UNIVERSITI PUTRA MALAYSIA

SPECIES AUTHENTICATION OF THERMALLY PROCESSED MEAT AND MEAT PRODUCTS BY PROTEOMICS APPROACH

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By

SITI AIMI SARAH BT ZAINAL ABIDIN

Thesis Submitted to the School of Graduate Studies,
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

SPECIES AUTHENTICATION OF THERMALLY PROCESSED MEAT AND MEAT PRODUCTS USING PROTEOMICS APPROACH

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December 2014

Chairman: Awis Qurni bin Sazili, PhD
Institute: Halal Products Research Institute

The present study focused on the development of a protein-based method which could differentiate pork from beef, chevon and chicken meat. Current protein-based detection methods such as ELISA and western blot have been extensively used for such species determination. However, heating of meat brings extensive changes in target antigen appearance and contributes to false detection. Hence, species authentication using proteomics approach was chosen as this state-of-the-art technology offers high discriminating power, specificity and selectivity, while the detection could be carried out on denatured protein. The purpose of this study was to determine porcine-specific peptide biomarker from thermally processed meat using combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry approach. In the initial phase, proteome extract of raw and cooked pork were profiled using two-dimensional gel electrophoresis to screen for thermally stable proteins available in the meat which has been subjected to 3 different heat regimes (1) chilled at 4°C, (2) boiled at 100 °C for 30 min and (3) autoclaved at 121°C, 15 psi for 20 min. Following to that, all thermal stable protein spots were identified using the MALDI-TOF-MS. In the second phase, selection of porcine-specific peptide was carried out using bioinformatics tool (CLUSTAL W and MS-Digest) and followed by a verification using the LC-QTOF-MS. In the final phase, the porcine-specific peptides were tested and verified on commercial meat products using the multiple reactions monitoring (LC-ESI-QQQ-MS). The 2-DE separations of autoclaved pork samples revealed only 43 spots in the gel, in which their presence and minimal changes following the thermal treatment confirmed that these spots are less susceptible to heat based on its high abundance in muscle. The aforementioned 43 spots were then compared with spots from the gel images of other species (cattle, goats and chickens). Prominent molecular weight differences were observed among the species studied, while no differences in isoelectric points were noted among the meat species. Through the protein identification of heat treated meat using MALDI-TOF/TOF mass spectrometry, only 13 proteins were consistently detected and found to be thermally stable throughout the treatment. Subsequently, interspecies comparison of amino acid sequence for each protein was carried out to search for porcine-specific peptides. The bioinformatics analysis revealed 35 potential peptides which are unique to pork only. However, a thorough investigation of LC-QTOF-MS data presented that only seven porcine-specific peptides were consistently detected by the mass spectrometry system. Specifically, two peptides were found to be derived from lactate dehydrogenase, one from creatine kinase, and four from serum albumin protein. Then, the multiple reactions monitoring method was

established for verification whereby only four of the peptides biomarkers were detected in commercial meat samples. Peptides EVTEFAK, LVVITAGAR, FVIER and TVLGNFAAFVQK were found to be consistently detected in the commercial meat products and displayed species-specific properties. In conclusion, meat species authentication through the combined platforms of 2DE and mass spectrometry is highly potential to offer scientifically valid and reliable results even at peptide level. Besides, the specificity and selectivity offered by the proteomics approach also provide a robust platform for halal authentication of thermally processed meat and meat products.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah.

PENGESAHAN SPESIES DAGING DAN PRODUK DAGING PERMPROSESAN HABA MENGGUNAKAN KAEDAH PROTEOMIK

Oleh

SITI AIMI SARAH BT ZAINAL ABIDIN

Disember 2014

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Penyelidikan ini memberi tumpuan kepada pembangunan kaedah berasaskan protein yang dapat membezakan daging babi dari daging lembu, kambing dan ayam. Kaedah pengesanan berasaskan protein masa kini seperti ELISA dan pemendapan western telah digunapakai secara meluas dalam menentukan spesis. Namun begitu, pemanasan daging akan memberi banyak perubahan terhadap rupa bentuk antigen yang disasarkan dan sekaligus menyumbang kepada pengesanan palsu. Pengesahan spesies menggunakan pendekatan proteomik telah dipilih kerana teknologi moden ini menawarkan kuasa pembezaan, pengkhususan dan pemilihan protein, tambahan juga pengesanan boleh dilakukan ke atas protein yang ternyahasli. Kajian ini bertujuan untuk menentukan penanda bio peptida yang spesifik bagi spesies babi daripada daging yang melalui proses perlakuan haba, menggunakan gabungan kaedah gel elektroforesis dua dimensi (2-DE) dan spektrometri jisim. Di peringkat awal, ekstrak proteome daging babi mentah dan yang dimasak telah diprofilkan menggunakan 2-DE untuk mengesan protein yang stabil kepada haba, juga boleh didapati dalam daging selepas tertakluk kepada 3 rejim haba yang berbeza (1) suhu sejuk pada 4°C, (2) suhu didih pada 100 °C selama 30 min dan (3) autoklaf pada suhu 121°C, 15 psi selama 20 min. Seterusnya, semua titik protein yang stabil terhadap rejim haba telah dikenal pasti menggunakan MALDI-TOF-MS. Pada peringkat kedua, pemilihan peptida spesifik bagi spesies babi telah dijalankan dengan menggunakan kaedah bioinformatik (CLUSTALW dan MS-Digest) dan peptide tersebut telah disahkan menggunakan LC-QTOF-MS. Pada langkah terakhir, peptida babi khusus telah diuji pada produk daging komersial menggunakan pemantauan tindak balas berbilang (LC-ESI-QQQ-MS). Hasil pemisahan protein menggunakan 2-DE dari sampel daging babi autoklaf mendedahkan hanya 43 titik protein pada gel yang mengalami perubahan yang minimum selepas rawatan haba sekaligus mengesahkan bahawa titik-titik tersebut kurang terkesan kepada haba disebabkan bilangan protein yang banyak dalam otot. Kesemua 43 titik protein kemudiannya dibandingkan dengan titik yang terdapat pada imej gel spesies lain (lembu, kambing dan ayam). Perbezaan berat molekul yang ketara telah ditunjukkan di antara spesies yang dikaji, manakala tiada perbezaan nilai isoelektrik diperhatikan. Proses pengenalan protein menggunakan spektrometri jisim MALDI-TOF/TOF hanya mengenalpasti 13 protein secara konsisten dan protein-protein ini didapati stabil terhadap perlakuan haba sepanjang tempoh rawatan. Seterusnya, perbandingan urutan asid amino antara spesies bagi kesemua 13 protein telah dilakukan untuk mencari cebisan peptida yang khusus bagi

babi. Analisis bioinformatik mendedahkan 35 peptida unik berpotensi sebagai penanda bio spesifik. Walau bagaimanapun, penyiasatan secara menyeluruh terhadap data LC-QTOF-MS menunjukkan bahawa hanya tujuh peptida khusus-babi telah dikesan secara konsisten oleh sistem spektrometri jisim, lebih spesifik, dua daripada mereka diperoleh dari lactate dehydrogenase, satu dari creatine kinase, dan empat daripada serum albumin protein. Seterusnya, kaedah pemantauan reaksi berbilang telah mengesahkan bahawa hanya empat daripada penanda bio peptida yang dapat dikesan dalam sampel produk daging komersial. Justeru, disahkan bahawa peptida EVTEFAK, LVVITAGAR, FVIER dan TVLGNFAAFVQK telah dikesan secara konsisten dalam produk daging komersial dan mempamirkan sifat spesies spesifik. Kesimpulannya, penentuan spesies daging melalui gabungan platfom 2-DE dan spektrometri jisim mempunyai berpotensi yang besar dan menawarkan hasil kajian secara saintifik yang sah dan boleh dipercayai walaupun pada tahap peptida. Selain itu, pengkhususan dan pemilihan spesies yang ditawarkan oleh pendekatan proteomik juga menyediakan platfom bagi analisis pengesahan daging dan produk daging halal terutamanya produk yang diproses menggunakan haba.

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I certify that a Thesis Examination Committee has met on December 2014 to conduct the final examination of Siti Aimi Sarah bt Zainal Abidin on her Doctor of Philosophy thesis entitled "Species Authentication of Thermally Processed Meat and Meat Products using Proteomics Approach" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

%	percentage
g	gram
kg	Kilogram
µg	microgram
ml	millilitre
w/v	weight per weight
v/v	volume per volume
kDa	kilo daltons
M	molar
mM	millimolar
µl	microlitre
µm	micron
°C	degree celsius
cm	centimetre
nm	nanometre
ng	nano gram
mA	milli ampere
V	volts
h	hour/hours
min	minute
g	gravitational force
psi	pounds per square inch
psig	pounds per square inch gauge
keV	kiloelectron volt
1-DE	one-dimensional electrophoresis
2-DE	two-dimensional gel electrophoresis
APS	ammonium persulphate
ACN	acetonitrile
BPC	base peak chromatogram
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CID	collision induced dissociation
DTT	dithiothreitol
EIC	extracted ion chromatogram
EDTA	ethylenediamine tetra acetic acid
FA	formic acid
HCl	hydrochloric acid
IAA	iodoacetamide
IEF	isoelectric focussing
LC-ESI-QQQ-TOF	liquid chromatography electrospray ionization triple quadrupole mass spectrometer
LC-QTOF-MS	liquid chromatography quadrupole time of flight mass spectrometer
LOD	limit of detection

MALDI-TOF	matrix assisted lased desorption ionization-time of flight
MW	molecular weight
MS	mass spectrometer / mass spectrometry
MRM	multiple reaction monitoring
NaCl	sodium chloride
NCBI	National Centre of Biotechnology Information
NCBInr	National Centre of Biotechnology Information non-redundant
pI	Isoelectric point
QTOF	quadrupole time of flight
QTrap	quadrupole ion trap
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	tetrametylethylenediamine
TCA	trichloroacetic acid
Tris-HCl	tris in hydrocholic acid
TIC	total ion chromatogram
Vh	volt hour
TPM1	tropomyosin-1 alpha chain
ACTC	actin alpha cardiac muscle
MLC3F	myosin light chain 3
MLC2F	myosin regulatory light chain 2
TnTf	troponin T, fast skeletal muscle
M-CK	creatine kinase M-type

CHAPTER 1

INTRODUCTION

Meat and meat products are one of the essential dietary components as they are important source of proteins, vitamins, minerals, fat, saturated fatty acids, cholesterol and many other nutrients. The main fact that leads to high consumption of meats is they have higher protein content than fruits, vegetables and breads, plus there are some peptides and amino acid that is essential in human diet such as carnosine, anserine and histidine (Boldrev and Severin, 1990). There are a variety of sources of red and white meat such as beef, mutton, chevon, venison, chicken and pork. Fresh meat can be cooked and served as it is or further processed into palatable products that are available in the market such as sausages, patties, corned beef and frankfurters. As a matter of fact, meat products such as emulsified or coarsely ground sausages may contain mixed meat from different species such as mixing pork with chicken, beef with pork as well as beef with horse. However, in some countries, not all meat species are acceptable for consumption and among other reasons are also due to ethical objections to killing animals for food, health concerns, environmental concerns or religion dietary laws.

Prohibition of pork consumption has been made compulsory to the Muslims and Jewish. The Islamic and Jews law forbid the consumption of pork for thousands of years and hence only pork-free food products are permissible for members of these religions (Regenstein *et al.*, 2003). However, the act of incorporating pork in the manufacturing of meat and meat products has been deliberately carried out by irresponsible producers in order to increase profit since pork is cheaper than any other type of meat. All the labelling has also been falsified and consequently misleads the consumers (Sentandreu and Sentandreu, 2014). Some cases of meat adulteration were either intentionally or unintentionally added during the processing (Primrose *et al.*, 2010), but both conducts are considerably unethical. As a matter of facts, meat adulteration cases are no longer a domestic issue as it has spread beyond boundaries and becoming an international concerns.

In 2012, seven frozen meat products imported from Thailand were shown to contain pig DNA (Berita Harian, 2012). The year 2013 uncovered a major international fraud case in the food industry whereby in March 2013, halal chicken sausages served to pupils in central London schools and nurseries were revealed to contain traces of pork (BBC News, 2013). In a different case, the Swedish National Food Agency reported that pork meat from Slovenia has been found in halal-marked salami sold in Sweden markets (The Local, 2013). All the cases highlighted above indicate the severity of the food scandal.

Identifying meat adulteration in mixtures of meat is nearly impossible with the naked eyes. Therefore, with the aid from modern technology, authentication techniques were developed from various sample state which range from small to molecular level (Nakyinsige *et al*., 2012; Montowska and Pospiech, 2010). Currently, genomic-based method particularly species-specific PCR and real time-PCR have become the preferred method to detect adulteration of processed meat products with porcine

sources as DNA can be detected at extremely low quantities and amplified up to thousand copy number (Che Man *et al.*, 2007). It would be advantageous to use DNA detection method since DNA is more stable, hence it allows traceability in pre-cooked or ready-to-eat products. However, DNA based detection method has a serious limitation when it comes to contamination, whereby it happen at any stage from food production, preparation as well as transportation of products.

Heat treatment such as cooking and sterilization of meat and meat products are among the other concerns arise in selecting a suitable method for species determination in meat products. It makes the identification of contaminating meat even more challenging. The analysis of pork adulteration as well as species authentication of meat is rather more challenging when dealing with processed meat products and this is mainly due to their composition, complexity and very often inhomogeneity. Moreover, fat, spices, various salts, including commonly used sodium chloride and sodium nitrite, antioxidants, vegetable additives or milk proteins are commonly added into the products and these have contributed to low extractability of DNA (Montowska and Pospiech, 2012).

Hence, proteomics-based analysis that uses the principle of protein was pursued. Although denaturation of protein might be the major limitation in the present experiment, the application of peptide biomarker from denatured proteins may not hinder the conduct of such analysis. A proteomic-based method applies the principle of chromatographic and electrophoretic techniques which have been proven to be useful in food components identification (Han and Wang, 2008). Moreover, it is a reliable method for meat speciation since meat products are muscle tissue mixtures built primarily from proteins (Montowska and Pospiech, 2010). Based on the molecular weight, isoelectric point and ion mass characteristics, it has been possible to distinguish differences in proteins separation among samples studied. For instance, the two-dimensional electrophoresis (2-DE) is a well-established electrophoresis technique for species identification of meat from various species of meat animals including fish species (Montowska and Pospiech, 2013; Martinez and Friis, 2004), through which, the proteins separation and their profile can be investigated comprehensively. Furthermore, Montowska and Pospiech (2011), reported differences in 2-DE profile of skeletal muscle myosin light chain isoform between cattle, pig, chicken, turkey duck and goose with regards to their molecular weight and isoelectric point. In addition, the mass spectrometry based analysis was proven to be suitable platform for species identification. A previous research by Sentandreu *et al.* (2010) reported that quantitative detection of chicken meat using stable isotope peptides was able to detect the presence of chicken in mixture of meat. Moreover, apart from meat research, peptide biomarker for distinguishing porcine and bovine gelatine has been successfully developed using NanoUPLC-ESI-Q-TOF-MS (Yilmaz *et al.*, 2013).

The great potentials offered by the proteomics method need to be fully explored and further developed particularly for halal meat authentication. However, to the best of our knowledge, there is insufficient information on heat stable protein from meat and meat products. Furthermore, the identification of proteins and peptide biomarkers following heat treatment of meat and meat products is yet to be documented. Research by von Bargen *et al*. (2013) only reported the application of peptide biomarker on mixture or raw meat while study conducted by Sentandreu *et al.*

(2010) was able to identify peptide marker from thermally treated meat, but instead of pork, chicken was selected as their main focus. Hence, this study was conducted to determine porcine-specific protein biomarker from thermally processed meat and meat products using proteomics approach. Hence, it could conceivably be hypothesised that porcine-specific peptide biomarker can be identified from denatured protein and specificity of the peptide is influenced by amino acid differences. The first phase of this project focused on the comparison of proteins profile of thermally processed pork, beef, chevon and chicken followed by identification of thermally stable protein in pork. The terms 'thermo stable' and heat stable are frequently used in this thesis when referring proteins remained after thermal treatment or cooking process. The terms generally understood as an ability of a single protein from thermophilic organism that could remain unfolded and maintain their activities in extremely high temperature (100°C). However, proteins identified in this study were described as having 'thermo stable'-like properties, which have been contributed by high amount of particular protein in the complex muscle component such as myosin and actin polymer. But thermo stability will not be observed when subjecting heat to individual meat protein.

The information provided by the thermally stable proteins were used to obtain porcine-specific peptide which was further verified using the LC-MS/MS. The second phase of the experiment involved the application of identified porcine-specific peptide biomarker for the detection of pork in commercial meat products. The search for biomarker was conducted from initial screening through gel-based proteomics, MS-based peptide biomarker discovery and verification of potential biomarker. Validation experiment was conducted in separate project apart from what have been presented in this thesis due to several reasons. Biomarkers have to be validated as a true biomarker for intended condition and their sensitivity and specificity must be established. Sensitivity test was not conducted due the fact that biomarker validation requires a very large number of sample (e.g. 100-500 for clinical sample) compared to the discovery and verification stage (10-50 samples only). Moreover, the validation of biomarkers is a very long and expensive process, in which a synthetic peptide or stable isotope labelled peptide must be generated to function as an internal standard. Limit of detection (LOD) and limit of quantitation (LOQ) must be established ranging from several hundred part per million (ppm) to part per billion (ppb) and hence justifies the absence of validation experiment. However, verification or refinement of potential biomarkers through multiple reaction monitoring (MRM) into some credible peptides that are in fact specific indicators for pork contamination is enough to prove that the listed biomarkers qualified to be used for species authentication method.

General objective

- To determine porcine-specific peptide biomarker from thermally processed meat and meat products using proteomics approach.

Specific objectives

1. To determine 'thermo stable' proteins present in thermally processed pork, beef, chevon and chicken that potentially be used as biomarker.
2. To evaluate the interspecies differences in tryptic digested peptide of 'thermo stable' protein among thermally processed pork, beef, chevon and chicken using mass spectrometry.
3. To verify presence of pork in meat and meat products using multiple reaction monitoring.

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