IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEIN FROM ELECTRICAL STUNNING OF BROILER CHICKENS MEAT PROTEIN

IDENTIFIKASI PERBEDAAN TINGKAT EKSPRESI PROTEIN PADA DAGING AYAM BROILER YANG DIBERI PERLAKUAN *ELECTRICAL STUNNING*

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ABSTRACT

Identification of differentially expressed protein from the muscle tissue of broiler chicken meat with different conditions of pre-slaughter has been done. Each sample (6 broilers aged 21 days, 1 kg of weight) was prepared through the process of pre-slaughter with 3 conditions, the first sample slaughtered in a conventional way which untreated electrical stunning, while the second and third sample of the chicken was prepared by using electrical stunning with 1 A and 25 Volts for 5 seconds and 1 A, 125 Volts for 30 seconds. Two biological replicate were done for each of samples. Muscle tissue protein extracted in Tris HCl pH 8.0 and the proteins separation by using SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). Identification of differentially expressed protein performed by densitometry to identify the profile of the resulting proteins. The results of this study showed that the protein bands constructed in the range of 8.5-140 kDa and 9 dominant protein bands with different relative intensities. Densitogram analysis results showed there are two specific protein bands appear on the results of the electrical stunning which more extensive over expression. This indicates the electrical stunning of slaughter process may triggered the expression levels of certain proteins that do not occur in the non-electrical stunning.

Keywords: broiler meat, densitometry, electrical stunning, protein expression, SDS-PAGE

ABSTRAK

Penelitian identifikasi protein yang terekspresi dalam jaringan otot daging ayam broiler pada kondisi pra penyembelihan yang berbeda telah dilakukan. Masing-masing sampel (6 ayam broiler usia 21 hari bobot $\pm$1 kg) disiapkan melalui proses pra-penyembelihan dengan 3 kondisi yang berbeda yaitu sampel 1 disembelih dengan cara konvensional dan tidak diberi perlakuan *electrical stunning* (kontrol). Sedangkan sampel 2 dan 3 diberi perlakuan *electrical stunning* dengan arus 1 A 25 Volt selama 5 detik dan sampel ayam 5 dan 6 diberi perlakuan *electrical stunning* 1 A, 125 Volt selama 30 detik. Masing-masing perlakuan diulang sebanyak dua kali. Ekstraksi protein dilakukan dalam buffer tris HCl pH 8.0 dan pemisahan profil protein menggunakan elektroforesis SDS-PAGE (*Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis*). Identifikasi profil protein dilakukan dengan metode densitometri untuk mengidentifikasi profil dan karakteristik masing-masing protein yang dihasilkan. Hasil penelitian menunjukkan pita-pita protein yang tersebar pada kisaran 8.5– 140 kDa dan 9 pita protein dominan dengan intensitas yang relatif berbeda. Hasil analisis densitogram menunjukkan bahwa terdapat 2 pita

protein yang spesifik dengan intensitas yang lebih tinggi pada *electrical stunning* dibandingkan dengan penyembelihan *non electrical stunning*.

Kata kunci: daging ayam broiler, densitometri, *electrical stunning*, protein, SDS-PAGE.

INTRODUCTION

The potential of non halalness food from food processing or food production especially in fresh meat need to be considered. Broiler meat is one of the fresh meat product that is much preferred by the people of Indonesia because of quality taste and price is relatively cheap, rich in protein nutrients and relatively easy in processing. According to Director General of Animal Husbandry and Health (2011), the supply of animal food products, especially meat commodities must meet safety requirements, hygiene and halal status. The meat consumed should be healthy, safe and lawful and comes from abattoirs (slaughter houses) have been halal certified (Dirjen Peternakan dan Kesehatan Hewan, 2011)

Today, there are a lot of Slaughtering Houses still less attentions of correct procedures in slaughtering accordance with Islamic Shari'a. This condition is exacerbated by the attitude of manufacturers or traders that are often harmful to consumers by selling a chicken carcass or chicken meat is preserved with formalin (preservatives that are not allowed for food). Chicken meat is one of product that is a doubtful of halal status, hence the need for research on the slaughter procedures accordance with Islamic Shari'a and its relation to the assessment of the concept of halal assurance system is very important to be developed (Wulandari, Estuti, & Gunawan, 2010). In connection with the development of analytical halal food methods, particularly for food products derived from fresh meat could be done based on the study of proteomics (Bendixen, 2005; Montowska and Pospiech, 2013; Ballin, 2010; Kelly, et al., 2006; Kim, et al., 2007).

Several studies have explained the existence of some differences in the expression levels of intracellular proteins in chicken meat slaughtered in different ways (Doherty, et al., 2004; Amid, Samah, & Yusof, 2012; Samah, Amid, & Yusof, 2011). Other research has been done by Zaman, et al. (2012), states that the different methods of slaughter in some of broilers slaughtered by a sharp knife and cut off the neck to separate from his body and the bodies of animals tied up animals died were then compared with the slaughter syar'i where the method of slaughter is not to break the animal's head from his body (just cut off the three main channels) and the animals allowed to roam free are not bound after slaughter. His experiments showed that the characteristic protein expressed in relatively large amounts, especially in the molecular weight of 45-66 kDa (Zaman, et al. 2012). Its obtained based on the analysis of SDS-PAGE. The results of subsequent studies using 2D-PAGE (polyachrylamide 2-dimensional gel electrophoresis), showed that a specific protein profiles that appear only in chickens results in a non-Islamic slaughter where there is a protein spot near pH 5.0 with a 116 kDa molecular size (Zaman, et al. 2012).

López, Carrilho, Campo, & Lafuente (2008) has also been reported that the technique of slaughtering with the separation of the jugular veins and carotid arteries by leaving the function of nerves intact to shorten the time of death when compared with other methods in which the separation of the entire head can disrupt the nervous system (spinal cord damage) that can cause asphyxia and breathlessness in these animals. Besides these differences also affect neurophysiological responses mediated by muscle movement. On the other hand, the external pressure on the muscles such as tying up animals after

slaughter dead animals can affect the level of suffering in animals that could ultimately affect the quality of meat produced (Josepth, et al., 2013). Besides pre-slaughter method using electric current (Amid, et al. 2012), showed that electrical stunning with current and 70 V 0.75 A able to produce cardiac muscle cell protein spots were relatively different when compared with controls which not to be stunned. It shows that the high voltage is able to induce some kind of protein that is expressed in heart muscle such as actin, and troponin I, where the samples were not induced (do not stunning), two types of protein is not found. However, research on the effects of electrical stunning of the protein expression levels of muscle tissue of broiler chicken meat is relatively not much done.

In order to develop an analytical methods of halal food products of meat studies have been conducted to explore the possibilities of specific proteins expressed in the muscle tissue of animal cells, especially in broiler chickens were slaughtered in different ways. This study focused on proteomic analysis of broiler meat products from slaughtering with the technique of electrical stunning pre-slaughtering and non electrical stunning. The analysis was conducted through an approach using SDS-PAGE where muscle tissue proteins were analyzed by the molecular weight of each protein levels expression in muscle tissue (Montowska and Pospiech, 2007; Ballin, 2010). From the analysis of proteomics is expected to obtain information regarding protein profiles of broilers slaughtered meat in different ways. In addition, proteomics analysis also aimed to map the specific protein profiles that can be used as a candidate biomarker for the development of the analysis of halal food products, especially for broiler meat product treated by electrical stunning.

EXPERIMENTAL

Material and Instruments

Six broiler chickens (age 21 days) weight of ~1 Kg obtained from slaughterhouse in Bekasi District, West Java. Other reagents consisting of sodium dodecyl sulfate (SDS) 10%; Lowry Lowry solution I and II, Gel Acrylamide solution (30% T; 2,67C) Bio-Rad; Bis-Acrylamide (Sigma), Resolving Buffer (1.5M Tris-HCl pH 8.8 Bio-Rad); Stacking Buffer (0.5M Tris-HCl pH 6.8 Bio-Rad); Ammonium peroxide disulfate (APS) 10%; N, N, N'N'-Tetramethylethylene-diamine (TEMED); Sigma Tris HCl pH 8.0; Running buffer (Sigma); Staining solution coomasie blue R-250 (Bio-Rad); Blenders, griding mill, knife for chopping and crushing meat. homogenizer (Tokebi), Eppendorf tubes and pipettes, High centrifuge Sorvall SC35, Microsentrifuge Sorvall and sonicator Branson 2210 for the isolation of proteins. Spectrophotometer UV/Vis Lambda 25 Perkin Elmer and MiniProtean III Cell electrophoresis (Bio-Rad) for the separation and characterization of proteins. Molecular weight analysis and protein profiles performed by using Images J 1:46 free software.

Experimental Animals

Six broiler chickens age of 21 days (± 1 Kg) prepared through the different of pre-slaughter process. Two chickens (1-2) were slaughtered by the conventional method (non electrical stunning; 0 A and 0 Volt) by using a sharp knife and according to the islamic shar'i (negative control). While another chickens (3-4) slaughtered by electrical stunning with electrical current of 1 A, 25 Volt for 5 seconds and the two last sample (5-6) were slaughtered by electrical stunning with a current of 1 A, 125 Volts for 30 seconds. The current and voltage used monitored by amperometer. Each treatment was repeated two replications for each sample. Amenities electrical stunner designed with power supply with current and voltage can be set manually. Positive electrode (copper plate)

immersed in an aquarium filled with water while 25x25x25 cm^3 negative terminal attached to the body of the chicken (Samah, et al. 2011). After the treatments, each chickens slaughtered by using a sharp knife and left a few minutes until it is completely dead and plucked and cleaned of offal and blood are still sticking with warm water (45 ^oC).

Preparation of Samples

The slaughtered chickens meat separated tightly and chopped with a knife to a smaller size. Each of the samples from broiler muscle tissue were homogenized in ice using 10 mL of tris HCl buffer solution pH 8.0 by addition of 0.1% triton X-100. The solution was then centrifuged at 6000 × g for 30 min at 4 °C and the supernatant was collected and stored in 1 mL aliquots at -80 °C until further use (Samah, et al. 2011).

Quantification of Muscle Proteins

Determination of total muscle proteins was performed by Lowry method, by using 1 mL solution of A (20 mm and 30 mm $CuSO_4.5H_2O$ Na-citrate) and 50 mL of solution B (0.1 M Na_2CO_3 and 0.1 M NaOH). The reaction mixture was homogenized with a vortex and allowed to incubated for 10 minutes. Furthermore, a solution of D (reagent Folin Ciocalteu 1 N) 0.25 mL was added to the reaction mixture, homogenized by vortex and allowed to incubated for 30 minutes. The reaction mixture was measured at λ 750 nm and total protein concentrations were determined by a standard curve of bovine serum albumin (BSA) (Lowry, Rosebrough, Farr, & Randall, 1951).

SDS-PAGE Electrophoresis

SDS-PAGE was performed by standard methods (Laemmli, 1970), using a Mini-Protean II Slab Electrophoresis Cell (Bio Rad). Each proteins (muscle tissue extracts) denatured with sample buffer (Tris-Cl pH 6.8 to 150 mm, 6:25% SDS, β-mercaptoethanol, 25% glycerol, 2.5 mM Bromophenol blue) with a ratio of protein and buffer 2:1, and boiled for 5min and centrifuged for 15 minutes. Gel polyacrylamide electrophoresis prepared from acrylamide and bis-acrylamide solution (30% T, 2,67C), stacking buffer (0.5 M Tris-HCl pH 6.8), resolving buffer (1.5 M Tris-HCl pH 8.8), 10% SDS, APS and TEMED as catalyst. After the separating gel (the bottom gel) is formed, stacking gel is put on the top and created the mold for placing the protein samples. Gel formulations for separating gel was 12% while for stacking gel was 4%. Electrophoresis of samples was performed on the voltage 150 Volts for 60 minutes following marker proteins (Bio-Rad) as a standard. Coomasie brilliant blue 0.1% (w/v) used for protein staining solution and staining gels were washed in a mixture of methanol: acetic acid solution (40%: 7.5%). Proteins form staining gel then photographed with a digital camera for further analysis (Hermanto & Meutia, 2009).

Determination of Protein profile by Densitography

To performed the protein profile information from the molecular weight of each proteins were analyzed by using the software ImageJ 1.46. The separation of each proteins were analyzed by the migration distance or the value of Rf (retention factor). Rf analysis results from ImageJ compared to each protein band with a protein marker of known molecular weights through the linear regression equation and the molecular weight determined of each protein done by its Rf value. The next steps, protein profiles of each sample was analyzed by comparing the intensity peaks generated from each of sample treatments (Samah, et al., 2011).

RESULTS AND DISCUSSION

Protein Level Expressed in Muscle Tissue

Proteins extract from the muscle tissue isolated by mechanical lysis using

a homogenizer and combination with sonication for ±10 minutes. The protein extract concentrations of muscle tissue were analyzed quantitatively by Lowry methods (Lowry, et al., 1951).

Based on the protein level obtained from each of different type of samples (**Table 1**), it was found that slaughter method with electrical stunning 25 Volt generating the highest protein expression with level of protein 0.67 mg/mL. This is possibly due to the slaughtering procedure with electrical stunning allegedly able to increase the expression of certain proteins in the cell as a result of stress or treatment given electric stimulation, causing their particular activity metabolism in the cell. In addition, the extraction process is also greatly influenced by composition of the buffer, the type of extraction and the sensitivity of the cell membrane.

To obtain a high protein content of cell destruction process should be done in conditions of free metal ions and salts interferences. It should be done to avoid the effect of precipitation due to denaturation of proteins by salt molecules. Wahniyathi and Ali (2005), said that the destruction of chicken meat aims to break the cell membrane of muscle fibers so that muscle tissue proteins can be extracted with a buffer solution. Friction with smoothing apparatus may result in inhibition of protein extraction, causing coagulation of proteins. For the process of cell lysis is preferably carried out in low temperature conditions (Wahniyathi and Ali, 2005). In the process of proteins extraction from muscle tissue, it should be carried out at a temperature of 4-10 ^{o}C on ice bath.

Proteins Expression Profile from SDS-PAGE

The separation of the proteins profile of broiler chicken meat muscle tissue expression with electrical stunning and non electrical stunning (controls) were obtained from SDS-PAGE (Laemmli, 1970). **Figure 1** show the protein profiles from chicken muscle tissue at three different conditions.

Tabel 1. Protein level of broiler muscle tissue

No	Treatments	Proteins level (mg/mL)
1.	Slaughtering by non electrical stunning(control)	0.35 ± 0.02
2.	Slaughtering by electrical stunning (25 Volt, 5 seconds,)	0.67 ± 0.23
3.	Slaughtering by electrical stunning (75 Volt, 30 seconds)	0.58 ± 0.07

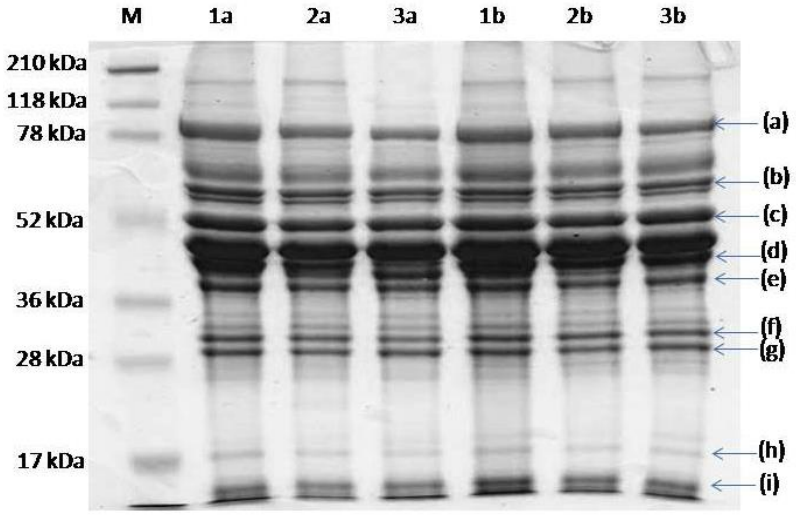
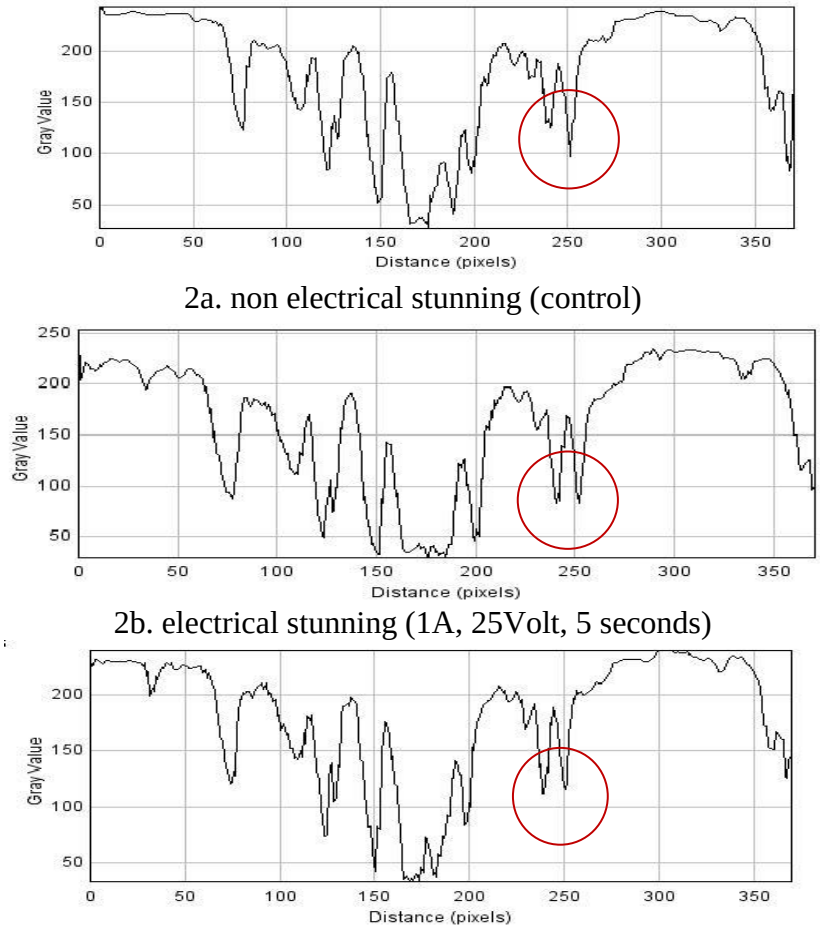


Figure 1. SDS-PAGE profile of broiler chicken proteins muscle tissue. Description: M = Marker, 1a & 1b = electrical stunning 1 A, 25 Volt (5 seconds) 2a & 2b = electrical stunning 1 A, 125 Volt (30 seconds) 3a & 3b = non electrical stunning (control). (Treatment slaughtering is done in duplicate).

Based on **Figure 1**, shows that the protein profile of muscle tissue of chicken meat protein overall produce bands with relative intensity vary in the range of 8-140 kDa. Each protein extract both in the treatment of electrical stunning and non electrical stunning showed 9 protein bands appear with a different relative intensity at molecular weight approximately 80 kDa (a), 62 kDa (b), 52 kDa (c), 45-48 kDa (d-e) and below 36 kDa (2 bands/f-g) and one band in the 17 kDa (h) and 8.5 kDa (i). In the protein extracts intensity of chicken meat slaughtered by non electrical stunning quite similar to the slaughtered by electrical stunning but with a different intensity, especially on protein bands emerged in the range 36 kDa to 52 kDa and below 36 kDa. Some of the specific proteins that appear in this range thought to be a specific protein markers that is expressed in the muscle tissue of chicken meat. The protein most likely is albumin, pyruvate kinase, beta-enolase and creatine kinase (Zaman, et al., 2012). This protein has also been reported as a dominant part of the expression level of chicken skeletal muscle. The pattern of protein expression and intensity were found before by Zaman et al. (2012), have similarities when compared with the resolution of the bands between the two gels but with different relative expression level.

The protein profile obtained from SDS-PAGE converted by image converter software and then analyzed by using the ImageJ 1:46 software (http://imagej.nih.gov/ij). The results of converting image can be seen in **Figure 2**.



2a. non electrical stunning (control)

2b. electrical stunning (1A, 25Volt, 5 seconds)

2c. electrical stunning (1A, 125Volt, 30 seconds)

Figure 2. The difference of proteins intensity pattern of chicken muscle from electrical stunning compared to non electrical stunning

The three pictures in **Figure 2** shows that intensity of each different protein bands relative to one another. The intensity difference is thought to result from the expression level of each protein which were different in each treatment. It was found that 2 protein bands in the range of 28-36 kDa were expressed with different intensities, where the electrical stunning treatment produces higher protein band intensity than the control. Electrical stunning with a high current allegedly trigger releasing of protein into the metabolic system as a result of the stress response given from the outside, such as over-expression of the protein IGF-1 (insulin growth factor) in the regeneration of muscle cells due to injury or strenuous exercise (Brighton, Wang, & Clark, 2008).

Samah, et al. (2011), explain the relationship between the electrical stunning with the expression of certain proteins in the muscle tissue of chicken meat. Based on his results, chickens treated with electrical stunning 0.75 A, 70 Volt, produces a protein spots were expressed in relatively large quantities and is not found in chickens untreated (control). Furthermore, the X protein expression is closely associated with the process stunning where the level of protein expression in line with effects of electrical stunning treatment. The results of protein X spot analyzed by MALDI-TOF MS showed that the protein X unidentified as Voltage Dependent Anion Chanel 2 (VDAC2) with a molecular weight of 30.293 kDa and reinforced the results with the confirmation of the level of transcription of genes VDAC2 through approach to Real Time PCR (Samah, et al. 2011). From those research, protein VDAC2 suggested as a candidate of biomarker to identified the differences in chicken treated with electrical stunning.

According to Samah, et al. (2011), VDAC2 expression levels are affected by iron deficiency in K562 cells (Human erythromyeloblastoid leukemia) obtained from two-dimensional electrophoresis followed by Western blot analysis. Lack of iron is known to stimulate hypoxia and hypoxia can induce membrane lipid peroxidation. Other research results show that the membrane lipid peroxidation is a result of the activation of VDAC-mediated transport. VDAC protein is also very useful in regulating the functions of enzymes involved in the control of redox reactions, as in the reaction of the enzyme superoxide dismutase containing copper and zinc (Cheng, Sheiko, Fisher, Craigen, & Korsmeyer, 2003; Budzinska, Galganska, Wojtkwska, Stobienia, & Kmita, 2007; Stark, 2005). With these results, we expect the candidate biomarker proteins can be analyzed further after the purification and separation of proteins, so it will be contribute especially in the development of halal food product analysis.

CONCLUSION

Based on these results, we can conclude that the difference in treatment of electrical stunning of broilers chicken pre-slaughtering process could trigger the expression level of certain proteins that ultimately result in differences in protein profiles generated. The results showed that two protein bands in the range of 28-36 kDa were higher expressed in the electrical stunning treatment in comparison with non-electrical stunning. These specific proteins are expected to be used as candidate of biomarkers for the detection of halal food products especially in broilers meat were slaughtered by overvoltage electrical stunning.

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