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ARTICLE

Analysis of Myosin Heavy Chain Isoforms from *Longissimus Thoracis* Muscle of Hanwoo Steer by Electrophoresis and LC-MS/MS

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Abstract

The purpose of this study was to analyze myosin heavy chain (MHC) isoforms in bovine *longissimus thoracis* (LT) muscle by liquid chromatography (LC) and mass spectrometry (MS). LT muscles taken from Hanwoo (Korean native cattle) steer (n=3) used to separate myosin bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The peptide queries were obtained from the myosin bands by LC-MS/MS analysis following in-gel digestion with trypsin. A total of 33 and 43 queries were identified as common and unique peptides, respectively, of MHC isoforms (individual ions scores >43 indicate identity or extensive homology, $p<0.05$). MHC-1 (IIx), -2 (IIa), -4 (IIb), and -7 (slow/I) were identified based on the Mowse score (5118, 3951, 2526, and 2541 for MHC-1, -2, -4, and -7, respectively). However, more analysis is needed to confirm the expression of MHC-4 in bovine LT muscle because any query identified as a unique peptide of MHC-4 was not found. The queries that were identified as unique peptides could be used as peptide markers to confirm MHC-1 (14 queries), -2 (8 queries), and -7 (21 queries) in bovine LT muscle; no query identified as a unique peptide of MHC-4 was found. LC-MS/MS analysis is a useful approach to study MHC isoforms at the protein level.

Keywords: myosin heavy chain, LC-MS/MS, cattle, *longissimus thoracis*

Introduction

The metabolic and contractile properties of muscle fiber types depend on the different myosin heavy chain (MHC) isforms, because myosin ATPase and actin-binding sites are localized at the globular head of the MHC (Rayment *et al.*, 1993; Tokunaga *et al.*, 1987). The MHC exists as multiple isoforms encoded for by the gene family, and nine MHC isoforms have been observed in mammalian cardiac and skeletal muscles (Berg *et al.*, 2001; Qin *et al.*, 2002; Weiss *et al.*, 1999). Previous studies (Acevedo and Rivero, 2006; Chikuni *et al.*, 2004; Kim *et al.*, 2005; Kim *et al.*, 2013a, b; Kjellgren *et al.*, 2003; Lefaucheur *et al.*, 2002) reported three or four MHC isoforms in mammals, inculding human, horse, and cattle muscles (MHC-I, -IIa, and -IIx), porcine muscle (MHC-I, -IIa, -IIx, and -IIb), and canine muscle (MHC-I, -IIa, and IIx). The relative proportions of the MHC isoforms are closely related to the differences in functional properties of muscle and in quality properties of meat both among the species and between the cuts. Efforts to analyze the expression of the MHC isoforms have been hindered be- cause of the high homology and identity among the MHC isoforms, regardless of species.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the most commonly used technique for MHC analysis, enables the separation of MHC isoforms. Until now, the method presented by Talmadge and Roy (1993) was adapted to MHC separation in various mammalian skeletal muscles. However, this method did not always clearly separate the MHC isoforms and since then many modified protocols have been introduced (Kim *et al.*, 2013a; Kohn *et al.*, 2007; Picard *et al.*, 1999). Nevertheless, to confirm the specific MHC isoforms from each separated band, additional analyses, such as immunoblotting and immunohistochemistry with monoclonal antibodies, are needed.

In the present study, liquid chromatography (LC) and mass spectrometry (MS) were used for the analysis of bovine MHC isoforms. LC-MS/MS analysis allows confirmation of the unique peptides of each isoform by comparing identified peptides among the MHC isoforms. This technique does not require separation of the MHC isoforms on SDS-PAGE gel. Therefore, the purpose of this study was to develop the method to identify bovine MHC

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isoforms using LC-MS/MS analysis.

Materials and Methods

Sample preparation

Muscles (*Longissimus Thoracis*, LT) were taken from three Hanwoo steers (842±25 d of age, 653±15 kg of live weight) and promptly frozen in liquid nitrogen. Myofibrillar protein extraction and preparation were performed using the method described by Talmadge and Roy (1993). In brief, samples were mixed with sample buffer containing 4% (w/v) SDS, 1 M Tris-HCl, 20% (w/v) glycerol, 1% (v/v) mercaptoethanol, and small amount of bromophenol blue as a trace. Protein concentration was determined according to the Bradford (1976) method with bovine serum albumin as a standard.

SDS-PAGE

The stacking gels were composed of 4 mM EDTA, 0.4% (w/v) SDS, 4% (w/v) acrylamide/Bis (50:1), 30% (w/v) glycerol, and 70 mM Tris-HCl (pH 6.70). The separating gels were composed of 0.1 M glycine, 0.4% (w/v) SDS, 0.2 M Tris-HCl (pH 8.80), 10% (w/v) acrylamide/Bis (50 :1), and 45% (w/v) glycerol. SDS-PAGE was conducted in a mini-gel (0.75-mm-thick) system (SE-250, Amersham Biosciences, USA) at 50 mA/gel for 2 h, and the bands were visualized by Coomassie Brilliant Blue (CBB) staining. The result for SDS-PAGE was presented in Fig. 1.

In-gel digestion

The protein bands were excised from the CBB stained gel, destained with 50 mM NH_4HCO_3 buffer (pH 7.8) containing 30% (v/v) acetonitrile, and dried completely in a SpeedVac (SPD1010, Thermo Fisher Scientific Inc., USA) for 10 min. The dried gel pieces were rehydrated in 10 μL (2.5 ng/μL) of trypsin solution (Promega, UK) in 50 mM NH_4HCO_3 buffer (pH 7.8) at 4°C for 2 h. After 10 μL of 50 mM NH_4HCO_3 was added, the gel slices were incubated at 37°C for 12 h.

LC-MS/MS and data searching

LC-MS/MS analysis was performed with a nano-LC and LTQ mass spectrometer (Agilent 1100, Thermo Electon, USA). The capillary column was obtained from Proxeon (150 mm × 0.075 mm, Odense M, Denmark) and slurry packed in-house with 5 μm, 100 Å pore size Magic C18 stationary phase (Michrom Bioresources, USA). The mobile phase A for the LC separation was 0.1% formic acid in deionized water, and mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was set up to give a linear increase from 5% B to 35% B in 50 min, from 40% B to 60% B in 20 min, and from 60% B to 80% B in 5 min. The flow rate was maintained at 300 nl/min after splitting. Mass spectra were acquired using data-dependent acquisition with a full mass scan (400-1800 m/z) followed by MS/MS scans. Each MS/MS scan acquired was an average of one microscan on the LTQ. The temperature of the ion transfer tube was controlled at 200°C and the spray was 1.5-2.0 kV. The normalized collision energy was set at 35% for the MS/MS. Sequences of the MS/MS spectra were identified by NCBInr database search using the MASCOT search engine (Matrix Science MASCOT software). Database search criteria were as described: fixed modification, no; carboxyamidomethylated at cysteine residues, variable modification, oxidized at methionine residues; maximum allowed cleavage, 1; MS tolerance, 1.2 Da; MS/MS tolerance, 0.6 D. Only peptides resulting from trypsin digests were considered.

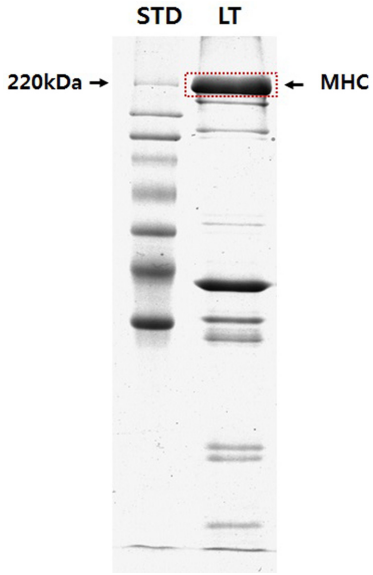

Fig. 1. The result of one-dimensional gel electrophoresis for separate the myosin heavy chain (MHC) isoforms from bovine *longissimus thoracis* (LT) muscle. STD, standard marker.

Probability based Mowse score

Ions score is -10×log(P), where P is the probability that the observed match is a random event. Individual ions scores >43 indicate identity or extensive homology ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. Mowse scores of the MHC isoforms were 5118, 3951, 2526, and 2541 for MHC-1 (IIx), -2 (IIa), -4 (IIb), and -7 (slow/I),

respectively.

Results

As shown in Fig. 1, MHC was separated from the sample mixture by SDS-PAGE analysis and LC-MS/MS analysis was performed following in-gel digestion of the MHC band with trypsin. Four MHC isoforms (three MHC-II types and one MHC-I type) including MHC-1 (2x, II type), -2 (2a, II type), -4 (2b, II type), and -7 (slow, I type) were identified (Table 1). MHC-1, which has p*I* of 5.57, molecular weight of 223,900 Da, and 1938 amino acids of pep-

Table 1. Proteins identified as bovine myosin heavy chain (MHC) isoforms by LC-MS/MS analysis

MHC[a)]	Accession number[b)]	Species	Theoretical MW (Da)[c)]	p*I*	Size (aa)[d)]	Sequence coverage (%)	Queries matched	Queries with identity[e)]
MHC-1 (IIx)	gi|21743235	*Bos taurus*	223900	5.57	1938	49	524	53
MHC-2 (IIa)	gi|261245063	*Bos taurus*	224243	5.63	1943	45	397	39
MHC-4 (IIb)	gi|296476617	*Bos taurus*	223875	5.62	1938	33	263	27
MHC-7 (slow/I)	gi|13560273	*Bos taurus*	224026	5.58	1935	37	225	28

[a)]Myosin heavy chain.
[b)]Accession numbers were taken from the NCBI database.
[c)]Theoretical molecular weight.
[d)]Peptide size (amino acids) recorded in NCBI database.
[e)]Individual ions scores greater than 43 indicate identity ($p<0.05$).

Table 2. Queries expressed in common in two or more myosin heavy chain (MHC) isoforms

Sequence	Ion score[a)]	Mr (expt)	Mr (cal)	MHC
ALQEAHQQTLDDLQAEEDKVNTLTK	71	2839.7	2839.0	MHC-1, MHC-2, MHC-4
ANSEVAQWR	57	1060.7	1060.1	MHC-1, MHC-4, MHC-7
HADSVAELGEQIDNLQR	73	1895.5	1895.0	MHC-2, MHC-4
HADSVAELGEQIDNLQRVK	76	2123.4	2122.3	MHC-1, MHC-2, MHC-4
IAEKDEEIDQLKR	69	1587.3	1586.7	MHC-1, MHC-2, MHC-4
IAEQELLDASER	67	1373.5	1373.5	MHC-1, MHC-2
IKEVTERAEDEEEINAELTAK	71	2418.6	2417.6	MHC-1, MHC-2
KALQEAHQQTLDDLQAEEDKVNTLTK	83	2967.8	2967.2	MHC-1, MHC-2
KHADSVAELGEQIDNLQR	46	2023.2	2023.2	MHC-1, MHC-2, MHC-4
LAQLITR	50	815.2	814.0	MHC-1, MHC-4
LASADIETYLLEK	72	1465.7	1465.6	MHC-1, MHC-2, MHC-4, MHC-7
LQHELEEAEERADIAESQVNKLR	82	2708.5	2707.9	MHC-1, MHC-2, MHC-4
LYEQHLGK	54	988.1	987.1	MHC-1, MHC-4
SALAHALQSAR	47	1125.4	1124.3	MHC-1, MHC-4
VGNEFVTK	65	893.7	893.0	MHC-1, MHC-4
VLNASAIPEGQFIDSK	50	1688.8	1688.9	MHC-1, MHC-4
DLEEATLQHEATAAALR	93	1838.0	1839.0	MHC-1, MHC-2, MHC-4
DLEEATLQHEATAAALRKK	46	2095.8	2095.3	MHC-4, MHC-7
ELEGEVESEQKR	54	1433.5	1432.5	MHC-1, MHC-2
IEELEEEIEAERASR	66	1803.6	1802.9	MHC-1, MHC-2, MHC-4
ILYADFKQR	48	1154.1	1153.3	MHC-1, MHC-2, MHC-4
INQQLDTKQPR	62	1341.4	1340.5	MHC-1, MHC-2, MHC-4
IQLELNQVKSEIDR	54	1684.6	1684.9	MHC-1, MHC-2, MHC-4
KIAEQELLDASERVQLLHTQNTSLINTK	53	3194.0	3193.6	MHC-1, MHC-2
KKHADSVAELGEQIDNLQR	83	2152.1	2151.3	MHC-1, MHC-2, MHC-4
LQDLVDKLQAK	63	1270.6	1270.5	MHC-1, MHC-2
LQTESGEFSR	67	1153.0	1153.2	MHC-1, MHC-2
NVEAVKGLR	69	984.6	985.1	MHC-1, MHC-2
RDLEEATLQHEATAAALR	66	1996.1	1995.2	MHC-1, MHC-2, MHC-4, MHC-7
TKYETDAIQR	58	1223.7	1224.3	MHC-1, MHC-2, MHC-4, MHC-7
VIQYFATIAVTGEK	46	1540.2	1539.8	MHC-2, MHC-4
VRELEGEVESEQKR	62	1688.8	1687.8	MHC-1, MHC-2
YKVLNASAIPEGQFIDSK	50	1981.0	1980.2	MHC-1, MHC-4

[a)]Individual ions scores greater than 43 indicate identity ($p<0.05$).

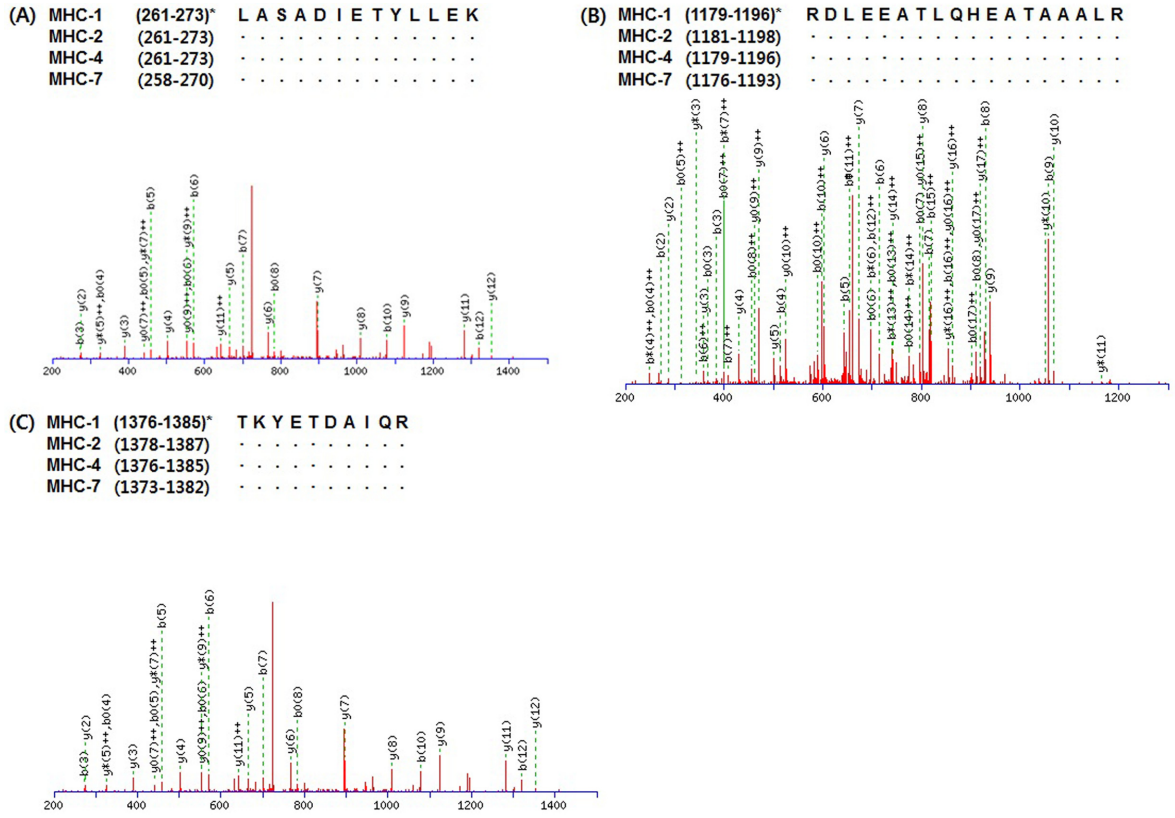

Fig. 2. Representative MS/MS spectra identified as common peptides of myosin heavy chain (MHC) isoforms in bovine *longissimus thoracis* muscle. *, residues of amino acid sequence of each MHC isoform.

tide size had 524 matched queries. The sequence coverage showed 49%, and there were 53 queries with identity (the scores greater than 43 indicate identity with $p<0.05$). MHC-4 also has 1938 amino acids of peptide size but showed matched queries, queries with identify, and sequence coverage of 263, 27, and 33%, respectively. The other MHC-II type, MHC-2, which has p*I* of 5.63, molecular weight of 224,243 Da, and 1943 amino acids of peptide sequence, was identified by 397 matched queries, 39 queries with identity, and 45% sequence coverage. MHC-7, a MHC-I type, has p*I* of 5.58, molecular weight of 224,026 Da, and 1935 amino acids of peptide size was identified by 225 matched queries, 28 queries with identity, and 37% sequence coverage. MHC-1 had the highest sequence coverage, matched queries, and queries with identity among the MHC isoforms, whereas MHC-4 had the lowest sequence coverage and queries having identity (ion score >43).

Peptides expressing in common in two or more MHC isoforms are shown in Table 2. A total of 33 queries were detected as common peptides and only three queries such as HADSVAELGEQIDNLQR, DLEEATLQHEATAAAL RKK, and VIQYFATIAVTGEK did not match the amino acid sequence of MHC-1. However, MHC-7 had just five queries (ANSEVAQWR, LASADIETYLLEK, DLEEATLQHEATAAALRKK, RDLEEATLQHEATAAALR, and TKYETDAIQR) matching its amino acid sequence. A total of 25 and 24 queries were observed as common peptides that matched MHC-2 and -4, respectively. As presented in Fig. 2, three queries (LASADIETYLLEK, RDL EEATLQHEATAAALR, and TKYETDAIQR) were found as common peptides expressing in all MHC isoforms. LASADIETYLLEK corresponds to residues 261-273 of MHC-1, -2, and -4 and to residues 258-270 of MHC-7. RDLEEATLQHEATAAALR corresponds to residues 1179-1196 of MHC-1 and -4, residues 1181-1198 of MHC-2, and residues 1176-1193 of MHC-7. TKYETDAIQR corresponds to residues 1376-1385 of MHC-1 and -4, residues 1378-1387 of MHC-2, and residues 1373-1382 of MHC-7.

Unique peptides matched the sequence of each MHC isoform presented in Table 3. A total of 43 peptides were detected for MHC-1 (14), -2 (8), and -7 (21). LQTESGEFSRQLDEKDALVSQLSR corresponds to residues 1283-1306 of MHC-1. This is the same sequence area of residues 1285-1308 of MHC-2; however, MHC-2 has “E (glutamic acid)” instead of “D (aspartic acid)” in residue

Table 3. Queries identified as unique peptides of each myosin heavy chain (MHC) isoform

MHC	Sequence	Residue number	Ion score[a)]	MW (expt)	MW (calc)
MHC-1	TSVFVADPKESFVKATVQSR	36-55	62	2197.6	2196.5
MHC-1	VGNEFVTKGQTVEQVYNAVGALAK	409-432	54	2523.2	2522.8
MHC-1	GQTVEQVYNAVGALAK	417-432	71	1648.6	1647.8
MHC-1	SSVKTLALLFSGPASGEAEGGPK	615-637	60	2203.4	2203.4
MHC-1	ARLQTESGEFSR	1281-1292	71	1380.1	1380.5
MHC-1	LQTESGEFSRQLDEKDALVSQLSR	1283-1306	83	2737.8	2736.9
MHC-1	QLDEKDALVSQLSR	1293-1306	88	1602.9	1601.8
MHC-1	LAQRLQDAEEHVEAVNAK	1396-1413	44	2021.9	2021.2
MHC-1	LQDAEEHVEAVNAKCASLEK	1400-1419	51	2242.1	2241.4
MHC-1	QKYEETHAELEASQKESR	1461-1478	44	2163.9	2163.3
MHC-1	IVESMQSTLDAEIR	1596-1609	49	1607.8	1607.8
MHC-1	SYKRQAEEAEEQSNVNLSK	1880-1898	82	2210.2	2210.3
MHC-1	RQAEEAEEQSNVNLSK	1883-1898	64	1832.4	1831.9
MHC-1	QAEEAEEQSNVNLSK	1884-1898	90	1676.6	1675.7
MHC-2	SAETEKEMATMKEEFQK	847-863	47	2050.0	2049.2
MHC-2	LQTESGEFSRQLDEKEALVSQLSR	1285-1308	60	2751.5	2751.0
MHC-2	QLDEKEALVSQLSR	1295-1308	52	1615.5	1615.8
MHC-2	ALSKANTEVAQWR	1365-1377	58	1473.5	1473.6
MHC-2	ANTEVAQWR	1369-1377	69	1074.3	1074.1
MHC-2	LQAAEEHVEAVNAK	1402-1415	80	1508.4	1508.6
MHC-2	RQAEEAEEQSNTNLSK	1885-1900	59	1834.5	1833.9
MHC-2	QAEEAEEQSNTNLSK	1886-1900	73	1677.4	1677.7
MHC-7	DVFVPDDKEEFVKATILSR	36-54	46	2209.1	2208.5
MHC-7	EQATGKGTLEDQIIQANPALEAFGNAK	208-234	81	2815.4	2815.1
MHC-7	GTLEDQIIQANPALEAFGNAK	214-234	80	2199.5	2200.4
MHC-7	VGNEYVTKGQNVQQVVYAK	406-424	69	2124.6	2124.4
MHC-7	GQNVQQVVYAK	414-424	58	1234.2	1233.4
MHC-7	LFDNHLGK	552-559	51	943.8	943.1
MHC-7	ILNPAAIPEGQFIDSR	724-739	69	1740.4	1741.0
MHC-7	LKEALEKSEAR	859-869	60	1273.4	1273.4
MHC-7	ALQEAHQQALDDLQAEEDKVNTLTK	998-1022	48	2809.0	2809.0
MHC-7	KKHADSVAELSEQIDNLQR	1194-1212	67	2182.0	2181.4
MHC-7	SKAEETQRSVNDLTSQR	1261-1277	47	1950.2	1949.0
MHC-7	SVNDLTSQRAK	1269-1279	45	1218.2	1218.3
MHC-7	LQTENGELSR	1280-1289	55	1146.1	1146.2
MHC-7	QLDEKEALISQLTR	1290-1303	48	1643.8	1643.8
MHC-7	VLSKANSEVAQWR	1360-1372	69	1487.5	1487.7
MHC-7	LAQRLQDAEEAVEAVNAK	1393-1410	54	1955.6	1955.1
MHC-7	SNAAAAALDKK	1435-1445	44	1059.3	1059.2
MHC-7	VVDSLQTSLDAETR	1593-1606	57	1533.3	1533.6
MHC-7	VRELENELEAEQKR	1819-1832	51	1743.4	1742.9
MHC-7	QAEEAEEQANTNLSK	1881-1895	98	1662.3	1661.7
MHC-7	VQHELDEAEERADIAESQVNKLR	1899-1921	77	2680.2	2679.9

[a)]Individual ions scores greater than 43 indicate identity ($p<0.05$).

1300 (Fig. 3). MHC-7 has different amino acids in residues 1284 (N, asparagine), 1287 (L, leucine), 1298 (I, isoleucine), and 1302 (T, threonine) instead of S (serine), F (phenylalanine), V (valine), and S of MHC-1, respectively. Unique peptides for MHC-7 were found as two queries including LQTENGELSR and QLDEKDALVSQLSR (Fig. 3C and 3D). The queries presented in Fig. 4 have different sequence length, but LQAAEEHVEAVNAK corresponds to residues 1402-1415 of MHC-2 is peptide located in the same area of the amino acid sequences in MHC-1, -2, and -7. LQDAEEHVEAVNAKCASLEK corresponds to residues 1400-1419 of MHC-1 and includes residues 1400-1413, which correspond to residues 1402-1415 of MHC -2. Only one amino acid (D) on residue 1402 is different from A (alanine) on residue 1404 of MHC-2. LAQRLQDAEEAVEAVNAK which matched to residues 1393-

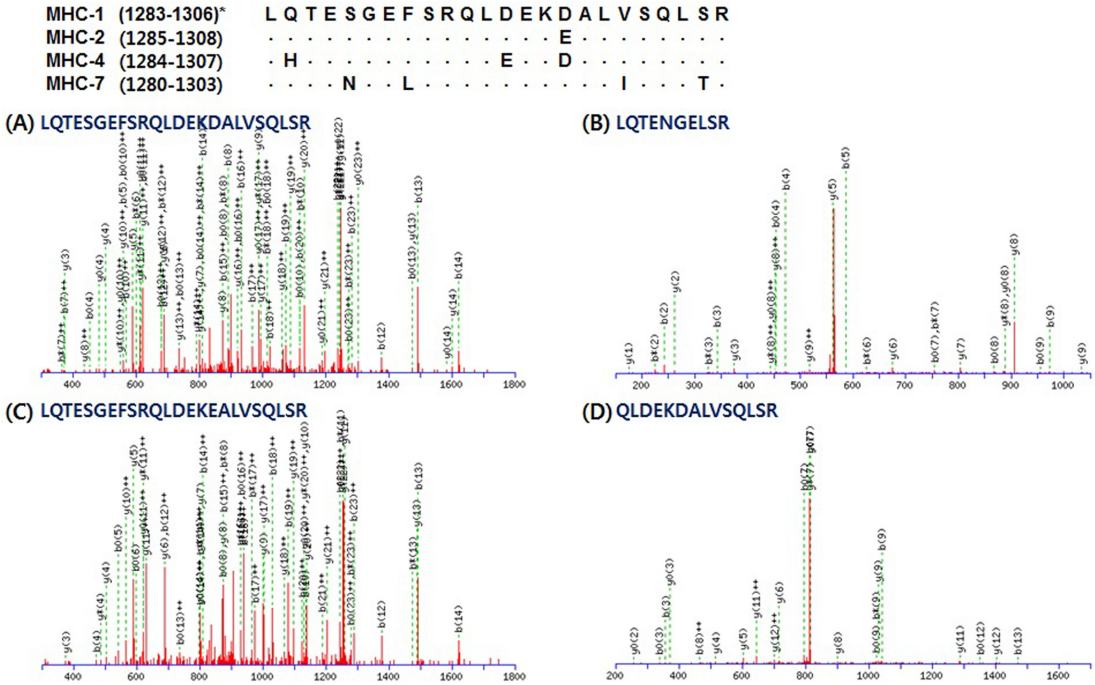

Fig. 3. Representative MS/MS spectra identified unique peptides of each myosin heavy chain (MHC) isoform: LQTESGEFSRQLDEKDALVSQLSR for MHC-1, LQTESGEFSRQLDEKEALVSQLSR for MHC-2, and LQTENGELSR and QLDEKEALISQLTR for MHC-7, respectively. *, residues of amino acid sequence of each MHC isoform.

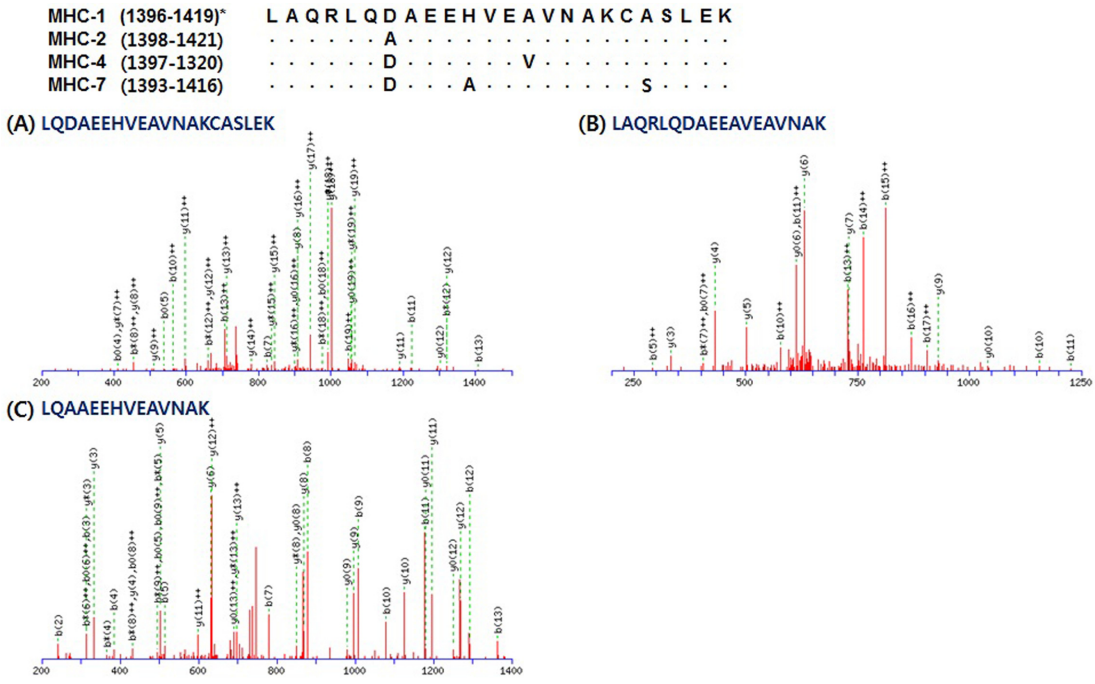

Fig. 4. Representative MS/MS spectra identified unique peptides of each myosin heavy chain (MHC) isoform: LQDAEEHVEAVNAKCASLEK for MHC-1, LQAAEEHVEAVNAK for MHC-2, and LAQRLQDAEEAVEAVNAK for MHC-7, respectively. *, residues of amino acid sequence of each MHC isoform.

1410 of MHC-7 showed that D on residue 1399 and A on residue 1403 were different from A on residue 1404 of MHC-2 and H on residues 1406 of MHC-1 and 1408 of MHC-2. Lastly, QAEEAEEQSNVNLSK, which corre-

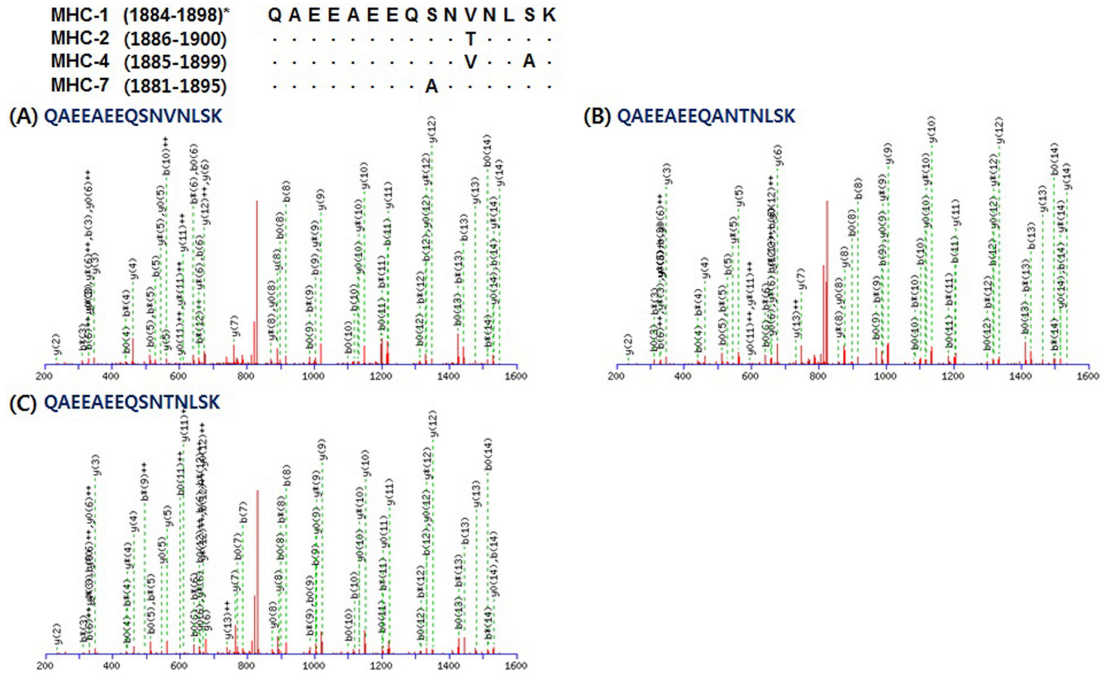

Fig. 5. Representative MS/MS spectra identified unique peptides of each myosin heavy chain (MHC) isoform: QAEEAEEQSNVNLSK for MHC-1, QAEEAEEQSNTNLSK for MHC-2, and QAEEAEEQANTNLSK for MHC-7, respectively. *, residues of amino acid sequence of each MHC isoform.

sponds to residues 1884-1898 of MHC-1, 1886-1900 of MHC-2, 1885-1899 of MHC-4, and 1881-1895 of MHC-7, was observed as a unique peptide of MHC-1, as presented in Fig. 5A. MHC-2 has T on residue 1896 instead of V on residue 1894 of MHC-1 (Fig. 5B). QAEEAEEQ SNTNLSK, which was observed in MHC-7, showed a different amino acid (A) on residue 1889 with S on residues 1892 of MHC-1 and 1894 of MHC-2 (Fig. 5C). No unique peptides were observed for MHC-4.

Discussion

The comparative sequence analysis of MHC isoforms in bovine skeletal muscle was conducted in a previous study by Chikuni *et al.* (2004). They demonstrated species-specific amino acid sequences of bovine skeletal muscle compared to porcine skeletal muscle by RT-PCR analysis of MHC mRNA. Picard and Cassar-Malek (2009) also reported MHC isoform expression in bovine skeletal muscle at the gene level. Unlike these previous reports, the findings of the present study allow us to confirm the expression of MHC isoforms in bovine skeletal muscle at the protein level. A number of peptides digested with trypsin were obtained from the MHC band that separated from the bovine LT muscle. Three MHC isoforms: MHC-1 (IIx), -2 (IIa), and -7 (I/slow), were confirmed by identification of their own unique peptides after amino acid sequence matching. MHC-4 (IIB) was also found, and its Mowse score (2526), sequence coverage (33%), and the number of queries with identity (27) were high (Table 1). However, MHC-4 had no unique peptide, as shown in Table 3. Chikuni *et al.* (2004) also reported three MHC isoforms, including MHC-slow (-7), -2a (-2), and -2x (-1) expression in bovine skeletal muscle. MHC-4 has been found in porcine skeletal muscle (Graziotti *et al.*, 2001; Kim *et al.*, 2013b, 2014; Lefaucheur *et al.*, 2002, 2004). Although Picard and Cassar-Malek (2009) demonstrated MHC-IIb (-4) expression in bovine *semitendinosus* and LT muscles, the authors recommended additional studies on MHC-4 gene transcription and any other mechanism.

The analysis of MHC isoforms at the protein level has been usually performed by electrophoresis, immunoblotting, and immunohistochemistry (IHC), regardless of the animal species (Abreu *et al.*, 2006; Kim *et al.*, 2013a; Kohn *et al.*, 2007; Lefaucheur *et al.*, 2004; Picard *et al.*, 1999). Methods including immunoblotting and IHC need three or four antibodies with specificities for MHC isoforms. Moreover, electrophoresis does not clearly separate MHC isoforms because the molecular weights among MHC isoforms are similar (223.947, 223.924, 224.010, and 223.657 kDa for porcine MHC-1, -2, -4, and -7, respectively; 223.900, 224.243, 223.875, and 224.026 kDa

for bovine MHC-1, -2, -4, and -7, respectively) (Kim *et al.*, 2013a; Table 1). In the present study, 43 queries were identified as unique peptides of each MHC isoform (excluding MHC-4) by LC-MS/MS analysis, as shown in Table 3. These peptides could be markers for MHC isoforms. For example, LQTESGEFSRQLDEKDALVSQLSR can be detected in MHC-1, whereas the others have one (E on residue 1297 of MHC-2), three (H, E, and D on residues 1285, 1296, and 1299 of MHC-4), or four (N, L, I, and T on residues 1284, 1287, 1298, and 1302 of MHC-7) different amino acids compared to MHC-1 (Fig. 3). Thus, 14, 8, and 21 queries identified as unique peptides could be identification markers for MHC-1, -2, and -7, respectively. Because unique peptides of MHC-4 were not observed, it is not possible to confirm the existence of MHC-4 in bovine LT muscle. However, the high Mowse score, high sequence coverage, and the number of queries with identity for MHC-4 indicate that additional analyses, such as immunoblotting and IHC, are needed to identify this isoform.

According to a review by Pette and Staron (2000), MHC transition occurs as MHC-I (-7) MHC-2a (-2) MHC-IIx (-1) MHC-IIb (-4), and age, nutrition, and stress can change MHC expression. Picard and Cassar-Malek (2009) found MHC-IIb (-4) in a French beef breed. If we suppose that MHC-4 did not originally exist in bovine LT muscle, some variations, such as breed (Hanwoo cattle), age (842 d old), and muscle (LT), could be reasons of it.

Conclusions

The expression of bovine MHC-1, -2, and -7 was confirmed by LC-MS/MS following electrophoresis analysis and in-gel digestion with trypsin. The unique peptides identified as part of each MHC isoform could be used as peptide markers for confirmation of MHC isoforms. It is unclear if MHC-4 expresses in bovine LT muscle because no query identified as a unique peptide of MHC-4 was found.

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