BA-F8 and BF-32), and fibers staining red were considered type IIa (positive for only BF-32). Fibers without stain were considered type IIx. A total of 400 to 900 fibers were evaluated per muscle. Composition was determined by calculating the ratio of each fiber type relative to total fibers analyzed. Fiber cross-sectional area (CSA) was assessed using ImageJ software (US National Institutes of Health, Bethesda, MD), and mean CSA was determined for each fiber type in each muscle. The frequency and mean CSA were utilized to calculate relative area, which was used for direct comparison with percentage of MyHC composition determined using SDS-PAGE.

Statistical Analysis

A paired-sample *t*-test was used to test that MyHC isoform relative abundance is the same when assessed by IHC and gel electrophoresis. For each MyHC isoform, the difference (*d*) in relative abundance determined by the 2 methods was calculated. The Shapiro-Wilk test was used to validate that differences were normally distributed for each isoform. Bland-Altman difference plots were constructed to evaluate agreement between 2 methods. For each MyHC isoform in each muscle sample, limits of agreement were calculated using Eq. 1:

$$\bar{d} \pm 1.96 \times SD_{\bar{d}}. \quad (1)$$

RESULTS AND DISCUSSION

Glycerol SDS-PAGE provides a relatively rapid means of quantifying MyHC composition in large experiments or functionally distinct muscles. This approach has been used to separate MyHC

isoforms of mice, rats, and other species (Talmadge and Roy, 1993; Blough et al., 1996; Mizunoya et al., 2008), but these protocols are not effective for separating bovine MyHC isoforms. Others have separated bovine MyHC isoforms using large format gels (Toniolo, 2005) or gradient gels (Picard et al., 1999) though neither provided validation of bands

with western blotting or histochemistry. Another protocol using small format gels without a gradient is more practical (Picard et al., 2011), but was not reproducible by our lab.

Using the protocol of Talmadge and Roy (1993) as a foundation, parameters of gel composition were varied in order to improve separation of IIA and IIX isoforms. Several factors, such as glycerol composition, cross-linker content (or acrylamide:bis-acrylamide), and acrylamide percentage, influence protein migration. Increasing the glycerol content of the stacking and separating gels to 37% and including β -mercaptoethanol in the upper running buffer resulted in clear separation of all 3 bovine MyHC isoforms (Fig. 1). Muscles included in the analysis represent wide variation in MyHC isoform composition. Based on band intensity and expected MyHC composition, type I migrated at the lowest apparent molecular weight; type IIA and IIX appeared at a greater apparent molecular weight, with IIA migrating slightly faster than IIX.

The identity of MyHC isoforms was confirmed using western blotting with 2-color detection (Fig. 2). Antibodies for detecting specific MyHC isoforms were used in conjunction with an antibody for all muscle MyHC isoforms (sarcomeric

Figure 1. Separation of bovine myosin heavy chain (MyHC) isoforms (type IIX, IIA, and I) in functionally distinct muscles. Proteins were detected using coomassie-based protein stain and imaged with a LI-COR Odyssey (gray scale, top) or with a camera in color (bottom). Stm = sternomandibularis; CT = cutaneous trunci; Ma = masseter; D = diaphragm; LL = longissimus lumborum.

Figure 2. Detection of bovine myosin heavy chain (MyHC) isoforms using 2-color western blotting. The left column (B, E, H, K) shows detection of all 3 MyHC isoforms using MF-20 antibody. The middle column (A, C, F, I, L) represents detection using antibodies specific for various MyHC (BA-F8, A4.840, BF-32, SC-71, and 6H1, respectively). The last column (D, G, J, M) shows the merged pictures of those images in the same row. Antibody BA-F8 could not be used with MF-20 since both are IgG2b isotypes. Within each image, lanes 1 to 5 represent sternomandibularis, cutaneous trunci, masseter, diaphragm, and longissimus lumborum, respectively.

Figure 3. Immunofluorescent staining of myosin heavy chain (MyHC) isoforms in masseter (A), longissimus lumborum (B), and cutaneous trunci (C). Myosin heavy chain isoforms were detected using antibodies specific for BA-F8 (type I) and BF-32 (I + IIa). Fibers appearing purple were designated MyHC I, while red fibers were IIa, and those with no stain were considered IIx. Cell membranes are labeled with wheat germ agglutinin 488 conjugate (green). The white bar (B) is 400 µm.

myosin) (Table 1) to easily validate relative positions of MyHC isoforms. Antibodies for MyHC I (BA-F8, Fig. 2A and A4.840, Fig. 2C) consistently recognized only the band with the lowest molecular weight, and as expected, the band for the masseter (lane 3) appeared the most intense, and the cutaneous trunci was least intense. Antibody BF-32 was used to establish MyHC IIa position. This antibody

Table 1. Antibodies used for western blotting and/or immunohistochemistry

Antibody ¹	MyHC target ¹	Host and isotype
MF-20	All (sarcomeric)	Mouse IgG2b
BA-F8	I	Mouse IgG2b
A4.840	I	Mouse IgM
BF-32	I + IIa	Mouse IgM
SC-71	IIa	Mouse IgG1
6H1	IIx	Mouse IgM

¹Source: Developmental Studies Hybridoma Bank (Iowa City, IA).

recognizes both MyHC I and IIa; the merged image (Fig. 2G) supports that MyHC IIa migrates slightly faster than IIx and its relative position is in the middle.

Additional antibodies were also tested to determine if they would be suitable for detecting only IIa or IIx using western blotting. Antibody SC-71 is recommended for identifying bovine MyHC IIa using western blotting, immunofluorescence, and immunohistochemistry (Developmental Studies Hybridoma Bank). However, for western blotting, SC-71 reacts more strongly with IIx (Fig. 2I and 2J), and to a lesser extent, MyHC IIa. In preliminary trials for IHC, SC-71 was not successful at immunofluorescent detection of bovine MyHC IIa. Lastly, antibody 6H1 has been used to detect MyHC IIx in other species. This antibody detected MyHC IIx by western blotting, but it also cross-reacted with MyHC I (Fig. 2L and 2M).

Subsequently, MyHC bands from gel electrophoresis were quantified and compared to IHC. Composition of MyHC isoforms determined by gel electrophoresis (Table 2) was consistent with results of previous studies using myosin ATPase staining or myosin ATPase combined with metabolic enzyme staining. Based on previous reports, masseter is 100% type I MyHC; sternomandibularis contains roughly 55% type I and 45% type IIa and 0% IIx; longissimus contains all 3 MyHC isoforms, with a greater percent IIx (roughly 20% type I, 25% type IIa, and 55% type IIx); and cutaneous trunci is comprised of little to no type I MyHC (0% to 10%), approximately 20% type IIa, and 70% to 80% type IIx MyHC (Totland and Kryvi, 1991; Kirchofer et al., 2002).

Next, MyHC isoform composition was determined by gel electrophoresis and IHC (Fig. 3) using the same samples. Methods were evaluated for agreement and bias. According to the paired *t*-test, IHC and gel methods generated similar percentages for each MyHC type ($P > 0.2$; Table 3). Mean difference between methods was less 1% for all 3

Table 2. Mean myosin heavy chain (MyHC) isoform composition determined using gel electrophoresis and immunohistochemistry (IHC)

Muscle	<i>n</i> ¹	Type I, %		Type IIa, %		Type IIx, %	
		IHC	Gel	IHC	Gel	IHC	Gel
Masseter	3	100	100	0	0	0	0
Diaphragm	2	ND ²	76.5	ND	23.5	ND	0
Sternomandibularis	2	ND	41.4	ND	58.6	ND	0
Longissimus lumborum	36	17.6	17.4	23.3	23.2	59.1	59.4
Cutaneous trunci	3	3.1	2.1	21.4	19.5	75.5	78.4

¹Number of muscles analyzed per method.²Not determined.**Table 3.** Comparison between immunohistochemistry (IHC) and gel electrophoresis methods for determination of myosin heavy chain (MyHC) isoform composition

MyHC isoform	Mean difference ¹	SE	<i>P</i> -value ²	Points within limits of agreement ³
I	0.24	0.47	0.60	42/42 (100%)
IIa	0.63	0.58	0.28	40/42 (95.2%)
IIx	−0.88	0.74	0.24	38/42 (90.5%)

¹Difference in percent MyHC composition, IHC - Gel.²Evaluated using paired *t*-test.³Limits of agreement determined by Eq. 1.

MyHC types. Bland–Altman difference plots were used to estimate an agreement interval and evaluate bias between mean differences. The Bland–Altman plot is considered superior to correlation analysis since it measures agreement rather than the strength of the relationship; 2 measurements can be highly related but may also include bias that is constant or bias associated with specific values. For 2 methods to agree, it is recommended that 95% of the data points lie within 2 SDs of the mean difference, referred to as the limits of agreement of the Bland–Altman plot (Bland and Altman, 1986). For both type I and IIa, >95% of the points fell within the limits of agreement, whereas only ~90% (38/42) were within limits for type IIx.

Biological variation and methodological factors may explain differences between the 2 methods for type IIx. First, the existence of hybrid fibers may affect classification of MyHC type and MyHC content assessed by immunohistochemistry, but not SDS–PAGE. For example, a fiber that co-expresses both IIa and IIx would stain positive for IIa (red), leading to classification as MyHC IIa by IHC and an overestimation of IIa content; conversely, it is expected that SDS–PAGE would separate these MyHC isoforms thereby allowing for more accurate quantification. However, the proportion of hybrid

fibers is likely relatively low in “normal” muscles compared to transitioning muscles that are undergoing environmental or experimental manipulation. In pork longissimus, hybrid fibers represented approximately 10% of the total (relative) area (Kim et al., 2014). Thus, hybrid fibers may partially explain differences between the 2 methods, but likely are not a large source of variation between methods for estimation of MyHC composition.

CONCLUSIONS

Using the parameters outlined herein, gel electrophoresis provides a reliable and rapid means of separating and quantifying bovine MyHC isoforms. Although IHC is necessary for determining fiber type frequency or cross-sectional area, gel electrophoresis may be more practical for analyzing larger data sets or as an initial method for documenting differences in protein abundance of bovine MyHC isoforms. Furthermore, we have quantified MyHC composition using small initial muscle samples (2 mg), making the gel method ideally suited to studies with limited tissue availability.

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