UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF THERMOSTABLE PEPTIDE MARKERS IN MEATS USING GEL-BASED FRACTIONATION COUPLED WITH MASS SPECTROMETRY

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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IDENTIFICATION OF THERMOSTABLE PEPTIDE MARKERS IN MEATS USING GEL-BASED FRACTIONATION COUPLED WITH MASS SPECTROMETRY

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

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by mass spectrometry is widely used in proteomic study mainly in meats due to their effectiveness and efficiency in generating reliable data. An alternative method such as enzyme-linked immunosorbent assay (ELISA), although known to be highly-specific but it bears the risk of cross-reactivity. The genomic approach is another commonly used method but suffers from potential deoxyribonucleic acid (DNA) contamination from other organism and due to its structure, DNA molecule is less resistant to heat treatment. The limitation of these methods may lead to a false positive result in detection analysis. First, the purpose of this study was to compare the electrophoretic pattern of proteins in one-dimensional (1DE) and two-dimensional (2DE) gel electrophoresis in meat derived from cow, water buffalo, pig and wild boar upon different heat treatments. Then, the next aim was to screen the species-specific thermostable protein markers in all species using principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA). The third aim was to identify the thermostable protein and peptide markers obtained from electrospray ionization liquid chromatography-mass spectrometry (ESI-LC-MS) and matrix-assisted laser desorption ionization time-of-flight/time-of-flight tandem mass spectrometry (MALDI-TOF/TOF-MS/MS). The meats were subjected into several heat treatments which were (1) chilled at 4℃ for 30 min, (2) roasted at 150℃ for 20 min, and (3) fried at 160℃ for 6 min, before subjecting to two different combinations of proteomic approaches i.e. SDS-PAGE coupled with ESI-LC-MS and 2DE coupled with MALDI-TOF/TOF-MS/MS. The extracted proteins were fractionated using 1DE gel electrophoresis coupled with combined multivariate analysis of PCA and PLS-DA for grouping and discriminative analysis. The pattern of electrophoretic proteins in all species was similar but differences appeared between the raw and cooked meats. The potential thermostable protein markers derived from all species were determined using ESI-LC-MS. At the molecular weight of 55.06 kDa, proteins that have been identified from cow samples were tropomyosin, moesin, cadherin, and septin. As for water buffalo, 5-oxoprolinase has been identified at the molecular weight of 181.22 kDa. In the pig, serum albumin, calpain-7, and ATP synthase subunit alpha, mitochondrial were identified with the molecular weight of 77.02 kDa while wild boar has shown to have

cathepsin K and calcium/calmodulin-dependent protein kinase type II subunit delta at the molecular weight of 48.61 kDa. These approaches were successful in providing preliminary data analysis for the screening of thermostable protein markers with species-specificity. For the second approach, tropomyosin was selected and analyzed using 2DE gel electrophoresis followed by MALDI-TOF/TOF-MS/MS. Minor differences were observed in the amino acids sequences in both tropomyosin (TPM) isoforms i.e. TPM2 and TPM1 for each species. Moreover, several thermostable peptides that were found to belong to the Bovidae family were HIAEDSDR and LDKENAIDR and Suidae family, RIQLVEEELDR. These potential peptides could be used as markers to discriminate between Bovidae and Suidae families. Thus, the result indicated that these isoforms have the potential to be selected as thermostable species-specific protein and peptide markers in meat authentication.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

MENGENAL PASTI PENANDA PEPTIDA YANG TAHAN HABA DI DALAM DAGING DENGAN MENGGUNAKAN PEMISAHAN BERDASARKAN GEL DAN SPEKTROMETRI JISIM

Oleh

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Kaedah natrium dodesil sulfat-elektroforesis gel poliakrilamida (SDS-PAGE) berserta dengan spektrometri jisim digunakan secara meluas dalam bidang proteomik bagi kajian daging kerana ia berkesan dan cekap dalam menghasilkan data yang dipercayai. Antara kaedah lain yang diguna pakai adalah antibodi menangkap enzim-esei imuno serapan (ELISA) yang mempunyai kespesifikan yang tinggi tetapi berisiko untuk berlakunya reaksi silang. Pendekatan genetik adalah antara kaedah lain yang digunakan, namun berpotensi untuk berlakunya pencemaran antara asid deoksiribonukleik (DNA) dengan organisma lain dan strukturnya sendiri yang tidak tahan dengan rawatan haba. Kelemahan dalam kaedah-kaedah ini boleh mengakibatkan penghasilan keputusan pengesanan analisis yang kurang tepat. Tujuan kajian ini dijalankan, pertama adalah untuk membandingkan corak elektroforetik protin dalam satu dimensi (1DE) dan dua dimensi (2DE) gel elecktoforesis dalam daging daripada lembu, kerbau, babi dan babi hutan setelah dikenakan rawatan haba yang berbeza. Kemudian, tujuan kedua adalah untuk melakukan pemeriksaan saringan bagi penanda protin yang stabil haba dan spesifik spesis bagi setiap spesis dengan menggunakan analisis komponen utama (PCA) dan analisis diskriminasi sebahagian kecil persegi (PLS-DA). Tujuan ketiga adalah untuk mengenal pasti penanda protin dan peptida yang stabil haba yang diperoleh daripada elektrospray pengionan kromatografi cecair spektrometri jisim (ESI-LC-MS) dan matrik dibantu laser desorpsi ionisasi-masa penerbangan/masa penerbangan spektrometri jisim (MALDI-TOF/TOF-MS/MS). Setiap daging dikenakan kepada beberapa rawatan haba iaitu (1) penyejukkan pada suhu 4℃ selama 30 min, (2) panggang pada suhu 150℃ selama 20 min, dan (3) goreng pada suhu 160℃ selama 6 min sebelum melalui dua kaedah gabungan proteomik yang berbeza, SDS-PAGE berserta dengan ESI-LC-MS dan 2DE berserta dengan MALDI-TOF/TOF-MS/MS. Protin yang telah diekstrak, dipisahkan dengan menggunakan 1DE gel elektroforesis berserta dengan gabungan analisis multivariat, PCA untuk pembahagian kepada kumpulan dan PLS-DA untuk diskriminasi. Corak bagi elektroforetik protin bagi setiap spesis adalah sama tetapi berbeza antara daging yang tidak dimasak dan dimasak. Beberapa protin yang stabil haba telah dikenal pasti dengan menggunakan ESI-LC-MS dan berpotensi untuk dijadikan penanda bagi setiap spesis. Lembu mempunyai tropomyosin, moesin, kaderin dan septin

pada berat molekular, 55.06 kDa. Kerbau mempunyai protin 5-oxoprolinase dengan berat molekular, 181.22 kDa. Babi pula memiliki serum albumin, calpain-7 dan ATP sintase subunit alpha, mitokondria pada berat molekular, 77.02 kDa manakala babi hutan memiliki cathepsin K dan calcium/calmodulin-dependent protein kinase type II subunit delta pada berat molekular, 48.61 kDa. Kaedah ini menyediakan data analisis awalan bagi penanda protin yang stabil haba untuk spesifik spesis. Bagi pendekatan kedua, tropomyosin telah dipilih dan dianalisis dengan menggunakan 2DE gel elektroforesis diikuti dengan MALDI-TOF/TOF-MS/MS. Perubahan kecil dalam urutan asid amino dapat dilihat dalam kedua-dua isoform tropomyosin (TPM), TPM2 and TPM1 bagi setiap spesis. Tambahan pula, beberapa peptida yang stabil haba yang dijumpai milik keluarga Bovidae adalah HIAEDSDR dan LDKENAIDR manakala keluarga Suidae adalah RIQLVEEELDR. Kesemua peptida ini berpotensi untuk dijadikan penanda bagi mendiskriminasikan antara keluarga Bovidae and Suidae. Keputusan menunjukkan kedua-dua isoform mempunyai potensi untuk dipilih sebagai penanda protin dan peptida yang tahan haba bagi spesifik spesis dalam pengesahan daging.

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

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1DE	One-dimensional
2DE	Two-dimensional
ACN	Acetonitrile
ADP	Adenosine diphosphate
APCI	Atmospheric pressure chemical ionization
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CaMKII	Ca^{2+}calmodulin (CaM)-dependent protein kinase
CHAPS	3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfate
CI	Chemical ionisation
CID	Collision-induced dissociation
CP	Creatine phosphate
CWF	Compassion in World Farming
DHAP	2,5-dihydroxyacetophenone
DHB	2,5-dihydroxybenzoic acid
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
ERM	Ezrin/radixin/moesin
ESI	Electrospray ionization
ESI-LC-MS	Electrospray ionization liquid chromatography-mass spectrometry
FAB	Fast atom bombardment
FAO	Food and Agriculture Organization
FD/FI	Field desorption/Field ionization
GC	Gas chromatography

HCBs	Halal Certification Bodies
HCCA	4-hydroxy-α-cyanocinnamic acid
HCL	Hydrochloric acid
HPA	3-hydroxypicolinic acid
IAA	Iodoacetamide
IDA	Information Dependent Acquisition
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IUPAC	International Union of Pure and Applied Chemistry
JAKIM	Jabatan Kemajuan Islam Malaysia
K_2HPO_4	Dipotassium hydrogenphosphate
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
m/z	mass to charge ratio
MALDI	Matrix-assisted laser desorption ionisation
MALDI-TOF-MS/MS	Matrix-assisted laser desorption/ionization time of flight tandem mass spectrometry
MLC1f	Myosin light chain 1 isoform
MLC2f	Myosin light chain 2 isoform
MLC3f	Myosin light chain 3 isoform
MRM	Multiple monitoring reaction
MUI	Majelis Ulama Indonesia
MUIS	Majlis Ugama Islam Singapura
OPLS-DA	Orthogonal projections to latent structure discriminant analysis
PC1	Principal component 1
PC2	Principal component 2
PCA	Principal component analysis

PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism
PLS-DA	Partial least square-discriminant analysis
PMF	Peptide finger printing
Q	Quadrupole
Q-TOF	Quadrupole time of flight
RAPD	Random amplified polymorphic deoxyribonucleic acid
SA	Sinapinic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SES	Socio-economic status
S-S	Sulphur-sulphur
TEMED	N,N,N',N'-tetramethylethylenediamine
TI	Thermospray ionization
TPM	Tropomyosin
TPM1	Tropomyosin alpha-1 chain
TPM2	Tropomyosin beta chain
UAE	United Arab Emirates
UK	United Kingdom
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

In recent decades, livestock production has shown to be increasing dynamically due to the high demand for meat. The situation is a result of global population's modern lifestyle where food preparation has to be done quickly and easily, hence consumers incline to include in their diet with fast food which contains mainly vegetable oils, sugar and animal products over the carbohydrate-rich staple food and this transitional phenomenon is known as the substitution stage (Vranken, Avermaete, Petalios, & Mathijs, 2014). This transition in the diet which mainly consists of fast food and frozen food, as well as the increasing purchasing power and urbanization process has directly influenced the demand for meat supply. In general, livestock production in developing countries is considered to be growing rapidly. However, the situation is contradicting in developed countries where the production of livestock is slow or stagnant even at the high-income levels (Thornton, 2010; Vranken et al., 2014).

Referring to a report released by World Bank 2009, the total number of meat production in the developing countries was tripled from 45 to 134 million tons since the year 1980 to 2002 (Thornton, 2010). Its number is expected to be increased in 2030 by achieving 37 kg from a modest average annual per capita consumption of 10 kg in the 1960s (Heinz & Hautzinger, 2007). In general, the pattern of meat consumption is differed geographically, due to diet regulation, religion or the economic situation. In developed countries such as the United State and the United Kingdom, the vital meat sources are mostly obtained from pig, sheep, and cattle. Moreover, almost half of all meat being consumed is derived from meat products (Kearney, 2010). In the developing country, the greatest livestock production in East Asia region is revolved between poultry and pig (Thornton, 2010). In other regions such as Malaysia, poultry is the major proportion of meat consumption followed by pig and cattle (Sheng, Shamsudin, Mohamed, Abdullah, & Radam, 2010).

Recent years, consumers' awareness of the authenticity of meat being sold in the market has been evolving ever since the horsemeat scandals have been publicized in the media and followed by other authentication issues. According to BBC News (2013b), halal chicken sausages which were served in several primary schools in London was claimed to contain pork DNA. Moreover, The Local (2013) has also reported that pork has been found in halal-marked salami in Swedish markets with surprisingly more than 10% of the meat content came from pork. In Jakarta, bakso, which is the local traditional delicacy, which it serves together with meatballs, has been publicized in the media that the meatballs contained beef contaminated with pork. It was reported that such cases emerged as a result of the increasing price of beef being sold in Indonesia (ABC Rural, 2012). The price of pork is comparatively cheaper than other livestock animals, as result, pork is commonly used as an alternative to replace other meats such as chicken, goat, and beef (Mutalib et al., 2012; Von Bargen, Dojahn, Waidelich, Humpf, & Brockmeyer, 2013). In Malaysia, the issue has been raised on the way of the storage of lamb and pork in one container, although the meats had been packed individually according to their species in different boxes (BH Online, 2017).

The awareness is emerged due to cultural practice, religious beliefs, gender, health and socio-economic status (SES) (Kearney, 2010; Vranken et al., 2014). For example, pork and beef are forbidden in Hinduism and Buddhism while pork is forbidden in Islam and Judaism (Bonne & Verbeke, 2008). The global population of Muslims is estimated to be around 1.6 to 1.8 billion and the figure is expected to be growing every year. In 2030, it is expected that the population to be increased up to 27% of the total global population. The growing population of Muslims will have a direct impact on the growth of the Halal industry (Farouk, 2012; 2013). A large number of Muslim population predetermined the importance of meat authenticity among consumers as they do not consume pork and its derivative in their diet. These issues must be taken seriously and to be addressed accordingly to regain consumers' trust in local meat supply and products. In addition, consumers nowadays are well-aware of their rights and it is their right to demand that the food that they consumed must be accurately labelled and the correct ingredients must be declared on the packaging of the products.

The development of an efficient and sensitive method for tracing the origin of the food products is of utmost importance as the total negligence on the issue has a big impact on many aspects. Various studies have been performed to find the most efficient, highly sensitive, time-saving and cost-effective methods for food authentication purposes. The desirable methods should be applicable to both heat-treated and nonheat-treated meat products. It is difficult to differentiate the species through naked eyes such as cow, pig, sheep, and poultry when they are mixed together. Meat authentication can be performed by using genomic and proteomic approaches such as polymerase chain reaction (PCR), ELISA and mass spectrometry.

The genomic approach is commonly practiced by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), real-time PCR, species-specific PCR and random amplified polymorphic DNA (RAPD) to determine the species identification (Nakyinsige, Man, & Sazili, 2012). It is selected due to stable molecules and the ability to withstand heat processing (Chisholm, Conyers, Booth, Lawley, & Hird, 2005). However, the extraction method for PCR is time-consuming and the potential for DNA contamination with other organisms (Montowska, Alexander, Tucker, & Barrett, 2014). Moreover, DNA analysis also may provide low-reliability result towards processed food as its structure will be degraded at the temperature above 100℃ and provide the opportunity for having nonspecific fragments (Ebbehoj & Thomsen, 1991; Sentandreu, Fraser, Halket, Patel, & Bramley, 2010).

Limitations in the genomic approach have encouraged researchers to choose the proteomic approach due to the stability of the primary amino acid sequence (Sentandreu et al., 2010). Several common methods used in proteomic approach are ELISA, electrophoretic and chromatographic. ELISA, although possess a high-specify, it has the risk for cross-reactivity (Chen & Hsieh, 2015). Furthermore, target proteins may denature during food processing and subsequently destroyed the target protein epitope which is the binding domain for the antibodies (Asensio, González, García, & Martín, 2008). Most of the studies are preferred electrophoretic approach using SDS-PAGE followed by mass spectrometry because they provide more extensive, thorough and comprehensive data and information for meat authentication analysis (Montowska & Pospiech, 2012; 2013b; Sarah, Karsani, Amin, Mokhtar, & Sazili, 2014). In order to improvise the previous method, multivariate analysis was integrated into this study. It provides preliminary screening data which further facilitate the downstream analysis (Nur Azira, Che Man, Raja Mohd Hafidz, Aina, & Amin, 2014).

This study proved the hypothesis the types of proteins that have been present in 1DE and 2DE in meats derived from cow, water buffalo, pig and wild boar upon heat treatments, although between same species, were varied due to the different chemical solutions and mechanical treatments have been used in the extraction and fractionation steps in both electrophoresis and the mass spectrometries. This study also demonstrated that multivariate analysis using PCA and PLS-DA have facilitated the screening of species-specific thermostable protein markers prior to the mass spectrometry analysis. Two types of mass spectrometries i.e. ESI-LC-MS and MALDI-TOF/TOF-MS/MS were identified the thermostable protein and peptide markers from these species.

1.1 Research Objectives

General objective:

- To determine the potential thermostable protein and peptide markers from meats via two different approaches i.e. (1) SDS-PAGE coupled with ESI-LC-MS and (2) 2DE coupled with MALDI-TOF/TOF tandem mass spectrometry.

Specific objectives:

1. To compare the electrophoretic pattern of proteins in 1DE and 2DE gel electrophoresis in meats derived from cow, water buffalo, pig and wild boar upon different heat treatments.
2. To screen the species-specific thermostable protein markers in all species using PCA and PLS-DA.
3. To identify the thermostable protein and peptide markers obtained from two mass spectrometries:
    a. ESI-LC-MS.
    b. MALDI-TOF/TOF-MS/MS.

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