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Analysis of Differentially Expressed Proteins in Bovine Longissimus Dorsi and Biceps Femoris Muscles

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ABSTRACT : Skeletal muscle contains slow and fast twitch fibers. These skeletal muscle fibers express type I and type II myosin, respectively, and these myosin isoenzymes have different ATPase activity. The aim of this study was to investigate protein profiles of bovine skeletal muscles by proteomic analysis. Fifty seven spots of distinct proteins were excised and characterized. The expression of sixteen spots was differed in longissimus dorsi muscle with a minimal 2-fold change compared to biceps femoris muscle. The majority of differentially expressed proteins belonged to metabolic regulation-related proteins such as glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase and carbonic anhydrase 3. The real time-PCR assay confirmed an increase or induction of specific genes: RGS12TS isoform, GAPDH, triosephosphate isomerase and carbonic anhydrase. These results suggest that the expression of metabolic proteins is under a specific control system in different bovine skeletal muscle. These observations could have significant implications for understanding the physiological regulation of bovine skeletal muscles. (**Key Words :** Longissimus Dorsi, Biceps Femoris, Skeletal Muscle, Proteome, Cattle)

INTRODUCTION

Skeletal muscles are composed of various fibers to fulfill functional needs. This diversity is caused by specific myofibrillar protein isoforms that result in different fiber types (Barany, 1967). The bovine longissimus dorsi comprises a higher proportion of total carcass value than other muscle and there is more carcass-to-carcass tenderness variation in longissimus than in other muscles (Shackelford et al., 1995).

Proteomics is a novel area of research that involves the global analysis of cellular proteins using diverse technologies, such as 2-D gel electrophoresis, mass spectrometry and bioinformatics. Two-dimensional gel electrophoresis (2-DE) with its recent development has been seen as an ideal tool for proteome analysis. Although it has shortcomings, for example, poor ability to separate hydrophobic proteins and trace quantity expressed proteins, immobilized pH-gradient strips used in the first-dimensional gel electrophoresis provide a basis for reproducible separation according to protein's isoelectric points. Mass spectrometry such as electrospray ionization mass spectrometry and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Yan et al., 2001; Jung et al., 2005), can routinely identify low femtomole quantities of proteins. Recently, diseases, especially cancer and obesity, have been studied by using proteomics in human and mouse (Jones et al., 2002). However, there is limited study for determining the expression of specific proteins in bovine skeletal muscles (Hwang, 2004).

In the present study, to understand the molecular basis of physiological regulations in bovine skeletal muscle, we undertook to compare protein patterns of the longissimus dorsi and biceps femoris muscles in cattle by using proteomic analysis.

MATERIALS AND METHODS

Sample preparation of longissimus dorsi and biceps femoris muscle

The bovine longissimus dorsi and biceps femoris muscles taken from five cattles were obtained from

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Table 1. Primer sequences for PCR amplification of the specific genes

Gene		Primer	T_m (°C)*
Actin	Forward	5'-CAA GGA CCT CTA CGC CAA CAC-3'	56
	Reverse	5'-GAG CCG CCA ATC CAC ACG-3'	55
GAPDH	Forward	5'-TTC AAC GGC ACA GTC AAG G-3'	55
	Reverse	5'-ACA TAC TCA GCA CCA GCA TCA C-3'	57
RGS12TS isoform	Forward	5'-AGG AGG AGA CGC CCA AAG C-3'	56
	Reverse	5'-AGC CCA GGT AGC CCA CAA C-3'	54
Triosephosphate isomerase	Forward	5'-GGA ACT CAT CAA CAC TCT GAA CGC-3'	55
	Reverse	5'-CGC AGC CAC GGC AAT CTT G-3'	56
Jmjd1a protein	Forward	5'-TCG GGG TTG GAG CCA GTG AC-3'	55
	Reverse	5'-CTG CCA GCC AGT TCA AAG GAG ACG-3'	55
Carbonic anhydrase 3	Forward	5'-CGT GAG AAA GGC GAG TTC CAG-3'	56
	Reverse	5'-CCT TCA GCA GCA GCC ACA C-3'	55

* T_m indicates primer melting point.

slaughterhouse. Bovine longissimus dorsi and biceps femoris muscles isolated from 13th rib were frozen directly after dissection and stored at -196°C until protein analysis. Before homogenization bovine longissmus dorsi and biceps femoris were weighed, pooled and thawed in the buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT). The homogenization (30 mg/ml) was performed at room temperature with a rotor blade homogenizer, 4,000 rpm, followed by centrifugation at 15,000 rpm, at 4°C, for 30 min. The supernatant was aliquoted and stored at -80°C until the 2-DE analysis.

Two-dimensional polyacrylamide gel electrophoresis

Protein extracts (80 μg) were applied to Immobiline Drystrip™ pH 3-10 L (Amersham Biosciences Inc., Sweden). Isoelectrofocusing was conducted using pH 3-10 Pharmalytes 12 h rehydration, 1 h at 500 V gradient, 1 h at 8,000 V gradient, and 13 h at 8,000 V steady-state level. Upon completion of the first dimension, strips were incubated with gentle shaking in an equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% SDS and trace amount of bromophenol blue) containing 1% DTT for 15 min and 2.5% iodoacetamide for 15 min. For second dimension, strips were transferred to the top of 12.5% polyacrylamide gels containing SDS. Gels were run for 12 h using the Ettan DALT system (Amersham Biosciences Inc., Sweden) with 2 W/gel and 20°C. After fixation, gels were stained with silver staining kit (Amersham Biosciences Inc., Sweden).

Image acquisition and data analysis

Gels were scanned using an ImageScanner flatbed scanner. Computer-assisted image analysis was performed using ImageMaster 2-D Elite software package (Amersham Biosciences Inc., Sweden). The relative spot volume was directly related to protein concentration. All master images were obtained from 2-DE analysis of real samples from a different tissue.

Protein identification by tandem mass spectrometry

Spots were excised from the 2-DE gel. After washing with H_2O, gels were vortexed in 30 mM potassium ferricyanide and 100 mM sodium thiosulfate until stained bands were changed to translucent. After washing with H_2O, proteins in gel were reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 30 min at 56°C and then S-alkylated with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 25 min at 25°C. Gel fragments were washed with 50 mM NH_4HCO_3 and acetonitrile, and rehydrated with the digestion solution (10 ng/μl of trypsin in 50 mM NH_4HCO_3). After incubation at 4°C for 45 min, the supernatant was replaced by 50 mM NH_4HCO_3 solution and gel particles were incubated overnight at 37°C to elute peptides.

Peptide extracts and the matrix solution (5 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA) were loaded onto a teflon masked MALDI-TOF target. The MALDI-TOF MS analysis was performed with a voyager-DETM STR Biospectrometry workstation (Applied Biosystems Inc., USA). The proteins were identified by searching against NCBI non-redundant database using MS-FIT software (http://prospector.ucsf.edu/ucsfhtmal4.0/msfit.htm).

RNA extraction and reverse-transcription

Total RNA was isolated from 100 mg of longissimus dorsi and biceps femoris using the TRizol Reagent (Invitrogen Corp. USA). The RNA was eluted with diethyl pyrocarbonate-treated water. The cDNA was synthesized in 20 μl of a reaction mixture containing 3 μg of eluted RNA, 200 units of M-MLV reverse-trancriptase (Promega Corp, USA) and first strand buffer solution supplied with the enzyme, 0.5 mM of each dNTP mix, 50 pmol of oligo dT (Promega Corp, USA), and 25 units of RNasin ribonuclease inhibitor (Promega Corp, USA). The mixture was incubated at 42°C for 1 h, followed by inactivated at 95°C for 10 min.

Real-time PCR

The PCR primers were designed from using the

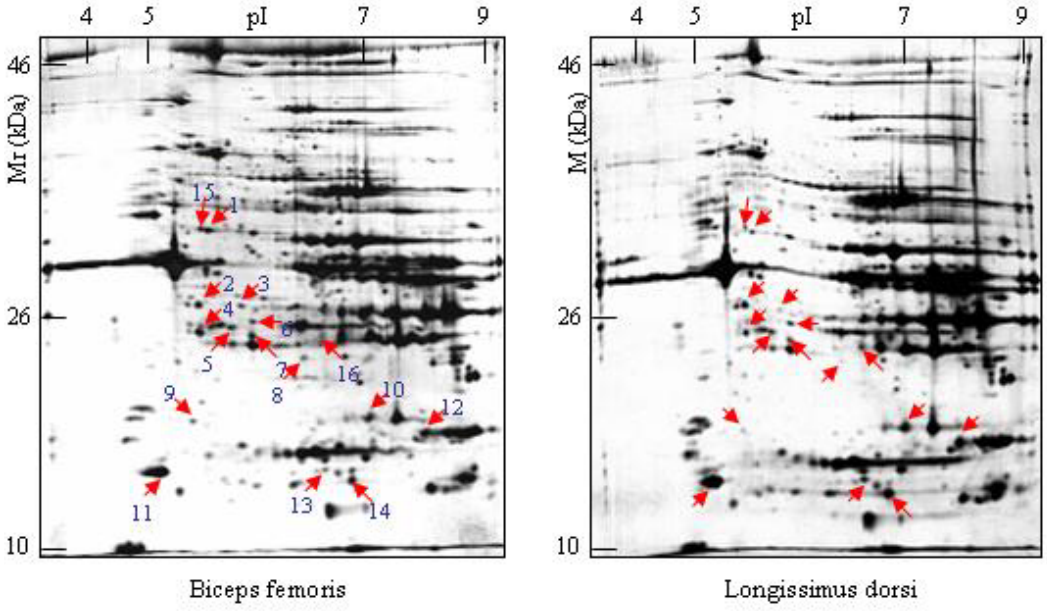

Figure 1. 2-DE protein patterns from bovine longissimus dorsi and biceps femoris muscles showing positions of putative markers identified by differential expression. Eighty μg of protein extracts were separated by 2-DE and stained with silver.

Table 2. List of identified proteins that were changed in longissimus dorsi muscle and rump tissue samples

Spot No.	Protein Name	Accession No.	Sequence Coverage (%)	Estimated MW (Da)/pI	Degree of expression in long. dorsi*
1	RGS12TS isoform	AAL69959	13	73,457/6.43	↓
2	Unidentification				↑
3	Glyceraldehyde 3-phosphate dehydrogenase	BAC56475	43	7,751/6.06	↓
4	Prx3A	CAA05283	24	24,477/9.57	↓
5	Similar to ribosomal protein S3a	Q862X0	47	15,440/9.64	↓
6	Hypothetical 49.5 kDa protein	Q8R3U9	19	50,041/5.73	↑
7	Unidentification				↓
8	Triosephosphate isomerase	1HTI_A	42	26,807/6.51	↓
9	Myosin light chain 1	2112200A	55	18,785/4.72	↓
10	Unidentification				↑
11	Carbonic anhydrase III	P07450	11	29,647/7.84	↓
12	Unidentification				↑
13	Unidentification				↑
14	Glutathione S-transferase pi	NP_803482	45	23,826/6.89	↑
15	Jmjd1 protein	AAH31158	16	86,016/7.96	↓
16	Gonadotropin inducible ovarian transcription factor 1	NP_598247	11	78,354/8.90	↑

Spot numbers refer to the location of the protein on the 2-DE gel as shown in Figure 1.

* Degree of protein expression in longissimus dorsi compared with biceps femoris (↑, increase; ↓, decrease).

program Beacom Designer 4.02 (http://www.premierBiosoft.com) designed by PREMIER Biosoft International. The amplification mixture contained 1 μl cDNA, 300 nM of each primer (see Table 1), and components of the iQ SYBR Green Supermix supplied by Bio-Rad (100 mM KCL, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP, iTaq DNA polymerase, 50 units/ml, 6 mM $MgCl_2$, SYBR Green 1, 20 nM fluorescein and stabilizers) in a final volume of 20 μl. Real-time PCR was performed with an iCycler iQ Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Inc, USA). The PCR program was consisted of an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, and primer annealing/extension at the specific annealing temperature for 30 seconds during fluorescence was measured. A melt curve was produced to confirm a single gene-specific peak and to detect primer/dimer formation by heating the samples from 50 to 95°C in 0.5°C increments with a dwell time at each temperature of 10 seconds while continuously monitoring the fluorescence. The generated melt peak represented the specific amplified product. Three independent experiments were conducted.

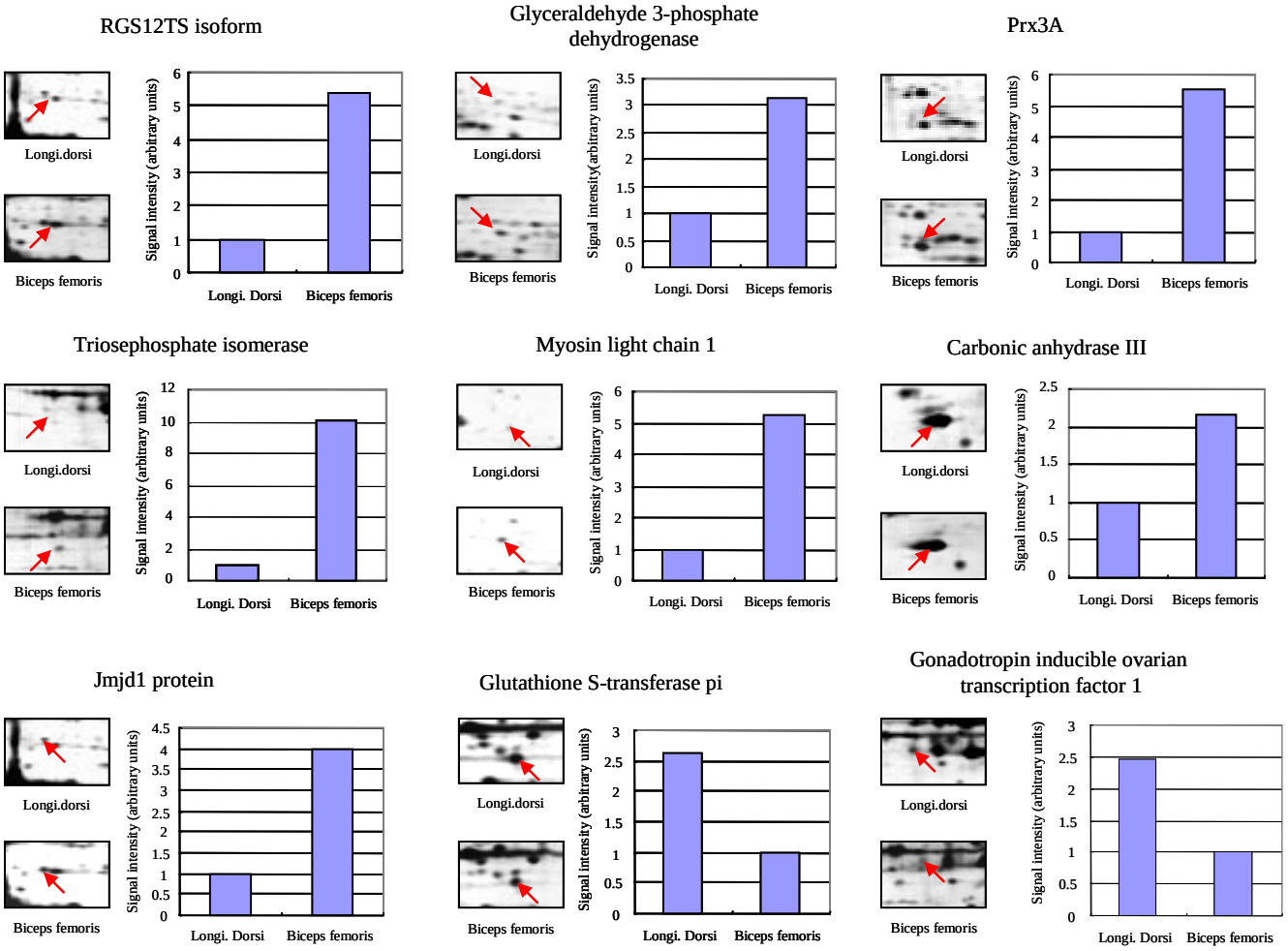

Figure 2. The magnified comparison maps of the different regions in 2-DE protein patterns between longissimus dorsi muscle and biceps femoris muscle. The arrows highlight the differentially expressed spots in tissues.

RESULTS

Proteomic analysis of bovine longissimus dorsi and biceps femoris muscle

In this study we conducted to identify differentially expressed protein in bovine longissimus dorsi and biceps femoris muscle. All differentially expressed protein spots in longissimus dorsi and biceps femoris are shown in Figure 1. We detected 286±12 and 293±14 protein spots in gels prepared from bovine longissimus dorsi muscle and biceps femoris muscle, respectively. For several of them, good MS data were collected but no identity could be assigned. This may be due to unknown genes or overlapping spots. For other spots, the MS data were not sufficient for protein identification (usually because of a low signal-to-noise ratio of the mass spectra and a small number of peptides derived from faint spots). Eighty-two percentage of protein spots in gels derived from the bovine longissimus dorsi muscle could be matched to that of biceps femoris gels. Sixteen protein spots were found to have their volumes changed in bovine longissimus dorsi muscle gels in comparison with bovine biceps femoris gels (Figure 1, Table 2). Among them, 4 protein spots had increased their expressions while 12 spots were decreased in bovine longissimus dorsi muscle compared with biceps femoris.

Identification of differentially expressed proteins by MALD-TOF MS

The selected protein spots were excised from the silver-stained gels and subjected to in-gel trypic digestion. Total sixteen differentially expressed protein spots were identified. The identified protein spots from bovine longissimus dorsi muscle and biceps femoris muscle are presented in Table 2. As shown in Figure 2, representive seven spots decreased in bovine longissimus dorsi muscles were identified as RGS12TS (regulator of G protein signaling) isoform, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Prx3A, triosephosphate isomerase (TIM), myosin light chain 1, Jmjd1 protein and carbonic anhydrase 3. Two spots increased in bovine longissimus dorsi muscles were identified as gonadotropin inducible ovarian transcription factor 1 and glutathione S-transferase pi.

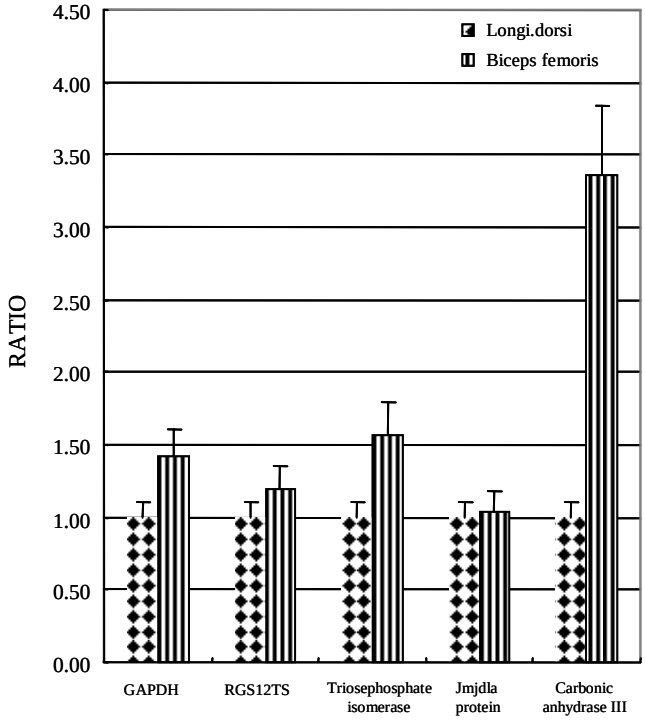

Figure 3. Transcriptional levels of differentially expressed proteins in longissimus dorsi and biceps femoris muscle. After the extraction of RNA, a real-time PCR was carried out as described in Material and Methods. The integrity of the RT reaction was confirmed by amplification using primers to the constitutively expressed beta-actin gene. Transcriptional relative intensity represents the ratio of actin intensity over that of differentially expressed proteins with regard to the transcriptional activity.

In order to determine the mRNA expression of differentially expressed proteins in longissiumus dorsi muscle compared with biceps femoris, we performed RNA quantitative real-time PCR assay. As shown in Figure 3, although there is not much change in mRNA levels, a substantial increase in carbonic anhydrase 3 protein level was observed in biceps femoris relative to longissimus dorsi muscle.

DISCUSSION

Differential proteome analysis in this study shows that sixteen proteins are quantitatively changed between the bovine longissimus dorsi muscle and biceps femoris muscle. Here we used in-gel digestion and MALDI-TOF MS to identify 16 proteins from bovine muscle: 11 of them were clearly assigned to known or homologous proteins in the SWISS-PROT non-redundant protein database. Of the identified proteins, three belong to glycolytic enzymes, two were signal transduction proteins, others were structural proteins or unknown proteins.

Spot 3 as shown in Figure 1, which was decreased in the bovine longissimus dorsi, was identified as GAPDH. GAPDH is a critical enzyme in the glycolytic pathway. Another protein decreased in the bovine longissimus dorsi muscle was TIM, spot 8. TIM catalyzes the fifth reaction of the glycolytic pathway, namely the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, two ketose-aldose isomers (Rieder and Rose, 1959). These enzymes are involved in cellular energy metabolism of skeletal muscle (Dubowitz et al., 1960). Cytosolic GAPDH and TIM catalyze a key step in anaerobic regeneration of ATP. The reduced glycolytic enzymes in longissimus tissue are strongly associated with increased intramuscular fat content (Larzul et al., 1997; Jeukendrup, 2002). These metabolism differences are associated with different mechanical properties that allow muscles to meet various functional demands.

The classification of muscle fibers into at least three types has been based on the identification of their contractile and/or metabolic properties (Cornforth et al., 1980; Kirchofer et al., 2002). The most basic classification involves slow-twitch oxidative (type I), fast-twitch oxidative glycolytic (type IIa) and fast-twitch glycolytic (type IIb) fiber types (Pearson and Young, 1989). Type II fibers are known to have lower oxidative metabolism than type I fiber (Brooke and Kaiser, 1970; Ashmore et al., 1972; Solomon et al., 1986). In the present study, the observed increase in the expression of mRNA and protein for glycolytic enzymes in the biceps femoris was consistent with the increase in the ratio of type II-to-type I fiber number and volume density seen in the muscle (Kichofer et al., 2002). In biceps femoris muscle the increased expression of glycolytic enzymes may link to reduced oxidative capacity and increased glycolytic and anaerobic capacities of skeletal muscle. It was suggesting that biceps femoris muscle contains an overall low proportion of type I (slow-twitch-oxidative) fibers compared with type II fibers. This result is also consistent with previous reports that the longissimus muscle contains more type I (slow-twitch oxidative) and fewer type IIB (fast-twitch glycolytic) fibers than biceps femoris muscles (Vestergaard et al., 1994). In addition, type I fibers have a higher density of glucocorticoid receptors (Dubois and Almon, 1984) and insulin receptors (Lefancheur et al., 1986) than do type II fibers. Therefore, the longissimus dorsi may be influenced by the concentration or metabolism of hormones such as tyroxine (Scheidegger et al., 1984), insulin (Webster et al., 1986; Vestergaard et al., 1994), and glucocorticoids (Dubois and Almon, 1984). Changes in muscle fiber characteristics and metabolic enzyme capacities should be evaluated in combination with hormone metabolism in next experiment.

Spot 11 identified as carbonic anhydrase 3 (CA3) was increased in the bovine biceps femoris muscle compared with the bovine longissiums dorsi muscle. Carbonic anhydrase plays a significant role in regulation of

cytoplasmic pH in skeletal muscle cells. Carbonic anhydrase 3 is a cytoplasmic enzyme that exhibits a relatively low carbon dioxide hydratase activity. It is well known that CA3 is present in high concentration in the cytoplasm of skeletal muscle type I to facilitate rapid conversion of glycolytic intermediates to oxaloacetate and citrate, and to stimulate in the rate of ATP synthesis (Chegwidden et al., 2000). However, our study shows that biceps femoris had dominant capacity for carbonic anhydrase to stimulate energy metabolism, suggesting that CA3 may have another role in biceps femoris. Interestingly, several studies have provided another function of CA3 to possess tyrosine phosphatase activity (Cabiscol and Levine, 1996; Kim et al., 2000). Therefore, further study would be necessary to analyze the role of CA3 in bovine rump tissue.

In the conclusion, on the basis of the present study the identification of distinct proteins shows different expression patterns in bovine longissiums dorsi and biceps femoris muscle.

IMPLICATIONS

This study presents the first investigation of the bovine longissimus dorsi muscle using a proteomic approach. The comparison of bovine longissimus dorsi muscle with biceps femoris muscle led to the identification of about 16 proteins that were specifically induced in either tissue. While we confirmed the regulated expression of many known proteins, we also found novel proteins with a potential role in either tissue such as Jmjd1 protein, gonadotropin inducible ovarian transcription factor 1, and RGS 12TS protein. Functional analysis of unknown proteins and predicted regulatory proteins would be of special interest as they will advance the understanding of the tissue-specific metabolism in cattle. Thus, differential proteome profiling is an extremely valuable tool for the identification of useful protein targets in bovine skeletal muscles

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