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COMPARING THE EFFECT OF HEAT ON TROPOMYOSIN ISOFORMS PATTERNS FROM WATER BUFFALO AND WILD BOAR MEAT BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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ABSTRACT

Tropomyosin is one of the most abundant proteins in meat; however, very little is known about it due to the lack of scientific literature. In this study, the spot volume of tropomyosin (TPM) isoforms, TPM2 and TPM1, in meat from water buffalo and wild boar subjected to various cook treatments were compared. We hypothesized that primary structures of the tropomyosin isoforms from both species would remain stable despite the application of heat. Proteins extracted from the treated meats were analyzed using two-dimensional gel electrophoresis and mass spectrometry. A Kruskal-Wallis test showed that there were no significant differences in protein spot volumes for all treatments; however, a significant difference was observed between species. Changes in the amino acid sequence of TPM1 were observed between the two species, indicating that the isoforms could be used as thermostable proteins or peptide markers for species identification because of their resistance to high temperatures.

Key words: Meat, tropomyosin isoforms, spot volume, proteomic approaches

INTRODUCTION

In Southeast Asia, wild boar is consumed by indigenous communities and is considered to be one of the main food source (Sales & Kotrba, 2013). However, in recent years, the meat from wild boar, which is popular as game meat, has become highly favored among mainstream consumers due to its nutritional benefits and desirable flavor. This meat is considered to be of immense nutritional value because of its low fat and cholesterol content. Its consumption is believed to be beneficial for human health, and thus, there has been a subsequent increase in its demand (La Neve *et al.*, 2008). Mainstream consumers have also begun to favor water buffalo meat, as it is claimed to be one of the healthier red meats, which is low in both calories and cholesterol (Abdolghafour & Saghir, 2014). Since meat is a product of special economic interest and skeletal muscles form the foundation of meat, we believe that there is a need to focus on this muscle using scientific studies as it is difficult to discriminate both red meat based on their appearances.

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Skeletal muscle, which is rich in blood, is the main constituent of meat. The primary function of skeletal muscle is to transport oxygen and nutrients across the body system via the arteries and through the contraction of muscles (Pearson & Young, 1989). Contraction of muscles occurs when tropomyosin, which is a part of the thin filaments work together with the thick filaments of the muscle. Tropomyosin is soluble in concentrated salt solutions and accounts for almost 7% of the total protein within the myofibrils of the muscle. It consists of two chains which are known as the α and β chains, having molecular weights of 34 and 36 kDa, respectively (Pearson & Young, 1989). After an animal is slaughtered, the oxygen supply to its muscles diminishes and adenosine triphosphate (ATP) levels drop, causing the formation of rigid actomyosin as a result of the interaction between the thin and thick filaments of the muscle tissue. These processes lead to protein denaturation (Warriss, 2000).

Cooking at high temperatures also causes protein denaturation by rupturing the weak intramolecular forces that hold the proteins together (Yu *et al.*, 2017). Myofibrillar proteins lose their native structure at 50–65°C (Davis & Williams, 1998). However, many studies have reported that myofibrillar proteins retain their structure even after being subjected to several heat treatments (Claydon *et al*., 2015; Montowska & Pospiech, 2013; Vujadinovic *et al*., 2014). These proteins could, thus, be used as thermostable makers for meat speciation. A previous study conducted by Sentandreu *et al.* (2010) reported that the myosin light chain 3 (MLC3) was consistently present in poultry meat after oven cooking it at 180°C for 1 hr. This finding was supported by Montowska and Pospiech (2012) who reported that MLC isoforms from several animals were retained after the meat was finely ground and smoked or cooked. In addition to MLC, actin (Sarah *et al.,* 2013), tropomyosin (Sarah *et al.,* 2014) and troponin (Von Bargen *et al.,* 2014) have also been studied as potential thermostable markers.

It has been previously shown that proteomic approaches are preferable for meat authentication, compared to DNA-based analyses, due to the stability of primary amino acid sequences (Sentandreu *et al*., 2010) and because there is a lower risk of cross-contamination during analysis (Soares *et al*., 2013). Other commonly used proteomic methods include enzyme-linked immunosorbent assay (ELISA), and electrophoretic or chromatographic techniques. Although analysis using ELISA ensures high specificity, it is also associated with an increased risk of cross-reactivity (Chen & Hsieh, 2015). Furthermore, target proteins may denature during food processing and subsequently, the target protein epitope, which is the binding domain for antibodies, may be destroyed (Asensio *et al*., 2008). Alternatively, an electrophoretic approach, such as two-dimensional gel electrophoresis (2DE), followed by mass spectrometry, can provide a significant amount of information about a protein (Montowska & Pospiech, 2012, 2013; Sarah *et al*., 2013). These approaches provide useful quantitative and qualitative results. Currently, to the best of our knowledge, studies on the present of tropomyosin isoforms following heat treatment in the meat of water buffalo and wild boar are limited. Many studies focused on species such as cow, pig and chicken (Kim *et al*., 2017; Sarah *et al*., 2016; Zvereva *et al*., 2015). This study will prove our hypothesis that the primary structure of tropomyosin isoforms from both species is maintained following the application of heat. Additionally, this study also compares the spot volumes of tropomyosin isoforms present in water buffalo and wild boar meat samples subjected to different cook treatments, using two-dimensional gel electrophoresis and mass spectrometry approaches.

MATERIALS AND METHODS

Samples

Meat from water buffalo (*Bubalus bubalis*) and wild boar (*Sus scrofa vittatus*) were purchased from local markets in Peninsular Malaysia. Three biological replicates were analyzed for each species. Muscle samples were located anatomically. Any visible fat was removed from the meat before storing it at –80°C until subsequent analysis.

Heat treatment of the meat samples

The meat samples were subjected to the following rounds of cook treatments: 1) chilling at 4°C for 30 min (control), 2) roasting at 150°C for 20 min, and 3) frying at 160°C for 6 min. The samples were minced using a meat mincer (Philips, UK). A total of 40 gm of each minced meat sample was used to form patties. The patties were roasted for 20 min in a preheated oven (Memmert, Germany) at 150°C, and then fried in cooking oil heated to 160°C using induction heating (ELBA, Malaysia). After cooking, all meat samples were cooled. The samples were then crushed into a powder, using liquid nitrogen to avoid protein degradation. A total of 1 gm of each meat sample subjected to each treatment was stored at –20°C until subsequent analysis.

Meat protein extraction

A total of 1 gm of sample was homogenized in 10 mL extraction buffer 1 (50 mM Tris-HCl, pH 8) and vortexed (Eppendorf Mixmate, Germany) for 2 min. Next, the homogenate was centrifuged (Eppendorf Centrifuge 5810R, Germany) at 10,000 g for 10 min at 4°C. The supernatant was collected, and the pellet was resuspended in 10 mL extraction buffer 2 (50 mM Tris-HCl (pH 8) supplemented with 6 M urea and 1 M thiourea) and homogenized in a vortex for 3 min for solubilization. The homogenate was centrifuged at 10,000 g for 10 min and the resulting supernatant was collected for further analysis.

Two-dimensional gel electrophoresis using immobilized pH gradient-sodium dodecyl polyacrylamide gel (IPG-SDS/PAGE)

For 2DE analysis, the second supernatant, consisting of myofibrillar extracts, was selected. About 100 µL of the sample was precipitated using 400 µL acetone. The mixture was vortexed and stored overnight at –20°C. The mixture was centrifuged at 12,000 g for 10 min and air dried to form a pellet for collection. The pellet was solubilized in 100 µL urea extraction buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.4% (v/v) bio-lyte (pH 3–10) ampholyte, and 50 mM DTT). Based on the results of the Bradford protein assay, a total of 1300 µg sample was mixed with the rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.4% (v/v) bio-lyte (pH 3–10) ampholyte, and 50 mM DTT) and loaded into a focusing tray of up to 125 µL. Rehydrated IPG ReadyStrips (Bio-Rad, USA) (pH 3–10) were positioned in the focusing tray and covered with mineral oil. Twelve hours of passive rehydration were followed by isoelectric focusing (IEF) using the Protean IEF cell system (Bio-Rad, USA).

IEF was carried out at 250V for 20 min at linear ramp. Next, the voltage was increased, also at linear ramp, to 4,000V for 2 hr. The voltage was maintained at 4,000V for 10,000Vh at rapid ramp before maintaining at 500V (for maximum 24 hr) at rapid ramp. Oil was drained from the strips by placing a plastic backing on a wet filter paper and pressing firmly along the length of the gel. The strips were flushed with deionized water before being incubated with the reducing buffer for 10 min. Next, they were incubated with an alkylation buffer for 10 min. The strips were agitated gently throughout incubation. IPG strips were dipped into the running buffer prior to SDS-PAGE. They were placed on a 12% polyacrylamide gel and sealed with 1% agarose. The second-dimension separation was performed using SDS-PAGE by applying a constant voltage of 120V until the dye reached the bottom of the gel. Protein spots were stained overnight and subsequently destained.

Image analysis

The gel was visualized using a densitometer (Bio-Rad, USA) in digital format and analyzed using the Quantity One software (Bio-Rad, USA) and PDQuest® 2DE image analysis software (Bio-Rad, USA). The images obtained for each spot were analyzed and compared within and between the image groups. The suggested matches were automatically defined by automated image analysis. The spot volumes of tropomyosin isoforms from every treatment for each species were analyzed. Spot volume refers to the total intensity of a defined gel spot (Zhan & Desiderio, 2003). The spots were identified in Raw 2D image by using spot boundary tools.

In-gel digestion

A blade was used to cut out the bands of interest from the preparative gel, as described previously (Dahlan *et al*., 2012). Gel bands were washed repeatedly with 200 µL of 50 mM ammonium bicarbonate/50% acetonitrile (ACN) (1:1 (v/v)) for 15 min at room temperature until they were completely destained. The washed gel bands were incubated in 200 µL of 10 mM DDT/100 mM ammonium bicarbonate for 30 min at 60°C before alkylating in 200 µL of 55 mM IAA/100 mM ammonium bicarbonate for 20 min at room temperature in the dark. The gel bands were washed twice with 50% (v/v) ACN/100 mM ammonium bicarbonate. They were incubated in 100% ACN for 15 min and dried in a vacuum centrifuge (SpeedVac, Thermo Scientific, Savant DNA 120). Tryptic digestion was performed by soaking the dried gel bands in 25 µL of 6 ng/µL trypsin digestion buffer overnight at 37°C under slow shaking conditions. Tryptic peptides were subsequently extracted by incubating with 50% ACN for 15 min followed by incubating with 100% ACN for 15 min. The extracted solutions were placed in separate tubes before being dried in a vacuum concentrator and solubilized with 200 µL of 10% ACN/40 mM ammonium bicarbonate.

Tropomyosin isoforms identification by mass spectrometry

Peptides extracted from the 2DE spots were first desalted using ZipTip C18 (Millipore, USA), according to the manufacturer's instructions. Equal parts of the peptide samples and α-cyano-4-hydroxycinnamic acid (10 mg/mL) were mixed. Next, 0.7 mL of this mixture was immediately spotted on an Opti-TOF 384 well insert (Applied Biosystems/MDS Sciex, Toronto, Canada) and analyzed with the 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems/MDS Sciex) using the mass standard kit (Applied Biosystems/MDS Sciex). This kit served as a calibrator for the mass

spectrometry (MS) and MS/MS mass spectra results (Seriramalu *et al.*, 2010).

Databank search and sequence analysis

Contamination peaks for keratin and auto-proteolysis peaks for trypsin were removed prior to sequence analysis. The MASCOT (Matrix Science, UK) program was used to match MS and MS/MS data against database information using the following parameters: mammalian taxonomy, mass tolerance of 100 ppm, fixed modification of carbamidomethyl, variable modification of oxidation of methionine, one missed cleavage, +1 peptide charges, and monoisotopic mass. The data obtained were screened against the mammalian database downloaded from the UniProtKB/Swiss-Prot homepage. Pairwise sequence alignment using EMBL-EBI was performed to compare the isoforms sequences from different species. The score of pairwise sequence alignment was calculated to determine the number of similarities.

Statistical analysis

The spot volumes were further analyzed using the XLSTAT software (XLSTAT Base, 2017). The aim was to compare the spot volumes of tropomyosin isoforms if any significant changes occurred either by species or heat treatments. The calculated *p*-value was observed to be less than 0.05 according to the Anderson–Darling test, suggesting that the data distribution was not normal. The non-parametric Kruskal–Wallis test was used to measure the mean volume of spots. A confidence level of 95% was used while evaluating the significant difference of tropomyosin isoforms for different species and heat treatments.

RESULTS AND DISCUSSION

Differences between isoforms analyzed using 2DE

In general, meat is consumed after cooking not only because of its improved palatability but also due to the resulting reduction in the presence of potentially harmful bacteria. In this study, we subjected the meat samples to different heat treatments, using dry-heat cookings which were frying and oven roasting. Dry-heat cooking produces cooked meat with unique properties in terms of appearance and flavor (Kerry, 2011). Oven cooking is a preferred method because it produces a uniform and consistent meat texture and makes the meat tender (Davis & Williams, 1998). Changes were observed in the meat after cooking in terms of protein denaturation, water-binding capacity, and physical appearance. Protein denaturation reduces the solubility of proteins (Cruz-Romero & Kerry, 2011). These changes continue as the temperature increases. Different proteins become insoluble at different temperatures according to their location. These changes are also dependent on the duration and temperature of cooking (Breidenstein *et al*., 1994).

Tropomyosin has been reported to be one of the most heat stable proteins in several studies (Huang & Ochiai, 2005; Montowska *et al.*, 2014; Sarah *et al*., 2014; Williams & Swenson, 1981). This protein consists of several isoforms derived from four TPM genes (Kim *et al*., 2017; Oe *et al*., 2007). It is a dimeric and coiled coil protein, which interacts with troponin and actin, contributing to skeletal muscle contraction and stabilization of the thin filament (Perry, 2001). In this study, TPM2 and TPM1 were consistently present in high abundance following heat treatments. TPM2 was similar to TPM1 in its amino acid composition but differed slightly in its cysteine content. TPM2 consisted of two cysteine residue, while TPM1 contained one cysteine residues. However, there is no marked difference in the biological properties of these isoforms (Perry, 2001).

In this study, in 2DE, the TPM2 and TPM1 spots for each species were clearly separated from each other (Figure 1). TPM2 and TPM1 were located at positions corresponding to a molecular weight of approximately 37 kDa, with a pI of 4.3 and 4.5, respectively. TPM2 was located slightly higher than TPM1 on the gel, showing that TPM2 was heavier than TPM1. Interestingly, the spots intensified and were constantly present after each treatment. A previous study conducted by Sarah *et al.* (2014) showed that TPM1 from goats was stable in 2DE even after being exposed to high temperatures. This was supported by another study by Kim *et al.* (2017) who reported that TPM2 and TPM1 were strongly present in beef, pork, duck, and poultry. This study also showed that TPM2 was weakly present in poultry meat using gel electrophoresis. Thus, TPM2 has the potential of being used as a marker for discriminating between poultry and other meats.

The Kruskal–Wallis test was used to compare the spot volumes for TPM2 and TPM1. The spot volumes showed no significant difference for proteins subjected to different treatments when the *p*-value was greater than 0.05. This result was similar to that reported by a previous study (Sarah *et al.*, 2014). However, there were significant differences in spot volumes for the chilled meats of water buffalo and wild boar (Figure 2). Thus, we concluded that although heat treatment did not affect the protein, but rather gave significant values to differentiate between water buffalo and wild boar meats. In order to identify the difference, the primary structure of these isoforms was investigated. The primary structures of some proteins present in the meats of different mammals have been

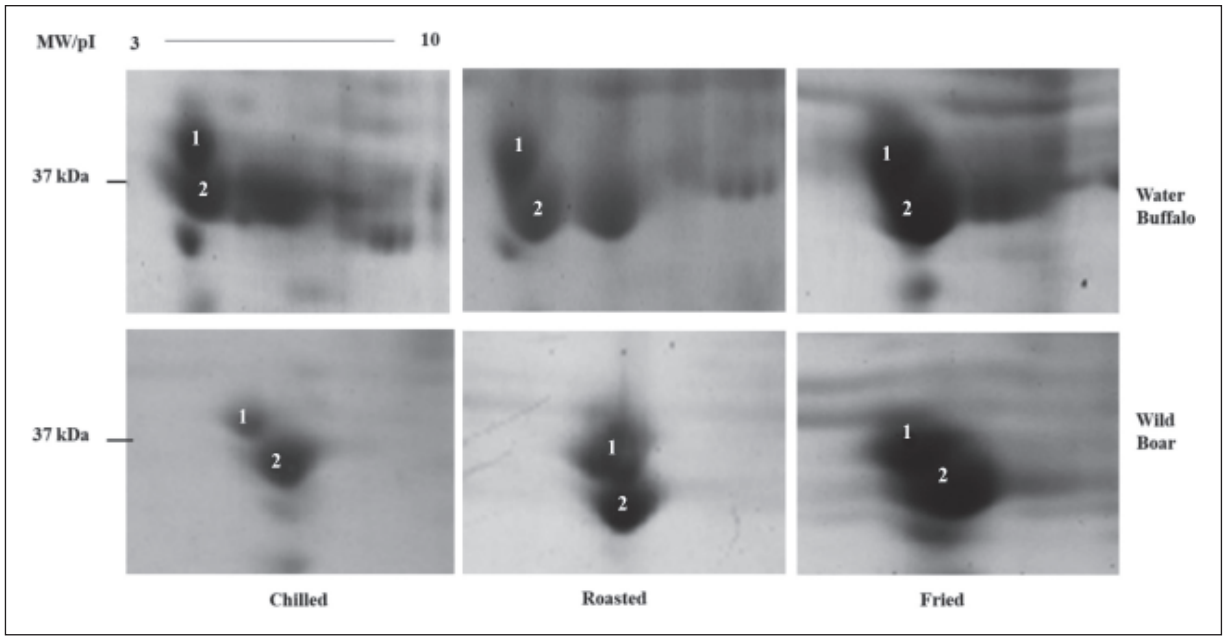

Fig. 1. Image of the gels showing results of the 2DE analysis for raw and cooked meats from water buffalo and wild boar: 1: TPM2; 2: TPM1.

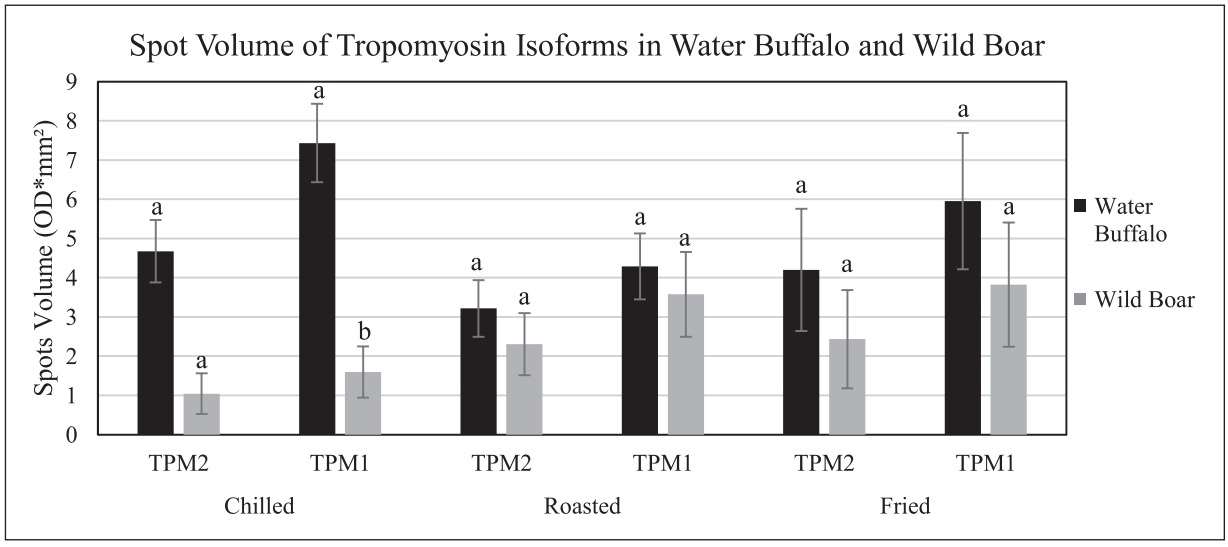

Fig. 2. Spot volumes for TPM2 and TPM1 from water buffalo and wild boar meat subjected to different heat treatments. The spot volumes were calculated using three replicates. Different alphabets represent a significant difference ($p < 0.05$) between spot volumes.

previously found to be relatively resistant to heat and tissue-specific in nature, which is useful for species identification (Montowska *et al.*, 2015). The spots from the 2DE analysis were further investigated using MALDI–TOF/TOF-MS to determine the amino acid sequences of proteins.

Differentiating between isoforms based on molecular properties

TPM2 (spot 1) and TPM1 (spot 2) sequences from the MALDI–TOF/TOF analysis were confirmed through a comparison using the MASCOT database. To compare the proteins, the sequences from cow (beef) and pig (pork) were selected from the database for comparison with those from the water buffalo and wild boar meats, respectively. These were selected because of the close relationship between the respective species since sequences for wild boar and water buffalo were not available. The MOWSE score for the majority of proteins was significant, except that for TPM2 derived from the chilled wild boar meat, which was less than 61, with the least sequence coverage of 17% (Table 1). The significant value of MOWSE score indicates the good quality

Table 1. Protein spots corresponding to TPM2 and TPM1 from water buffalo and wild boar identified using MALDI–TOF/TOF mass spectrometry

Spot no.	Species	Protein name	Treatment	Accession number[a]	Match peptide	Theoretical MW/pI	Experimental MW/pI[b]	MOWSE score[c]	Sequence coverage (%)
1	Water Buffalo	Tropomyosin beta chain	Chilled	TPM2_BOVIN	26	32931/4.66	36470/4.3	226	46
2	Water Buffalo	Tropomyosin alpha-1 chain	Chilled	TPM1_BOVIN	24	32732/4.69	33860/4.5	255	37
1	Water Buffalo	Tropomyosin beta chain	Roasted	TPM2_BOVIN	28	32931/4.66	36640/4.3	284	50
2	Water Buffalo	Tropomyosin alpha-1 chain	Roasted	TPM1_BOVIN	29	32732/4.69	34770/4.5	214	52
1	Water Buffalo	Tropomyosin beta chain	Fried	TPM2_BOVIN	26	32931/4.66	36630/4.3	160	46
2	Water Buffalo	Tropomyosin alpha-1 chain	Fried	TPM1_BOVIN	28	32732/4.69	34260/4.5	221	51
1	Wild Boar	Tropomyosin beta chain	Chilled	TPM2_BOVIN	12	32931/4.66	37700/4.3	49	17
2	Wild Boar	Tropomyosin alpha-1 chain	Chilled	TPM1_PIG	19	32732/4.69	35550/4.5	121	30
1	Wild Boar	Tropomyosin beta chain	Roasted	TPM2_BOVIN	23	32931/4.66	36710/4.3	206	40
2	Wild Boar	Tropomyosin alpha-1 chain	Roasted	TPM1_PIG	28	32732/4.69	34550/4.5	286	54
1	Wild Boar	Tropomyosin alpha-1 chain	Fried	TPM1_PIG	23	32732/4.69	36010/4.3	175	34
2	Wild Boar	Tropomyosin alpha-1 chain	Fried	TPM1_PIG	27	32732/4.69	35700/4.5	265	52

[a]Accession numbers derived from the UniProtKB database.
[b]Value of molecular weight and isoelectric point from the conducted experiments.
[c]Protein scores greater than 61 considered to be significant ($p < 0.05$).

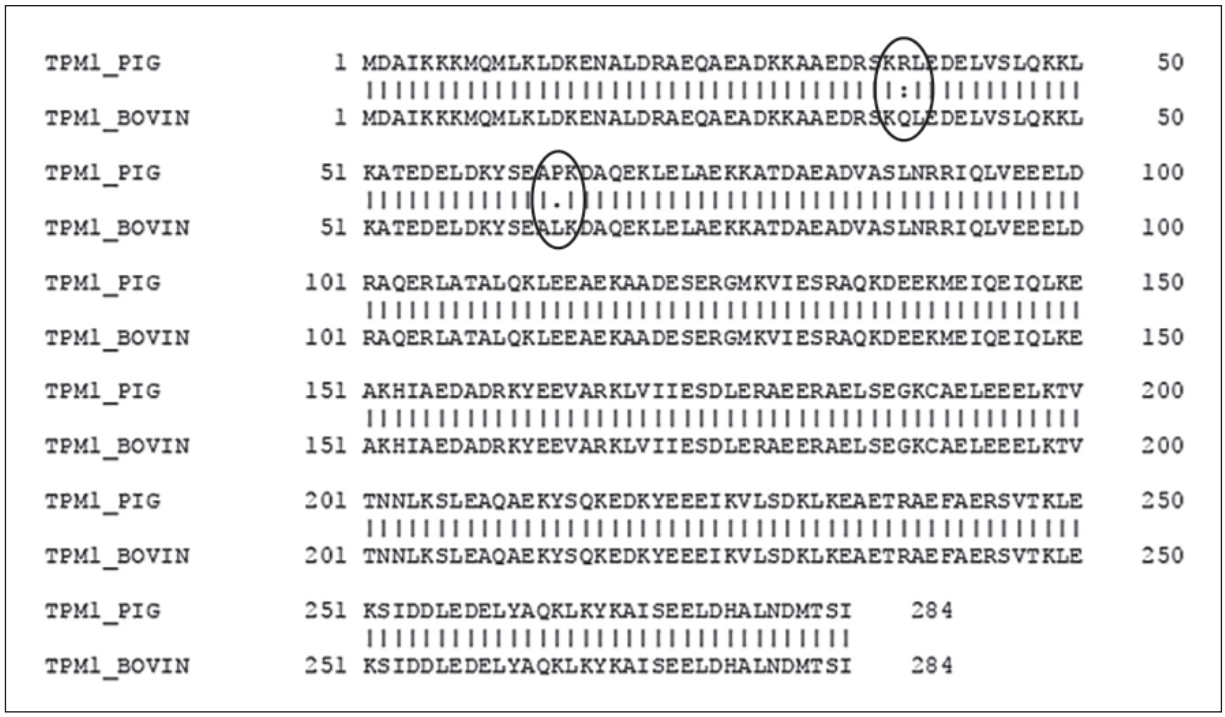

Fig. 3. Pairwise sequence alignment of TPM1 for pig and bovine (using cow database for bovine). The circle areas represent the amino acid differences between pig and bovine for TPM1 at position 38 and 64 along with the sequences.

of the data. Moreover, the high value of MOWSE score also determines a valid match of mass values with low probability. Thus, most of the spots present in this study were matched as TPM2 and TPM1.

The sequence coverage for TPM2 in water buffalo was derived from the roasted meat sample, which had the highest percentage at 50%, followed by the fried and chilled meats with 46% for each and in wild boar with 40, 34, and 17%, respectively. Similarly, the sequence coverage for TPM1 after each treatment was 52, 51, and 37% for water buffalo, and 54, 52, and 30% for wild boar respectively. Sequence coverage serves as an indicator for the completion of protein digestion. However, the lower sequence coverage of proteins from meats possibly due to the difficulty in extracting hydrophobic peptides from the gel. Moreover, sufficient trypsin may also fail to penetrate the gel matrix and cause low digestion efficiency of proteins (Rosemberg, 2006).

There was no significant difference was observed between spot volumes in TPM2. The spot volumes of TPM1 showed significant differences in the chilled meats of both species; however, the TPM1 primary structure remained intact. Further analysis of molecular aspects plays a significant role in discriminating between these species. Based on the pairwise sequence alignment results (Figure 3), the difference in amino acid sequences of TPM1 from cow and pig was low, with a score of 0.4%. The differences were present at positions 38 and 64 in the amino acid sequence. Thus, the present study showed that TPM2 and TPM1 were not affected by different heat treatments.

CONCLUSION

This study reported that tropomyosin isoforms, TPM2 and TPM1, from water buffalo and wild boar were consistently present following heat treatments, indicating the thermostability of both proteins. TPM1 can be potentially used as a protein marker for species identification using processed meat samples. The spot volumes from 2DE showed that there was no significant difference in samples from the two species following the application of heat; however, under chilled conditions, there was a significant difference between samples from these species, based on the volume of spots. Molecular analysis revealed that sequence coverage for every protein from each species was relatively high. The limited information present in databases regarding TPM2 and TPM1 proteins from both species studied restricted our analysis of the primary amino acid sequence of the tropomyosin isoforms. A general recommendation for future study is to identify thermostable peptide sequence by using tandem mass spectrometry. The unique peptide sequence between these species has the potential to be selected as thermostable peptide marker for species authentication.

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