EFFECTS OF DIFFERENT METHODS OF SLAUGHTERING ON PROTEIN EXPRESSION IN CHICKEN MEAT

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ABSTRACT: This study investigated the variation in total protein profile in chicken skeletal muscle which is thought to be influenced by variations in the method of slaughtering. Two types of samples, depending on the method of slaughtering using a sharp knife, were examined. These were: (i) CT- neck was cut off completely and body was tied until the animal expired, and (ii) PR- neck was cut off partially leaving the spinal cord intact and the body was released immediately after slaughtering. Proteins, expressed in higher amounts, were mostly found to be distributed in the range of Mw 45-66 kDa -- as resolved using SDS-PAGE. 2D-PAGE resolution showed differences in protein expression between the samples. Protein spotted on the gel near pH 5.0 and Mw 116 kDa was found in the skeletal muscle from CT samples but were absent in the PR samples.

ABSTRAK: Variasi profil jumlah protein di dalam otot skeletal telah dikaji dengan jangkaan bahawa ianya dipengaruhi oleh kaedah penyembelihan yang berbeza. Berdasarkan kaedah penyembelihan, dua jenis sampel telah diperiksa: (i) CT – leher telah dipotong sepenuhnya dan badan haiwan diikat hingga mati and (ii) PR – leher dipotong separuh meninggalkan kord tulang belakang dalam keadaan sempurna dan badan dilepaskan serta-merta selepas penyembelihan. Kandungan protein didapati tinggi dalam taburan Mw 45-66 kDa sebagai terurai menggunakan SDS-PAGE. Sementara itu, kandungan protein yang berbeza diperhatikan di dalam penguraian 2D-PAGE. Tompok protein kelihatan pada gel sekitar pH 5 dan Mw 116 kDa telah dijumpai pada CT otot skeletal, tetapi tidak kelihatan pada sampel PR.

KEYWORDS: *2D-PAGE; slaughtering; protein; slaughtering*

1. INTRODUCTION

Methods of poultry slaughtering varies depending on the concern over painless sacrifice of the animal applying pre-slaughtering stunning; ease of handling in case of industrial slaughtering by applying physical restriction such as shackling; and consumers' safety. Animals are also sacrificed based on ritual slaughtering laws such as halal (for Muslims) and kosher (for Jews). Recommended codes for halal and kosher slaughtering involve cutting off the carotid arteries, jugular vessels, esophagus and trachea in the throat, without any specific reference to pre-slaughter stunning. In halal slaughtering practice, stunning is allowed with certain restrictions [1]. Variation in slaughtering eventually

determines the time needed to die and it varies enormously, depending on which blood vessels in the neck are cut [2].

In this study, chickens are slaughtered manually by cutting ventrally both of the jugular veins and carotid arteries without pre-slaughtering stunning. Possible changes in total protein profile were observed in skeletal muscle due to variation in handling the chicken during slaughtering. i.e., whether or not (i) the chicken will be released immediately after slaughtering and (ii) the neck is completely or partially severed effects the expression of protein. Beheading or complete severing of the neck raptures the spinal cord while releasing the animal immediately after slaughtering allows free muscle movements until the death.

Earlier it was reported that, separation of the jugular vein and carotid arteries leaving the nerves intact to function, shortens the time of death compared to other methods; and separation of whole head disrupts the nervous system (spinal cord damage) by causing asphyxia and suffocation [2]. These differences influence neurophysiologic response mediated muscular movement. On the other hand, existence of external pressure on muscle such as keeping the animals tied up until the animal dies might affect the suffering level.

Therefore, it has been hypothesized that those variations during slaughtering might affect the neurophysiological parameters of the chicken that could be reflected by the variation in protein expression. 1D and 2D polyacrylamide gel electrophoresis (PAGE) are used to study differential expression of total protein. In this study, possible variations in differentially expressed proteins in chicken skeletal muscle were assessed using 1D and 2D-PAGE**.** Chickens were slaughtered in either way: (i) CT- neck was cut off completely and body was tied until the animal died and (ii) PR- neck was cut off partially leaving the spinal cord intact and body was released immediately after slaughtering.

2. MATERIALS AND METHODS

2.1 Sample Preparation

Slaughtering method applied in this study was conventional manual slaughtering which is practiced universally. All experiments were conducted at research facilities at Kulliyyah of Science, International Islamic University Malaysia. Animal handling and management procedures were carried out in accordance with the ethical guidelines of International Islamic University Malaysia (IIUM).

Twenty one (21) days old chickens (weight 750-850 g), purchased from local farm, were slaughtered in either of the following conditions: (i) necks were partially cut-off (by cutting both of the jugular veins, carotid arteries and oesophagus without cutting the spinal cord) and birds were released immediately after slaughtering (PR); (ii) necks were completely cut-off and birds remained restrained immediately after slaughtering (CT). Approximately 1 g of muscle sample was homogenized on ice in 10 ml of 0.25 M Phosphate buffer (pH 7.5) with 0.1% triton X-100. Solution was then centrifuged at 6000×g for 30 min at 4° C and supernatant was collected and preserved in 1ml aliquots at -80° C until further use. Protein quantification was then performed with spectrophotometer at 595 nm.

2.2 Protein Quantification

Solubilized protein was quantified using the Bradford assay. Five milliliter of Bradford reagent (GE Healthcare, Uppsala, Sweden) was mixed with 100 µl of sample solution.

Prior to that, the sample solution was diluted (1/10 dilution). Absorbance value was measured at 595 nm. A standard curve was generated with serial dilutions of bovine serum albumin (BSA) to get the concentrations of the samples.

2.3 SDS-PAGE

To resolve the variation in total protein profile in terms of their molecular weight equal amount of protein (120 µg) were electrophorsed on 12.5% polyacrylacimde gel in denaturing condition in a 6×8cm^2 dimensional gel (Mini-PROTEAN 3 cell, Biorad, Hercules, USA) with 250 V. To further verify the result two other samples were ran on 12.5% gel of 8×12 cm^2 dimensions in SE 600 Ruby™ Vertical Unit (GE Healthcare) with 450 V. All the gels were stained with Coomassie Brilliant Blue.

2.4 Two-Dimensional Gel Electrophoresis

To further resolve the variation in total protein profile, samples were also electrophoresed in 2D gel essentially followed by manufacturer's instruction (GE Healthcare). Briefly, sample was mixed by vortexing with rehydration buffer and incubated at room temperature (RT) for 30 min. Sample (200 µl) containing 120 µg of protein was loaded to 11 cm of Immobilized pH Gradient (IPG) strip, pH 3-10 in Immobiline DryStrip Reswelling Tray and left on desk at room temperature overnight ensuring proper rehydration of the strip. Isoelectric focusing (IEF) was conducted with Multiphor II (GE Healthcare). Strips were placed on the plastic aligner on top of the glass tray, gel faced up. IEF was conducted at 1 mA and 10° C in 3 steps 300 V for 3 h and 30 min, 3500 V for 1 h and 30 min and 3500 V for 1 h and 45 min. The strips were then equilibrated in two steps each for 15 min at room temperature using 10 ml SDS equilibration buffer containing 6 M urea, 50 mM Tris-HCl (pH 8.8), 30% glycerol, 2% SDS and 0.002% bromophenol blue, and either 50 mg DTT (for first step) or 125 mg iodoacetamide (for 2nd step). The strips were then applied to 12.5% SDS polyacrylamide gel for electrophoresis using SE 600 Ruby™ Vertical Unit (GE Healthcare) at 45 V overnight at 16° C. At the end of the run, proteins in gel were visualized by silver staining using PlusOne Silver Staining kit (GE Healthcare). Each sample was run in duplicate to minimize the gel to gel biasness.

2.5 Gel Analysis

Gels were analyzed with Image Master 2D Platinum (v 7.0) (GE Healthcare). Parameters for spot detection were as follows: minimum area 100; smooth factor 4.0; saliency 115. A gel was used as reference gel from each group. Comparative analysis was done by comparing the intensity of individual spots within and between groups. Differential expression of a spot was considered when all four gels showed the up- or down-regulation of the protein.

3. RESULTS

3.1 Protein Quantification

Muscle proteins were solubilized in PB buffer with 0.1% triton X-100 and then assessed for protein concentration with spectrophotometer. Protein concentrations were found between 5.3-11.1 µg/µl (Table 1).

Table 1: Protein concentration in meat solubilized in PB buffer.

Sample ID	Protein Conc.(µg/µl)
PR	12.23 ± 4.14
CT	13.22 ± 7.79

3.2 Total Protein Profile Showed Differential Protein Expression due to Difference in Slaughtering

Samples were then run on polyacrylamide gel that separates proteins based on molecular weight (Mw) (Fig. 1). Protein profiles of CT and PR were found different both in terms of presence or absence of bands and on their intensity when resolved in a gel of smaller dimension (6×8 cm^2) (arrow marked in Fig. 1A). Further resolution in gel of higher dimension (8×12 cm^2) demonstrated similar variations in protein profile (arrow marked in Fig. 1B). Notably the variations were identified with the proteins having Mw in the range between 45-66 kDa.

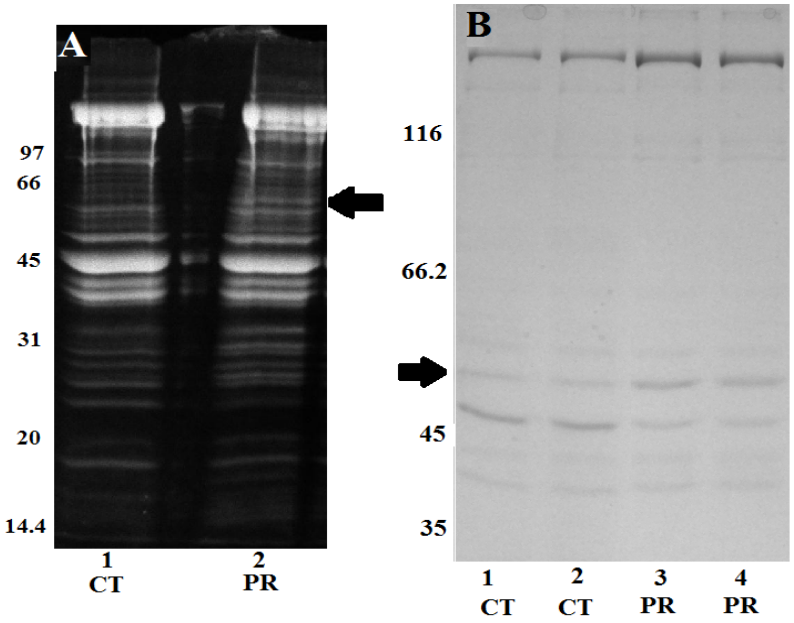

Fig. 1 Chicken total skeletal muscle protein expression in 12.5% SDS-PAGE gel. (A) Protein samples were resolved on gel (6×8 cm^2) PR (lane 1) and CT (lane 2). Difference in band intensity are marked with arrow between 66-45 kDa. (B) Proteins from each sample were run in duplicates: PR (lane 1 &2); CT (lane 3 & 4) on gel (8×12 cm^2). The arrow identifies band intensity difference in similar region between 66- 45 kDa.

3.3 Total Protein Profile Showed Differential Protein Expression due to Difference in Slaughtering

For 2D-gel electrophoresis, 1st dimension was carried out in precast IPG strips, pH range 3.0-10. After first dimension separation based on protein pI value, proteins were transferred to 2nd dimension, 12.5% polyacrylamide gel which separates protein based on Mw. Figure 2 shows two representative gels from two different types of sample, PR and

CT. Gels were matched with the help of specialized image analysis software, Image Master 2D Platinum (v 7.0) (GE Healthcare). The differentially expressed protein was marked with circle. In CT, it has been expressed near pH 5.0 and Mw 116 kDa while it is absent in PR (Fig. 2). Comparison between the differentially expressed proteins with their magnified and cropped image and respective 3D view that were generated (Fig. 3) with the software also demonstrated the proteins spotted in the said location from the sample CT.

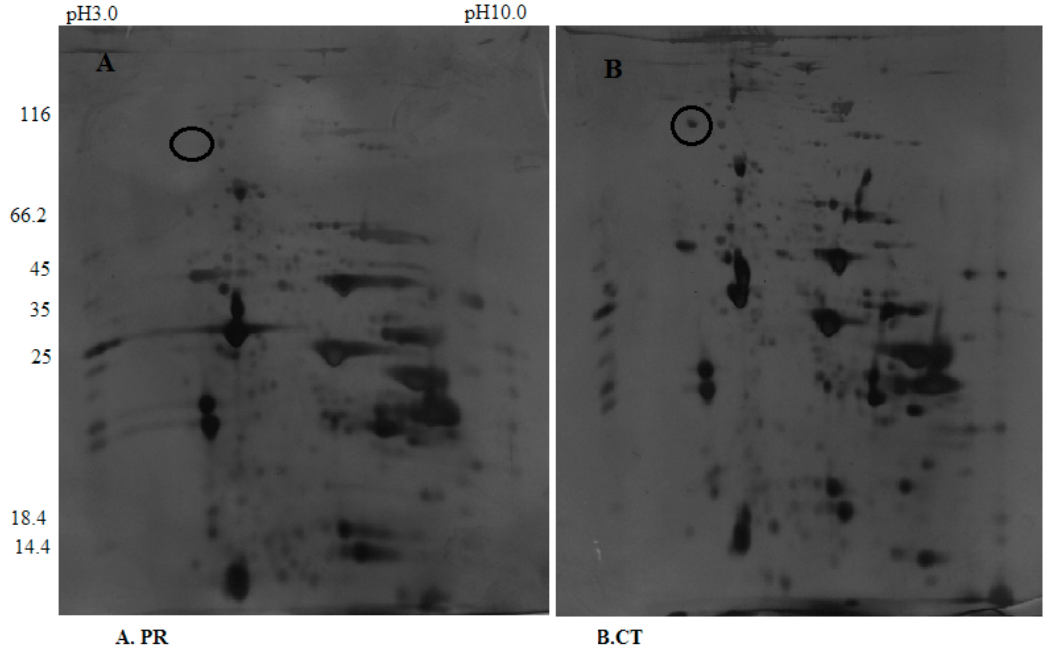

Fig. 2 Differential protein expression in samples PR (A) and CT (B) in 2D-gel electrophoresis. Protein spot identified with differential expression is marked in circle. Protein spotted in CT near pH 5.0 and Mw 116 kDa which is absent in PR.

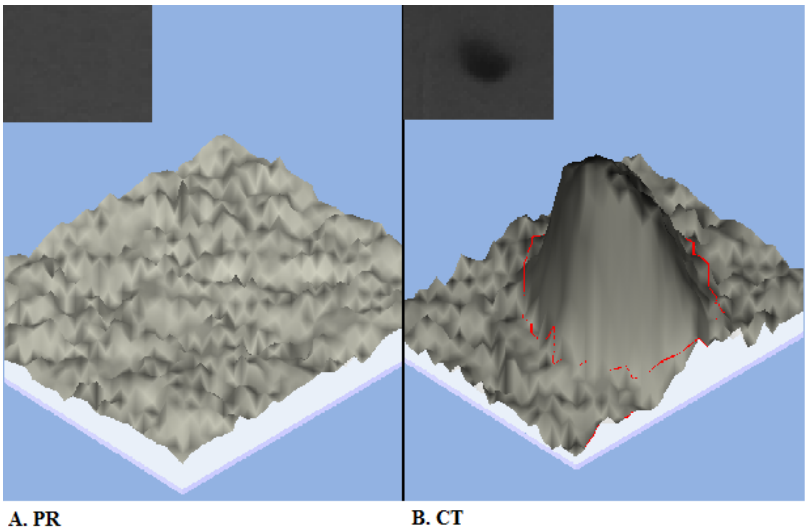

Fig. 3 Image analysis of the differentially expressed proteins.
3D view using Image Master 2D Platinum (v 7.0) (GE Healthcare) of the spots identified from the 2-D gels (Fig 2) shows the 3-dementional image manifestation of the area for the protein in sample CT (B) compared to that from sample PR (A).

4. DISCUSSION

Poultry slaughtering may involve physical restriction of the bird by shackling. Difference in poultry slaughtering practices is also observed in terms of cutting the neck, with or without injuring spinal cord during slaughtering. Variation in these two parameters i.e., physical restriction and spinal cord damage is expected to affect the neurophysiogcal in/activation hence alter the expression of protein at neuromuscular junction. This study investigated the changes in protein expression, due to the variations in slaughtering techniques, as mentioned above, in muscle samples collected from slaughtered chickens. Total proteins were resolved by SDS-PAGE in gels with two different dimensions: 6×8 cm^2 (Fig. 1A) and 8×12 cm^2 (Fig. 1B). Protein bands were found to be distributed between higher (116 kDa) and lower (14 kDa) molecular weights. Highly expressed proteins are distributed within Mw of 35-66 kDa. The protein bands between Mw 35-66 kDa were previously identified as albumin, pyruvate kinase, beta-enolase and creatine kinase [3]. These proteins are also reported as highly expressed in chicken skeletal muscle. Expression pattern and intensity of the proteins were found similar when compared the resolution of the bands between two gels having different dimensions (Fig. 1).

The differentially expressed proteins were identified when total proteins were resolved in 2D-PAGE based on the pI and Mw of the proteins. Proteins differentially expressed in different conditions of slaughtering have been marked with circles in reference gels (Fig. 2). Quantitative and qualitative assessment of the protein expressed in low level by 2D-PAGE has been considered as a common drawback of the technique [4]. However when a large number of proteins are expressed in higher amount it is also hard to identify the variation in expression using 2D-PAGE. Despite all the shortcomings, it is considered as one of the best methods to get a glance of soluble proteins which may form a ground for further studies on differentially expressed protein [5]. Therefore, variation in protein expression in the current study as observed in SDS-PAGE was analyzed by 2-D gel electrophoresis and matched within the set of four gels and then between pair of slaughtering techniques. Protein spotted near pH 5.0 and Mw 116 kDa in CT were absent in PR. One of the possible candidate for this protein to be the 20S catalytic subunit of the enzyme acetylcholine esterase (AChE) having Mw 110 kDa [6], expression of which is expected to be altered at neuromuscular junction (NMJ) due to variation in neurophysiological in/activation. Notably, the 20S subunit is the heavier, collagen tailed, asymmetric form of AChE in birds which was found to be a hybrid molecule with three subunits- AChE catalytic subunit (apparent Mw 110 kDa), BuChE catalytic subunit (72 kD) and a collagenous tail subunit (58 kDa) [6]. This preliminary observation of the total protein expression with the differentially expressed proteins as spotted will be further analyzed for identification and characterization.

ACKNOWLEDGEMENT

This research was supported by the research grant from the Ministry of Higher Education, Malaysia (FRGS 01-06-24), Basic and Applied Biomedical Research Cluster Unit and Faculty of Science, IIUM. Authors are grateful to Assoc. Prof. Dr. Azura Amid, Shahidah Abu Samah and Maliza Azrain for their support and advices.

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ABBREVIATIONS

2D-PAGE: Two Dimensional Polyacrylamide Gel Electrophoresis

IEF: Isoelectric Focusing