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Liquid Chromatography-Tandem Mass Spectrometry for Detecting Myosin Light Chain 3 in Dry-Aged Beef

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Abstract: Liquid chromatography-tandem mass spectrometry (LC/MS/MS) is a more accurate technique for detecting proteins than electrophoresis-based methods such as western blotting. Because of its convenience, western blotting is commonly used for protein analysis in beef. We developed a method for detecting myosin light chain 3 (myl3) in beef samples, particularly dry-aged beef, using LC/MS/MS for quality testing. *Musculus longissimus dorsi* of Holstein was aged for 0, 2, 4, 5, 9, 11, 17, 20, and 24 weeks and used to measure the myl3 concentration. Because of the high molecular weight of myl3, the limitations of LC/MS/MS were overcome by implementing immunoprecipitation and digestion steps. Ultimately, a tryptic fragment of myl3 (13-mer), generated using immunoprecipitation and digestion by a biotinylated antibody, was detected using LC-MS/MS in positive ion mode through multiple reaction monitoring and analyte separation on a C18 column. Our method showed limits of detection and quantification of less than 0.3 and 0.8 µg/kg, respectively. However, differences in the myl3 concentrations according to the aging time were not significant ($p > 0.05$). After 12 weeks, myl3 disappeared in tested all samples, thus our analytical method can be used for accurate measurement of muscle protein in beef samples.

Keywords: myosin light chain 1; liquid chromatography-tandem mass spectrometry; food quality; dry-aged beef; tenderness

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1. Introduction

Dry aging has been a common method to preserve and tenderize beef for centuries [1,2]. Dry-aged beef is prepared by unpacking and hanging or racking in a chiller with controlled temperature and relative humidity [3]. In general, beef quality, including flavor and tenderness, is improved by aging [4–7]. Meat tenderness is primarily influenced by the composition and contractile state of the muscle fiber, amount and solubility of connective tissue, and extent of post-mortem proteolysis [8].

Although the exact mechanism of the beef tenderizing process is not completely clear, the tenderness of dry-aged beef is largely dependent on protein degradation. During aging, many changes occur in the structure of muscle fibers. The first change is collapse of the Z-lines, followed by weakening and fragmentation of the muscle fiber [9]. Previous studies have focused on detecting protein degradation over the aging time; Phelps et al. suggested that degradation of desmin and troponin-T in beef muscle occurred when aging time was increased [10]. However, collapse of the Z-lines (protein degradation) results in the production of a polypeptide with a molecular weight of approximately 28–98 kDa [9]. Therefore, western blotting is widely used to detect degraded protein in dry-aged beef.

Liquid chromatography-tandem mass spectrometry (LC/MS/MS)-based methods are more sensitive and stable than western blotting. Moreover, LC/MS/MS can distinguish variations that cannot be detected using western blotting [11]. Thus, LC/MS/MS is widely used in the field of clinical science, particularly immunology, when precision is required.

Despite these advantages, unfortunately quadrupole scan-based detection can only detect small molecules (<m/z 8500) [12].

Myosins, which belong to a superfamily of motor proteins in muscles, are widely known for their roles in muscle contraction and numerous other motility processes in eukaryotes [13,14]. Most myosin molecules are composed of head, neck, and tail domains. Myosin light chain 3 (myl3), a component of the myosin head encoding a deduced protein of 195 amino acids, is a 23 kDa isoform protein [15]. As muscle proteins typically decompose into low-molecular-weight proteins during the meat aging process, myl3 may be an appropriate quality factor for determining the maturity of aged beef.

Chromatography-based analytical methods for the detection of muscle proteins have been commonly used in the field of immunology, but few studies have been conducted in the field of food science and technology. Humans and cattle are both mammals; thus, their muscle connective tissues may be similar. Myl3 in human serum is considered as a biomarker for cardiac necrosis [15] and, recently, a method to determine myl3 in human serum samples was developed using LC/MS/MS [16]. Although these studies were performed in the field of medical diagnosis, this method shows potential for applications in the field of food science and technology for determining meat tenderness.

Therefore, the objective of this study was to develop methods for detecting myl3 using LC/MS/MS for beef as a dry-aging quality test.

2. Materials and Method

2.1. Chemicals and Reagents

The reference material for the peptide 13-mer (CM102356v.1.1, ALGQNPTQAEVLR, full peptide sequence more than 50% crude) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Sodium fluoride (CAS No 7681-49-4; Catalogue No S7920), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES; CAS No 7365-45-9; Catalogue No H3375), sodium orthovanadate (CAS No 13721-39-6; Catalogue No 450243), ethylene diamine tetra acetic acid (EDTA; EDS; CAS No 60-00-4), protease inhibitor tablets (Catalogue No S8830-20TAB), Tris hydrochloride solution (pH 8.0, 1 M, Tris-HCl buffer; CAS No 1185-53-1; Catalogue No T3038), trypsin (CAS No 9002-07-7; Catalogue No T1426), and papain (CAS No 9001-73-4; Catalogue No P4762) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phenylmethylsulfonyl fluoride (CAS No 329-98-6; Catalogue No 11359061001) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). The EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit (Catalogue No 1854210) and magnetic beads (Dynabeads™ M-280 streptavidin, Catalogue No 11206D) were purchased from Thermo Fisher Scientific. Myl3 antibody (Catalogue No SC-58805) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and the His-tagged myl3 protein standard (purity >90%, Catalogue No MYL3-8027H) was obtained from Creative Biomart (Shirley, NY, USA). All organic solvents such as methanol, acetonitrile, 2-propanol, and trichloroacetic acid were obtained from Sigma-Aldrich. Distilled water was obtained using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Dry-Aged Beef Sample Preparation

Musculus longissimus dorsi of Holstein cattle was purchased at a local market in Seoul, Republic of Korea. The beef samples were cut into 35 × 20 × 15 cm^3 (ca. 6.0 kg) sections and subjected to dry aging with a DRY AGER® (DX1000, Landig + Lava GmbH & Co. Bad Saulgau, Germany) (temperature: 1.5 °C ± 1.0 °C; relative humidity: 82 ± 5.0%; air flow velocity: 2.5 m/s). The myl3 concentration was measured in beef samples after 0, 2, 4, 5, 9, 11, 17, 20, and 24 weeks. On the sampling day, the external crust (non-edible part) was trimmed, and only the internal meat (edible part) was used for experiments. Each sample (ca. 200 g) was minced with a Food Processor (CH580, Hai Xin Technology Company, Shenzhen, China) and stored at −18 °C prior to analysis.

2.3. Protein Extraction and Myl3 Separation

Protein was extracted from minced dry-aged beef samples as described by Chen et al. with minor modifications [16]. Each sample (1 g) was placed in a 22 mL amber vial (Supelco, Bellefonte, PA, USA) containing a magnetic stir bar (10 mm length, 6 mm i.d.) (Bel-Art Products, Wayne, NJ, USA) and treated with 10 mL of clean-up solvent containing 25 mM HEPES, 50 mM sodium fluoride, 0.25 mM sodium orthovanadate, 0.25 mM phenylmethylsulfonyl fluoride (in isopropanol), 2.5 mM EDTA, and half of a protease inhibitor tablet. The vials were tightly capped and mixed with a rotator (Twister, GERSTEL, Rotterdam, The Netherlands) at 25 °C for 60 min under agitation at 1300 rpm. Subsequently, the samples were centrifuged at 3000× *g* for 30 min (Centrifuge 5810R, Eppendorf, Hamburg, Germany). The supernatant was discarded, and the pellet was resuspended in 8.8 mL of Tris-HCl buffer (pH 8.0) containing 2 mM EDTA.

To separate the myosin head from the extracted protein, 100 µL of trypsin (20 mg/kg) was added, and the resulting solution was incubated in a shaker at 37 °C and 300 rpm for 7 h. To extract myl3 from the myosin head, 100 µL of papain (56 mg/kg) was added, followed by overnight incubation at 65 °C and 300 rpm. After incubation, 1.0 mL of 5% trichloroacetic acid was added to terminate the papain reaction. Finally, the supernatant was filtered through a 0.45 µm Nylon syringe filter (Whatman, Maidstone, UK).

2.4. Immunoprecipitation and Digestion

Myl3 is a 23 kDa protein. Because of its large size (13-mer), a tryptic fragment of myl3 was used for detection. The peptide 13-mer from myl3 was produced as reported by Berna et al. with minor modifications [15]. Prior to use, the magnetic beads were washed twice with 1 mL of buffer 1 (pH 7.5) containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.1% bovine serum albumin. To produce biotinylated antibody, 2.2 mg of 10 mM Sulfo-NHS-LC-biotin was dissolved in 400 µL of distilled water, and 8.1 µL of the Sulfo-NHS-LC-biotin solution was added to 1 mL of myl3 antibody, followed by incubation for 24 h at 4 °C. After incubation, the biotinylated antibody was added to magnetic beads at a ratio of 8 µg of antibody/mg of beads. The myl3 antibody-complexed beads were then incubated at room temperature for 4 h while rotating (rotation speed at 150 rpm). After incubation, residual unbound antibody was removed by washing with buffer 1, followed by resuspending with buffer 1 to obtain 10 mg of beads/mL at a final concentration. Next, 0.5 mg (50 µL) of the myl3/antibody-complexed beads was mixed with a 100 µL aliquot of the extracted protein samples described in Section 2.3 and the total volume was adjusted to 1 mL with buffer 1. The samples were incubated and rotated at 150 rpm overnight at 4 °C. After incubation, the sample beads were washed twice with 1 mL of buffer 2 (buffer 1 without bovine serum albumin) and then resuspended in 50 µL of methanol/water (10:90, *v/v*) solution containing 5 mM ammonium bicarbonate and 2 µg/mL trypsin. The samples were digested overnight at 37 °C while rotating at 150 rpm. Following digestion, the beads were removed using a magnet, and the sample supernatant (3 µL) was used for LC/MS/MS analysis.

Recovery of the target compounds during the immunoprecipitation (IP) step was verified as described by Berna et al.; this approach was reported to have accuracy values (percent relative error, % RE) of less than 12.0 [15]. We performed a recovery test using the His-tagged myl3 protein standard by analyzing two concentration levels of the target compound at a final concentration of myl3 (10.0 and 50.0 µg/kg).

2.5. Quantification and Analytical Parameters

To determine the linearity of the developed method for the peptide 13-mer, seven standards (100.0, 50.0, 10.0, 5.0, 1.0, 0.5 and 0.2 mg/kg) were analyzed.

2.6. Tryptic Fragment of Myl3 (13-mer) Detection Using LC/MS/MS

The tryptic fragment of myl3 (13-mer) was analyzed using an Agilent LC 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a SCIEX Triple Quad

4500 mass spectrometer equipped with a turbo ion spray ionization source (Applied Biosystems, Foster City, CA, USA). A standard stock solution of the peptide 13-mer reference material was prepared by dissolution in 10% methanol. Working standard solutions in the concentration range of 0.20–100.00 μg/kg were prepared by dilution. Chromatographic separation was performed on a reverse-phase C18 CAPCELL CORE ADME column (2.1 mm i.d., 150 mm length, 2.7 μm particle size, Hitachi-High Technologies Corp., Hitachinaka, Japan). Chromatographic elution was performed with a mobile phase consisting of 0.1% formic acid in distilled water (A) and methanol-acetonitrile−0.1% formic acid in distilled water (8:2:1, *v/v/v*) (B). The gradient conditions were as follows: 0–1 min, 98% A (2% B); 1–3 min, 50% A (50% B); 3–3.75 min, 98% A (2% B); and 3.75–7 min, 98% A (2% B). The column flow rate was 0.5 mL/min, and injection volume was 3 μL. The mass spectrometer was operated using a turbo ion spray ionization source configured for electrospray ionization in positive mode, and acquisition was conducted using multiple reaction monitoring. The two-pair multiple reaction monitoring transition for the peptide 13-mer, *m*/*z* 699.01–913.48 (collision energy (CE) 35 V) and *m*/*z* 699.01–1027.58 (CE 34 V) was used.

3. Results and Discussion

The product ion mass spectrum (MS/MS spectrum) of the 13-mer peptide is presented in Figure 1A. The product ion spectrum corresponding to the total ion chromatogram (sum of *m*/*z* 913.48 and 1027.58) is presented in Figure 1B–G. Only detected the 13-mer peptide in beef samples were presented in Figure 1C–G. The following general settings were used: curtain gas, 30 psi; ion source gas (1), 50 psi; ion source gas (2), 55 psi; temperature 550 °C; and ion spray voltage, 5500 V.

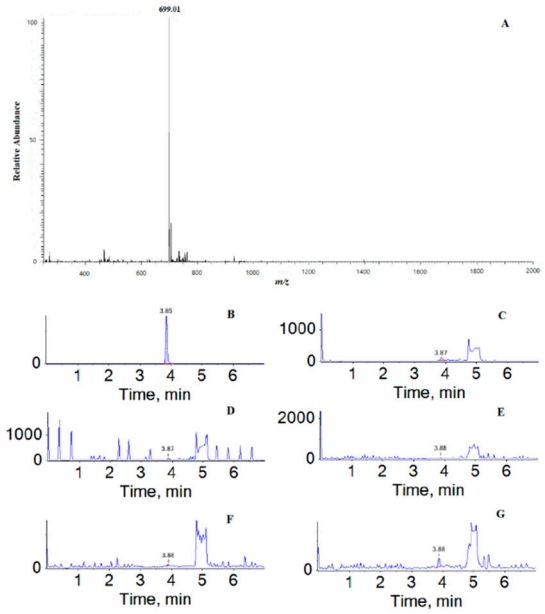

Figure 1. Product ion mass spectrum (MS/MS spectrum) of the 13-mer peptide (**A**), TIC of 13-mer (*m*/*z* 699.01–913.48, 1027.58) in standard solution (100.0 μg/kg) (**B**) and beef samples aged for 0 (**C**), 2 (**D**), 4 (**E**), 5 (**F**), and 9 (**G**) weeks, respectively.

We developed a precision analysis method using LC/MS/MS to measure the myl3 concentration in beef samples as a quality test for dry aging. Myofibrillar proteins, mainly myosin and actin, are the primary components of the skeletal muscle [17]. We hypothesized that myosin isolated from muscle skeletal protein is related to beef tenderness. However, the molecular weight of myosin is approximately 500 kDa [18]; to detect isolated myosin in beef samples, it must be broken down into small molecules. Myosin is composed of two subunits, a heavy chain, which is the protein tail, and a light chain, which is the protein head. Natural enzymes such as trypsin and papain can degrade myosin. The myosin

molecule is separated into heavy and light chains by trypsin. The light chain (myosin head) can be separated into small fragments (including myosin light chain 3) by papain, indicating beef tenderness [18]. Therefore, in this study, trypsin and papain were used to extract myl3 from the myosin head.

Nevertheless, myl3 also has a large molecular weight. To measure the my13 concentration using LC/MS/MS, digestion and IP were required. After digestion and IP, myl3 was detected with its standard (tryptic fragment of myl3, 13-mer). The analytical results are presented in Table 1. Good linearity was achieved, as revealed by the correlation coefficients (r = 0.9900). The limit of detection and limit of quantification for the method were estimated at SD/b ratios of 3.3 and 10, where SD and b represent the standard deviation of the intercept and slope of the regression line, respectively. The limit of detection and limit of quantification were 0.3 µg/kg and less than 0.8 µg/kg, respectively, indicating that the developed method for evaluating beef aging is reliable. To verify recovery for an IP procedure using a His-tagged Myl3 protein standard, a spiking recovery test was performed. Recovery test method was used in previous study by Berna et al. [15]. These tests yielded recoveries of 81.1% and 84.5%, which are proximate values compared to the recovery (12.0, percent relative error, % RE) described by Berna et al. [15].

Table 1. Linearity and sensitivity of the developed method for the peptide 13-mer [(1)].

Analyte	Linear Range (µg/kg)	Slope	Intercept	Correlation Coefficient (r)	LOD (µg/kg)	LOQ (µg/kg)
Peptide13-mer [(1)]	0.2–100.0	1.57×10^5	$-1.71 \times 10^2 \pm 13.09$	0.9990	0.3	0.8

[(1)] All values were calculated using intraday (n = 5) analyses of standard solutions.

The myl3 contents in dry-aged beef are presented in Figure 2. In non-dry-aged beef (fresh beef), the myl3 content was approximately 6 µg/kg. The myl3 content decreased to approximately 3 µg/kg after 2 weeks; this level was maintained until 5 weeks. The myl3 content nearly recovered to the initial value of 6 µg/kg at 9 weeks but the data showed a few variations. Myl3 was not detected after 11 weeks. These results indicate that residual myosin, including the myl3 myosin fragment, is degraded to an undetectable level after 11 weeks. The absence of myl3 after 11 weeks can be evidence to judge the degree of aging degree in evaluating dry aging beef.

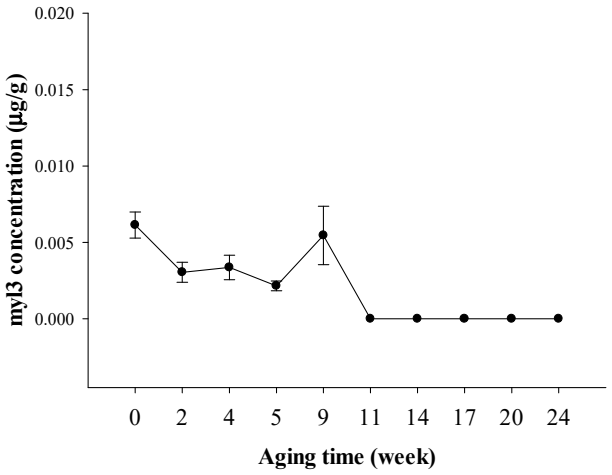

Figure 2. Analytical values for myl3 (as the peptide 13-mer) contents in beef samples as a function of aging period (intraday, n = 3).

4. Conclusions

In summary, we established a method for detecting the muscle protein fragment myl3 using LC/MS/MS in beef, which is more accurate than western blotting. The developed

analytical method is the first approach useful for analyzing myl3 in beef using LC/MS/MS. In addition, this method can be used to measure protein more accurately in beef samples than western blotting.

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