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Comparative proteome analysis of skeletal muscle between Merino and Tsigai lambs

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ABSTRACT

Proteomic methods allow detecting breed-specific differences at protein level. The objective of the present study was to compare muscle proteome profiles of Hungarian Merino and Tsigai sheep breeds by two dimensional gel electrophoresis and mass spectrometry based protein identification. Approximately 327 protein spots were detected in *musculus longissimus dorsi* and 14 protein spots, identified as 12 proteins, showed significant differences in their intensity ($P < 0.05$) between breeds. Seven and five proteins had higher expression in Merino and Tsigai skeletal muscle, respectively. Identified proteins were classified as structural, carbohydrate metabolism-related and miscellaneous ones. Six genes, which represent the three protein groups by function, were analysed by quantitative real-time PCR. All of the identified structural proteins have shown higher intensity in Merino, while the expression of identified glycolytic enzymes and myoglobin were higher in Tsigai lambs. One of the structural and one of the carbohydrate metabolism-related protein showed differences in expression at mRNA level by quantitative real-time PCR, as well. To the best of our knowledge, it was the first differential proteome analysis of sheep muscle and these results contribute to a better understanding of molecular differences between breeds.

1. Introduction

Although numerous papers related to proteomic study on production of meat and milk, animal welfare and health have been published recently (Lippolis and Reinhardt, 2008; Bendixen et al., 2010), the application of proteomics is still limited in animal sciences, especially in studies related to small ruminants. Proteomic methods allow the detection of breed-specific differences at protein level: whether different selection strategies and breed improvements have had measurable effects on skeletal muscle proteome. Breed-specific protein markers may help to better understand the biochemical pathways which influence meat quality and animal performance. Moreover, such studies can elucidate the significant metabolic differences between breeds which were selected for different purposes (Timperio et al., 2009).

Merino sheep is the dominant breed in many countries worldwide due to its high adaptability. In Hungary, 80% of sheep flocks belong to the Hungarian Merino sheep (Nagy et al., 2011). Hungarian Merino is originated from local native sheep, they were graded up with Rambouillet, Precoce, German Mutton Merino and Russian Merino (Porter et al., 2016). Originally, this breed is triple or dual-purpose, but nowadays meat production is the main objective. In breeding programmes the focus is on increasing prolificacy, and lamb rearing ability. The Hungarian Merino stock used in our study was formerly improved by crossing with Booroola Merino carrying the prolific allele of FecB gene to increase prolificacy (Veress et al., 1999).

Tsigai sheep is originated from Asia Minor; the first animals arrived to Central Europe at the end of 18th century through Balkan countries. Tsigai became widespread and popular, because it is a triple-purpose breed and has high adaptability to different climatic conditions (Kusza et al., 2008). This traditional breed had a larger ratio in Hungarian sheep population in the last century, but only small flocks exist at present time (Kusza et al., 2010). The Cokanski type improved for milk production at one time was included in the trial.

The aim of this study was to investigate the differences in the muscle proteome of Hungarian Merino and Tsigai sheep breeds by two dimensional gel electrophoresis and mass spectrometry-based protein

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identification.

2. Materials and methods

2.1. Animals and sampling

Twelve lambs were involved in the study, but two Hungarian Merinos were left out due to health problem, thus four Hungarian Merinos and six Tsigai lambs were used in proteomic analyses. Housing and feeding conditions were the same for each animal. They were fed *ad libitum* with concentrate and hay, fattened in a stable, kept in small groups. 4.5 months old Hungarian Merino and Tsigai lambs were sacrificed at the weight of 33.0 ± 1.4 kg and 34.4 ± 2.0 kg, respectively. Dressing weight was measured as 16.6 ± 1.3 kg and 16.4 ± 1.4 kg, dressing percentage was calculated as 48.2 ± 2% and 49.6 ± 2% in Hungarian Merino and Tsigai, respectively. None of the phenotypic traits differed significantly ($P < 0.05$) between breeds. Animals were slaughtered at a commercial slaughterhouse according to EU regulations. 2 g of *musculus longissimus dorsi* (between the 12th and 13th ribs) per samples in three replicates was harvested within 20 min after slaughter into cryotubes. Muscle samples were snap frozen in liquid nitrogen, and then kept at −80 °C until further analyses. The three replicates of muscle samples were pooled before protein isolation.

2.2. Extraction of proteins

Sample preparation and solubilisation was performed from sheep muscle tissue as follows: tissue samples were placed into liquid nitrogen and crushed in a mortar with a pestle to a very fine powder. 50 mg of tissue powder was transferred to sterile tubes containing 500 µl of lysis buffer (8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 50 mM DTT, 0.2% (v/v) Bio-Lyte 4/6 and 6/8 ampholyte at a ratio of 1:2) and 5 µl protease inhibitor cocktail (Promega). The mixture was incubated for 60 min on ice with occasional vortexing and centrifuged at 15 000*g* for 40 min at 4 °C. The supernatant was collected and stored at −80 °C until subsequent analysis. The protein concentration was determined using RC DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as standard.

2.3. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

For the first dimension (isoelectric focusing) of two-dimensional gel electrophoresis, 17 cm immobilized pH gradient strips (pH 5–8, linear, Bio-Rad) were rehydrated by passive rehydration using samples dissolved in 300 µl rehydration buffer (2 M thiourea, 8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 0.2% (v/v) Bio-Lyte 4/6 and 6/8 ampholyte at a ratio 1:2, 0.002% (w/v) Bromophenol Blue) for 15 h at room temperature. 150 µg of protein was loaded and the isoelectric focusing was conducted in Protean IEF Cell (Bio-Rad). Low voltage (250 V) was applied for 20 min, than the voltage was gradually increased to 10 000 V over 2.5 h, and maintained at that level until a total of 50 000 Vh. The current limit was adjusted to 50 mA per strip, and the run was carried out at 20 °C. Focused IPG strips were equilibrated for 10 min in 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris pH 8.8 and 2% (w/v) DTT, and then for an additional 10 min in the same buffer except that DTT was replaced by 2.5% (w/v) iodoacetamide. After equilibration, proteins were separated in the second dimension using Protean II XL vertical electrophoresis system (Bio-Rad). Second dimension was performed on 160 × 200 mm, 13% polyacrylamide gels (37.5:1 acrylamide:bis-acrylamide ratio). Gels were run using 16 mA for 30 min and then 24 mA until the bromophenol blue dye marker reached the end of the gels. A cooling system provided constant 20 °C running temperature. The gels were stained with silver staining as described by Shevchenko et al. (1996).

2.4. Image and data analysis

Gel images were recorded using PharosFX Plus (Bio-Rad) fluorescent scanner and image analysis was performed using Delta2D software (Decodon™ GmbH, Germany). For gel analysis, all gel images within a group were warped to the first standard gel image. A master gel was created by fusing all images using union fusion. Every spot on each gel was quantified and normalized according to the total intensity of all spots in each gel. Student's *t*-test was performed to assess the statistical significance of differentially expressed proteins at 95% confidence level (*t*-test; $P < 0.05$). For subsequent mass spectrometric analysis significant spot coordinates were transferred to the gels for spot picking.

2.5. Protein identification

Protein identification was carried out in the University of Debrecen Proteomics Core Facility. The spots were cut out and destained using a 1:1 mixture of $K_3(Fe(CN)_6)$ and $Na_2S_2O_3 \times 5H_2O$ and throughout washed with 50% acetonitrile in 25 mM ammonium bicarbonate solution. After stain removal trypsin digestion was performed using stabilized MS grade bovine trypsin at 37 °C overnight. The digested peptides were extracted, concentrated with speed-vac and redissolved in 10 µl 1% formic acid.

The LC-MS/MS analysis was carried out on a 4000 QTRAP (ABSciex) mass spectrometer coupled to an Easy nLC II nanoHPLC (Bruker). The peptides were desalted on a Zorbax 300SB-C18 precolumn (Agilent) and separated on a reverse phase Zorbax 300SB-C18 analytical column (Agilent) using a 90 min water/acetonitril gradient and 300 nl/min flow rate. Information Dependent Acquisition was performed and the two most intensive ions were used for MS/MS. During analyses the spray voltage was 2800 V, the nebulizing gas 50 psi, the curtain gas 10 psi, the source temperature 70 °C and the declustering potential was 50 V.

The acquired LC–MS/MS spectra were used for protein identification; the ProteinPilot 4.5 (ABSciex) search engine, the Uniprot/SwissProt database with no species restriction, trypsin as the modifying enzyme and the Biological modification table included in the ProteinPilot 4.5 were administrated. For protein identification minimum two peptides with more than 95% confidence were required.

2.6. RNA isolation and reverse transcription

For the efficient lysis, muscle tissue samples (25 ± 2.5 mg) were grinded in liquid nitrogen using a mortar and pestle, followed by lysis in 600 µl TRIzol Reagent (Thermo Fisher Scientific) with a rotor-stator homogenizer (Ultra-Turrax T10, IKA) for 90 s at max speed. Total RNA was extracted from lysate with a Direct-zol RNA MiniPrep (Zymo Research) with a DNase I digestion step. Isolated RNA concentration and purity were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was checked with agarose gel electrophoresis. 1.5 µg of total RNA was reverse transcribed using a qPCRBIO cDNA Synthesis Kit (PCR Biosystems). Reaction conditions were 42 °C for 30 min and 85 °C for 10 min. After reverse transcription, cDNA samples were diluted tenfold and stored at −20 °C.

2.7. Quantitative real-time PCR assays

Target cDNA specific, intron spanning primers (Table 1) were designed using Primer Express v3.0.1 software (Applied Biosystems). Relative quantification of target genes was based on the detection with SYBR Green (SG) non-specific dye. Real-time PCR reactions were run in triplicates (all biological replicates and both reference and target genes on the same plate) on 96 reaction plates using an ABI 7300 real-time PCR (Applied Biosystems). Thermal profile were: 95 °C for 2 min, 40 cycles of 95 °C for 5 s and 60 °C for 30 s. PCR reactions (20 µl)