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Comparisons of Chicken Muscles between Layer and Broiler Breeds Using Proteomics

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ABSTRACT : The present study was carried out to investigate differentially expressed chicken muscle proteins using proteomics approach. More than 300 protein spots were investigated for the muscle samples in 2DE gels and the differentially expressed protein spots between *pectoralis* and *peroneus longus* muscles from Cornish and White Leghorn breeds were characterized by MALDI-TOF. In *pectoralis* muscles, PGAM1 protein was detected as differentially expressed between White Leghorn and Cornish breeds. On the other hand, 4 protein spots (SP22, nxf-2, SOD1, TNNI2) were differentially expressed between White Leghorn and Cornish breeds in *peroneus longus* muscles. These proteins assumed to be related with muscle development, growth, stress, and movements in chicken. In this experimental process, 2D reference map of the chicken muscle proteins was needed and 25 proteins, which were commonly expressed in both *pectoralis* and *peroneus longus* muscles in both breeds, were selected and characterized. Upon finishing the exact roles of the differentially expressed proteins, the identified 5 proteins will be used as valuable information for the fundamental mechanisms of muscle biology and underline genetics. (**Key Words :** White Leghorn, Cornish, Muscles, 2-DE, MALDI-TOF MS)

INTRODUCTION

Among livestock species, chicken is the first animal species to enter the post genomics era and the full genome sequence information in chicken makes possible for the functional genomics approaches in order to understand molecular basis of various traits (Burt, 2005). Currently, large-scale expression analyses, such as cDNA microarrays, have been being carried out in chicken. However, recent studies indicate that there have been less correlations between mRNA and protein expressions. Thus, proteomics plays more important role to delineate the gaps between genome sequence information and phenotypes (Burgess, 2004).

Modern poultry industry has dramatically increased productivity, especially for growth and egg production, mainly based on the improvement of animal breeding tools (Møller and Manning, 2003). Cornish is one of the well-known broiler breeds, originally derived from fighting chickens imported from India, and had many desired carcass characteristics as a meat type chicken including body type, growth rate, feed conversion ratio. These characteristics have been being improved dramatically in response to selection. However, these improvements had side effects for low egg production, low fertility, poor hatchability, and reduced viability. On the other hand, White Leghorn is a famous commercial egg-laying breed and the egg productivity has been dramatically increased for the past 60 years (Arthur, 1986). These two breeds, Cornish and White Leghorn, were believed to be the descendants of their common ancestor, Red Jungle Fowl, which is still found in wild parts of India and Southeast Asia (Crawford, 1990; Fumihito et al., 1994). Even though Cornish and White Leghorn have the same genetic background before the divergent of the breeds, different breeding objectives for meat and egg production gave huge differences in productivity for these two breeds. Large participation of commercial poultry breeding company makes these breeding goals in the shortest possible time (Emsley, 1993).

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In the present study, we aimed to investigate differentially expressed muscle proteins between Cornish and White Leghorn breeds in order to address the possibility for improving meat quantity and quality in chicken.

MATERIALS AND METHODS

Sample preparations

Muscle samples from three mature birds each for Cornish and Large White breeds were used in this study. These two breeds of chickens were raised in the National Livestock Research Institute (NLRI), Korea. The skeletal muscles from these birds were rapidly removed and immediately frozen in -80°C. Two grams of chicken breast (*pectoralis* muscle) and drum (*peroneus longus* muscle) samples were crushed in a mortar containing liquid nitrogen and mixed with 10 ml of solution containing 0.3% SDS, 50 mM Tris-HCl pH 8.0, 1 mM PMSF (phenylmethylsulfonyl fluoride, Roche, Germany) for protease inhibitor and 200 mM DTT. The mixtures were incubated at room temperature for 30 min and boiled at 100°C for 10 min. Then, these mixtures were transferred to ice and incubated with solution containing 40 U DNase I (Roche, Germany), 14 U RNase A (Roche, Germany), 50 mM Tris-HCl pH 8.0, 0.1 mM $MgCl_2$ for 10 min. After centrifugation at 15,000 g at 4°C for 30 min, the supernatant was precipitated with 50% Trichloroacetic Acid (TCA) solution for 1 h at -20°C. The protein pellet was washed with ice-cold acetone at least four times to remove contaminants and lyophilized. The washed pellet was dried using vacuum centrifuge prior to 2-DE analysis.

Electrophoresis

Muscle supernatants containing proteins were solubilized in rehydration solution containing 8 M urea, 2% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, Amersham Pharmacia, Sweden), 60 mM DTT, 0.5% IPG buffer (Amersham Biosciences, Sweden) with a trace amount of bromophenol blue. This solution was used to rehydrate the IEF strips at 20°C overnight.

Protein concentration was determined according to Bradford (Bradford, 1976) using BSA as a control. The protein solution containing 1 mg of protein was applied on immobilized pH 3-10, 18 cm nonlinear gradient strips and rehydrated for 16 hr. Automatic isoelectric focusing was carried out for voltage started at 50 V and gradually increased to a final voltage of 8,000 V at 20°C. After IEF separation, the gel strips were equilibrated in a tube containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) DTT for 15 min with gentle shaking, followed by 15 min in the same solvent containing 2.5% (w/v) iodoacetamide in place of the DTT. The second dimension was run on a 12% polyacrylamide SDS gel using a PROTEAN II xi electrophoresis kit (Bio-Rad, USA). After the gel strips were placed into a 12% polyacrylamide SDS gel and sealed with agarose, electrophoresis was carried out at 15 mA per gel until the dye front reached the lower end of the gel. After completion of the second dimension, the gel was placed into the fixing solution (30% methanol, 10% acetic acid) for 1 h and stained with colloidal CBB G250 (Fluka, Switzerland) for 24 h. After staining, the gel was destained with 1% acetic acid or distilled water for the visualization of protein spots.

Image analysis and in gel digestion

After scanned using Powerlook III image scanner (UMAX data system, Taiwan), gels were compared using Progenesis Workstation (Nonlinear, USA). Six gels in each group were compared in order to minimize the variation of individual gels. Among 300 spots that were detected in each gel, 45 candidate spots in the comparisons of *pectoralis* muscles and 44 spots in *peroneus longus* muscles were further analyzed. Ultimately, 5 significantly different spots were selected. For 2DE reference map of chicken muscle, 25 spots, which were commonly expressed of all gels, were analyzed. The differentially expressed muscle protein spots were used for further analysis. After washing with 30% methanol and 10 mM ammonium bicarbonate and 50% acetonitrile, gel pieces were dried in a vacuum centrifuge. Approximately 50 μl of 10 mM DTT and 100 mM ammonium bicarbonate was added, and the samples were incubated for 45 min at 56°C. After that, 50 μl of 55 mM ammonium bicarbonate was added and the samples were incubated for 40 min in dark room. A total of 150 μl of distilled water was then added. After a brief shaking, the supernatant was discarded and 100% acetonitrile was added. The gel pieces were dried in a vacuum centrifuge for 20 min. The gel pieces were swollen in digestion solution (50 mM ammonium bicarbonate, 5 mM $CaCl_2$, and 12.5 ng/μl trypsin) and incubated at 37°C for 15 h. The peptides were recovered by stage extraction using 50 mM ammonium bicarbonate and 100% acetonitrile. The resulting peptide extracts were pooled, lyophilized in a vacuum centrifuge and stored at -20°C.

Characterization of protein spots by MALDI-TOF MS

To improve the ionization efficiency of MALDI-TOF MS, sample peptides were purified with ZIP-tip C18 (Millipore, USA) before MS analysis. Equal volumes of peptide and matrix solution were mixed and crystallized on the sample plate. The matrix solution consisted of 10 mg α-cyano-4-hydroxycinnamic acid dissolved in 1 ml of a solution containing 50% acetonitrile and 0.5% TFA. The

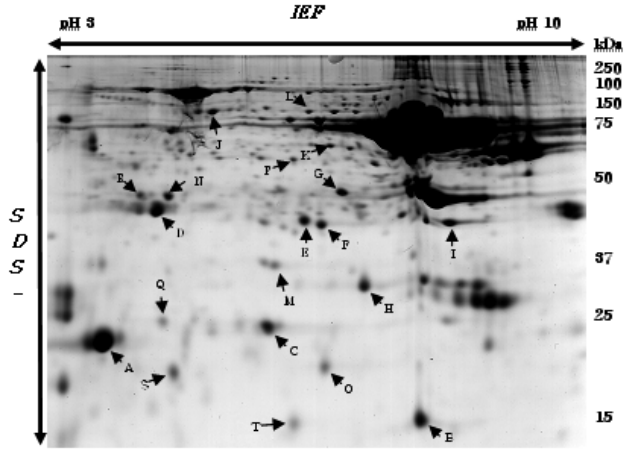

Figure 1. Identification of commonly expressed proteins in *pectoralis* and *peroneus longus* muscles for 2DE reference map.

peptide solution was analyzed using a modified Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, USA). Desorption and ionization of samples was performed using a nitrogen laser (λ = 337 nm). Proteins were detected by searching against NCBI non-redundant database using MASCOT peptide mass fingerprint software (http://www.matrixscience.com).

RESULTS AND DISCUSSION

In order to detect differentially expressed proteins for muscle growth and meat quality, *pectoralis* and *peroneus longus* muscles from Cornish and White Leghorn breeds were compared. More than 300 protein spots in each coomassie blue stained 2DE gel were detected with the pH range between 3 to 10 and molecular weight range between 15 to 250 kDa (data not shown). In order to use as a standard of the gel images, the common protein spots appeared in both *pectoralis* and *peroneus longus* muscles in Cornish and White Leghorn were investigated and indicated in Figure 1. These commonly expressed proteins were detected using MALDI-TOF MS and the detected proteins were listed in Table 1. The 2DE gel images are almost identical with our previous experiments (Jung et al., 2005) indicating the high reproducibility of the experiments.

When we compared the *pectoralis* muscle with the *peroneus longus* muscle, Parvalbumin was over expressed in *peroneus longus* muscle compared with *pectoralis* muscle in both Cornish and White Leghorn breeds. In

Table 1. Identification of proteins for making the 2DE reference map

Spot[1]	Description of protein	Mowse score	Coverage (%)	Mass	pI	Species	NCBI accession No.
A	Parvalbumin (PVALB)	31	37	11,934	4.94	Gallus gallus	gi 131121
B	Polyubiquitin	304	6	73,905	8.7	*Cricetulus sp.*	gi 940395
C	Fatty acid-binding protein, smooth muscle (SM-FABP)	117	66	2,321	9.99	*Gallus gallus*	gi 6015127
D	Myosin light chain 1, skeletal muscle isoform (A1 catalytic)(Alkali) (MLC-1)	379	29	20,684	4.96	*Gallus gallus*	gi 127127
E	Nuclear export Factor family member (nxf-2)	53	2	48,103	6.71	*Caenorhabditis elegans*	gi 17562778
F	Parkinson disease (autosomal recessive, early onset) 7 (DJ-1)	425	35	19,930	6.32	*Gallus gallus*	gi 17974316
G	Peroxiredoxin 6 (Prdx6)	330	17	24,855	5.71	*Mus musculus*	gi 3219774
H	Copper/zinc superoxide dismutase (SOD1)	318	35	15,549	6.12	*Gallus gallus*	gi 2134412
I	Phosphatidylethanolamine-binding protein (PEBP)	88	7	20,842	7.38	*Bos taurus*	gi 89407
J	Serum albumin precursor (ALB)	290	7	69,872	5.51	*Gallus gallus*	gi 113575
K	Laminin-binding protein (RPSA)	452	25	31,774	4.84	*Homo sapiens*	gi 34234
L	Cytochrome c oxidase polypeptide Va (COX5A)	94	22	12,428	5.01	*Bos taurus*	gi 117097
M	Copper/zinc superoxide dismutase (SOD1)	76	13	15,549	6.12	*Gallus gallus*	gi 2134412
N	Parkinson disease (autosomal recessive, early onset) 7 (DJ-1)	425	35	19,930	6.32	*Gallus gallus*	gi 17974316
O	Prolyl-4-hydroxylase (P4HB)	394	10	54,831	4.66	*Gallus gallus*	gi 63739
P	Interleukin 5 (IL5)	47	-	15,197	-	*Rattus norvegicus*	gi 27670678
Q	Peroxiredoxin 6 (Prdx6)	299	17	24,855	5.71	*Mus musculus*	gi 3219774
R	SERine Proteinase Inhibitors (SERPIN)	45	-	44,059	-	*Brugia malayi*	gi 433400
S	Cathepsin D precursor (CTSD)	165	8	43,270	5.90	*Gallus gallus*	gi 461696
T	Pyruvate dehydrogenase E1-beta subunit (PdhE1beta-1)	178	7	39,037	5.95	*Xenopus laevis*	gi 2134143

[1] The spot locations were indicated in Figure 1.

Table 2. Identification of differentially expressed proteins from *pectoralis* and *peroneus longus* muscles between White Leghorn and Cornish

Spot ID	Description of protein	Mowse score	Coverage (%)	Mass	pI	Species	NCBI accession No.
pectoralis muscle							
1004	Phosphoglycerate mutase 1 (PGAM1)	137	16	28,786	6.67	*Homo sapiens*	gi 4505753
peroneus longus muscle							
827	Substrate protein of mitochondrial ATP-dependent proteinase (SP-22)	149	10	21,595	5.73	*Bos taurus*	gi 627764
866	Nuclear export factor family member (nxf-2)	42	2	48,103	6.71	*Caenorhabditis elegans*	gi 17562778
932	Copper/zinc superoxide dismutase (SOD1)	91	13	15,549	6.12	*Gallus gallus*	gi 2134412
1109	Troponin I, fast skeletal muscle (TNNI2)	138	14	21,221	9.19	*Gallus gallus*	gi 136216

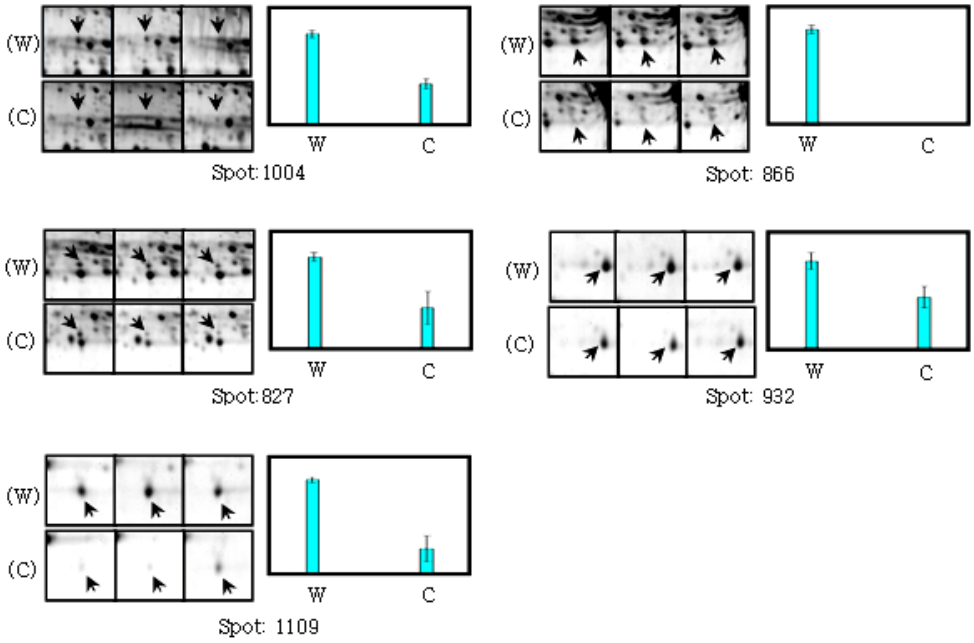

Figure 2. Gel images of the five differentially expressed protein spots between muscles from White Leghorn (W) and Cornish (C) breeds. Spot volumes are indicated on the right.

muscle tissue, parvalbumin is the high affinity Ca^{2+} binding protein and plays a crucial role in muscle contraction by translocation Ca^{2+} from the myofibril to the sarcoplasmic reticulum (Berchtold et al., 2000). Therefore, the high expression of the parvalbumin indicates frequent use of *peroneus longus* muscle than *pectoralis* muscle. The protein spots in the same muscles between Cornish and Leghorn breeds were also investigated. As a result, five differentially expressed protein spots were identified. These proteins were further analyzed by MALDI-TOF MS and their molecular weights, *pI*, were estimated (Table 2). In *pectoralis* muscles between the two breeds, phosphoglycerate mutase 1 (PGAM1) was up regulated in White Leghorn. When we compared *peroneus longus* muscles, four proteins, namely substrate protein of mitochondrial ATP-dependent proteinase (SP-22), Nuclear export factor family member (nxf-2), Copper/zinc superoxide dismutase (SOD1), and Troponin I, fast skeletal muscle (TNNI2), were up regulated in White Leghorn (Figure 2). Even though many protein spots showed variations in the muscle samples between these two breeds, they were excluded for the further analysis because of lacks of consistency among replicate samples.

The identified proteins were carefully investigated in order to examine their biological roles in chicken muscles. SP-22 gene was first found as a substrate protein for mitochondrial ATP-dependent protease (Watabe et al., 1994). Recently, SP-22 was detected as Prx III, one of peroxiredoxins (Prxs) in mammals (Wood et al., 2003). Prxs are thiol-specific antioxidant enzymes that reduce reactive oxygen species including hydrogen peroxide or superoxide anion mediated from signal transduction of some growth factors or cytokines such as platelet-derived growth factors, tumor necrosis factor α, and interleukin 1 (Schulze-Osthof

et al., 1992; Sundaresan et al., 1995; Monteiro and Stern, 1996; Wood et al., 2003).

SODs (superoxide dismutase) are among the most important antioxidant metalloenzymes protecting cells against oxidative stress arising from reactive oxygen species produced during aerobic respiration. Four major classes of SODs (MnSOD, FeSOD, Cu/ZnSOD and NiSOD) have been identified and distinguished by the protein fold and by the nature of the catalytic metal ion (Whittaker, 2003). Previous investigation in mice indicated that the CuZn-SOD overexpression have been shown to be protected against vascular dysfunction in models of subarachnoid hemorrhage (Kamii et al., 1999) and hypoxia with reoxygenation as well as in response to ceramide and lipopolysaccharide (LPS) (Didion, 2004), and overexpression of β-amyloid precursor protein (Iadecola et al., 1999). The different response of oxidative stress in *peroneus longus* muscles may suggest the White Leghorn seems to be more sensitive to oxidative stress than the Cornish breed.

Troponine I was composed of three isoforms containing slow skeletal muscle (TNNI1), fast skeletal muscle (TNNI2) and cardiac muscle (TNNI3) (Mullen and Barton, 2000). Among these isoforms, TNNI2 was firstly investigated in human fast skeletal muscle cDNA library (Zhu et al., 1994). However we could not know why the TNNI2 protein is highly expressed in *peroneus longus* muscle in White Leghorn. Also we detected that the high expression of nxf-2 protein was observed in *peroneus longus* muscle of the White Leghorn. Until recently, the DNA and amino acid sequences of the nxf-2 gene were only investigated in *C. elegans* and *Canis familiaris* species. The function of this gene has to be investigated more in the future.

Proteomics have been being regarded as a powerful tool in biological research. The most generally used method includes the protein separation by 2DE and dye staining. Following this procedure, target proteins were identified by mass spectrometry. However, there are difficulties in identifying the proteins having low molecular weight and very acidic/basic proteins. In livestock animals, the proteomics is launched recently and the protein databases are ultimately needed. The chicken genome sequences published on March 2004 will give good information for the improvement of chicken proteomics research and make bridges between genomics and proteomics.

In conclusion, 25 common proteins, both appeared in *pectoralis* and *peroneus longus* muscles in Cornish and White Leghorn, have been identified as the reference of muscle proteins in this study. These proteins can be used as landmark protein markers for the further studies. We also found that some of the differentially expressed proteins act as important roles in the muscle metabolism. Based on the known functions of the identified proteins, they are related to the muscle development, growth, stress and movement in chicken with some unidentified functions of the proteins. Upon tracing exact biological roles for the identified proteins, they can be used as valuable biomarkers for meat quality and productivity in chicken.

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