UNIVERSITI PUTRA MALAYSIA

SHOTGUN PROTEOMICS APPROACH FOR THE ESTABLISHMENT OF PEPTIDE MARKERS FOR PORK

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

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DEDICATION

"And if whatever trees upon the earth were pens and the sea [was ink], replenished thereafter by seven [more] seas, the words of Allah would not be exhausted. Indeed, Allah is Exalted in Might and Wise."

(Quran 31: 27)

"Allah will raise those who have believed among you and those who were given knowledge, by degrees. And Allah is Acquainted with what you do."

(Quran 58: 11)

Special dedication to:

My beloved wife Nurul Farhana Abdullah
My adorable son Muhammad Taufiq Farish
My charming son Muhammad Umar Farish
My future children

&

Parents

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

SHOTGUN PROTEOMICS APPROACH FOR THE ESTABLISHMENT OF PEPTIDE MARKERS FOR PORK

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MOHD HAFIS YUSWAN BIN MOHD YUSOFF

July 2018

Chairman : Dhilia Udie Lamasudin, PhD
Institute : Halal Products Research

Statistically, in 2015 the largest population in the world is the Christians, nevertheless, by 2060 the population of Muslim is expected to be nearly equal to the Christians. This indicates that the halal status of any particular food as a future global concern. According to the Food and Agricultural Organisation of the United Nations 2009, the demand for meat is expected to increase drastically for the developing countries, from 26 to 37 kg of average annual per capita consumption from the year 2000 to 2030. Consequently, certain manufacturers have unethically adulterated the meat owing to the desire to generate a high-profit margin as well as to fulfil the market demand, whereby pork is added to beef. These consequences highlight a requirement for meat authentication analysis. Recently, qPCR is the most famous genomic-based method that has been employed in the routine laboratories worldwide; owing to the lower limit of detection method as compared to any other proteomic-based methods such as SDS-PAGE and ELISA. In contrast to the DNA, the peptide sequences are extremely stable, in which their intactness remain against chemical or mechanical processes. The objective of this study, therefore, is to establish the peptide markers for pork by a newly shotgun proteomics approach, which those peptide markers shall be related to the contractile proteins of meat such as myosin, actin, tropomyosin, or troponin complexes. Initially, the peptide masses of proteolytic peptides, generated from peptide mass fingerprinting of LC-MS analysis, were analysed by principal component analysis (PCA) to overview the distribution pattern of 577 peptide masses among pork, beef, and broiler. Then, the most significant peptide masses for pork were determined from a validated PCA model through a discriminant analysis of orthogonal partial least square. Consequently, only seven potential peptide markers for pork were identified, but only five peptide markers were true-positive, as confirmed by another independent tandem LC-MS/MS analysis. Subsequently, the MS/MS spectra of true-positive peptide markers were annotated for their peptide sequences and inferential proteins through *de novo* database search engine (MS-Taq tool of ProteinProspector 5.20.0). Furthermore, a validation study was conducted to measure the precision, detection limit, and specificity of the peptide markers in relation to their reliability and applicability. In summary, only three reliable and applicable peptide markers from

contractile proteins of pork had successfully established through an advanced shotgun proteomics approach by using Agilent 1200 Series high-performance liquid chromatography hyphenated with AB Sciex 4000 QTrap mass spectrometer with a detection limit was 10% of pork. Unfortunately, the three peptide markers are not applicable to processed pork. This finding might be owing to a modification of certain amino acid in the peptide marker sequence through deamination or oxidation due to the extreme processing. Therefore, further comprehensive study is warranted for processed pork, as the established peptide markers from this study were successfully developed for raw pork not processed pork. Moreover, an advanced method validation for individual peptide marker is required, before it can be routinely implemented in the laboratory as a standard procedure for meat authentication.

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

PENDEKATAN PROTEOMIK *SHOTGUN* BAGI PENGHASILAN PENANDA PEPTIDA DAGING BABI

Oleh

MOHD HAFIS YUSWAN BIN MOHD YUSOFF

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Menurut statistik, pada tahun 2015 penduduk terbesar di dunia adalah Kristian, namun, menjelang 2060 penduduk Islam dijangka hampir sama dengan Kristian. Hal ini menunjukkan status halal bagi makanan tertentu menjadi isu global pada masa hadapan. Menurut Pertubuhan Makanan dan Pertanian bagi Pertubuhan Bangsa-Bangsa Bersatu 2009, permintaan terhadap daging dijangka meningkat secara drastik bagi negara-negara membangun, daripada 26 kepada 37 kg purata penggunaan per kapita tahunan sepanjang tahun 2000 hingga 2030. Akibatnya, terdapat pengeluar memalsukan daging, ekoran mendapatkan keuntungan lumayan serta memenuhi permintaan pasaran, lantas daging babi dicampurkan ke dalam daging lembu. Keadaan ini menunjukkan keperluan analisis ketulenan daging. Kini, kaedah qPCR yang berasaskan pengenalpastian gen adalah yang terkenal dan digunakan di dalam makmal seluruh dunia; disebabkan had pengesanan yang rendah berbanding kaedah-kaedah berasaskan protein seperti SDS-PAGE dan ELISA. Namun, urutan peptida adalah sangat stabil terhadap proses kimia atau mekanik. Oleh itu, objektif kajian ini adalah untuk menghasilkan penanda peptida bagi mengenal pasti daging babi, di mana penanda peptida tersebut berkait dengan protein daging seperti myosin, actin, tropomyosin, atau kompleks troponin. Pada mulanya, jisim-jisim peptida daripada proses proteolitik dikesan menerusi pencapjarian peptida melalui analisis LC-MS, kemudian dianalisis melalui *Principal Component Analysis* (PCA) untuk melihat corak taburan bagi 577 peptida antara daging babi, lembu, dan ayam. Seterusnya, jisim-jisim peptida yang paling ketara terhadap daging babi ditentukan daripada model PCA yang disahkan melalui *Orthogonal Partial Least Square – Discriminant Analysis*. Hasilnya, hanya tujuh penanda peptida yang berpotensi bagi daging babi telah dikenal pasti, tetapi hanya lima penanda peptida sahaja yang positif-benar, disahkan melalui analisis LC-MS/MS. Selanjutnya, spektrum MS/MS bagi penanda peptida positif-benar di jujuk untuk urutan peptida dan protein inferensnya melalui pencarian pangkalan data *de novo* (MS-Taq, ProteinProspector 5.20.0). Selain itu, kajian validasi telah dijalankan untuk mengukur ketepatan, had pengesanan, dan kekhususan penanda peptida berhubung kestabilan dan keberkesanannya. Kesimpulannya, hanya tiga penanda peptida yang stabil dan spesifik terhadap daging babi telah berjaya dikenal

pasti melalui kaedah pendekatan proteomik *shotgun* dengan menggunakan kromatografi cecair berprestasi tinggi Agilent 1200 Series bersama spektrometer jisim AB Sciex 4000 QTrap dengan had pengesanan adalah 10% daging babi. Namun, tiga penanda peptida positif-benar tersebut tidak berkesan terhadap daging babi proses. Penemuan ini mungkin disebabkan oleh pengubahsuaian asid amino tertentu dalam urutan penanda peptida tersebut melalui deaminasi atau pengoksidaan akibat pemprosesan melampau. Oleh itu, kajian komprehensif diperlukan untuk daging babi proses, kerana penanda peptida yang dikenal pasti daripada kajian ini dibangunkan untuk daging babi mentah dan bukan untuk daging babi proses. Selain itu, pengesahan lanjutan bagi setiap penanda peptida diperlukan, sebelum diguna pakai secara rutin di makmal sebagai prosedur standard untuk pengesahan daging.

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APPROVAL

I certify that a Thesis Examination Committee has met on 6 July 2018 to conduct the final examination of Mohd Hafis Yuswan Bin Mohd Yusoff on his thesis entitled "Shotgun Proteomics Approach for The Establishment of Peptide Markers for Pork" 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 in Halal Product Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microliter
µm	Micrometre
µL/min	Microliter Per Minute
1DE	One-dimensional Gel Electrophoresis
2D-PAGE	Two Dimensional Polyacrylamide Gel Electrophoresis
amu	Atomic Mass Unit
ACN	Acetonitrile
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ATPase	Adenosine Triphosphate Enzyme
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
c	Intercept
cm	Centimetre
CID	Collision-induced Dissociation
cps	Count Per Second
CV	Coefficient of Variance
Da	Dalton
Da/s	Dalton Per Second
DDA	Data-dependent Acquisition
DIA	Data-independent Acquisition
DNA	Deoxyribose Nucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
e.g.	*Exempli Gratia* (For Example)
EIC	Extracted Ion Chromatogram
ELISA	Enzyme-linked Immunosorbent Assay
EMS	Enhanced Mass Spectrometry
EPI	Enhanced Product Ion
ER	Enhanced Resolution
ESI	Electrospray Ionisation
FA	Formic Acid
FASTA	Fast Alignment
FDA	Food and Drug Administration
FDR	False Discovery Rate
g	Gram
GeLCMS	Gel-enhanced Liquid Chromatography-Mass Spectrometry
GHP	Good Hygiene Practice
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GPM	Global Proteome Machine
h	Hour
HACCP	Hazard Analysis Critical Control Point
H_o	Null Objective
H_a	Alternative Objective
HMM	Heavy Meromyosin
HPLC	High-performance Liquid Chromatography

HSD	Turkey's Honest Significant Difference
IAA	Iodoacetamide
ICH	International Conference of Harmonisation
IEF	Iso-electrical Focusing
IUPAC	International Union of Pure and Applied Chemistry
JAKIM	Islamic Development Malaysia
kDA	Kilo Dalton
kg	Kilogram
L	Litre
LC	Light Chains
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LMM	Light Meromyosin
M	Molarity
MALDI	Matrix Assisted Laser Desorption Ionisation
mM	Mili Molar
M1	First Mass Peak
M2	Second Mass Peak
mg	Miligram
mg/mL	Miligram Per Mililitre
min	Minute
mL	Mililitre
MS	Mass Spectrometry/Mass Spectrometer
MS/MS	Tandem Mass Spectra
m/z	Mass-to-charge Ratio
MW	Molecular Weight
ND	Not Detected
NH_4HCO_3	Ammonium Bicarbonate
NIST	National Institute of Standard and Technology
OPLS-DA	Orthogonal Partial Least Square – Discriminant Analysis
PAGE	Polyacrylamide Gel Electrophoresis
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PMF	Peptide Mass Fingerprinting
PPG	Polypropylene glycol
ppm	Part Per Million
psi	Pounds Per Square Inch
PSMs	Peptide-spectrum Matches
Q^2	Prediction Variance
Q1	Quadrupole 1
Q2	Quadrupole 2
qPCR	Quantitative Polymerase Chain Reaction
QTof	Quadrupole Time-of-flight
QTrap	Quadrupole Linear Ion-Trap
R^2	Linear Regression / Total Variance
RAPD	Random Amplified Polymorphic DNA
rcf	Relative Centrifugal Force
RFLP	Restriction Enzyme Fragment Length Polymorphism

RT-PCR	Real-time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl sulphate – Polyacrylamide Gel Electrophoresis
SNPs	Single Nucleotide Polymorphisms
TDS	Target-decoy Database Searching
TIC	Total Ion Chromatogram
ToF	Time-of-flight
US	United State
USA	United State of America
V	Volt
VIP	Variable influence on projection
v/v	Volume Per Volume
w/v	Weight Per Volume
UPM	Universiti Putra Malaysia

CHAPTER 1

INTRODUCTION

1.1. Research Background

Meat is considered as a main source of proteins owing to the proteins as the second-largest components in the meat (Huff-Lonergan, 2010). The proteins are also substantial components for the manufacturing and refurbishing of damaged muscle or tissue in our body. Furthermore, proteins are subjected to various post-translational modifications that confer numerous biological functions such as hormones, enzymes and haemoglobin (Hoffman & Falvo, 2004); whereas some other proteins act as bioactive components with properties such as antithrombotic, anti-microbe, and as an immunomodulation (Kilara & Panyam, 2003).

Owing to the meat nutritional value, demand on meat is increased drastically, especially in the developing countries from 26 kg of average annual per capita consumption in the year 2000 to 37 kg around the year 2030 (Kearney, 2010). The main reasons for this circumstance are resulted from the proliferation of human population, fast progression of urbanisation, and improvement in the lifestyle; moreover, it is a trend in the developing countries that eating or dining are merely for pleasure rather than for survival (Kwon, 2015).

According to the Food and Agriculture Organisation of the United Nations (UN), 37% of the world meat source consumption is pork with China as the main producer, whereas broiler and beef contribute for 35% and 23%, respectively (McGlone, 2013). Despite the pork as a main source of meat worldwide, consumption of pork is totally prohibited among Muslim owing to the strict Islamic dietary laws (Farouk, 2013; Farouk et al., 2014; Fazryatul, Nor Aini, & Faridah, 2017; Fischer, 2016; Kwon & Tamang, 2015; Regenstein, Chaudry, & Regenstein, 2003).

Consequently, the Department of Islamic Development Malaysia (JAKIM) issues the halal (an Arabic word that translates as lawful and wholesome) certification to ensure that each of food premise that applies for halal certification fulfils the halal dietary laws. However, the halal certification is not compulsory, but a voluntary basis for the manufacturers. Nevertheless, halal certification will be a benefit especially in the Muslim majority countries. In a case of fast food restaurant in Malaysia such as McDonald, for instance, JAKIM has fully certified the restaurant in 1995; consequently, gain attraction among Muslim consumer (Fischer, 2016).

Nonetheless, some unethical manufacturers produce meat products adulterated with non-halal (forbidden and not wholesome) meats to gain more profit and fulfil the market demand (Ali et al., 2014; Ali, Nina Naquiah, Mustafa, & Hamid, 2015). In

2013, for instance, both pork and equine have been purposely mixed with beef products in Europe (Stanciu, Stanciuc, Dumitrascu, Ion, & Nistor, 2013). Even though the equine is considered halal by some Islamic schools of thought such as Syafie, the followers of Hanafi and Maliki schools prohibit the consumption of equine (Averroës, Nyazee, & Abdul-Rauf, 1999). Additionally, meat fraudulent is also reported in China, wherein a chemically treated pork and rat meat have been sold as beef and lamb, respectively (Ali, Razzak, & Hamid, 2014). These non-halal meat products, even in trace amount are problematic especially for Muslim as such products are totally prohibited, known as haram (an Arabic word that translates as forbidden and not safe).

1.2. Problem Statements

In order to protect consumer rights and prevent the adulterated meats with pork widespread, meat authentication requires a sophisticated analytical method; wherein an inspection by the naked eyes is definitely impossible. Currently, the established methods for meat authentication depend on the targeted biomolecules. These include genomics and proteomics. In genomics, the DNA is detected by hybridisation or amplification; instead, the proteomics is commonly implemented by chromatography, electrophoresis or immunoassay technique to detect the proteins (Ballin, 2010; Rahmati, Muhd Julkapli, Yehye, & Basirun, 2016).

For the genomic-based methods, the techniques are sensitive, selective and rapid; moreover, the limit of detection has been reported to be as low as 0.01% of pork (Ballin, Vogensen, & Karlsson, 2009). Instances for genomic-based methods are polymerase chain reaction (PCR), real-time or quantitative PCR (RT-PCR/qPCR), random amplified polymorphic DNA (RAPD), and restriction enzyme fragment length polymorphism (RFLP) (Rahmati et al., 2016). However, all of these genomic-based methods have limitations in relation to expensive reagents, laborious procedures, contamination, degradation at extreme processing temperature, and requirement of experienced, trained as well as skilful personnel to conduct the methods (von Bargen, Brockmeyer, & Humpf, 2014; von Bargen, Dojahn, Waidelich, Humpf, & Brockmeyer, 2013).

Alternatively, enzyme-linked immunosorbent assay (ELISA) is a common immunoassay in proteomics. Currently, there are four commercial ELISA kits available used to authenticate the meat, which are (i) Reveal for Ruminant in MBM (Neogen Corporation), (ii) MELISA-Tek (ELISA Technologies), (iii) FeedCheck (Strategic Diagnostics Inc.), and (iv) Tepnel Biosystem Biokit (Stamford, Conn.) (Ballin, 2010). However, these commercial ELISA kits impotent to fulfils the requirement set up by the US FDA's Centre for Veterinary Medicine Office of Research for selectivity, sensitivity, ruggedness, and specificity. Furthermore, ELISA is not multiplex and if it is, costing is an additional limitation (Ballin, 2010). Other proteomic methods such as sodium dedocylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectrical focusing (IEF) on the PAGE (commonly known as 2D-PAGE) have the same limitations as genomic-based methods (Montowska & Pospiech, 2007).

Despite the limitations of ELISA, SDS-PAGE, and 2D-PAGE method, most analyst still prefers proteomics to complement with genomics owing to the undeniable fact of numerous proteins construct the meat. Thus, it is crucial to develop an analytical proteomic-based method that reliable and efficient to authenticate meat species routinely. As compared to the ELISA, liquid chromatography-mass spectrometry (LC-MS) has a potential to substitute the ELISA by overcoming all the immunoassay's limitations as the technology advances. Generally, the limitations of ELISA are basis for the advantages of LC-MS such as high sensitivity, high selectivity, fast, high-throughput, low sample volume, low running cost, reproducible, multiplexing, and wide analyte range (Cross & Hornshaw, 2016). Moreover, the LC-MS is a powerful technique as its resolvable and analysable capacity allows the identification of proteins unbiasedly towards their molecular weight, isoelectrical point and hydrophobicity as compared to SDS-PAGE and 2D-PAGE (Pedreschi, Hertog, Lilley, & Nicolaï, 2010).

1.3. Novelty of Research

Based on the previous studies (Claydon, Grundy, Charlton, & Romero, 2015; Jira & Schwägele, 2017; Montowska, Alexander, Tucker, & Barrett, 2014, 2015; Ruiz Orduna, Husby, Yang, Ghosh, & Beaudry, 2015; Sarah et al., 2016; von Bargen et al., 2014, 2013), trypsin is a common endoproteinase enzyme used in the shotgun proteomics. This technique is also known as a bottom-up proteomics as the proteins have been digested by the trypsin to produce short fragment of peptides, subsequently, being detected by the LC-MS/MS to deduce the inferential proteins through protein database (Gregorich, Chang, & Ge, 2014; Moradian, Kalli, Sweredoski, & Hess, 2014; Zhang, Fonslow, Shan, Baek, & Yates, 2013). Moreover, advanced in *in-silico* is implemented in another study to identify the peptide markers via computer simulation prior to the shotgun proteomics (Ruiz Orduna et al., 2015).

In this study, endoproteinase Glu-C has been used instead of trypsin, as other study claimed that the Glu-C produces large fragment of peptides (Moradian et al., 2014), thus, significantly reduce the redundancy to identify the potential peptide markers. The peptide markers from this study, therefore, are novel owing to different cleavage side, to be specific cleaves at the carboxyl side of glutamic acid and 3,000 times slower at aspartic acid except followed by proline (Zhang et al., 2013). In addition, other study claims that the Glu-C eliminates deamidation artefact during proteolysis (Liu, Moulton, Auclair, & Zhou, 2016), consequently, producing stable peptide markers.

Moreover, this study introduces a new approach of shotgun proteomics by combining a multivariate analysis consists of principal component analysis and orthogonal partial least square discriminant analysis to assist in the selection of potential peptide markers statistically. In contrast to *in-silico*, this novel approach is considered as a real-time analysis, wherein the *in-silico* requires confirmation that could be tedious and required special skill. This novel approach is never being applied in a common practice of shotgun proteomics.

1.4. Significance of Research

Having considered the emergence of many cases of meat adulterated with pork as well as religion concern and ethics, there is undeniable fact for the establishment of peptide markers for pork as 20% of meat constituent are proteins. Moreover, this study can be an alternative authentication method that compliment with DNA based method. From this study, the established peptide markers for pork can be used specifically to identify raw pork for meat speciation. These peptide markers can assist any government institution, in this case JAKIM or enforcement laboratory under Jabatan Kimia Malaysia to authenticate meat species routinely.

1.5. Research Hypotheses

The fundamental is referred back to the central dogma of molecular biology, wherein the information from the DNA is passed to the RNA through a transcription process; subsequently, the information from the RNA is translated into a protein through a translation process. Every contractile protein in meat species, therefore, is different from each other in term of certain amino acids in the protein sequence. Generally, meat contains various types of contractile proteins such as actin, myosin, tropomyosin, troponin I, troponin C, and troponin T (Listrat et al., 2016). These contractile proteins are also known as myofibrillar proteins, which have an intact primary structure (amino acid sequence); thus, stable from degradation through an extreme condition or processing (Ali et al., 2015; Buckley, Melton, & Montgomery, 2013). This advantage gives an opportunity to authenticate the meats based on the primary structure of proteins through available cutting-edge liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis by a shotgun proteomic approach. In shotgun proteomics, specific peptide markers for meat authentication can be established from the proteolytic peptides of extracted proteins mixture among different type of biological samples through the LC-MS/MS assisted by protein database searching (Pedreschi et al., 2010).

1.6. Research Objectives

The main objective of this study was to establish the peptide markers for pork between beef and broiler using shotgun proteomics approach.

In order to achieve the main objective, the specific objectives of this study were:

i. To measure the yield and quality of proteins extracted from pork, beef, and broiler by Bradford assay and gel-enhanced LC-MS.

ii. To identify the potential peptide markers for pork between beef and broiler by chemometric-assisted exploratory shotgun proteomics.

iii. To annotate the peptide sequences and inferential proteins of the identified peptide markers by tandem LC-MS/MS through confirmatory proteomics.

iv. To validate the applicability of identified peptide markers by data-dependent acquisition of tandem LC-MS/MS against raw and processed pork.

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