



## Developmental Proteomic Profiling of Porcine Skeletal Muscle during Postnatal Development

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**ABSTRACT :** In this study, we have compared the skeletal muscle proteome at various stages of porcine postnatal development. Korean native pigs were divided into five postnatal stages of 30, 70, 130, 170 and 300 d and their loin muscles were analyzed for muscle proteome by using two-dimensional electrophoresis and mass spectrometry. We found 5 proteins showing a consistent pattern during skeletal muscle growth. Four proteins were identified as myosin light chain 1 slow-twitch (MLC1sa) isoform, troponin T, triosephosphate isomerase (TIP) and DJ-1 protein. The remaining protein was not identified. Two muscle fiber proteins of MLC1sa isoform and troponin T showed a high expression level at an early postnatal stage and then their levels were decreased markedly during growth stages. On the other hand, the expression of TIP and DJ-1 protein, which are well known as catalysis enzyme and antioxidant-related protein, respectively, were linearly increased during growth stages. Thus, the stage-related muscle proteins may be useful as parameters for understanding the developmental characteristics of biochemical and physiological properties in Korean native pig skeletal muscle. (**Key Words :** Muscle Proteome, Growth, Two-dimensional Electrophoresis)

### INTRODUCTION

During development, skeletal muscle characteristics change greatly. Beside the growth of muscle fiber, changes in the concentration of chemical component and the protein content in the muscles have been reported (Dickerson and Widdowson, 1960; Mahan and Shields, 1998; Bertram et al., 2002). In particular, the increase in muscle mass as a major proportion of the whole body results in a 30-fold increase in muscle protein with only a 13-fold increase in body weight (Millward et al., 1975), indicating that muscle is one of the most developmentally and metabolically active tissues in the body.

In skeletal muscles, myosin and actin are responsible for generating the physical movement of muscle fibers, while

tropomyosin and troponin are concerned with regulating this movement (Sharma, 1996). Also, skeletal muscle contains various proteins that are essential for the maintenance of homeostasis, metabolism, membrane transport and nucleic acid synthesis. As a result, muscle protein will be the important target for understanding the molecular mechanism and various proceedings on growth. The molecular mechanism of muscle growth is still unclear. Also, the changes of skeletal muscle proteome on growth have not been previously investigated.

Previously studies focus on the association of genetic variants with economic traits in pig (Kim et al., 2005; Choi et al., 2006). Proteins in muscle, not genes, sustain function. Proteomics is the large-scale study of the whole protein cell content or proteome. Recently the proteome analysis technique was used to application of meat production area (Bendixen, 2005). Growth rate and feed efficiency, as well as meat quality and body composition are important characteristics in livestock production. Korean native pig (KNP) has more attractive meat quality, but slowly growing in comparison with other commercial pig breeds. So, growth performance is an important factor for improving of KNP breed. For this reason, we have compared the proteome change of skeletal muscle of KNP breed

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throughout growth using the proteomic analysis approach. A great deal of useful information could be understood from studying the muscle proteome and its relationship with physiological growth.

## MATERIAL AND METHODS

### Animals and sample preparation

Fifteen male pigs of Korean native pig were divided into five postnatal stages of 30, 70, 130, 170 and 300 d (three animals in each stage); at which a live weight of pig is corresponding to approximately 5, 20, 50, 70 and 110 kg, respectively. The animals were provided by the National Institute of Animal Science, RDA in Korea. All pigs were stunned by an electronic stunner (230 V for 2.5 s) and conventionally slaughtered. Muscle samples were taken from the *longissimus dorsi* muscle of pig carcasses within 30 min after slaughter, respectively. The tissue samples were ground to a fine powder under liquid nitrogen using a mortar and pestle and stored at -80°C.

Frozen muscle tissue (100 mg) was incubated for 40 min in 1 ml of 8 M urea, 2 M thio-urea, 65 mM DTT, 2% CHAPS, 1% bio-lyte ampholyte (3-10, Bio-Rad, Hercules, CA, USA) and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Incubated samples were centrifuged at 10,000×g for 30 min and the resulting supernatants were used as the protein extract. Protein concentration determined using the protein assay system (Bio-Rad) and BSA as the standard.

### Two-dimensional electrophoresis

Samples of approximately 200 µg for analytical gels and 1 mg for preparative gels were applied on 17 cm immobilized pH gradient (IPG) strips (3-10 nonlinear, Bio-Rad). IPG strips were rehydrated overnight with rehydration solution containing of 8 M urea, 0.5% CHAPS, 0.28% DTT, 10% glycerol, 0.5% bio-lyte ampholyte (3-10, Bio-Rad), bromophenol blue (a few grains). After rehydration, isoelectric focusing (IEF) was performed for total 46,000 Vh and 82,000 Vh with a PROTEAN IEF Cell unit (Bio-Rad) at 100 V, 200 V, 500 V and 1,000 V for 1 h per step and then gradually increased to 8,000 V. The current limit was adjusted to 50 µA per strip, and the run was carried out at 20°C. After IEF, IPG strips were incubated for 20 min with 10 ml of equilibration solution consisting of 50 mM Tris-HCl (pH 8.8), 6 M urea, 2% SDS, 20% glycerol, bromophenol blue (a few grains) and 5 mM TBP. The IPG strips were transferred onto SDS-polyacrylamide gels (12.5%T, 2.67%C) with a run of 10 mA per gel for 1 h followed by 20 mA per gel until the dye front reached the bottom of the gel.

### Gel stain and image analysis

Gels were fixed 1 h (40% methanol, 10% acetic acid)

and silver stained (Hochstrasser et al., 1988) for analytical gels and coomassie brilliant blue stained using R-250 reagent (Bio-Rad) for analysis of mass spectrometry.

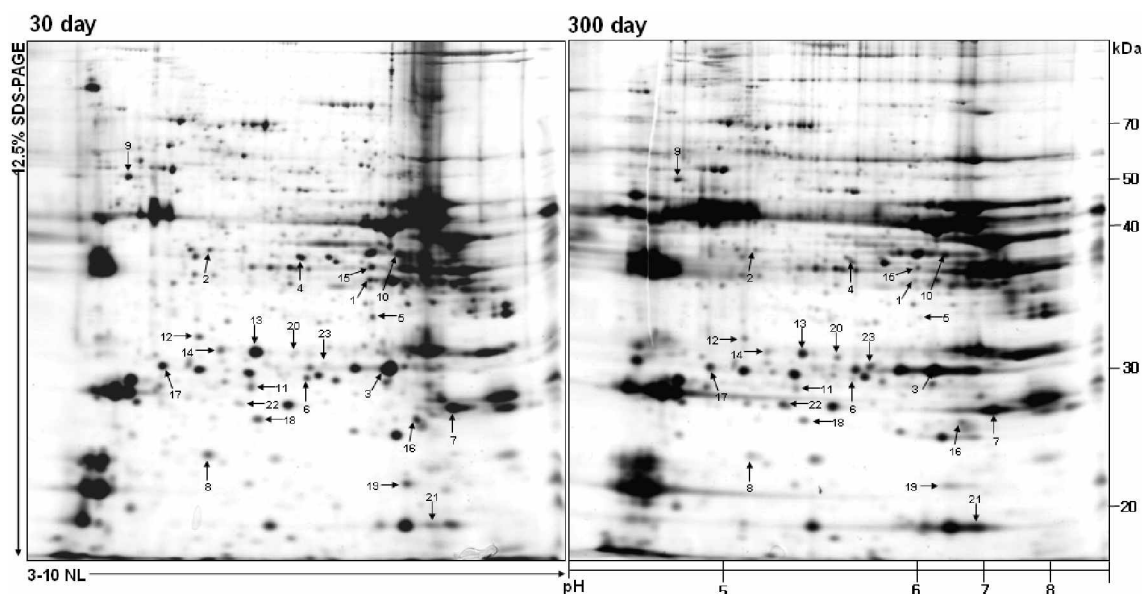
Stained gels were matched and analyzed with the PDQuest software (Bio-Rad). Three replicated gels obtained for each growth stage were normalized by total quantity in valid spots method and analyzed. The averaged spot densities were submitted to a one-way analysis of variation (ANOVA) using SAS software. A spot was considered significantly when it was associated to  $p < 0.05$  in ANOVA. The differentially expressed spots in all stage were selected and identified. In firstly, we selected that the spots of differentially expressed which change of densities more than 2-fold between two postnatal stages of the 30 and 300 d. Then, we analyzed that significantly changed spots among the growth stage within the selected protein spots. The densities of some spots were also significantly changed over growth stage. However, we have focused mainly on the major quantitative changes during the growth. The  $M_r$  and the  $pI$  were estimated by running 2-D SDS-PAGE standards (Bio-Rad).

### Protein identification

For protein identification, the screened spots were excised from stained gel and destined with deionised water. The gel slices were reduction with DTT and alkylation with iodoacetamine. The gel slices were washed with 15 µl of 0.1 M ammonium bicarbonate in 100% acetonitrile and repeated using 70 µl of 100% acetonitrile. Digestion was performed with 20 µl of trypsin solution (1 µg/ml trypsin in 50 mM ammonium bicarbonate, pH 7.8) for 45 min while keeping the tube on ice and incubated for overnight at 37°C with 30 µl of 50 mM ammonium bicarbonate. After enzymatic digestion, the supernatant was concentrated in a vacuum centrifuge and used for analysis. The analysis of MALDI-MS or ESI-MS was performed using a M@LDI-R and Q-TOF2 mass spectrometers (Micromass, Manchester, UK), respectively, and the proteins were identified by searching SWISS-PROT, EMBL and NCBI nr databases using the Mascot peptide mass fingerprint and ion search programs ([www.matrixscience.com](http://www.matrixscience.com)). Information of the peptide mass about experimental  $pI$  and  $M_r$  values and protein scores was used to search for protein by peptide mass fingerprinting.

## RESULTS

Proteomics is the large-scale study of proteins, usually by biochemical methods. The key elements of classical proteomics are the separation of proteins in a sample using two-dimensional gel electrophoresis (2-DE) and their subsequent quantitation and identification. Skeletal muscle characteristics change greatly during development. The postnatal development of Korean native black pigs was



**Figure 1.** Comparative analysis of the expressed protein patterns at different growth stages of porcine skeletal muscle. Protein (200  $\mu$ g) was loaded and separated in the IPG strip (3-10 nonlinear; Bio-Rad, USA) and an SDS gel (12.5%T). Arrows show 23 spots that were quantitatively changed more than 2-fold between two postnatal stages of the 30 and 300 d.

**Table 1.** List of proteins differentially expressed at two postnatal stages of the 30 and 300 d

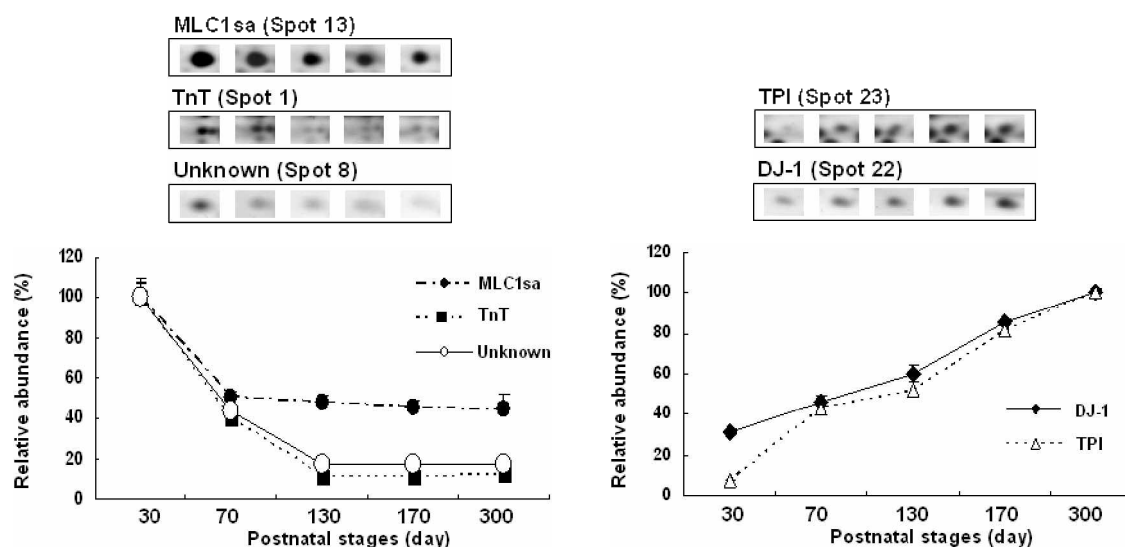
Spot No.	Mass <sup>a</sup>	pI <sup>a</sup>	Ratio <sup>b</sup>	Spot No.	Mass <sup>a</sup>	pI <sup>a</sup>	Ratio <sup>b</sup>
Downregulated proteins				Upregulated proteins			
1	37.20	5.96	0.07	13	30.95	5.46	0.36
2	39.94	5.25	0.10	14	31.14	5.30	0.36
3	25.03	6.16	0.13	15	38.28	5.97	0.38
4	39.21	5.66	0.16	16	20.58	6.54	0.38
5	33.80	5.98	0.16	17	26.80	5.04	0.39
6	23.58	5.68	0.18	18	20.58	5.46	0.42
7	20.90	7.95	0.19	19	18.86	6.41	0.47
8	19.61	5.24	0.27				
9	47.84	4.72	0.32	20	29.68	5.62	2.22
10	39.48	6.29	0.33	21	17.84	6.74	2.25
11	21.50	5.43	0.34	22	21.03	5.38	2.62
12	32.18	5.20	0.36	23	27.48	5.76	4.84

<sup>a</sup> Protein mass (kDa) and isoelectric point (pI) determined on 2-DE gel by PDQuest, which values were compared with 2D SDS-PAGE standard (Bio-Rad, USA).

<sup>b</sup> Ratio value of the stage of 300 d to the stage of 30 d in protein expression level.

classified as five postnatal of 30, 70, 130, 170 and 300 d. Loin muscle proteome from five developmental stages of pigs was analyzed by 2-DE technique. Approximately 600 spots were detected in each of the silver stained gels by the computer-assisted image analysis (Figure 1). We have detected 23 spots showing the quantitative changes more than 2-fold between two postnatal stages of the 30 and 300 d (Figure 1 and Table 1). Five proteins among 23 spots were highly expressed at the stage of 300 d, whereas the other 18 spots at the stage of 30 d. Furthermore, the expression profiling of the above 23 spots was examined at different five postnatal stages (30, 70, 130, 170 and 300 d). We finally selected 5 proteins of spots 1, 8, 13, 22 and 23 showing the consistent expression profiling throughout the muscle growth. Although the other 18 spots were expressed

differentially between two stages of the 30 and 300 d, the expression patterns were not observed consistently at various stages of muscle growth (data not shown). The protein profiling of spots 1, 8 and 13 showed a decreasing pattern, whereas the protein profiling of spots 22 and 23 an increasing pattern (Figure 2). Two proteins of spots 1 and 8 had the highest level at the stage of 30 d and these levels were decreased until undetectable level at the stage of 130 d. And then these decreased levels were continued until at the stage of 300 d. The protein of spot 13 had also the highest expression level at the stage of 30 d and its level was about 55% as comparing with at the stage of 70 d. This decreased level was maintained until at the stage of 300 d. On the other hand, the protein expressions of spots of 22 and 23 were increased linearly until at the stage of 300 d (Figure 2).



**Figure 2.** Relative changes of the identified 5 proteins in porcine skeletal muscle at various five postnatal stages of growth (30, 70, 130, 170 and 300 d). MLC1sa: myosin light chain 1 slow-twitch isoforms, TnT: troponin T, TPI: triosephosphate isomerase.

**Table 2.** Characterization of differentially expressed proteins during the growth stages by ESI-MS analysis

Spot No.	Protein	Accession No.	Theoretical Mw/pI	Observed Mw/pI	Mowse score <sup>a</sup>	Seq. cov. <sup>b</sup> (%)	Organism
13	Myosin light chain 1 slow-twitch isoform	P14649	22.76/5.51	30.95/5.46	69	6	Human
1	Troponin T	gi 92923	32.24/6.92	37.20/5.96	57	5	Rat
23	Triosephosphate isomerase	gi 68423	27.02/6.90	27.48/5.76	56	6	Mouse
22	DJ-1 protein	gi 7429593	20.06/6.33	21.03/5.38	49	7	Human
8	Unknown	-	-	19.61/5.24	-	-	-

Accession number is SWISS-PROT and NCBI database number.

<sup>a</sup> The Mascot baseline significant score is 48.

<sup>b</sup> Sequence coverage.

These expression levels at the stage of 30 d were increased more 5-fold than those at the stage of 300 d.

Five proteins showing the consistent changing patterns during growth stages were excised from the gels and analyzed by peptide mass fingerprinting (MALDI-MS). In order to identify proteins, we searched databases of SWISS-PROT and EMBL using Mascot program. However, all proteins had no good match in the protein database. Therefore, further identification was performed with other method (ESI-MS) and the searching programs of Mascot and NCBI database (Table 2). Two proteins of spots 1 and 13 were identified as myosin light chain 1 slow-twitch isoform and troponin T, respectively, which belong to muscle fiber proteins playing a role of muscle contraction and movement. On the other hand, two proteins of spots 22 and 23 showed similarities with the triosephosphate isomerase and DJ-1 protein, respectively. However, one protein of the spot 8 was not identified.

## DISCUSSION

Using the proteomic analysis we have compared loin

muscle proteins during porcine postnatal development. Proteome analysis is the only method that provides the possibility to quantitatively study global changes in expression profile of proteins, although it is somewhat limited in the identification of low abundant proteins. For comparing the developmental changes of muscle proteome levels, Korean native black pigs were divided into five postnatal stages (30, 70, 130, 170 and 300 d) and their loin muscle proteomes were analyzed by 2-DE and mass spectrometry. We finally screened 5 proteins demonstrating the consistent profiling such as increasing or decreasing patterns during developmental stages. Four of 5 proteins were identified as myosin light chain 1 slow-twitch isoform (MLC1sa), troponin T (TnT), triosephosphate isomerase (TPI) and DJ-1 protein. During muscle growth the developmental expression profilings of the screened proteins showed a decreasing pattern in the case of muscle fiber proteins of MLC1sa and TnT, whereas an increasing patterns in the case of TPI and DJ-1 proteins.

Postnatal growth of skeletal muscle was also accompanied by changes in the distribution of muscle fiber types. In pig and cattle, the conversion of type IIa (fast-

twitch; oxidative-glycolytic) fibers into type IIb (fast-twitch; glycolytic) fibers was increased during development (Lefaucheur and Vigneron, 1986; Jurie et al., 1995). Muscle fiber proteins consisting of actin, myosin, troponin and tropomyosin were determined to investigate alternative mechanisms for changes in contractile properties and myofibrillar ATPase activity with body size. The composition changes in myosin heavy and light chain isoforms result in a showing of the mechanical properties of muscle and changes in myofibrillar ATPase activity (Moss et al., 1995). Myosin consisting of heavy and light chains was a major component of the contractile proteins and has various isoforms within different fiber types by histochemical stains and electrophoretic techniques. The MLC1sa was a major isoform in rabbit and human skeletal muscle and expressed highly at early stage than at late stage during muscle growth (Hailstones and Gunning, 1990). This report corresponds with our present results about the expression profiling of MLC1sa during porcine skeletal muscle growth.

The troponin complex consists of three subunits such as troponin C (TnC), I (TnI) and T (TnT), and TnT is an important regulatory and structural component of skeletal muscle thin filaments (Perry, 1998). Troponin shows very specific manner in muscle type and development stage. For example, in the mouse adult the TnT-slow isoform was only present in muscles enriched with slow fibers (Jin et al., 1998), and TnT-cardiac isoform was solely expressed in the heart, whereas TnT-fast was expressed in almost all-skeletal muscles. Besides, TnT-fast isoform was abundant during later fetal development while TnT-slow isoform predominates in the skeletal muscle during early fetal development in the rat (Sabry and Dhoot, 1991; Kyprianou et al., 1997). In the case of porcine postnatal development, the TnT expression in skeletal muscle appeared very highly at early stage and then decreased until undetectable level with the stages of growth (Figure 2). These findings provide that during skeletal muscle differentiation and development, muscle fiber proteins such as myosin and troponin complex and other muscle proteins undergo complex stage-specific programs of isoforms switching and expression. Thus, the expression profiling of MLC1sa and TnT during porcine postnatal development may be used as a parameter to study factors affecting the skeletal muscle growth.

Triosephosphate isomerase (TPI) as an essential housekeeping enzyme catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehydes-3-phosphate, and plays an important role in both glycolysis and gluconeogenesis as well as in glyceride synthesis. The period of growth was characterized by increased glycolytic metabolism (Lefaucheur and Vigneron, 1986; Jurie et al., 1995). Also, other previous report showed the quantitative increases of the glycolytic enzymes such as TPI, enolase

and phosphoglucosmutase during the stages of growth (Doherty et al., 2004). Our result also showed that TPI expression in porcine skeletal muscle was gradually increased during postnatal development, which coincides with the finding of above reports. Moreover, TPI was well characterized and has been used as a model for age-related modified and accumulated forms of protein damage (Gracy et al., 1998). Various aging-dependent isoforms of TPI have been identified and may be derived from the protein degradation by deamination and oxidation (Pontier and Hart, 1981; Zhang et al., 1995). The present data indicated that the gradual increase of TPI level during muscle growth might be caused by the changed properties of glycolytic metabolism in porcine skeletal muscle.

Interestingly, we found DJ-1 protein showing a similar expression pattern with that of TPI protein. DJ-1 protein first was identified as an oncogene that was preferentially expressed in the testis and moderately in other tissues (Nagakubo et al., 1997). Nagakubo et al. (1997) suggested that this protein has a growth-related function, since it was translocated from the cytoplasm to nuclei during the cell cycle after mitogen stimulation. Recent study reported that DJ-1 may also function as an antioxidant because of playing a role as a hydrogen peroxide-responsive protein (Mitsumoto and Nakagawa, 2001; Taira et al., 2004). Cells obtain energy by the oxidation of materials obtained from the environment. In addition, biological growth demands countless energy for maintenance of homeostasis, metabolism and macromolecules synthesis, however, which leads to a progressively increasing formation of reactive oxygen and nitrogen species associated with increased oxidative stress (Stadtman, 1992; Beal, 2002). In order to prevent the increasing accumulation of oxidative stress during growth, cells need activate an antioxidant system. In our study, the increase of DJ-1 level during muscle growth may be necessary for increasing the resistance of muscle to oxidative stress.

This study is the first time about the proteome comparison of pig skeletal muscle during postnatal development. We found the several stage-related proteins such as myosin light chain, troponin T, triosephosphate isomerase and DJ-1 protein in porcine skeletal muscle, probably providing a basis for understanding the biochemical and molecular diversities of skeletal muscle during growth stages.

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