

Relationships between *Pectoralis* Muscle Proteomes and Shear Force in Thai Indigenous Chicken Meat

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ABSTRACT

Two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) were used to investigate the association of *pectoralis* muscle proteomes with Warner-Bratzler shear force (WBSF) values in Thai indigenous chicken meat. A total of 169 proteome spots were found in chicken muscle. Of these, three protein spots were significantly up-regulated and associated with high-WBSF values. These protein spots were characterized and showed homology with pyruvate kinase (PKM2), phosphoglycerate mutase1 (PGAM1) and triosephosphate isomerase1 (TPI1). Moreover, the protein expression levels were highly correlated to the WBSF values. The PKM2 and TPI1 proteins were positively correlated to the WBSF values ($r=0.71$, $p<0.05$ and $r=0.65$, $p<0.05$, respectively), whereas, the PGAM1 protein trended toward an association with the WBSF values ($r=0.49$, $p=0.15$). Additionally, the expression levels of PGAM1 were positively correlated to the TPI1 protein expression levels ($r=0.88$, $p<0.01$), whereas no significant correlation between the expression levels of PKM2 and PGAM1 ($r=0.43$, $p=0.21$) and PKM2 and TPI1 were found ($r=0.51$, $p=0.13$). The results indicated that these three proteomes of the glycolytic pathway are important in the energy metabolism processes of muscle. This finding promotes PKM2, PGAM1 and TPI1 as the functional protein markers for the tenderness trait in Thai indigenous chicken.

Key words: Thai indigenous chicken, proteomics, meat, shear force

INTRODUCTION

Meat quality is one of the economically important traits in chicken. The major determinants of meat quality consist of toughness, tenderness, juiciness and flavor. Moreover, the tenderness and toughness traits are a major aspect of consumption quality among consumers (Maltin *et al.*, 2003; Mullen *et al.*, 2006). Thai indigenous chicken is very popular among consumers in Thailand and the price of its meat is two or three times higher

than that of commercial broilers (Wattanachant *et al.*, 2004). Thai indigenous chicken has a unique taste, with strong and tough muscles, whereas the broiler chicken has an over-tender characteristic (Wattanachant *et al.*, 2004; 2005). The physical structure and chemical composition of Thai indigenous chicken muscle have been characterized (Wattanachant *et al.*, 2004). Proteomic technology is a powerful method to identify proteins that play a major role in the mechanism for controlling meat quality traits.

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Recently, the proteomics approach has been successful in discovering the functional protein marker associated with meat quality traits of livestock, such as chicken (Doherty *et al.*, 2004), cattle (Bouley *et al.*, 2005), sheep (Hamelin *et al.*, 2006) and pigs (Lametsch and Bendixen, 2001; Lametsch *et al.*, 2002; Sayd *et al.*, 2006). However, information on molecular markers for meat tenderness and toughness of chicken is limited. The aim of this study was to investigate the association of *pectoralis* muscle proteomes and WBSF values in Thai indigenous chicken meat.

MATERIALS AND METHODS

Animals

One hundred mixed-sex Thai indigenous chickens ("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 chickens were slaughtered at 16 weeks of age (market size) in an abattoir after a fasting period for 12 h. The carcasses were packaged in plastic bags, placed on ice and transported to the laboratory. The *pectoralis* muscles were dissected from the carcasses after chilling at 4°C for 24 h. A 5 g sample of *pectoralis* muscle (left side) was excised and immediately frozen in liquid nitrogen and subsequently stored at -80°C for proteomic analysis. The remaining part was used for Warner-Bratzler shear force measurement.

Warner-Bratzler shear force measurement

The Warner-Bratzler shear force (WBSF) was determined using a texture analyzer (Model: TA/XT plus, Texture Technology, USA) according to Laville *et al.* (2007). The samples were cooked in a water bath at 80°C to an internal temperature of 72°C and then cooled at room temperature. Five cylinders of 12.7 mm diameter and 20 to 25 mm length were taken from each muscle sample with

the main cylinder axis parallel to the direction of the muscle fibers, with sections dispersed across the whole muscle area. Each of the cylinders was then sheared across its main axis in a Warner-Bratzler cell. The operating parameters consisted of a cross-head speed of 100 mm/min and 5 kg load cell. The WBSF values were expressed in Newton (N).

Two-dimensional gel electrophoresis

Among 100 Thai indigenous chickens, the five with the highest WBSF values as well as the five with the lowest WBSF values were subjected to proteomic analysis. To prepare protein extracts, approximately 1 g of frozen *pectoralis* muscle was ground in liquid nitrogen using a pestle and mortar. The powdered tissue was homogenized (Ultra Turrax®, IKA works, Germany) 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) at 4°C for 10 min. The homogenate was centrifuged at 4°C at 10,000 x g for 10 min. The extracted protein was cleaned with a 2-D clean up kit (Amersham Biosciences, Thailand) according to the manufacturer's procedure. The concentration of the purified protein was determined with 2-D Quant kit (Amersham Biosciences, Thailand). Proteomic analysis was performed using two-dimensional gel electrophoresis (2-DE) as described by Kuo *et al.* (2005). 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°C for 12 h and first-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°C. The strips were 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 for protein characterization, respectively. The protein spots were scanned and the intensity of spots was analyzed as normal volume by using Dymension Revolutionary 2-DE software version 2.05a (Syngene, UK).

Protein identification

The protein spots of interest were excised from the gels using pipet 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 reflex IV model (Bruker Daltonics, Germany). Calibration was performed using the peptide standard (P/N 206195, Bruker Daltonik, Bremen, Germany). The spectra were acquired in 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 fingerprinting by searching on the NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>) and Swiss-prot (www.expasy.org) databases, both of which are protein sequence databases accessible using the MASCOT software (<http://www.matrixscience.com>). The initial search parameters allowed a single trypsin missed cleavage, carbamido-methylation of cysteine and partial oxidation of

methionine. Database searches were performed using parameter mass tolerances of ± 1 Da for peptide mass ions.

Statistics

The data were analyzed using the SAS package software, version 6.08 (SAS Institute, Inc., Cary, NC). The differences in protein spot intensity between high- and low-WBSF groups were analyzed with Student's t-test. Moreover, the Pearson linear correlation coefficients (r) of the protein expression levels and WBSF values were calculated as well as estimating the significance of differences among expression levels of target proteins. All statistical test were considered significant at $p < 0.05$.

RESULTS

Differentially expressed proteome in chicken meat

The average WBSF values of Thai indigenous chicken meat were 52.40 ± 2.40 N, whereas the two extreme shear force groups of Thai indigenous chicken meat were 99.43 ± 9.33 (high-WBSF, $n=5$) and 26.89 ± 1.42 N (low-WBSF, $n=5$), respectively. A total of 169 protein spots, ranging from 14.4-97.0 kDa, pH 3-10, were observed in this study. Figure 1 shows a proteome profiling of chicken muscle and the differentially expressed proteomes of the high- and low-WBSF values. These proteins were identified (Table 1) and showed homology with pyruvate kinase (PKM2), phosphoglycerate mutase 1 (PGAM1) and triosephosphate isomerase 1 (TPI1). Three protein spots were significantly up-regulated and associated with high-WBSF values (Figure 2). The expression levels of PKM2, PGAM1 and TPI1 proteins of the high-WBSF group were 0.98 ± 0.05 , 0.46 ± 0.02 and 0.95 ± 0.03 units, respectively, whereas the expression levels of the same proteins in the low-WBSF group were 0.79 ± 0.06 , 0.37 ± 0.03 and 0.74 ± 0.05 units, respectively.

Correlation between proteomes and shear force values

Figure 3 shows the relationships of the differentially expressed proteomes and the WBSF values. PKM2 and TPI1 were significantly correlated to the WBSF values ($r=0.71$, $p<0.05$ and $r=0.65$, $p<0.05$, respectively), whereas no correlation between the PGAM1 and WBSF values

was observed ($r=0.49$, $p=0.15$). Moreover, the expression levels of PGAM1 were significantly correlated to the TPI1 protein expression levels ($r=0.87$, $p<0.01$), whereas no correlation between the expression levels of PKM2 and PGAM1 ($r=0.43$, $p=0.21$), nor the expression levels of PKM2 and TPI1 ($r=0.51$, $p=0.13$) was observed.

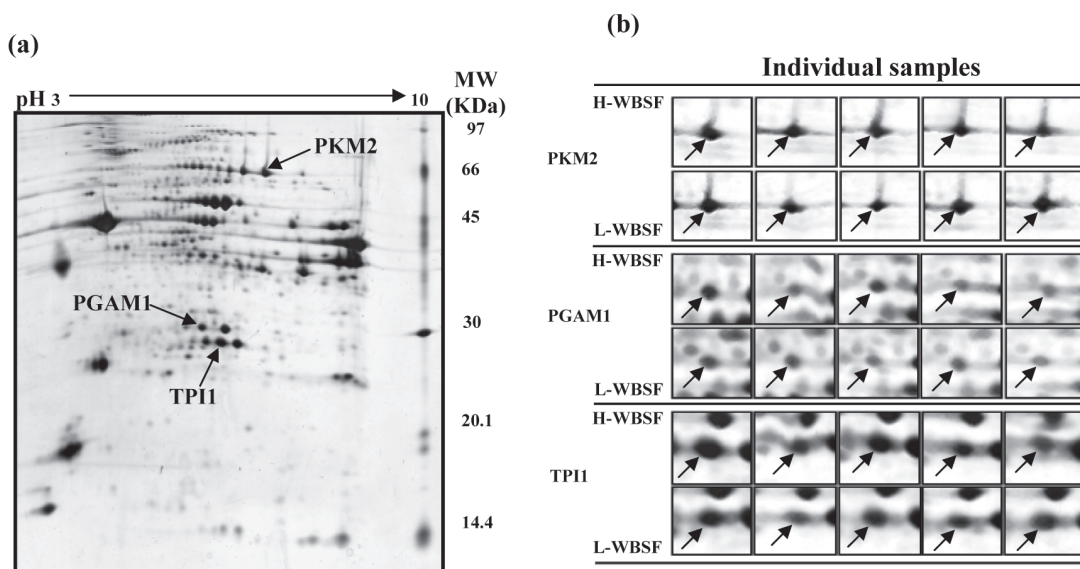


Figure 1 Two-dimensional gel electrophoresis (2-DE) of Thai indigenous chicken muscle: (a) differentially expressed proteome of high- and low-WBSF groups (arrows indicate the protein spots of PKM2, PGAM1 and TPI1); and (b) variation of expression levels among 10 individual samples of high- and low-WBSF (H-WBSF and L-WBSF) groups (arrows indicate the target proteins).

Table 1 Identification of proteome changes in chicken *pectoralis* muscle.

Protein identification	Accession number	Mowse score ^a	Mw ^b (kDa)	pI ^c	Sequence coverage ^d (%)	Matching ^e
Pyruvate kinase 2 muscle (PKM2)	NP_990800	202	58.43	7.29	43	21
Phosphoglycerate mutase 1 (PGAM1)	P84174	79	28.92	7.21	49	8
Triosephosphate isomerase 1 (TPI1)	NP_990782	116	26.70	6.79	84	19

^a Mowse scores greater than or equal to 69 are significant ($p<0.05$). ^b Molecular weight of spot. ^c Isoelectric point of spot.

^d Percentage of coverage of the entire amino acid sequence. ^e The number of matched peptides in the database search.

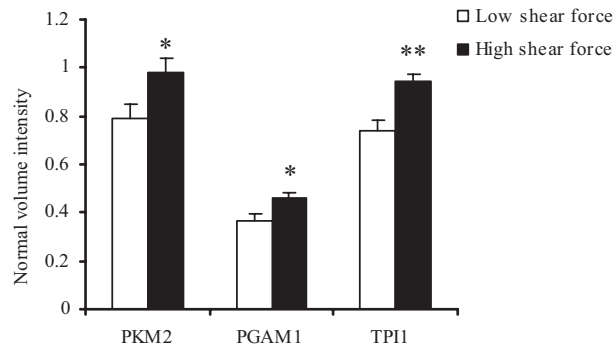


Figure 2 Average of normal volume intensity of the differentially expressed proteomes of high- and low-WBSF groups. Each bar represents the mean \pm S.D. of PKM2, PGAM1 and TPI1 protein spots, respectively (* $p < 0.05$ and ** $p < 0.01$).

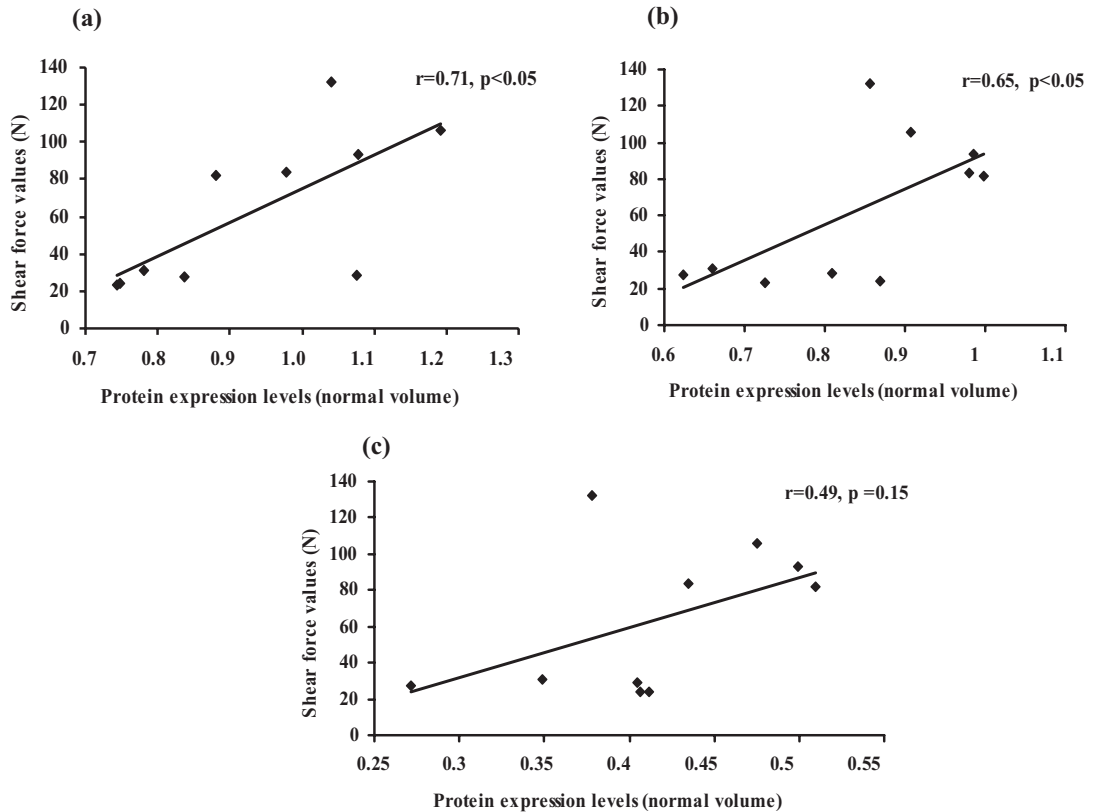


Figure 3 Relationships between *pectoralis* muscle proteomes and Warner-Bratzler shear force (WBSF) of Thai indigenous chicken meat: (a) PKM2 vs. WBSF ($p<0.05$); (b) TPI1 vs. WBSF ($p<0.05$); and (c) PGAM1 vs. WBSF ($p=0.15$).

DISCUSSION

Proteomic profiles and the association of protein markers with high- and low-WBSF values of Thai indigenous chicken meat were analyzed. Three protein markers (PKM2, PGAM1 and TPI1) showed differentially expressed high- and low-WBSF groups of chicken. These proteins are known to be involved in glycolytic metabolism. The PKM2 protein is a muscle isoform of the rate-limiting enzyme that catalyzes the conversion of phosphoenolpyruvate to pyruvate in the last step of the glycolytic pathway and plays a major role in muscle glyconeogenesis (Gleeson, 1996; Fontanesi *et al.*, 2008). In this study, the expression levels of the PKM2 protein were higher in the high-WBSF chicken than in the low-WBSF chicken (1.24 times, $p < 0.05$). Moreover, the expression levels of PKM2 were positively correlated to the WBSF values ($r = 0.71$, $p < 0.05$). However, association of the *PKM2* gene with estimated breeding values (EBVs) for average daily gain, lean cuts and feed:gain ratio and a tendency toward an association with meat quality in pigs has been reported (Fontanesi *et al.*, 2008). PGAM is the enzyme that catalyzes the inter-conversion of 2-phosphoglycerate and 3-phosphoglycerate in the glycolytic process to release energy (Fothergill-Gilmore and Watson, 1989; Doherty *et al.*, 2004). In the current study, the PGAM1 proteins were higher in the high-WBSF chicken than in the low-WBSF chicken (1.24 times, $p < 0.05$). No correlation between the expression levels of the PGAM1 protein and the WBSF values was observed. However, the polymorphisms of the *PGAM* gene were significantly associated with meat quality, growth and fat deposit, as well as muscle growth development in pigs (Fontanesi *et al.*, 2008; Qiu *et al.*, 2008).

The TPI1 protein is an enzyme that works in the process of glycolysis which catalyses the conversion of dihydroxyacetone phosphate (DHAP) to glyceraldehyde-3-phosphate (G3P).

The G3P is used as a starting substance in the synthesis of pyruvate (Solem *et al.*, 2008). In this investigation, the TPI1 protein was present in the high-WBSF chicken at a higher level than in the low-WBSF chicken (1.28 times, $p < 0.05$). Additionally, the expression level of the TPI protein was positively correlated to the WBSF value ($r = 0.65$, $p < 0.05$). The current results were consistent with previous studies (Hwang *et al.*, 2005). The TPI1 protein expression levels were positively correlated to the WBSF values of pork, whereas, Lametsch *et al.* (2003) reported that this protein was negatively correlated to WBSF values and its level decreased during aging (Park *et al.*, 2007). This conflict in results may reflect the expression of multiple isoforms of TPI1 in the muscle proteome profiling (Hwang *et al.*, 2005; Kwasiborski *et al.*, 2008; Hollung *et al.*, 2009). Moreover, aging periods after post-mortem obviously affected TPI1 protein expression levels (Lametsch *et al.*, 2002).

Several studies have demonstrated the role of glycolytic proteins in energy metabolism in muscle during the post-mortem period and it is believed to be closely related to meat tenderness and toughness traits. Lametsch *et al.* (2002) reported that six metabolic proteins, glycogen phosphorylase, creatin kinase, phosphopyruvate hydratase, myokinase, pyruvate kinase, and dihydrolipoamide succinyltransferase changed during the post-mortem periods in pigs. Moreover, the metabolic proteins of aldehyde dehydrogenase, enolase1, ADP-ribosylhydrolase like1, GPD1 protein, 3-hydroxyisobutyrate dehydrogenase, guanidinoacetate *N*-methyltransferase, adenylate kinase 1, creatine kinase M changed in bovine *longissimus thoracis* muscle during the early postmortem storage period (Jia *et al.*, 2007).

CONCLUSION

Proteomic analysis of *pectoralis* muscles in Thai indigenous chicken indicated that the

expression levels of the PKM2, PGAM1 and TPI1 proteins were significantly associated with the high- and low-WBSF values of the chicken meat. Moreover, the expression levels of the PGAM1 protein were correlated to the TPI1 protein levels. No correlation between the expression levels of PKM2 and PGAM1, or PKM2 and TPI1 were found. The results indicated that the importance of energy metabolism proteins in the glycolytic pathway is associated with the meat quality of chicken. Further study to identify the variations of the *PKM2*, *PGAM1* and *TPI1* genes is needed to develop DNA markers to help improve meat quality traits in Thai indigenous chicken.

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