Research Paper

Proteome Changes in Thai Indigenous Chicken Muscle during Growth Period

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Abstract

Proteomic profiling of the *pectoralis* muscle of Thai indigenous chickens during growth period was analyzed using two-dimensional gel electrophoresis (2-DE) and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS). A total of 259, 161, 120 and 107 protein spots were found to be expressed in the chicken *pectoralis* muscles at 0, 3, 6 and 18 weeks of age, respectively. From these expressed proteins, five distinct protein spots were significantly associated with chicken age. These protein spots were characterized and showed homology with phosphoglycerate mutase 1 (PGAM1), apolipoprotein A1 (APOA1), triosephosphate isomerase 1 (TPI1), heat shock protein 25 kDa (HSP25) and fatty acid binding protein 3 (FABP3). These five protein spots were categorized as follows: (i) the expression levels of PGAM1 and TPI1 proteins were positively correlated with chicken aging ($p<0.05$), (ii) the expression levels of APOA1 and FABP3 proteins were negatively correlated with chicken aging ($p<0.05$) and (iii) the expression levels of the HSP25 protein were up- and down-regulated during growth period. Moreover, the mRNA expression levels of the *FABP3* and *HSP25* genes were significantly decreased in muscle during the growth period ($p<0.05$), whereas no significant changes of the *PGAM1*, *TPI1* and *APOA1* gene expression from the chicken muscle was observed. The identified proteins were classified as metabolic and stress proteins. This demonstrates a difference in energy metabolism and stress proteins between age groups and shows that proteomics is a useful tool to uncover the molecular basis of physiological differences in muscle during the growth period.

Key words: chicken, gene expression, growth, muscle, proteome

INTRODUCTION

Thai indigenous chickens have a unique taste, tough and strong muscles. Such meat is very popular among consumers and the market price is two or three times higher than the commercial broiler chicken (1). Thai indigenous chickens have a slow growth rate and contain low fat. The chemical composition, color, texture and structure of the Thai indigenous and commercial broiler chicken meat have been characterized (1,2). Thai indigenous chicken has

a firmer texture and more flavor than the commercial broiler chicken (1).

Proteomic technology is a powerful method to identify proteins that play a major role in the mechanism for controlling meat quality traits. The proteomics approach has been successful in discovering the protein markers associated with meat quality traits in several livestock species such as cattle (3), sheep (4) and pigs (5-7). Moreover, the proteome of

muscle growth and development has been characterized in chicken and pigs (8,9). However, information on molecular markers for muscle development of Thai indigenous chickens is limited. The aim of this study was to analyze proteome changes in Thai indigenous chicken during the growth period. Moreover, the expression levels of these target genes were then further validated by quantitative real-time PCR.

EXPERIMENTAL PROCEDURES

Animals

Twenty mixed-sex Thai indigenous chickens (named "Pradhuhangdum") were obtained from the Livestock Breeding and Research center, Sanpatong, Chiang Mai and reared under the same conditions at the research farm of the Department of Animal Science, Chiang Mai University. The *pectoralis* muscles were collected from 5 individual chickens (3 females and 2 males) at 0, 3, 6 and 18 weeks of age. The tissue samples were kept at -80 ºC for proteomic and quantitative gene expression analysis.

Two-dimensional gel electrophoresis

To prepare protein extracts, *pectoralis* muscle samples were homogenized in 1.5 ml of lysis buffer (8M urea, 4% CHAPS, 40 mM Tris-HCL pH 8.8 and 0.5% IPG buffer pH 3-10). The extracted protein was cleaned using a 2-D clean up kit (Amersham Biosciences, Thailand) according to the manufacturer's procedure. The concentration of the purified protein was then determined using a 2-D Quant kit (Amersham Biosciences, Thailand). Proteomic analysis was performed using two-dimensional gel electrophoresis (2-DE). Briefly, 10 µg of the protein was diluted with 125 µl of rehydration buffer (8M urea, 4% CHAPS, 60 mM DTT, 0.5% IPG buffer and 0.002% bromophenol blue) and applied to the Immobiline DryStrips (pH 3-10, 7 cm, Amersham Biosciences, Thailand). The strips were rehydrated at 20 ºC for 12 hours and one-dimensional, isoelectric focusing (IEF) was carried out on an Ettan IPGphor II, IEF system (Amersham Biosciences, Thailand) using 300 V for 150Vh, 1,000 V for 300 Vh, 5,000 V for 4,000 Vh and 5,000 V for 500 Vh, 50 µA at 20 ºC. The strips were then subjected to two step equilibration in 5 ml of SDS equilibration buffer containing 6M urea, 75 mM Tris-HCL pH 8.8, 29.3% glycerol, 2% SDS and 0.002% bromophenol blue, 50 mg DTT for 15 min at room temperature for the first step and 125 mg iodoacetamide for the second step. The strips were then applied to 18.75% SDS polyacrylamide gel electrophoresis. The gels were silver and Coomassie blue stained for protein expression analysis and protein characterization, respectively. The protein spots were scanned and the

intensity of spots was analyzed using the Dymension Revolutionary 2-DE software version 2.05a (Syngene, UK).

Protein identification

The protein spots of interest were excised from the gels using pipette tips and subjected to in-gel tryptic digestion, which was performed in an Ettan Spot Handling workstation (Amersham Biosciences, Thailand). Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS) was performed at the Genome Institute, National Science and Technology Development Agency, Pathumthani, Thailand. The supernatant containing the peptide was mixed with α-cyano-4-hydroxycinnamic acid (CCA). Peptides were analyzed by a MALDI-TOF with the model reflex IV (Bruker Daltonics, Germany). Calibration was performed using the peptide standard (P/N 206195, Bruker Daltonik, Bremen, Germany). The spectra were acquired in the mass range 900-3500 amu summing 50 laser shots for each sample and an m/z error of ± 20 ppm. Proteins were identified from their peptide mass fingerprints, searching on the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) and Swiss-prot databases (www.expasy.org). Both of these databases contain publically accessible protein sequence which can be checked using the MASCOT software (http://www.matrixscience.com). The initial search parameters allowed a single trypsin missed cleavage, carbamido-methylation of cystein and partial oxidation of methionine. Database searches were performed using parameter mass tolerances of ± 1 Da for peptide mass ions.

Quantitative Real-Time PCR

To validate the results of the differential proteome analysis of chicken muscles at mRNA level, real time quantitative PCR was performed using cDNA from *pectoralis* muscles of Thai indigenous chickens at 0, 3, 6 and 18 weeks of age. Total RNA was isolated from the *pectoralis* muscles using Trizol reagent (Invitrogen, USA) according to the manufacturer's procedure and treated with DNase I (Promega, USA) for 1 h at 37 ºC. RNA purification was performed using an RNeasy mini Kit (Qiagen, Germany). Total RNA samples were reverse transcribed in 10 µl reaction volume containing 1 µg of total RNA, 100 pmol of oligo (dT)$_{12}$ primers, heated for 5 min at 70 ºC and placed on ice for 3 min. Then 10 µl of mixture components of 1x first strand buffer, 10 µM dNTPs, 10 mM DTT, 10 units RNase inhibitor (Promega, USA) and 200 units of SuperScript III re-

verse transcriptase (Invitrogen, USA) were added, incubated at 50 °C for 60 min and finally heated to stop reaction at 70 °C for 15 min and stored at -20 °C until analyzed.

The expression levels of the target genes were determined on a Chromo 4 Real-Time PCR Detector (MJ Research PTC-200). The PCR reactions were performed in 20 µl reaction volume containing 10 µl 2x QuantiTech SYBR Green master mix (Qiagen, Germany), 2 µl of muscle cDNA sample and forward and reverse primers (Table 1) in optimal combinations of concentrations (4 µM). The target genes were amplified in duplicate at the following conditions: an initial denaturing at 95°C for 15 min, 45 cycles of 95 °C for 15 sec, 58-62 °C for 30 sec, 72 °C for 1 min. The expressed target genes in muscles were reported as relative gene expression or n-fold difference to calibrator cDNA using the comparative threshold cycle (CT) method ($2^{-\Delta\Delta CT}$) (10).

Table 1. Primers used for the quantification of target genes.

Target genes	Sequence	GenBank accession no.	Annealing temperature (°C)	Amplicon size (bp)
PGAM1	5'-CCTGCCCTTCTGGAATGAG-3' 5'-TGGGCTTCAGGTTCTTGTCC-3'	NM_001031556	62	191
APOA1	5'- ATGCCATCGCCCAGTTCG -3' 5'- GAGCCTCGGTGTCCTTCA -3'	M17961	58	162
TPI1	5'-CTTGCCTATGAGCCAGTTTG-3' 5'-GTTCCTTACAGTTGCCACCA-3'	NM_205451	60	175
HSP25	5'- TGCTTCACCCGCAAATACAC -3' 5'- TCTTGGCTGGTTCTTCCTTC -3'	BM488075	60	175
FABP3	5'-GACCAAACCCACCACCATCA-3' 5'-CTCCTTCCCATCCCACTTCT-3'	NM_001030889	62	199
GAPDH	5'- CGTGTTATCATCTCAGCTCC-3' 5'- ACCCTCCACAATGCCAAAGT-3'	NM_204305	60	168

Statistics

The data were analyzed using the SAS package software, version 6.08 (SAS Institute, Inc., Carry, NC). The difference in protein spot intensity and relative mRNA expression of four age groups were analyzed using the proc GLM procedure. Moreover, correlation coefficients of the mRNA and their protein expression levels were calculated. Differences were considered significant at $p<0.05$.

RESULTS

Proteomic analysis

In order to identify differentially expressed proteomes in Thai indigenous chicken muscle during the growth period, the proteome profiles of chicken muscle were compared at 0, 3, 6 and 18 weeks of age using the 2-DE and MALDI-TOF/MS approaches. A total of 259, 161, 120 and 107 protein spots were found in chicken *pectoralis* muscles at 0, 3, 6 and 18 weeks of age, respectively. A representative example of the proteome profiles in chicken muscle is shown in Figure 1. Forty-six protein spots were found differentially expressed during the growth period. For these proteomes, five protein spots showed well-resolved spots and were selected for further protein characterization. These protein spots showed homology with phosphoglycerate mutase 1 (PGAM1), apolipoprotein A1 (APOA1), triosephos-phate isomerase 1 (TPI1), heat shock protein 25 kDa (HSP25) and fatty acid binding protein 3 (FABP3). Table 2 summarizes the mowse scores, estimated molecular weights (Mw), isoelectric points (pI), estimated minimum sequence coverage and matched peptides of the individual spots.

The expression levels of the protein spots between 0 and 18 weeks of age from Thai indigenous chicken muscle are indicated in Figure 2a. The expression levels of PGAM1 and TPI1 proteins were positively correlated with chicken aging ($p<0.05$). PGAM1 was expressed 3.9-fold at 3 weeks, 5.6-fold at 6 weeks and 5.1-fold at 18 weeks as compared to 0 weeks of age. TPI1 was expressed 2.1-fold at 3 weeks, 3.0-fold at 6 weeks and 2.5-fold at 18 weeks as compared to the levels at 0 weeks of age, whereas the expression levels of APOA1 and FABP3 proteins were negatively correlated with chicken aging ($p<0.05$). The APOA1 protein was only expressed 0.30±0.05 unit at 0 weeks of age. The FABP3 protein was expressed 0.5-fold at 3 weeks, 0.3-fold at 6 weeks and 0.1-fold at 18 weeks as compared to 0 weeks of age. The expression levels of the HSP25 protein were both up- and down-regulated during the growth period. This protein spot was expressed 0.4-fold at 3 weeks and 0.7-fold at 18 weeks as compared to 0 weeks of age, while no expression of HSP25 protein in week 6 was observed.
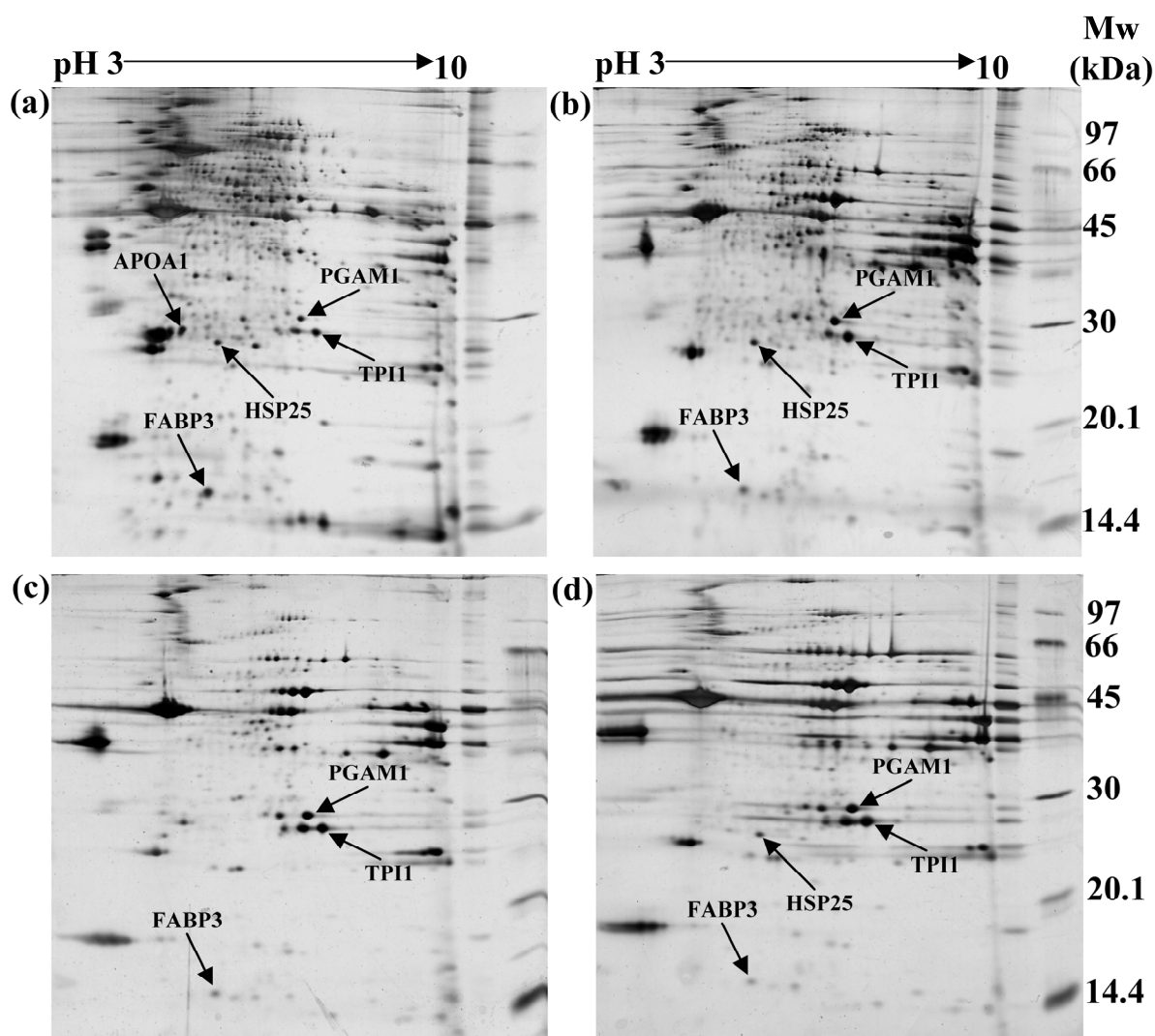
Figure 1. Representation of two-dimensional electrophoresis (2-DE gel) profiles of *pectoralis* muscle of Thai indigenous chickens at (a) 0, (b) 3, (c) 6 and (d) 18 weeks of age, respectively (arrows indicate the target proteins).

Table 2. Identification of proteome changes in Thai indigenous chicken *pectoralis* muscle.

Protein identification	Accession number	Mowse Score[a]	Sequence Coverage[b] (%)	Experimental		From databases	
				Mw[c] (kDa)	pI[d]	Mw (kDa)	pI
PGAM1	NP_990800	79	49	33.34	7.33	28.92	7.21
APOA1	NP_990856	82	33	31.23	5.28	30.66	5.58
TPI1	P84174	116	84	31.12	7.55	26.70	6.79
HSP25	NP_990621	74	43	29.98	5.91	21.72	5.77
FABP3	NP_001026060	70	43	14.49	5.71	14.81	5.92

Phosphoglycerate mutase 1 (PGAM1), apolipoprotein A1 (APOA1), triosephosphate isomerase 1 (TPI1), heat shock protein 25 kDa (HSP25) and fatty acid binding protein 3 (FABP3). [a]Mowse scores greater than or equal to 69 are significant ($p<0.05$). [b]percentage of coverage of the entire amino acid sequence. [c]Molecular weight of spot. [d]Isoelectric point of spot.

Quantitative gene expression analysis

To confirm differential expression of the muscle proteome in the Thai indigenous chicken, quantitative real-time PCR of individual mRNA from chicken muscle at 0, 3, 6 and 18 weeks of age was performed. The relative expression levels of the target genes are indicated in Figure 2b. Significant expressions of the *HSP25* and *FABP3* genes were observed. The *HSP25* gene was expressed 0.4-fold in 3 weeks, 0.2-fold in 6 weeks and 0.4-fold in 18 weeks as compared to 0 week of age. The *FABP3* gene was expressed 0.2-fold in 3 weeks, 0.1-fold in 6 weeks and 0.1-fold in 18 weeks as compared to 0 week of age. Whereas, no significant difference of the *PGAM1*, *APOA1* and *TPI1* genes were found among of age groups.
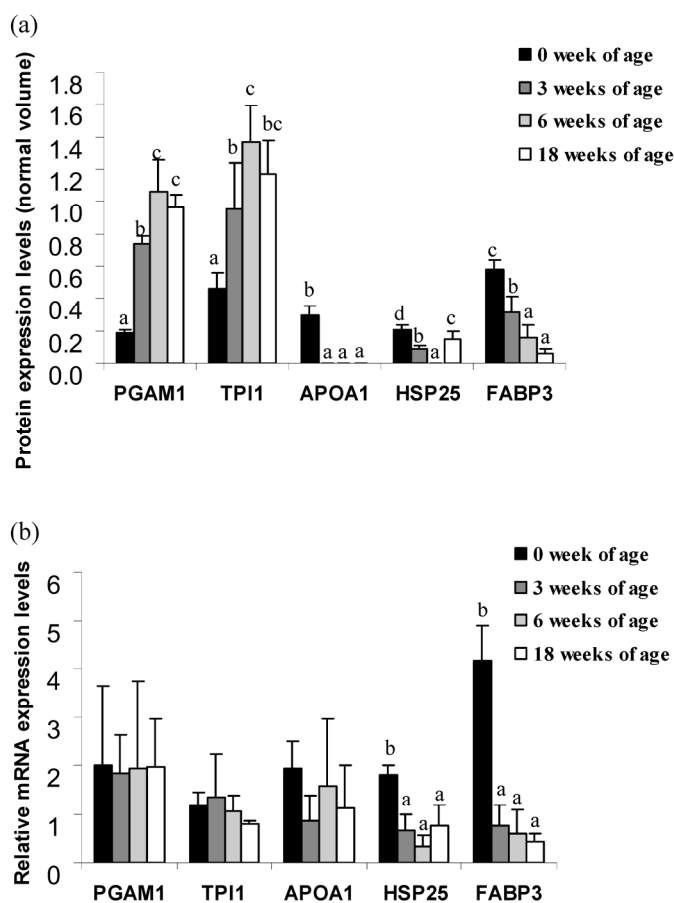

Figure 2. Normal volume intensity (a) protein and (b) relative mRNA expression levels of *PGAM1*, *APOA1*, *TPI1*, *HSP25* and *FABP3* genes of Thai indigenous chicken during the growth period (0, 3, 6 and 18 weeks of age). Each bar represents the mean ± S.E.M. of each protein or mRNA. [a-d]Means with differing superscripts in the same protein/gene are significantly different (*p*<0.05).

DISCUSSION

Understanding the molecular mechanism of muscle development in Thai indigenous chickens can be investigated by studying the proteome profile during the growth period to identify the important proteins that regulate this trait. In the present study, the proteome profiles from *pectoralis* muscles of Thai indigenous chickens at 0, 3, 6 and 18 weeks of age were investigated. These results were then further validated using quantitative real-time PCR. Five protein spots showed altered expression among age groups of Thai indigenous chicken. These proteins included the metabolic proteins (PGAM1, TPI1, APOA1 and FABP3) and the stress protein (HSP25). Thai indigenous chickens were found to have higher levels of the PGAM1 and TPI1 proteins in older than in younger chicken. This indicates that the differential expression, of these proteins, is related to chickens age. These results are consistent with previous studies which found that the PGAM1 and TPI1 proteins expression levels of layer chicken were positively correlated to the growth period (8) as well as the TPI1 protein expression levels in pigs which were associated with age (9). PGAM1 and TPI1 are glycolysis enzymes which play an important role in energy generation for muscle cells (11,12). Several studies have found glycolytic proteins responsible for energy metabolism in muscle to be closely related to meat tenderness and toughness traits (6,13-15). This may reflect a higher glycolytic activity in older chicken.

In contrast, Thai indigenous chickens have lower levels of the APOA1 and FABP3 in the older than in the younger chicken. This result is consistent with previous studies in layer chicken (8) which indicated that the animals aging had an effect on the expression levels of APOA1 and FABP proteins. Furthermore, the FABP3 protein was found to be associated with muscle growth in commercial pig breeds (9). The APOA1 protein is a main component of high density lipoprotein (HDL) in plasma which delivers cholesterol into cells (16). The fatty acid binding proteins (FABPs) play a role in the transfer of fatty acid into cells and delivering the inner cellular fatty acid to combust in mitochondria (17). Our results indicate that the APOA1 and FABP3 play an important function in the early stage of muscle development in Thai indigenous chicken. The expression levels of the small heat shock protein (HSP25) fluctuated during the growth period. Similarly, the small heat shock protein (HSP27) was found to be associated with breed characteristics of pigs (9) and meat quality of beef (18,19). In addition, a previous study (8) found

that the large heat shock protein (HSP90) was related to the growth period in layer chicken.

The mRNA expression levels of five genes (*PGAM1*, *TPI1*, *APOA1*, *HSP25* and *FABP3*) were evaluated. The expression levels of *HSP25* and *FABP3* genes are in agreement with the results obtained by proteomic analysis and tended toward an association with their protein expression levels ($r=0.88$, $p<0.11$; $r=0.92$, $p<0.07$, respectively). Whereas, no correlation between the expressions levels of the *PGAM1*, *TPI1* and *APOA1* genes and their proteins expression levels were found. It has been reported that there is no correlation between some mRNA expression levels and their protein expression in yeast or human liver (20,21). Genes may be present but not transcribed and the number of mRNA copies does not always reflect the number of functional proteins present (22). Protein levels are also regulated by posttranscriptional mechanisms, including transcript localization and stability, translational regulation, and protein degradation (23). A further validation of these proteomic results should be performed with Western blot analysis.

In conclusion a proteomic analysis, of the *pectoralis* muscle in Thai indigenous chicken, found five marker proteins (PGAM1, TPI1, APOA1, HSP25 and FABP3) which are associated with aging during the growth period and influenced muscle development. These results indicate that the metabolic energy and stress protein are important for muscle development of chicken. Further studies are needed to compare the muscle proteome characteristics of Thai indigenous and commercial broiler chickens and to find further protein markers for muscle development and meat quality.

Acknowledgements

Conflict of Interest

The authors have declared that no conflict interest exists.

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