Protein Markers Related to Non-halal Slaughtering Process of Rat as Mammal Animal's Model Detected Using Mass Spectrometry Proteome Analysis

Alvina Nur Aini[1], Claude Mona Airin[2], and Tri Joko Raharjo[1,3*]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia*

[2]*Department of Physiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Jl. Fauna No. 2 Karangmalang, Yogyakarta 55281, Indonesia*

[3]*Institute of Halal Industry and System (IHIS), Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*

* ***Corresponding author:***

email: trijr_mipa@ugm.ac.id

Received: March 18, 2022
Accepted: May 24, 2022

DOI: 10.22146/ijc.73656

Abstract: *Meat produced from non-halal slaughter is forbidden for Moslems. The slaughter methods contribute to the physiological response of animals expressed as different proteome profile. Proteome of two meat obtained from the halal and non-halal slaughter of Wistar rats (*Rattus norvegicus*) as an animal model was used to search for protein markers related to the slaughter method. The analysis employed Sodium Dodecyl Sulhate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and High-Resolution Mass Spectrometer (HRMS) assisted with Label-Free Quantification (LFQ) Proteome Discoverer software. The non-halal slaughter contributed to the changes in protein expression in animal meat where thirteen proteins were up-regulated and three proteins were specifically identified in the non-halal slaughter, these three proteins are NSFL1 cofactor p47, transketolase, and Von Willebrand. There are three stable peptides identified of those three proteins including SYQDPSNAQFLESIR (m/z = 1755, z = +1) part of NSFL1 cofactor p47, LGQSDPAPLQHQVDVYQK (m/z = 2023, z = +1) part of transketolase, and VPLLCTNGSVVHHEVINAMQCR (m/z = 2550, z = +1) part of Von Willebrand. Two of peptides can be targeted as markers in MRM mode LC-MS/MS routine analysis to authentication-halal slaughtering meat. The proposed MRM pair ions are 1755 to 1376, 1262, 1164, and 963, 2033 to 1355, 1016, and 762.*

Keywords: *non-halal slaughter; stress response; biomarker; mass spectrometry; label-free proteomics*

■ INTRODUCTION

The large Muslim population and the increasing need for meat lead the determining of halal properties of meat is very important. To date, halal analysis that has been studied is related to the counterfeiting of non-halal products. Adulteration of meat and meat products with non-halal components, such as pork meat, has been extensively studied, and several methods have been developed to detect such adulteration [1-3]. Furthermore, the halal status of the meat is not only determined based on the animal used but also on the producing process of the meat, including the slaughter process. To become halal meat, the meat must be obtained from the animal that is slaughtered according to the halal method. Therefore, the development of methods to determine whether the meat is halal or non-halal based on the slaughtering methods have become crucial.

In general, slaughter of an animal is performed by using stunning techniques before cutting the jugular vein and carotid artery on the neck near the head. Otherwise, halal slaughter is performed by severing the animal neck using a sharp knife and acting in the name of God. Islam does not allow any deliberate torture to injure the animal before or after slaughter. Halal slaughter requires gentle handling of the animal before

and during the slaughter process. Therefore, halal slaughter should omit the stunning process. Good handling of animals during pre-slaughter and slaughter contributes significantly to the good meat quality as a product. Harsh treatment in handling animals before slaughter causes stress and produces low meat quality [4]. An effective slaughter will cause 40–60% of blood volume lost from animal bodies [5]. One of the factors that affect meat quality, contamination, and deterioration is the amount of blood retained in the animal body [6]. Blood is an excellent medium for the growth of bacteria. Blood components, especially hemoglobin, promote the lipid oxidation and decrease the shelf-life of meat and fish products [7]. Long transit time before slaughter also influenced the stress response and protein expression of animals [8]. According to Huang et al., the difference in treatment in the slaughtering process caused a stress response related to the expression level of certain genes of the animal [9].

In line with the halal authentication efforts, several studies have been conducted to compare the meat characteristics of meat due to the different pre-slaughter methods, mainly between the stunning and the non-stunning methods [10-12]. The difference in meat quality of lamb meat was found between those methods [13]. The stunning treatment could affect the stress response of animals [14], through changing gene expressions leading to different proteome patterns of the meat compared to the untreated one [9]. A study using 2D-electrophoresis-MALDI TOF showed different proteins of chicken meat resulting from the electrical current pre-treatment slaughter compared to the non-stunning chicken meat protein [15]. The protein of Voltage-Dependent Anion Channel 2 (VDAC2) was identified as a protein marker of the chicken meat treated using electrical stunning before slaughter. Further study reported other biomarker proteins related to the same treatment, which were troponin I and actin, both known as alpha cardiac muscle [16]. The same technique successfully identified a different pattern of the proteome of sheep meat due to the electrical stunning before slaughter [17]. In the comparison of chicken treated with gas stunning against non-stunning, a proteome analysis using 2D electrophoresis-MALDI TOF revealed an increased expression of β-enolase, pyruvate kinase, and creatine kinase from the chicken meat treated by gas stunning [18].

Most of the reported techniques to distinguish the meat resulted from stunning and non-stunning slaughter used the laborious 2-D electrophoresis coupled with MALDI-TOF mass spectrometry. More sophisticated liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) instruments has been successfully used in proteomic studies due to their simplicity [19]. This method is interesting to use to replace the 2D-electrophoresis MALDI-TOF system in the identification of protein markers in meat related to the slaughter process. On the other hand, most studies on searching protein markers to differentiate meat resulting from halal and non-halal slaughter used poultry as the sample, which was not representative of all meat-producing animals. Mammals, such as cattle, could have different metabolism systems to the poultry leading to different protein markers. This study aimed to identify proteins marker of meat resulting from non-halal slaughter using LC-HRMS, with a rat (*Rattus norvegicus*) used as a mammal model. The protein markers are concluded not only to the protein that was specifically expressed related to the non-halal slaughter but also to that which was up-regulated due to the process.

■ EXPERIMENTAL SECTION

Materials

The male, three months old Wistar rat (*Rattus norvegicus*) with an average weight of 200 g was used as the subject of the research. The sequencing grade modified trypsin (Promega) together with ammonium bicarbonate (Merck, Germany), dithiothreitol (Promega, USA), and iodoacetamide (Merck, Germany) were used to digest the protein. Sodium dodecyl sulfonate (SDS) (Merck), acrylamide, bisacrylamide, and ammonium persulfate (APS) (Sigma, USA), Precision Plus Protein Dual Color protein (BioRad, USA), were used during the SDS-PAGE profiling. The LC-HRMS analysis used MS-grade acetonitrile (Merck, Germany) and double distilled water as mobile phase.

Instrumentation

The instrumentations used in this study were the Ultimate 3000 Rapid Separation Ultra-High-Performance Liquid Chromatography connected to the Q-Exactive Orbitrap Mass Spectrometer (LC-HRMS) (Thermo Scientific, USA) and SDS- electrophoresis PAGE Mini Protean® II Cell (Bio-Rad), shaker incubator (Thermo Scientific, USA), refrigerated centrifuge (High-Speed Sigma Sartorius 3-30K, Sigma, USA) electric scale (Denver AA-250), vortex (MX-S IKA), and shaker mixer (RATEK Instrument). The softwares used in this study included Xcalibur and Proteome Discoverer 2.2 (Thermo Scientific, USA), ImageJ 1.52a, and STRING v11 [20-21].

Procedure

Animal slaughter

The protocol was approved by the Ethical Committee of Integrated Research and Testing Laboratory of Universitas Gadjah Mada, Yogyakarta (Ethical Clearance No. 00058/04/LPPT/2018 dated 6th June 2018). Six rats (*Rattus norvegicus*) were divided into two treatment groups. The first group of 3 was slaughtered according to the halal slaughtering guideline, which basically, the animal should not be dead prior to slaughtering. The slaughtering was performed by cutting the jugular vein, artery carotid, trachea, and esophagus using a sharp knife. While the second group of 3 was slaughtered through the cervical dislocation without anesthesia, as the non-halal method. After being slaughtered, meat was pooled and chilled at -80 °C until the extraction of protein.

Protein extraction

Protein was extracted from 0.5 g of meat from each rat using 2.5 mL of 0.05 M Tris HCl buffer (pH 7–8). The meat was homogenized with the buffer at room temperature using a vortex. The suspension was then centrifuged using a refrigerated centrifuge at 10,000 rpm for 20 min. The protein concentration of the supernatant was estimated using Lowry's protocol. The protein supernatant was stored at -80 °C until the digestion.

SDS-PAGE profiling

Protein supernatant was mixed with loading buffer and heated at 95 °C for 5 min. As much as 25 μg protein for each sample was loaded into the gel. Protein was separated on 10% separating gel at a constant voltage of 125 V, 70 mA for 90 min. After the electrophoresis, the gel was stained using 10% of acetic acid, 45% of methanol, and 0.3% of Coomassie blue (v/v), followed by destaining using 10% of acetic acid and 20% of methanol. The interpretation of each protein band was carried out using ImageJ 1.52a [20].

Protein tryptic digestion

Two hundred μL of protein supernatant was transferred into a microcentrifuge tube and precipitated with 1,000 μL of ice-cold acetone at -20 °C for 24 h. The protein pellet was separated through centrifugation at 10,000 rpm for 20 min. The protein pellet was then dissolved in 0.05 M of ammonium bicarbonate. The protein solution was reduced and alkylated with 2 mM of dithiothreitol and 5 mM of iodoacetamide, respectively. As much as 2 μg of sequencing grade modified trypsin was added to the sample, and the enzymatic reaction lasted at 37 °C for 24 h. The digestion was stopped by adding 100% of formic acid to pH < 3. The digested protein was diluted with ultrapure distilled water: acetonitrile (95:5) and filtered through a nylon syringe filter before LC-HRMS analysis [22].

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis

Peptides obtained from the trypsin digestion, were separated using LC-HRMS equipped with a C18 trap column. As much as 5 μL of protein hydrolysate was eluted from the trap column and separated using Acclaim PepMap RSLC C18 column (75 μm × 150 mm, Thermo Fisher Scientific). A gradient system was adapted to separate the peptides. Mobile phase A consisted of water, and mobile phase B consisted of water containing 0.1% of formic acid and acetonitrile containing 0.08% of formic acid, with a ratio of 1:4. The initial gradient was 96% A and 4% B from 0 to 3 min. The portion of solvent B was increased linearly to reach 95% at 50 min and maintained at the same ratio until the end of analysis at 90 min. The flow rate was fixed at 0.3 μL/min. The Q Exactive Orbitrap Mass Spectrometer equipped with nano spray ionization was employed to detect the peptides operated under positive ion mode.

The MS parameters were set to analyze the m/z range of 200–2000, using full-MS/dd-MS2 mode. The resolving power of MS1 and MS2 were set at 140,000 and 17,500 (FWHM), respectively.

Protein identification and data analysis

The software Proteome Discoverer (PD) 2.2 was used to identify the protein based on the Uniprot database of *Rattus norvegicus*. The label-free quantification (LFQ) quantification workflow was performed through MS/MS signal intensity of identified protein. Calculation of the statistical significance was carried out using Student's t-test at level of p-value < 0.05. The differentially changed proteins were identified by performing a *Quan-ratio distribution* analysis between non-halal and halal slaughter identified proteins. For protein pathways, identification over the Kyoto Encyclopedia of Genes and Genomes (KEGG) was selected [23].

■ RESULTS AND DISCUSSION

Protein SDS-PAGE Profile

Protein profiling using the SDS-PAGE technique was carried out as the initial checking whether any clear differences in protein expression were caused by the different slaughter methods. Since the protein was isolated from meat, it was assumed that most of the protein belonged to the muscle proteins. According to Fig. 1, most of the protein observed in both group samples have sizes ranging from 17 to 115 kDa. Doherty et al., and the protein database The Uniprot Consortium have published the number of muscle proteins and their size [24-25]. The thick band with the size of approximately 17 kDa was identified most probably as myoglobulin, while the tiny band above 100 kDa was calculated to have 116.2 kDa size and presumably identified as 2-oxoglutarate dehydrogenase. Further identification using ImageJ 1.52a produced some protein bands with the size of approximately 55, 49, 46, 41, and 26 kDa. There is plenty of possible proteins within those ranges of sizes. However, some possible proteins involved in the metabolism process of animal cells are enolase, creatine kinase, pyruvate kinase, phosphorylase, and myoglobin which are the main sarcoplasmic proteins. Meanwhile, the possible proteins contained in the skeletal muscle are sarcoplasmic, myofibrillar, myosin binding, actin, and stromal proteins [26]. Some other possible proteins are dihydrolipoyl dehydrogenase, myosin-binding, calreticulin, and aspartate aminotransferase [24-25].

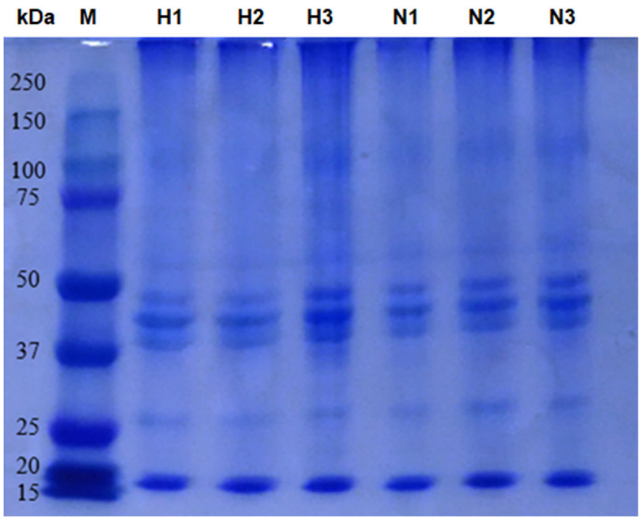

Fig 1. SDS-PAGE profile of the rat's muscle proteins for halal and non-halal slaughter. Each sample gave seven protein bands. M: protein marker; H1: halal method 1; H2: halal method 2; H3: halal method 3; N1: non-halal method 1; N2: non-halal method 2; N3: non-halal method 3

Comparison of the SDS-PAGE profile between proteins originating from halal and non-halal slaughter was proven to be difficult. The band with the size of 26 kDa shows a slight thicker for the non-halal slaughter protein. The lack of SDS-PAGE imaging comparison, such as an image analyzer for 2-D gel, hindered further investigation. At least, the SDS-PAGE data confirmed the success of protein isolation. Not only the main muscle proteins were successfully extracted, but also other proteins can be observed and interested to be further analyzed using mass spectrometry.

Up-Regulated Protein Abundance Due to Non-halal Slaughtering

A total of 253 proteins were identified from the LC-HRMS analysis from all samples, in which the abundance of several proteins was significantly different either in non-halal or halal slaughter. A *Quan-ratio* distribution was performed to determine the abundance ratio of the two slaughter groups. This comparison was

obtained through a ratio of non-halal/halal. Furthermore, the abundance of 13 proteins of non-halal slaughter was higher than the halal slaughter, as given in Table 1.

Most proteins (21.69% of the whole proteins) were associated with the metabolic process, while 29.97% of proteins acted as the protein binding. According to the KEGG database, some of these proteins were involved in the process of glucose metabolism in cells, such as β-enolase, dihydrolipoyl dehydrogenase, phosphoglucomutase 1, and malate dehydrogenase that present in glycolysis and gluconeogenesis process. Aspartate aminotransferase acted in the amino acid metabolism. Myc box-dependent-interacting protein, myosin-binding for fast-type C protein, tropomyosin alpha-1 chain, troponin I, and filamin were the proteins related to the structure and activity of muscle tissue. Calreticulin drove the calcium regulation in cells, while the superoxide dismutase and stress-70 protein used in the oxidative stress and stress response.

According to Astuti et al., the stress in the animal can lead to an increase in glycogen breakdown in muscle. Furthermore, glycogen would be converted into glucose to produce ATP as energy-saving [27-28]. Salwani et al. reported that stress could be in the form of high energy requirements [18]. The reduction of oxygen supply to the body during exsanguination increased the rate of glycolysis to produce ATP. This would cause some enzymes associated with the glycolysis process to be highly expressed. In non-halal slaughter, the stress response was thought to be greater because animals experienced greater physiological discomfort. In this study, cervical dislocation caused brain and spinal injury before death. The process of cervical dislocation provided two traumatic effects, not only the hematoma, brain damage, spinal fractures, and spinal cord disorders, but also severe lacerations (rips injuries) in the medulla oblongata [29].

In this study, some of the proteins that were significantly changed included glycolysis proteins, such as β-enolase, dihydrolipoyl dehydrogenase, phosphoglucomutase 1, and malate dehydrogenase. Referring to Nakyinsige et al., aspartate aminotransferase was also found to be higher in the non-halal group [30]. This indicated that the stress response of non-halal slaughter increased the glucose and energy metabolism of an animal so that several proteins associated with the glycolysis were higher expressed. The high energy required for animal muscle activity was obtained from the metabolism of glucose (from glycogen) in the muscle [31]. It was also supposed that some proteins related to the muscle tissue activity increased, such as myc box-dependent-interacting 1, myosin-binding protein C fast-type, tropomyosin alpha-1 chain, troponin I, and filamin-C. Calcium regulation in

Table 1. Proteins abundance and the ratio of non-halal to halal slaughter

Protein	Abundance (a.u.)		Ratio (non-halal/halal)	*p*-value
	Non-halal	Halal		
Mitochondrial aspartate aminotransferase (Got2)	163.6	36.4	4.495	0.00023
Beta-enolase (Eno3)	118.9	81.1	1.467	0.03162
Calreticulin (Calr)	116.6	83.4	1.398	0.03450
Dihydrolipoyl dehydrogenase (Dld)	155.9	44.1	3.535	0.02769
Filamin-C (Flnc)	162.1	37.9	4.277	0.00127
Phosphoglucomutase 1 (Pgm1)	123.4	34.4	1.611	0.00015
Mitochondrial malate dehydrogenase (Mdh2)	165.6	76.6	4.814	0.01434
Myc box-dependent-interacting 1 (Bin1)	133.9	66.1	2.026	0.04250
Myosin binding protein C, fast-type (Mybpc2)	149.1	50.9	4.865	0.00294
Stress-70 protein (Hspa9)	174.3	25.7	6.782	0.03666
Superoxide dismutase [Cu-Zn] (Sod1)	152.5	47.5	3.210	0.01466
Tropomyosin alpha-1 chain (Tpm1)	122.5	77.5	1.580	0.00154
Troponin I (Tnni2)	188.8	11.2	16.86	0.01736

the body was useful to maintain homeostasis. Loss of energy causes the loss of cellular homeostasis and damages Ca^{2+} signaling, which directed the endoplasmic reticulum stress response activity [32]. This is supposed to increase the expression of calreticulin protein. Furthermore, the proteins that were up-regulated in the non-halal slaughter could be proposed as the candidates for markers for non-halal animal slaughtering.

Specifically Expressed Proteins Caused by Non-halal Slaughtering

Although the up-regulated protein related to the non-halal slaughter has been identified, it cannot easily be used as a marker since it must employ the comparison study of each analysis. A protein marker specifically expressed only in non-halal slaughter was the best candidate for the marker. There were three proteins, NSFL1 cofactor p47 (Nsfl1c), transketolase (Tkt), and Von Willebrand factor (Vwf), consistently present only in the non-halal samples. Transketolase proteins were involved in the regulatory process, cell organization, biogenesis, and metabolism. Meanwhile, the Von Willebrand factor was a protein involved in the response to stimulus. This protein worked in the blood coagulation process caused by wounds or injuries on the blood vessels [33]. The process of cervical dislocation could be thought to stimulate the excretion of the Von Willebrand factor due to the occurrence of injuries in an animal body. The process of cervical dislocation-initiated hematoma, whereas the halal slaughter process created a large incision on the neck's blood vessels that caused the blood to quickly run out of the body [29]. Therefore, the blood clotting process might not occur. In contrast to cervical dislocation, wounds arise on the blood vessels of an animal body so that the blood clotting could occur as a response to stopping internal bleeding. Therefore, these proteins were proposed to be the potential candidates for markers for non-halal animal slaughtering.

Interactions of the up-regulated and specifically expressed proteins were analyzed using STRING v11. Fig. 2. shows the protein-protein interaction from 16 proteins. The network indicates 16 nodes and 55 edges with an average node degree of 6.88 and an average local clustering

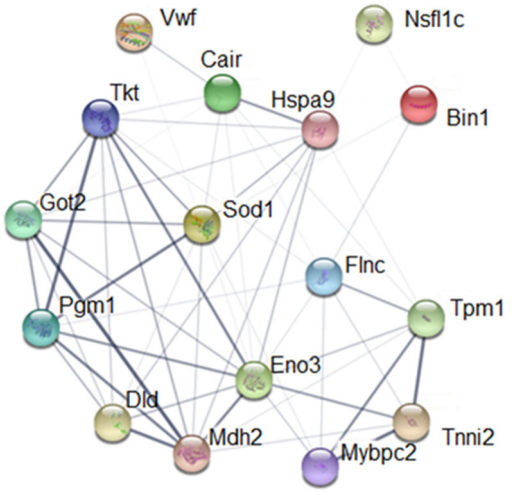

Fig 2. Protein-protein interaction from the up-regulated and specifically expressed protein in non-halal slaughter. The thickness of the line represents the confidence of the protein interaction

coefficient of 0.79. Proteins can interact and associate physically or functionally. From this result, it might be concluded that the identified proteins were related to each other. One protein expression would stimulate the expression of other proteins. Further studies were required to better understand the relationship between proteins and the slaughter process.

Peptide Target for Protein Marker Analysis of Non-halal Slaughtering

The common method to detect protein markers, in this case, the specific proteins present in non-halal slaughtering meat samples, is by detecting the specific peptide of the protein. Proteome discoverer analysis of HRMS data revealed several peptides specific to the proteins NSFL1 cofactor p47, transketolase, and Von Willebrand factor. The sequence of the peptides was confirmed by analysis of MS/MS data using the Proteome discoverer. Fig. 3(a) shows the MS/MS spectra of the NSFL1 cofactor p47 peptide obtained from the fragmentation of a peptide with the molecular mass (MH^+) = 1754.83213. However, the most stable ion of the peptide was detected as an ion with m/z = 877.92064 (z = +2). Fig. 3(b) confirmed the match of the fragment mass and the difference mass between the fragments to the mass database to possibly construct the peptide sequence.

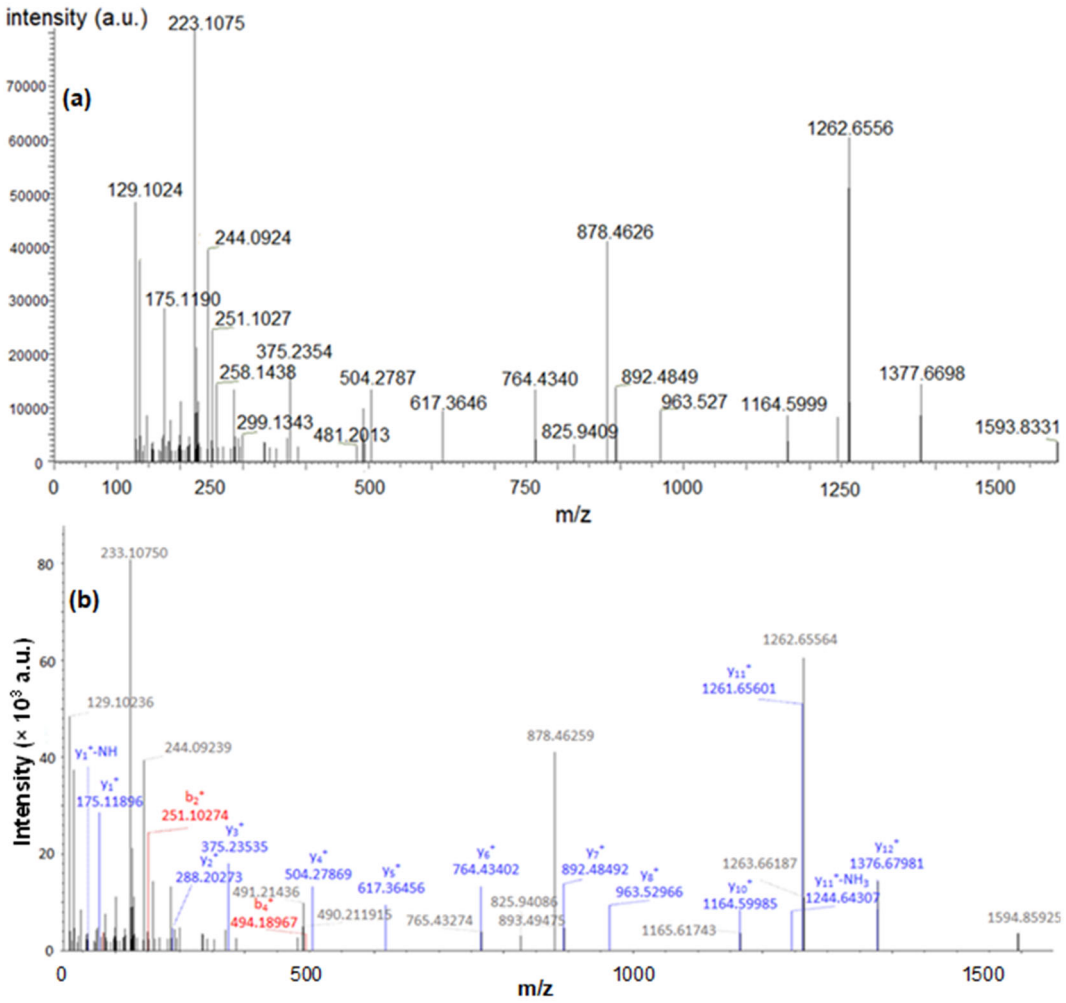

Fig 3. The Mass spectra of peptide-specific to NSFL1 cofactor p47 protein. (a) The raw MS/MS fragmentation of the peptide. (b) Proteome discoverer identification of the fragment b^+ and y^+ from raw MS/MS leading to a sequence of the peptide

The dominant ions were y^+ ions. To be mentioned, a few of the fragmentation analyses were the difference between the y_{12}^+ fragment (1376.67981) and the y_{11}^+ (1261.65601) was confirmed as amino acid D (115.03), the difference between y_{11}^+ with y_{10}^+ (1164.59985) was confirmed as P (97.05). By using the same calculation, DPSNAQFLESI was obtained. After comparing the sequence to the whole sequence of NSFL1 cofactor p47 protein and possible trypsin digestion product of the protein, it was concluded that the peptide sequence is SYQDPSNAQFLESIR.

Fig. 4(a) shows the MS/MS spectra of the transketolase protein-peptide as the result of peptide fragmentation with molecular mass (MH^+) of 2023.02262, which present as molecular ion with m/z of 675.01275 (z = +3). Interpretation of Fig. 4(b) using Proteome discoverer revealed several match ions with possible ion fragment peptides that were part of transketolase protein. Analysis of the difference among several detected y^+ fragments led to a sequence of the peptides. The difference between the y_8^+ ion (1016.52655) to the y_6^+ ion (751.39722) was equal to the mass of QH, then the difference of the y_6^+ to the y_5^+ ion (652.32953) led to V, followed by the mass difference of the y_5^+ ion to the y_4^+ ion (537.30408) was equal to the mass of D. Furthermore, the different of the y_4^+ ion to the y_3^+ ion (438.23602) could be read as V, while the difference between the y_3^+ ion to the y_2^+-NH_3 (258.14426) was equal to Y, and the y_2^+-NH_3 to the y_1^+ (147.11281) was equal to Q. The analysis of the y^+

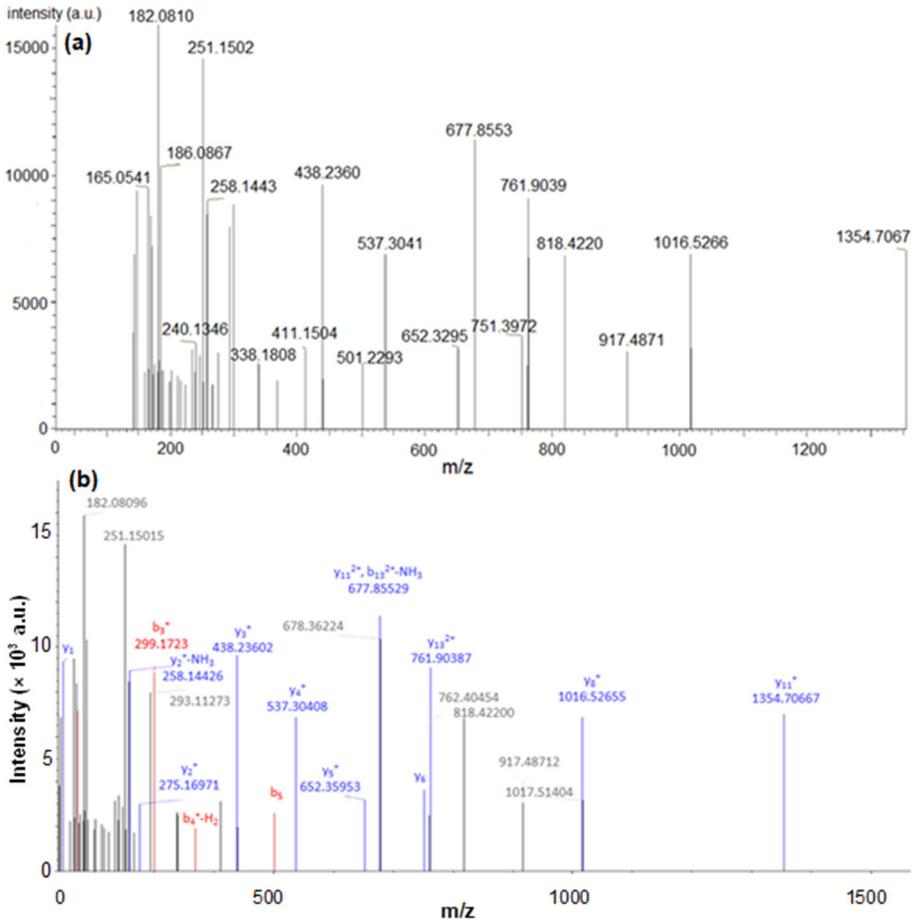

Fig 4. The Mass spectra of peptides specific to transketolase protein (a) The raw MS/MS fragmentation of the peptide (b) Proteome discoverer identification of the fragment b^+ and y^+ from raw MS/MS leading to a sequence of the peptide

ions fragment mass concluded the sequence QHQVDVYQK as part of the peptide sequence. The same approach to analyze the b^+ ions including b_5^+ (501.22931), b_4^+-H_2O (368.19409) b_3^+ (299.17123), and b_2^+ (171.11282) concluded the sequence QSD as part of the peptide. Based on this information and the protein database, the proteome discoverer confirmed the sequence of the peptide is LGQSDPAPLQHQVDVYQK.

The sequence of the specific peptide of Von Willebrand factor was revealed mainly based on the protein database and the high-resolution MS of the molecular ion of peptide, with MH^+ of 2550.23739 and m/z of 510.85397 (z = +5). Only a few of the fragmentation of MS2 was identified as the b_3^+ ion (195.11179) and the y_1^+ 175.11818. The proteome discoverer concluded the sequence as VPLLCTNGSVVHHEVINAMQCR.

These three peptides were suggested to be used as a target for routine analysis. The targeted analysis usually employed standard LC-MS/MS using an MRM (Multiple Reaction Monitoring) which was known as the best mode to perform both qualitative and quantitative analysis using LC-MS/MS. This approach required information regarding the mass of the selected peptide as the precursor peptide ion targets and the production of the MS2 fragmentation [34]. Based on the MS spectra in Fig. 3(b) and 4(b), several MRM pairs were proposed to identify each protein that was specifically expressed in the non-halal slaughter process. Those pairs of MRMs are presented in Table 2 selected based on certain criteria. According to You et al., the precursor ion must be the predominant specific peptide with a size of approximately 600–2000 [35]. Among the three protein-specific peptides, only the first two peptides met those criteria (peptide number one and two). Meanwhile, the

Table 2. The proposed MRM pairs to detect specifically expressed protein related to non-halal slaughter using LC-MS/MS

No	Specific peptides	MRM pair	
		Precursor ion mass	Fragment ion mass
1	SYQDPSNAQFLESIR	1755	1376 1262 1164 963
2	LGQSDPAPLQHQVDVYQK	2023	1355 1016 762

production ions should be the predominant single charged ions generated from the fragmentation, which were preferable for the ions with m/z higher than m/z of the precursor ion. The first peptide had m/z = 877.92064 (z = 2), meaning that the production higher than 877, which were 1376, 1262, 1164, and 963 will be the good options. The production of 892 was also shown predominant signal, but both were selected because they had an m/z value close to the precursor ion. The second peptide had m/z of 675.01275 (z = 3), making the production of 1355, 1016, and 762 met the criteria as MRM productions.

■ CONCLUSION

The study demonstrated that the different slaughter methods altered the proteome expression of animal meat. Thirteen proteins were identified to be up-regulated on non-halal slaughter. Proteome analysis also revealed that three proteins were specifically expressed on non-halal slaughter, which were NSFL1 cofactor p47, transketolase, and Von Willebrand factor, and proposed as the biomarker protein related to non-halal slaughter. The up-regulated and the specifically expressed proteins suggested that non-halal slaughter could contribute to stress response in the animal. The two stable peptides of SYQDPSNAQFLESIR (m/z = 1755, z = +1) part of NSFL cofactor p47, and LGQSDPAPLQHQVDVYQK (m/z = 2023, z = +1) part of transketolase can be targeted as markers in MRM mode LC-MS/MS routine analysis to distinguish non-halal slaughtering meat from halal slaughtering originated meat. The proposed MRM pair ions were 1755 to 1376, 1262, 1164, 963, and 2033 to 1355, 1016, 762.

■ ACKNOWLEDGMENTS

This research was financially supported by Institute Halal for Industry and System, Universitas Gadjah Mada. The author would like to thank the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada, Yogyakarta for providing the necessary facilities.

■ AUTHOR CONTRIBUTIONS

The first author conducted the experiment of protein extraction and protein digestion as well SDS-PAGE and some data analysis, the second author conducted the animal experiments including ethical clearance admission, the third author did the HRMS analysis. The third author conducted the data analysis, wrote and revised the manuscript together with the first author. All authors agreed to the final version of this manuscript.

■ REFERENCES

[1] Raharjo, T.J., Cahyaningtyas, W., Surajiman, S., Istini, I., and Pranowo, D., 2012, Validation of PCR-RFLP testing method to detect porcine contamination in chicken nugget, *Indones. J. Chem.*, 12 (3), 302–307.

[2] Rohman, A., Erwanto Y., Hossain, M.A.M., Rizou, M., Aldawoud, T.M.S., and Galanakis, C.M., 2021, "The Application of DNAZ-based methods for authentication analysis: Examples in halal and kosher food products" in *Food Authentication and Traceability*, Eds. Galanakis, C.M., Academic Press, Cambridge, US, 195–213.

[3] Nalazek-Rudnicka, K., Kłosowska-Chomiczewska, I., Wasik, A., and Macierzanka, A., 2019, MRM–MS of marker peptides and their abundance as a tool for authentication of meat species and meat cuts in single-cut meat products, *Food Chem*., 283, 367–374.

[4] Grandin, T., 2001," Antemortem handling and welfare" in *Meat Science and Applications*, Eds. Hui, Y.H., Nip, W.K., Rogers, R.W., and Young, O.A., Marcel Dekker, New York, 221–254.

[5] Bakhsh, A., Ismail, I., Hwang, Y.H., Lee, J.G., and Joo, S.T., 2018, Comparison of blood loss and meat quality characteristics in Korean black goat subjected to head-only electrical stunning or without stunning, *Korean J. Food Sci. Anim. Resour*., 38 (6), 1286–1293.

[6] Ali, S.A.M., Abdalla, H.O., and Mahgoub, I.M., 2011, Effect of slaughtering method on the keeping quality of broiler chicken meat, *Egypt Poult. Sci*., 31 (4), 727–736.

[7] Maqsood, S., and Benjakul, S., 2011, Effect of bleeding on lipid oxidation and quality changes of Asian seabass (*Lates calcarifer*) muscle during iced storage, *Food Chem*., 124 (2), 459–467.

[8] Mouzo, D., Rodríguez-Vázquez, R., Lorenzo, J.M., Franco, D., Zapata, C., and López-Pedrouso, M., 2020, Proteomic application in predicting food quality relating to animal welfare. A review, *Trends Food Sci. Technol.*, 99, 520–530.

[9] Huang, J.C., Huang, M., Yang, J., Wang, P., Xu, X.L., and Zhou, G.H., 2014, The effects of electrical stunning methods on broiler meat quality: Effect on stress, glycolysis, water distribution, and myofibrillar ultra-structures, *Poult. Sci*., 93 (8), 2087–2095.

[10] Addeen, A., Benjakul, S., Wattanachant, S., and Maqsood, S., 2014, Effect of Islamic slaughtering on chemical compositions and post-mortem quality changes of broiler chicken meat, *Int. Food Res. J*., 21 (3), 897–907.

[11] Nielsen, S.S., Alvarez, J., Bicout, D.J., Calistri, P., Depner, K., Drewe J.A., Garin-Bastuji, B., Rojas, J.L.G., Schmidt, C.G., Herskin, M., Michel, V., Chueca, M.A.M., Roberts, H.C., Sihvonen, C.G., Spoolder, H., Stahl, K., Velarde, A., Viltrop, A., Candani, D., Van der Stede, Y., and Winckler, C., 2020, Welfare of cattle during killing for purposes other than slaughter, *EFSA J*., 18 (11), e06312.

[12] Żurek, J., Rudy, M., Kachel, M. and Rudy, S., 2021, Conventional versus ritual slaughter–ethical aspects and meat quality, *Processes*, 9 (8), 1381.

[13] Velarde, A., Gispert, M., Diestre, A., and Manteca, X., 2013, Effect of electrical stunning on meat and carcass quality in lambs, *Meat Sci*., 63 (1), 35–38.

[14] Yardimci, M., 2019, Comparison of the stunning and non-stunning slaughtering methods in the light of the current knowledge, *J. Vet. Anim. Res*., 2 (3), 1–5.

[15] Samah, N.A., Amid, A., and Yusof, F., 2011, Overexpression of voltage-dependent anion channel 2 (VDAC2) in muscles of electrically stunned chickens, *IIUM Eng. J*., 12 (4), 213–222.

[16] Amid, A., Samah, N.A., and Yusof, F., 2012, Identification of troponin I and actin, alpha cardiac muscle 1 as potential biomarkers for hearts of electrically stimulated chickens, *Proteome Sci*., 10 (1), 1.

[17] Kiran, M., Naveena, B.M., Smrutirekha, M., Baswa Reddy, P., Banerjee, R., Praveen, K.Y., Venkatesh, C., and Rapole, S., 2019, Traditional halal slaughter without stunning versus slaughter with electrical stunning of sheep (*Ovis aries*), *Meat Sci*., 148, 127–136.

[18] Salwani, M.S., Adeyemi, K.D., Sarah, S.A., Vejayan, J., Zulkifli, I., and Sazili, A.Q., 2015, Skeletal muscle proteome and meat quality of broiler chickens subjected to gas stunning prior slaughter or slaughtered without stunning, *CyTA-J. Food*, 14 (3), 375–381.

[19] Yates, J.R., 2013, The revolution and evolution of shotgun proteomics for large-scale proteome analysis, *J. Am. Chem. Soc*., 135 (5), 1629–1640.

[20] Aini, A.N., 2019, Identifikasi Protein Penanda Spesifik Metode Penyembelihan Hewan Menggunakan Spektrometri Massa Resolusi Tinggi, *Thesis*, Universitas Gadjah Mada, Indonesia.

[21] Szklarczyk, D., Gable A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N.T., Morris, J.H., Bork, P., Jensen, L.J.,

and von Mering, C., 2019, STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets, *Nucleic Acids Res.*, 47 (D1), D607–613.

[22] Orduna, A.R., Husby, E., Yang, C.T., Ghosh, D., and Beaudry, F., 2017, Detection of meat species adulteration using high-resolution mass spectrometry and a proteogenomics strategy, *Food Addit. Contam., Part A*, 34 (7), 1110–1120.

[23] Kanehisa, M., and Goto, S., 2000, KEGG: Kyoto encyclopedia of genes and genomes, *Nucleic Acids Res.*, 28 (1), 27–30.

[24] Doherty, M.K., McLean, L., Hayter, J.R., Pratt, J.M., Duncan, H.L., Robertson, D.H., El-Shafei, A., Gaskell, S.J., and Beynon, R.J., 2004, The proteome of chicken skeletal muscle: Changes in soluble protein expression during growth in a layer strain, *Proteomics*, 4 (7), 2082–2093.

[25] The UniProt Consortium, 2019, UniProt: A worldwide hub of protein knowledge, *Nucleic Acid Res.*, 47 (D1), D506–515.

[26] Feng, Y.H., Zhang, S.S., Sun, B.Z., Xie, P., Wen, K.X., and Xu, C.C., 2020, Changes in physical meat traits, protein solubility, and the microstructure of different beef muscles during post-mortem aging, *Foods*, 9 (6), 806.

[27] Astuti, P., Putro, C.P.C., Airin, C.M., Sjahfirdi, L., Widiyanto, S., and Maheshwari, H., 2014, Comparison of process slaughtered on beef cattle based on level of cortisol and Fourier Transform Infrared Spectroscopy (FTIR), *Int. J. Anim. Vet. Sci.*, 8 (12), 876–879.

[28] Adeva-Andany, M.M., González-Lucán, M., Donapetry-García, C., Fernández-Fernández, C., and Ameneiros-Rodríguez, E., 2016, Glycogen metabolism in humans, *BBA Clin.*, 5, 85–100.

[29] Bader, S., Meyer-Kühling, B., Günther, R., Breithaupt, A., Rautenschlein, S., and Gruber, A.D., 2014, Anatomical and histological pathology induced by cervical dislocation following blunt head trauma for on-farm euthanasia of poultry, *J. Appl. Poult. Res.*, 23 (3), 546–556.

[30] Nakyinsige, K., Fatimah, A., Aghwan, Z.A., Zulkifli, I., Goh, Y.M., and Sazili, A.Q., 2014, Bleeding efficiency and meat oxidative stability and microbiological quality of New Zealand white rabbits subjected to halal slaughter without stunning and gas stun killing, *Asian-Australas. J. Anim. Sci.*, 27 (3), 406–413.

[31] Abdela, N., Jilo, K., Siraj, S., Adem, J., and Mohammed, A., 2016, Impact of stress on health and productivity of animal: A review, *J. Nat. Sci. Res.*, 6 (9), 45–51.

[32] Krebs, J., Agellon, L.B., and Michalak, M., 2015, $Ca^{(2+)}$ homeostasis and endoplasmic reticulum (ER) stress: An integrated view of calcium signaling, *Biochem. Biophys. Res. Commun.*, 460 (1), 114–121.

[33] Zeng, J., Shu, Z., Liang, Q., Zhang, J., Wu, W., Wang, X., and Zhou, A., 2022, Structural basis of von Willebrand factor multimerization and tubular storage, *Blood*, 139 (22), 3314–3324.

[34] Rauh, M., 2012, LC-MS/MS for protein and peptide quantification in clinical chemistry, *J. Chromatogr. B*, 883-884, 58–67.

[35] You, J., Willcox, M.D., Madigan, M.C., Wasinger, V., Schiller, B., Walsh, B.J., Graham, P.H., Kearsley, J.H., and Li, Y., 2013, Tear fluid protein biomarkers, *Adv. Clin. Chem.*, 62, 151–196.