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Differences in thermostable actin profile of goat meat as observed in two-dimensional gel electrophoresis (2DE)

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Abstract

Different heat treatments, (1) chilled, 4°C (2) boiled at 100°C for 30 min and (3) autoclaved at 121°C at 15 psi for 20 min were employed on goat meat to mimic domestic and industrial cooking. The effects on intensity of actin proteins was observed using two-dimensional gel electrophoresis where significant differences ($p>0.05$) were observed in the spot intensity between chilled and boiled samples, similarly in chilled and autoclaved samples. However, no significant difference was observed between boiled and autoclaved samples. The slight changes observed in the cooking of meat confirmed that actin protein is susceptible to denaturation cause by heat. MALDI-TOF/TOF analysis revealed the peptide-mass fingerprint between positions 21 – 374 that not affected by heat treatment. Peptides from this position merit the candidature of actin as putative thermostable marker for detecting goat meat (chevon) in food product.

Introduction

Generally, meat or meat products will be subjected to processing or cooking prior serving for its palatability and safety from microbial contaminant (Lawrie, 1992). Despite all the advantages of thermal processing, the severe condition produced by cooking and sterilisation temperature during the entire process can adversely affect the nutritional and sensory qualities of meat (Christensen *et al*., 2000; Gatellier *et al*., 2009; Horn and Voit, 2009; Oluwaniyi *et al*., 2010). Heating or cooking of meat and meat products will cause conformational changes or denaturation of muscle protein where it induces water loss in meat and denatured some protein component by breaking of hydrogen or electrostatic bonds (Tornberg, 2005).

When meat components were heated, proteins in the meat particularly actin lose its solubility and release from meat along with water. Levieux *et al*. (1995) and Xiong and Blanchard (1994) have demonstrated, by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), that myosin and actin are not presence in the supernatant when a suspension of myofibrils is heated to 65°C. Moreover, it was reported that actin denaturation is found between 80 and 83°C when investigated using differential scanning calorimetry (Contaxis *et al*., 1977; Wright *et al*., 1977). However, possibility that actin could remain in the muscle after the cooking process specifically at temperature beyond 65°C is still unknown.

We assumed that although actins are liberated from the actomyosin complex after being heated, there are still some actins left in the muscle. Thermal denaturation of actin has been numerously studied by using differential scanning calorimetry (Wright *et al*., 1977), ultraviolet difference spectroscopy and circular dichroism (Akihiro *et al*., 2009), but still, proteomic findings on the thermostable actin is limited particularly from chevon. Thus, we exploit the proteomics advances to see the influences of common processing, which are boiling and autoclaving, which have temperature beyond 80°C on actin proteins upon heating. The present study was conducted to screen and characterize the molecular weight and isoelectric point of actin using two-dimensional gel electrophoresis and mass spectrometry approaches.

Materials and Methods

Slaughtering and sampling procedures

A total of 10 male crossbred Boer goats of mean body weight from 35±2 kg were slaughtered at a research abattoir in the Department of Animal Science,

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Universiti Putra Malaysia. The animal handling and slaughtering were carried out following the guidelines of Research Policy of the Universiti Putra Malaysia on animal ethics and Malaysian Standard 1500:2009 on halal food production, preparation, handling and storage. Samples of longissimus muscle were specifically taken from between the 12th and 13th rib region and plunged into liquid nitrogen within 1 hr post-mortem. All samples were kept at -80°C until subsequent analysis.

Heat treatment of samples

One gram of pulverized frozen muscle tissue was placed individually in a heat stable container (capped glass tube) and allowed to thaw before being subjected to both heat treatments (100°C for 30 min and 121°C at 15 psi for 20 min). For boiling, the meat samples were heated by fully immersing the container in a boiling water bath (Memmert, Germany) for 30 min until internal temperature of 99±0.7°C was obtained. Meanwhile, the autoclave machine temperature was allowed to reduce until 90°C before taking out the samples. Samples were taken out and immediately chilled on ice before extraction to avoid any protein loss.

Extraction of proteins

Chilled and heat treated muscle tissue was homogenized (Wiggen Hauser D500, USA) in 3 mL of ice cold extraction buffer containing 7 M Urea, 2 M thiourea, 50 mM DTT (dithiothreitol), 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.4% (v/v) carrier ampholytes (pH 3-10, Bio-Rad, USA) and 50 µL of protease inhibitor cocktail (Catalogue No. 535140, Calbiochem, USA), followed by centrifugation at 12,000 g for 20 min at 4°C. The resulted supernatant was collected and subsequently subjected to clean-up procedure using ReadyPrep 2D Cleanup Kit as outlined in the manufacturer's protocol (Bio-Rad, Hercules, USA). Resulting supernatant was collected and stored at -80°C until subsequent two-dimensional electrophoresis. Total extractable protein concentration was determined using Bradford protein (Bradford, 1976).

Two-Dimensional Gel Electrophoresis (2DE)

100 µg of extracted proteins in rehydration buffer [7 M Urea, 2 M thiourea, 50 mM DTT, 4% (w/v) CHAPS and 0.4% (v/v) carrier ampholytes (pH 3-10, Bio-Rad)] was loaded onto a 7 cm, immobilized pH gradient strip, pH 3-10 (IPG, Bio-Rad, USA). The active rehydration process was performed at 50 V for 12 h using the Protean IEF cell system (Bio-Rad, USA). The isoelectric focusing was carried out at low voltage, where 250 V was applied initially followed by a stepwise increase increment to 8,000 V until it reach 15,500 Vh. The focused strips were then pre-equilibrated in reducing buffer for 15min [6 M Urea, 20% (v/v) glycerol, 0.375 M Tris-HCl pH 8.8, 2% sodium dodecyl sulfate (SDS) and 1% DTT] and followed by alkylating buffer for 15 min [6 M Urea, 20% (v/v) glycerol, 0.375 M Tris-HCl pH 8.8, 2% SDS and 1% iodoacetamide (IAA)]. The excess buffer was drained out before continuing with the second dimension electrophoresis. The IPG strips were placed on 15% polyacrylamide gel and sealed with 1% agarose. The second dimension separation was performed by initially applying a 10 mA/gel current for 15 min, followed by 15 mA/gel for the subsequent 90 min. Protein spots were stained with Coomassie Brilliant blue R-250 staining (Merck, Germany).

Image and statistical analysis of gels

The gels were visualized using QuantityOne® software (Bio-Rad, USA) and analysed using PDQuest® 2DE image analysis software (Bio-Rad, USA). Individual spots were analysed and compared within and between the image groups. The matches suggested by automated image analysis were individually inspected and confirmed. Following background subtraction, protein spots were automatically defined and the volume of each spot in a gel was normalized as a percentage of the total volume of all spots detected on the gel.

For statistical analysis, the spot volumes were imported into SAS software (SAS/STAT® Version 9.2, 2009). The mean of spot volumes was subjected to analysis of variance General Linear Models Procedure. The differences between means were analysed by Duncan's Multiple Range test. The aim was to check if the expression of the analysed actin protein differed significantly depending on the heat treatment. A protein spot was considered statistically significant across the treatment group when $p < 0.05$.

In-Gel tryptic digestion.

The protein spots of interest were cut out from the preparative gels using pipette tips and extracted from gels according to the published method (Dahlan *et al*., 2011). Briefly, gel pieces were washed repeatedly with 50 µL of 50 mM ammonium bicarbonate/50% acetonitrile (ACN), 1:1 (vol/vol) for 15 min at ambient temperature until completely destained. This was followed by incubation in 150 µL of 10 mM DTT/100 mM ammonium bicarbonate for 30 min at 60°C, and alkylation in 150 µL of 55 mM IAA/100 mM ammonium bicarbonate for 20 min at room

temperature (25°C) in the dark. The gel plugs were then washed twice with 50% (v/v) ACN/100 mM ammonium bicarbonate. They were then incubated in 100% ACN for 15min and dried in vacuum centrifuge (SpeedVac, Thermo Scientific, Savant DNA 120).

In-gel tryptic digestion was performed with 25 µL of 6 ng/ µL trypsin digestion buffer (Promega trypsin gold) overnight at 37°C. Tryptic peptides were then extracted using 50% acetonitrile for 15 min, followed by 100% acetonitrile for 15 min. The extracted solutions were then pooled into a single tube and dried in a vacuum concentrator and solubilized with 10µL of 10% acetonitrile/40 mM ammonium bicarbonate.

Protein identification with MALDI-TOF/TOF mass spectrometry

Extracted peptides were first desalted using ZipTip C18 (Millipore, USA) according to protocols described by the manufacturer. The final elution volume following ZipTip cleanup was 1.5 µL. The peptide samples were then mixed (1:1) with a matrix consisting of a saturated solution of CHCA (α-cyano-4-hydroxycinnamic acid, Sigma) prepared in 50% ACN/0.1% TFA. Aliquots of samples (0.7 µL) were spotted onto stainless-steel sample target plates. Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (ABI 4800 plus, Applied Biosystems) in the positive ion reflector mode. For precursor ion selection, all fractions were measured in single MS before MS/MS was performed. For MS/MS spectra, the peaks were calibrated by default. The 20 most abundant precursor ions per sample were selected for subsequent fragmentation by high-energy CID. The collision energy was set to 1 keV and air was used as the collision gas. The criterion for precursor selection was a minimum S/N of 5. The mass accuracy was within 50 ppm for the mass measurement and within 0.1 Da for CID experiments. The other parameters for searching were of trypsin, 1 missed cleavage, variable modification of carbamidomethyl and oxidation of methionine, peptide charge of 1+, and monoisotopic. For database searches, known contamination peaks such as keratin and autoproteolysis peaks for trypsin were removed before searching. Spectra were processed and analyzed by the Global Protein Server Explorer 3.6 software (Applied Biosystems). This uses an internal MASCOT (Matrix Science, UK) program for matching MS and MS/MS data against database information. The data obtained were screened against mammalian databases downloaded from the Swiss-Prot/TrEMBL homepage (*http://www.expasy.ch/sprot*).

Results and Discussion

The present study employed proteomics advances in determining thermal stability of actin. Application of SDS-PAGE in present study will result in a distinguish band that may contain two or more different protein in the same band. Hence, 2-dimensional electrophoresis was used to observe the effect of heat specifically onto the actin protein. Furthermore, in order to examine the effect of heat solely onto goat longissimus muscle without being influenced by early post mortem changes like pH, and aging time, all the skeletal muscle samples collected were immediately frozen in liquid nitrogen to avoid proteolytic degradation associated with it (Lawrie, 1998; Bendixen, 2005). In addition, meat samples were pulverized with liquid nitrogen to turn it into powder form as we aimed to observe the effect of heat thoroughly onto the meat. Common cooking method of meat cubes will result in different core temperature and might left some actin unaffected with heat (Warris, 2000).

Figure 1 shows the representative gel of actin spots after being heated. The spots consistently appeared at 42.7 kDa with pI 6.1 across chilled, boiled and autoclaved gels. Decreasing of spot intensity was consistently observed across 10 gels of each treatment. Spots intensity was used as an indicator to determine the level of denaturation of actin in the samples. The mean of spot intensity of chilled, boiled and autoclaved meat were 991.9, 549.2 and 484.8 respectively. ProcGLM analysis revealed that there is significance effect ($p<0.05$) of heat treatment towards actin denaturation which in good agreement with Mikhailva *et al*. (2006). Significant differences were observed in the spot intensity between chilled and boiled as well as chilled and autoclaved samples. However, no significant difference was observed between boiled and autoclaved samples. Although the result was not significant, the mean values shown

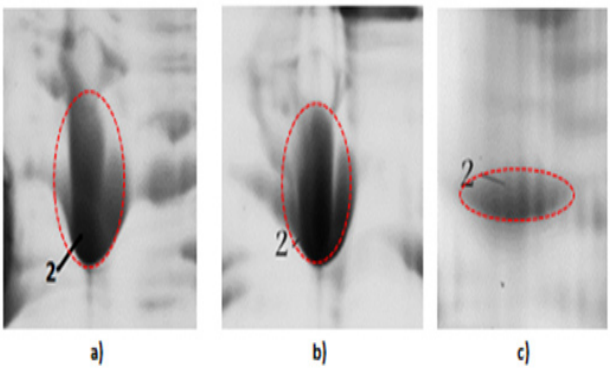

Figure 1. Image of actin spots from a) chilled, b) boiled and c) autoclaved samples of goat *Longissimus dorsi* muscle.

Table 1. Differences of mean of spot intensity between chilled, boiled and autoclaved samples of goat *Longissimus dorsi* muscle

Treatments (n=10)	Mean of spot intensity
Chilled (4°C)	991.9[a]
Boiled (99 ± 0.7°C)	549.2[b]
Autoclaved (121°C, 15 psi)	484.8[b]

[ab] means within a row with different superscripts are significantly different at p<0.05

Table 2. Amino acid sequences and molecular weights determined by MALDI-TOF/TOF mass spectrometry of trypsin digested peptides used to assemble the amino acid sequence of thermostable actin

No.	Sequence	Peptide sequence position	m/z values	Ion scores		
				Chilled	Boiled	Autoclaved
1	K.AGFAGDDAPR.A	21 – 30	976.50	67	15	19
2	R.AVFPSIVGRPR.H	31 – 41	1198.77	34	16	7
3	K.IWHHTFYNELR.V	87 – 97	1515.82	56	54	62
4	R.VAPEEHPTLLTEAPLNPK.A	98 – 115	1956.15	115	114	82
5	R.GYSFVTTAER.E	199 – 208	1130.62	44	57	38
6	K.SYELPDGQVITIGNER.F	241 – 256	1791.03	No match	81	103
7	K.QEYDEAGPSIVHR.K	362 – 374	1500.79	89	86	105

by autoclaved group were found to be numerically lower than the boiled samples and appeared to be consistent over the treatment.

Proteins separated by 2DE gels were identified by MALDI-TOF/TOF. The result confirmed the identity of protein is actin. However, the peptide mass detected has been matched with actin from bovine database since up to this point, database search for actin sequence from goat skeletal muscle is not available. Therefore, a comparison was made with the species that exhibit high homology with goat, which is bovine. Experimental molecular weight of actin from present study is 42.7 kDa with pI 6.1 which slightly differ from what has been reported in the MASCOT database. The theoretical values reported in the database were 42.451 kDa with pI 5.31 (Bjarnadóttir *et al*., 2010). Differences observed in experimental and theoretical molecular weight might cause by phosphorylation of actin upon heating and subsequently affect the shift in pI towards basic region (Bendixen, 2005). Furthermore, MALDI-TOF/TOF analysis consistently gave information on the peptide-mass fingerprint from position 21 – 374 which was assumed that these are the polypeptides that not affected by heat treatment.

During the course of this investigation, an attempt was made to figure out at which particular sequence of actin were affected by the heat. For this purpose, ion scores of actin were tabulate and shown in Table 2. The Ion Scores is a measure of how well the observed MS/MS spectrum matches to the stated peptide (Bantscheff *et al*., 2007). Clear distinction can be made from the result (Table 2) where there is reducing ion score of actin at position 21 – 208. The lower scores indicate that the peptides are suppressed by action of heat and remarkable influence of heat treatment is observed. Meanwhile, increasing ion score at position 241 to 374 shows actin is liberated from actomyosin complex (Akihiro *et al*., 2009). Heat denatured the protein and cause unfolding of tertiary structure into secondary and primary amino acid. These sequences might be the amino acid that not affected by heat. Stabilization of these peptides might contribute by the hydrophobic chain between amino acid residues. Further study has to performed in order to see the how each of the polypeptides can withstand thermal treatment.

Conclusion

Heat treatment highly influenced the protein composition remained after the heat treatments. Actin protein is one of the proteins affected by increasing temperature in meat. However, some poly peptides that still exist in the meat could be use as one of potential biomarker for detecting presence of goat meat in food product. It is expect that this research would facilitate research on protein structure prediction and molecular modelling of denatured actin.

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