Proteomic Comparison between Japanese Black and Holstein Cattle by Two-dimensional Gel Electrophoresis and Identification of Proteins

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ABSTRACT : Differences of meat qualities between Japanese Black and Holstein have been known in Japan, however, the causative proteins and/or the genetic background have been unclear. The aim of this study was to identify candidate proteins causing differences of the meat qualities between the two breeds. Using technique of two-dimensional gel electrophoresis, protein profiling was compared from samples of the *longissimus dorsi* muscle and subcutaneous adipose tissue. Five protein spots were observed with different expression levels between breeds. By using LC-MS/MS analysis and Mascot program, three of them were identified as ankyrin repeat protein 2, phosphoylated myosin light chain 2 and mimecan protein. Subsequently, we compared the DNA coding sequences of three proteins between breeds to find any nucleotide substitution. However, there was no notable mutation which could affect pI or molecular mass of the proteins. The identified proteins may be responsible for different characteristics of the meat qualities between Japanese Black and Holstein cattle. (**Key Words :** Cattle, Meat Quality, Two-dimensional Gel Electrophoresis, LC-MS/MS, Proteome)

INTRODUCTION

The Japanese Black breed of cattle is valued for its excellent meat quality such as marbling. softness and flavor, while most of the dairy cattle in Japan are Holstein breed, which are known for their excellent milk output capabilities. The male Holstein has been used as a source of domestic beef, although the meat quality is inferior to that of Japanese Black. Differences of the meat qualities and the characterization between Japanese Black and Holstein have been frequently reported, however, the mechanism and the genetic background is not well known.

Recently, a lot of proteomic researches using a two-dimensional gel electrophoresis (2DE) method have been reported (Edgar et al.. 1999; Gygi et al.. 2000). This method can separate all protein contained in sample by its specific isoelectric point and molecular mass. The 2DE method can be applied to various tissue or cell samples of animals. Comparing 2DE gel pattern among multi-samples. the

candidate proteins that are contributive to the characteristics may be revealed (Pyo et al.. 2003; Jung et al.. 2005; Yoon et al.. 2005).

The aim of this study was to identify candidate proteins causing differences of the meat qualities between two cattle breeds of Japanese Black and Holstein. The proteins with different expression level between two cattle breeds were compared for the *longissimus dorsi* muscle tissue and subcutaneous adipose tissue using 2DE electrophoresis. The candidate protein spots were identified by LC-MS/MS. Subsequently, we compared gene coding sequences of the proteins between two breeds.

MATERIALS AND METHODS

Animals

The breed comparison experiments were carried out using three steers from both Japanese Black and Holstein breeds. The animals were housed in adjacent pens in each breed group and fed under the same conditions (Taniguchi et al.. 2004). Samples of *longissimus dorsi* muscle and subcutaneous adipose tissue around the right neck were collected from each animals at 34-mo old and stored at -80°C prior to sample preparation for 2DE.
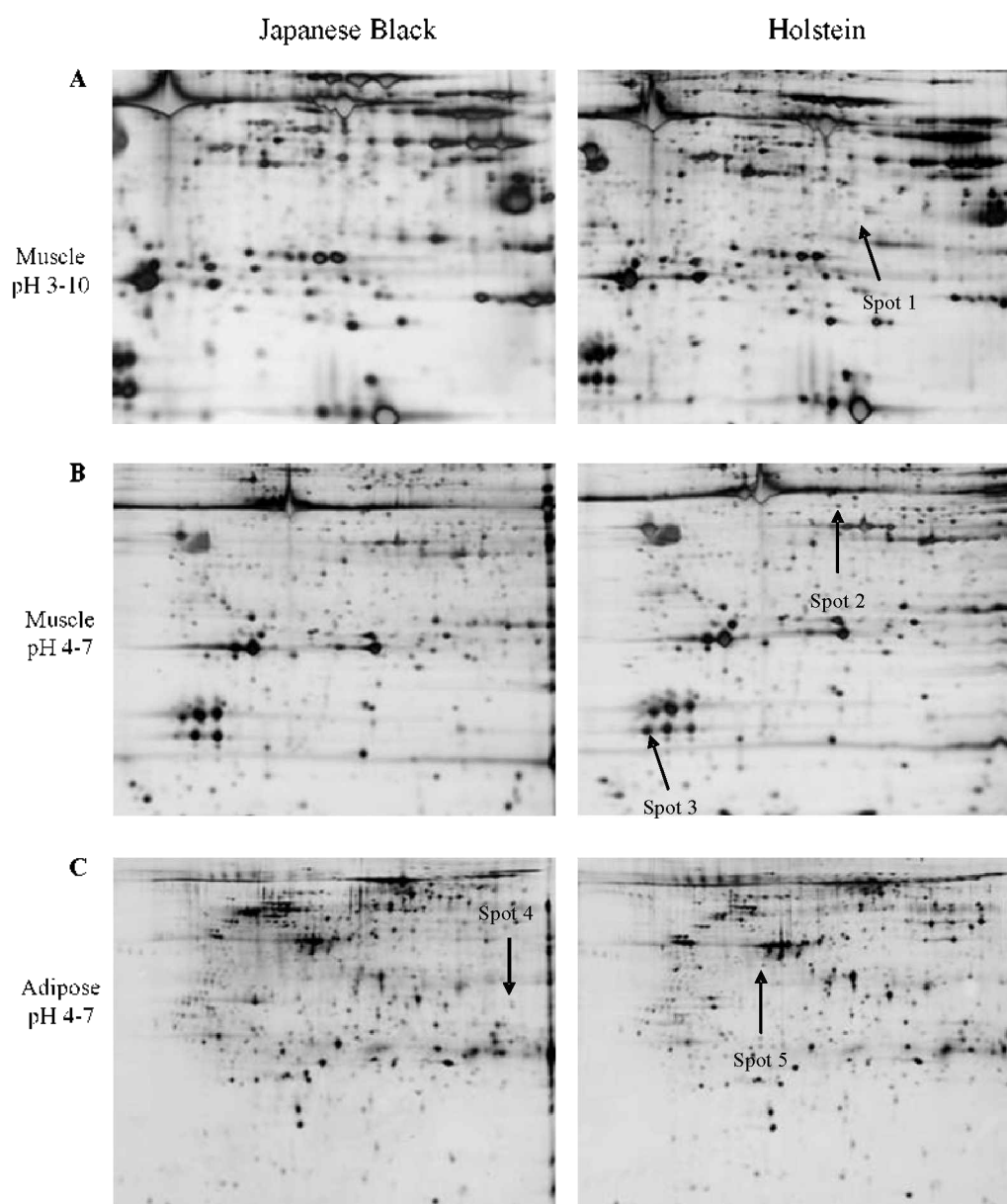
Two-dimensional gel electrophoresis

Muscle (1 g) and subcutaneous fat (2 g) were
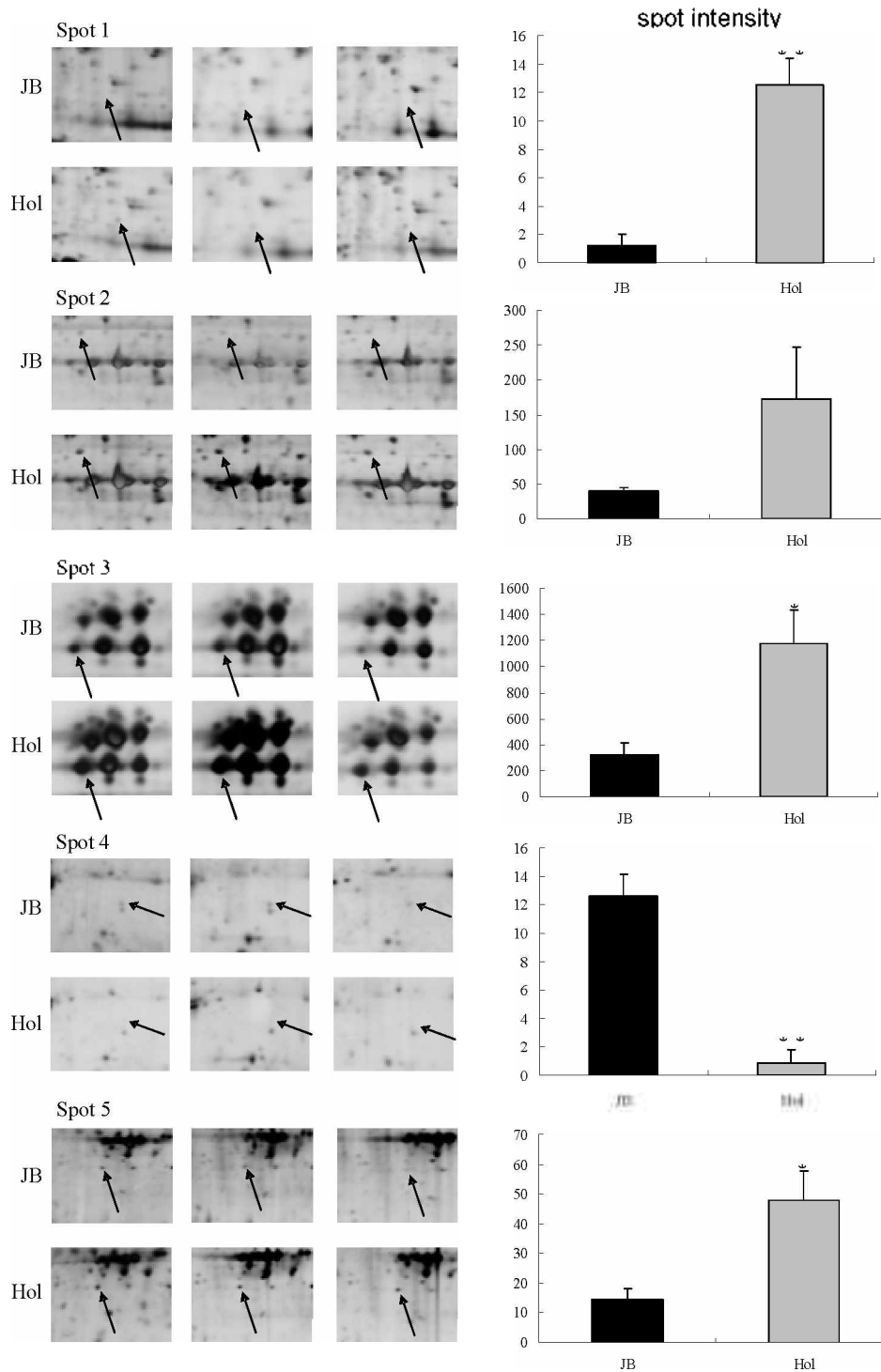
Japanese Black   Holstein

Figure 1. Comparison of 2DE map between Japanese Black and Holstein cattle. A: The 2DE gels of LD tissue using IEF of pH 3-10. B: The 2DE gels of LD tissue using IEF of pH 4-7. C: The 2DE gels of adipose tissue using IEF of pH 4-7. The black arrows indicate the spot with different expression level between two cattle breeds.

homogenized and lysed in 10 ml of Lysis Buffer (8 M urea, 2% CHAPS, 40 mM Tris-base). The lysates derived from muscle tissue were then centrifuged at 10,000 rpm for 1 min and then at 5,000 rpm for 10 min. The supernatants were collected and centrifuged at 42,000 rpm for 60 min and the resulting supernatants were stored. The lysates from adipose tissue were centrifuged at 15,000 rpm for10 min. The middle phases were again centrifuged at 15,000 rpm for 60 min and resulting middle phases were extracted.

IEF (Isoelectric Focusing) was performed with an IPGphor (Amersham Pharmacia Biotech, NJ) using 13 cm linear range of pH 3-10 and pH 4-7 IPG dry strip gels. Muscle (15 μl) and adipose (20 μl) protein samples were

applied to strip holder supplemented with swelling buffer (8 M urea, 2% CHAPS, 0.028% DTT with BPB) and IPG buffer (Amersham Pharmacia Biotech, NJ). The strip gels were then swelled for 12 h. IEF was performed at 500 V for 1 h, 1,000 V for 1 h and 8,000 V for 6 h. After IEF separation, the strip gel was equilibrated in equilibrium buffer A (50 mM Tris-HCl pH 6.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT with BPB) and equilibrium buffer B (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.25% iodoacetamide with BPB). The second dimension separation was performed on 12% SDS-PAGE gel using SE600Ruby (Amersham Pharmacia Biotech, NJ) with 10 mA for 15 min and 20 mA until the line of BPB reached the

Figure 2. Localization of protein spots (arrows) with different expression level between Japanese Black (JB) and Holstein (Hol), and the protein quantity comparisons of the spots. Measurement of spot intensity was performed triplicate, and the bar in each graph mean standard error of the intensity. Numbers of graph vertical scale are values measured by Gellab II plus ver. 2.0. * p<0.05, ** p<0.01.

bottom of the gel.

Silver staining

Silver staining of 2DE gels was performed to breed comparison analysis. Gels were fixed with 40% ethanol,

10% acetic acid for 30 min. Then, gels were sensitized with 30% ethanol, 0.125% glutaraldehyde, 0.2% sodium thiosulfate, 0.068 g/ml sodium acetate for 30 min. After washing three times with distilled water, gels were stained with 0.25% silver nitrate, 0.0074% formaldehyde for 20

Table 1. Three differentially expressed proteins identified by comparative analysis of 2DE protein profiling between Japanese Black and Holstein cattle breeds

Spot no.	Protein identified	Accession no.	Molecular mass	pI	Source	Peptides matched	Total score	Sequence coverage
2	similar to ankyrin repeat domain protein 2 isoform 1 (Arpp2)	XP_582383	36,836	5.65	*Bos taurus*	10	433	35%
3	similar to myosin light chain 2 (MLC2)	XP_591906	19,000	4.91	*Bos taurus*	23	466	75%
5	OGN protein (mimecan)	AAI02527	34,176	5.43	*Bos taurus*	1	72	5%

min, washed twice, and developed with 0.025 g/ml sodium carbonate, 0.0037% formaldehyde for 5 min. Reactions were stopped with 0.0146 g/ml EDTA-Na$_2$·H$_2$O. The stained 2DE gels were analyzed using Gellab II plus ver.2.0 (BD Biosciences, MD). A series of experiments were repeated three times for each samples.

Protein identification

In order to identify proteins whose expression levels were different between two cattle breeds, we entrusted Promega e-Service (Promega Corporation, WI) with LC-MS/MS analysis. Briefly, after 2DE, gel was stained with Coomassie Brilliant Blue (CBB) staining solution (25% ethanol, 8% acetic acid supplemented with a tablet of CBB R350 (Amersham Pharmacia Biotech, NJ)) for 30 min. Gels were bleached and washed for 1 h and, then, objective spots were picked up. The product ion data of MS/MS spectrum were assigned and searched against the NCBInr database using the Mascot search engine.

DNA sequencing

Total RNA were purified from muscle and adipose tissues using Sepasol-RNA I (nacalai tesque, Kyoto, Japan) and cDNA were synthesized using Super Script III reverse transcriptase (Invitrogen, CA). The coding region of each gene that codes the protein identified above were amplified using gene specific primers designed according to GenBank sequence. After the amplified PCR products were purified, the sequences of the products were determined by BigDye[3] Terminator v3.1 cycle sequencing kit with PRISM 3100 (Applied Biosystems, CA) according to our previous studies (Odahara et al., 2006; Sasazaki et al., 2006).

RESULTS

Comparison of 2DE patterns

Muscle and adipose tissues were collected from three of each Japanese Black and Holstein steers fed under the same conditions for 2DE comparative analysis. For 2DE separation, the first dimensional electrophoreses were performed using two types of IPGphor strips, 13 cm pH 3-10 IPG and pH 4-7 strips to observe total and intimate gel patterns. Each experiment was repeated three times for each sample. Figure 1 shows protein spot patterns of 2DE gel

stained by silver staining. Total numbers of the protein spots from muscle and adipose tissue were approximately 2,000 and 1,700, respectively. The 2DE gel patterns were almost same in each tissue between breeds (Figure 1). Three spots from muscle (spots 1, 2 and 3) and two from adipose tissue (spots 4, 5) were detected with different expression level between two cattle breeds (Figures 1 and 2). Spot 1 was found in the pH 3-10 gel and the other spots were found in pH 4-7 gel.

The spots 1, 2, 3 and 5 revealed higher expression level in Holstein than in Japanese Black, while spot 4 was a specific spot in Japanese Black. The protein spot quantities were measured using Gellab II plus ver.2.0 software (M&S Instruments Trading Inc.). Figure 2 indicates comparisons of protein spots with different expression level and the measured spot quantities between two cattle breeds. The differences of the spot quantities were significant in spots 1, 3, 4 and 5 (Figure 2). Although difference of spot 2 was not significant, Holstein cattle showed a tendency to have higher expression level (p = 0.12).

Protein identification

In order to identify the proteins with the intensity differences on the spots, we performed CBB staining for gels of candidate proteins and LC-MS/MS analysis with NCBInr database using the Mascot search engine. In consequence, the proteins contained in spots 2, 3 and 5 were identified to skeletal muscle ankyrin repeat protein 2 (Arpp2), myosin light chain 2 (MLC2) and OGN protein (mimecan), respectively (Table 1). Figure 3 shows the matched peptide sequences of each protein by LC-MS/MS analyses. All of these spots were highly expressed in Holstein cattle. The molecular mass and pI of these proteins were corresponded with the predicted these from the locations of spots in 2DE gels. The total scores of each protein were significant (p<0.05) analyzing on the basis of MOWSE algorithm incorporated in Mascot program (Pappin et al., 1993). The protein spots 1 and 4 could not be identified since these were undetectable spots with low quantities in CBB stained gel.

Sequencing of coding region

To investigate whether some nucleotide substitutions exist on these proteins coding sequences, DNA sequencing

Arpp2

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  1   MADSEEVQRATALIEERLAQEEENEKLRGTTHQKLPMEMLVLEDEKHHRP
 51   ESPSLQKVKGQERVRKTSLDLRREIIDVGGIQNLIQLRKKRKQKKREALA
101   ASQEPPPEPEEITGPVDEETFLKAAVEGKMKVIEKFLADGGSPDTCDQFR
151   RTALHRASLEGHMEILEKLLESGATVDFQDRLDCTAMHWACRGGHLEVVR
201   LLQSRGADTNVRDKLLSTPLHVAVRTGQVEIVEHFLSLGLDINAKDREGD
251   SALHDAVRLNRYKIIKLLLLHGADMMSKNLAGKTPTDLVQLWQADTRHAL
301   ENPEPGSEQNGLEGSTESGRETPQPVAAE
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MLC2

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  1   MAPKKAKRRAAAEGGSSSVFSMFDQTQIQEFKEAFTVIDQNRDGIIDKED
 51   LRDTFAAMGRLNVKNEELDAMMKEASGPINFTVFLNMFGEKLKGADPED
101   ITGAFKVLDPEGKGTIKKKFLEELLTTQCDRFSQEEIKNMWAAFPPDVGG
151   NVDYKNICYVITHGDAKD
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mimecan

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  1   MKTLQSTLLLFLFVPLIKPAPPSQQDSRNIYDYGTDNLEETFFSQDYEDK
 51   YLDGKSTKEKETMIIVPDEKSFQLQKDETITPLPPKKENDEMPTCLLCVC
101   LSGSVYCEEVDIDAVPPLPKESAYLYARFNKIKKLTAKDFADIPNLRRLD
151   FTGNLIEDIEDGTFSKLSLLEELTLAENQLLKLPVLPPKLTLFNAKYNKI
201   KSRGIKANTFKKLHNLSFLYLDHNALESVPLNLPESLRVIHLQFNNITSI
251   TDDTFCKANDTSYIRDRIEEIRLEGNPVILGKHPNSFICLKRLPIGSYI
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Figure 3. Amino acid sequences of three identified proteins and matched peptides sequences by LC-MS/MS analyses. The underlined bold letters indicate the matched peptides identified by LC-MS/MS.

for the coding region of Arpp2, MLC2 and mimecan genes were carried out using cDNA of two breeds. No mutation was observed in coding regions of Arpp2 and MLC2 between breeds. In mimecan gene, we observed one nucleotide substitution at bp 236 (C→A), which caused replacement of amino acid from threonine to asparagine. Both the amino acids are neutral and hydrophilic. Therefore, this amino acid replacement would not affect the molecular mass or pI in 2DE gel.

DISCUSSION

In this study, we found five protein spots (three from muscle tissue and two from adipose tissue) that revealed different expression levels between Japanese Black and Holstein cattle using 2DE electrophoresis. By LC-MS/MS analysis, spots 2, 3 and 5 were identified as Arpp2, MLC2 and mimecan proteins (Table 1), respectively, although the other two spots could not be identified because of their undetectable low expression level by CBB staining. The

accuracy of identification depends on Total score and identification is significant if Total score is over 49.

Spot 2 was identified to Arpp2 which is known to be expressed in skeletal muscle and also called skeletal muscle ankyrin repeat protein. The Arpp2 is related to skeletal muscle hypertrophy in mouse. Furthermore, this gene revealed very high expression level in slow-contracting soleus muscle of mouse and is associated with the slow muscle fibers, while this was almost undetectable in fast muscle (Kemp et al., 2000; Mckoy et al., 2005). The percentage of slow muscle in Japanese Black is higher than that in Holstein in almost all muscle types (Iwamoto et al., 1991). In this study, however, the protein expression level of Arpp2 was higher in Holstein cattle. Although they did not study using *longissimus dorsi*, our result may suggest different expression level of Arpp2 between ruminants (cattle) and monogastric (mouse) animals.

Spot 3 was determined to be MLC2. Bouley et al. (2005) analyzed bovine skeletal muscle hypertrophy using 2DE technique, indicating quite similar gel pattern with our

results. According to their result of protein profiling, this spot was MLC2 as was our result, moreover, they suggested this was phosphorylated MLC2. Phosphorylation of myosin light chain is associated with the transition of slow muscle to fast muscle in rat soleus muscle (Bozzo et al., 2003). Given the proportion of fast muscle in Holstein is higher than in Japanese Black (Iwamoto et al., 1991), high expression level of phosphorylated MLC2 in Holstein may be based on the proportion of muscle types.

Spot 5, which detected in adipose tissue, was identified to OGN protein that is also called mimecan/osteogoycin. It belongs to a family of small leucine-rich proteoglycans that are secreted into the extracellular matrix (Hu et al., 2005). Mimecan was initially isolated from bovine bone and subsequently characterized as one of the three major keratin sulfate-containing proteoglycan (Funderburgh et al., 1997). Tasheva et al. (2002) reported that the mimecan deficient mice have collagen fibril abnormalities in their skin. It suggests that mimecan have important role in collagen fibrillogenesis. Additionally, as with other types of proteoglycans, mimecan is also expressed in connective tissue. In our study, Holstein cattle revealed higher expression level of mimecan than Japanese Black. This result might be caused by the different expression level of this protein in fatty connective tissue and/or by higher proportion of the connective tissue in Holstein than is in Japanese Black.

Subsequently, we sequenced protein coding DNA sequences of these genes in order to search for nucleotide subsitutions, however, there was no notable mutation that could affect pI or molecular mass of these proteins. It suggests that different expression levels of these proteins between two breeds are consequence of the differences in the gene transcription or translation.

In conclusion, we found five protein spots that were differently expressed between Japanese Black and Holstein cattle breeds using 2DE. Three protein spots of them were identified by LC-MS/MS analysis. The proteomic approaches using 2DE technique would be useful to understand the complex mechanisms influenced on meat quality, although further studies are required to elucidate the differences between two cattle breeds.

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