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Comparison of myosin heavy chain content determined by label-free quantification between porcine *longissimus thoracis*, *psoas major* and *semimembranosus* muscles

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ABSTRACT

The present study was conducted to quantify MHCs in porcine *longissimus thoracis* (LT), *psoas major* (PM) and *semimembranosus* (SM) muscles through the label-free quantification (LFQ). To estimate the accuracy of LFQ, quantitative RT-PCR (qRT-PCR), immunohistochemistry (IHC), and Western-blotting (WB) were performed. MHCs 2x, 2a, 2b and slow were identified by LC-MS/MS analysis and 279 ion spectra were selected for LFQ analysis. The content of MHC 2b was higher in LT and in SM than in PM ($p < 0.05$), while the content of MHC slow was highest in PM among the muscles ($p < 0.01$) regardless of LFQ types. Positive correlation coefficients of MHC 2b and MHC slow between LFQ and IHC (relative area composition) and qRT-PCR results partially supported the LFQ results. Though low-abundant peptides should be considered to estimate MHC contents via the spectral count method, LFQ enables the determination of MHC contents at protein level regardless of LFQ types.

1. Introduction

Myosin is a molecular motor known for its role in muscle contraction and the superfamily is divided into twenty four classes (Foth, Goedecke, & Soldati, 2006). Myosin generally consists of three domains: a motor head responsible for ATPase activity, actin binding and movement; a neck domain that binds regulatory proteins; and a tail that binds various cargo associated proteins (Syamaladevi, Spudich, & Sowdhamini, 2012). Myosin found in mammalian skeletal muscles is class II and it has several isoforms which can be distinguished by the differences in head (myosin heavy chain, MHC) domain sequences (Rayment et al., 1993; Weiss, Schiaffino, & Leinwand, 1999). MHCs are associated with muscle fiber types, which are classified by the distribution of MHC isoforms (Bär & Pette, 1988; Schiaffino et al., 1989). Muscle fiber has its own metabolic and contractile properties because of different ATPase activity and the kinetics of MHC isoforms (Rayment et al., 1993; Reggiani, Bottinelli, & Stienen, 2000).

In muscle foods, MHC isoforms are considered the primary factor determining the physicochemical properties of muscle food as postmortem biochemical change is mainly affected by the composition of MHC isoforms or muscle fibers regardless of animal species (Choi & Kim, 2009). Furthermore, chemical components, which have significant implications for nutrition and quality characteristics, are closely related to muscle fiber type. Therefore, a number of studies have been conducted to investigate MHC or MHC-based muscle fiber compositions and their relationships with muscle foods (Chang et al., 2003; Choi, Ryu, & Kim, 2008; Eggert, Depreux, Schinckel, Grant, & Gerrard, 2002). The electrophoretic method introduced by Talmadge and Roy (1993) has been used to determine the MHC composition at the protein level and this technology was successfully applied to most species. Nevertheless, MHC quantification in porcine skeletal muscle is still challenging. The MHCs 2x, 2a, 2b and slow, distributed in porcine skeletal muscles have similar molecular weights (224.084, 224.061, 224.146, and 224.095 kDa, respectively) and amino acid sequences. The high homology makes clear separation of porcine MHCs difficult, especially among the fast isoforms (2x, 2a and 2b). Thus, most studies have been conducted without clear separation of fast MHCs (Choi et al., 2008; Wojtysiak & Połtowicz, 2014; Park, Gunawan, Scheffler, Grant, & Gerrard, 2009). In our previous study, we tried to develop better technology for porcine MHC analysis, but individual MHC separation failed despite success in obtaining clear separated gel bands (Kim, Jeong, Yang, & Joo, 2013).

In the present study, label-free quantification (LFQ) was employed to overcome problems in the quantification of porcine MHC isoforms. The LFQ technique is one of the quantitative proteomics technologies and has been applied to several protein and peptide studies in large

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areas to date (Nahnsen, Bielow, Reinert, & Kohlbacher, 2013). However, in muscle food systems, few studies have been conducted with this technology (Gallego, Mora, Aristoy, & Toldrá, 2016; Sayd, Chambon, & Santé-Lhoutellier, 2016). To verify the accuracy of this technology, relative MHC expression levels were analyzed using immunohistochemistry (IHC), WB, and qRT-PCR. These results were compared to LFQ results.

2. Materials and methods

2.1. Sample preparation and experimental design

Porcine *longissimus thoracis* (LT), *psoas major* (PM) and *semimembranosus* (SM) muscles were taken from five crossbred ((Landrace × Yorkshire) × Duroc) pigs (180 d of age, 109.2 ± 2.3 kg body weight) obtained from a commercial slaughter house at 24 h postmortem. All procedures involving animals were approved by the Animal Care and Use Committee of the Gyeongsang National University, Republic of Korea and the pigs were slaughtered according to the standard commercial procedures of the Korean livestock production system. Five pieces (1.0 × 1.0 × 1.5 cm) were cut from the central part of each muscle. Three of them were promptly frozen using 2-methylbutane cooled with liquid nitrogen for IHC and one-dimensional gel electrophoresis (1DE). The others were put into cryotubes, capped, and frozen using liquid nitrogen for qRT-PCR. The workflow for this study is presented in Fig. 1. For MHC quantification, myofibrillar proteins were fractionated by 1DE and then MHC bands were selected. Tryptic peptides obtained by in-gel digestion were separated by liquid chromatography (LC) and mass spectrometry (MS). Porcine MHC isoforms were identified from MS/MS and unique peptides for each MHC were selected for LFQ analysis. Spectral intensity and count were obtained for the LFQ results. The LFQ results were compared to the results of IHC, WB, and qRT-PCR.

2.2. Immunohistochemistry

Immune-peroxidase staining with avidin-biotin complex (ABC) was used for muscle fiber classification. Transverse serial sections (10 μm thick) were taken using a cryostat microtome (HM525, Microm GmbH, Walldorf, Germany) at −27 °C and blocked with goat serum. Four primary antibodies including BA-F8 (1:500), SC-71 (1:500), 10F5 (1:200) and BF-35 (1:200) were purchased from DSHB (Iowa City, IA, USA) and applied for 60 min at room temperature. The sections were rinsed three times using PBS and then incubated with secondary antibodies (biotinylated anti-mouse IgG and IgM, 1:500) for 30 min. The sections were rinsed again and incubated with ABC reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 30 min at room temperature. They were then rinsed with PBS three times and visualized after incubating in diaminobenzidine tetrahydrochloride (D3939, Sigma-Aldrich Corp., St. Louis, MO, USA) with 0.3% H_2O_2 for 10 min. Muscle fiber classification was performed according to our previous report (Kim, Ryu, Jeong, Yang, & Joo, 2013). In brief, MHC slow fibers were reacted with BA-F8 (anti-MHC slow) and BF-35 (anti-MHC slow and 2a) but not with 10F5 (anti-MHC 2b) and SC-71 (anti-MHC 2a). The fibers that reacted with SC-71 and BF-35 were classified as MHC 2a fibers, whereas fibers detected by SC-71 but not by the others were classified as MHC 2x fibers. MHC 2b fibers were reacted with only 10F5. In this study, these four pure types which contained only one MHC isoform were accepted, whereas hybrid fibers with two or more MHC isoforms were excluded because the amount of each MHC expressed in the hybrid fibers could not be estimated. The relative amounts of hybrid fibers were as follows: MHC IIAX fiber (consisted of MHCs 2a and 2x), 0.00–0.52% of relative number and 0.00–0.45% of relative area; and MHC IIXB fiber (consisted of MHCs 2x and 2b), 0.30–0.64% of relative number and 0.56–0.72% of relative area (Supporting Table 1).

Approximately 600 fibers per muscle piece were counted and the relative area composition (%) was considered the muscle fiber composition by estimating the ratio of the total cross-sectional area (μm^2). Image analysis was performed using Image-Pro® plus 5.1 (Media Cybernetics Inc., Rockville, MD, USA) and data was obtained from three pieces each of LT, PM and SM taken from 5 pigs.

2.3. mRNA extraction and qRT-PCR analysis

Total-RNA was extracted from muscle samples using TRIzol reagent

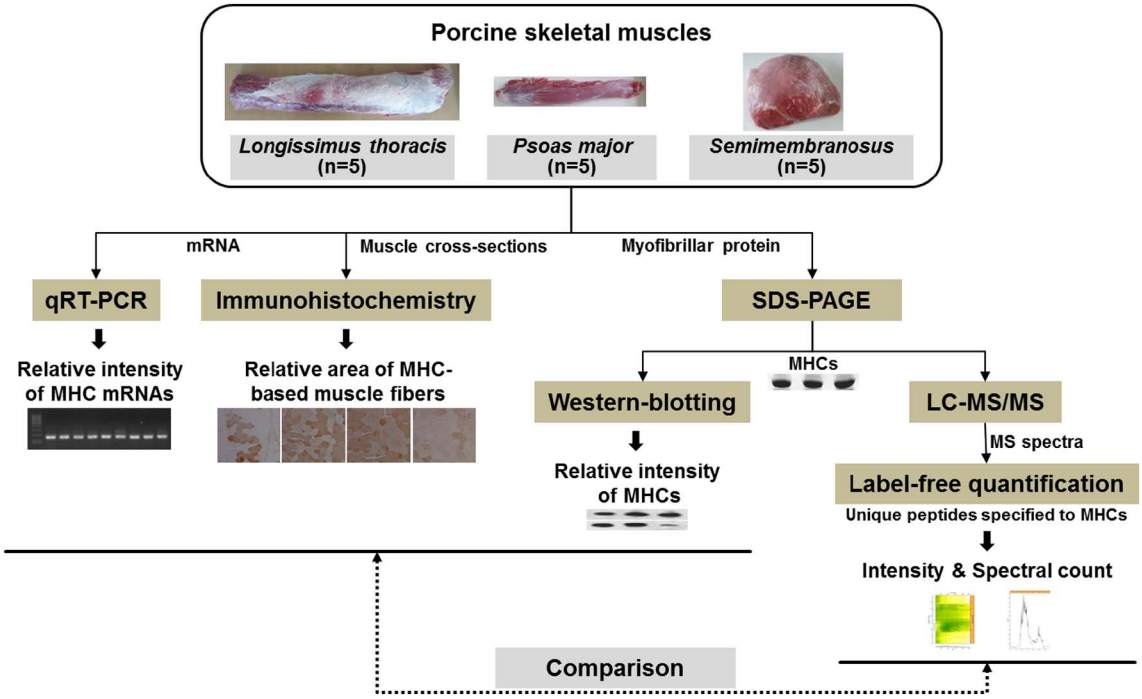

Fig. 1. Schematic representation of myosin heavy chain (MHC) quantification workflow.