UNIVERSITI PUTRA MALAYSIA

DETECTION OF PORK AND LARD ADULTERATION IN FOOD PRODUCTS USING MOLECULAR BIOLOGY TECHNIQUES FOR HALAL AUTHENTICATION

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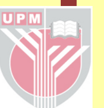

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By

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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

March 2007

ESPECIALLY DEDICATED TO MY BELOVED FAMILY

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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March 2007

Chairman: **Professor Raha binti Abdul Rahim, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Adulteration of food products has become a common problem in many countries. Adulteration may take the form of substitution of one species for another whereby the food products from one species have been mixed intentionally with either similar substitute material or cheaper species. In most cases, food manufacturers often choose lard as a substitute ingredient for oil because it is cheap and easily available. However, the usage of pork and lard is a serious matter in Islam because foods containing ingredients from pig sources are haram (unlawful or prohibited) for Muslims to consume. Therefore, a reliable technique for detection of pork and lard adulteration in food products is necessary in order to protect Muslim consumers from intentional or non-intentional fraud.

Polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) techniques have been developed for species identification in various types of food products such as canned fish, peanut and milk. These techniques are proven to be rapid and specific in detecting species adulteration. In this study, rapid methods using PCR and ELISA were utilized to detect pork and lard adulteration in selected food products.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and species-specific PCR detection of the conserved region in the mitochondrial (mt) cytochrome (cyt) *b* gene and mt 12S rRNA gene, respectively, for species identification from raw meat, fat and three types of food products were optimized and developed. Genomic DNA of raw meats, fats and sausages were successfully extracted and were found to be of good quality. Genomic DNA was not detected from the extraction of casing samples and the yield of genomic DNA extracted from bread and biscuit samples were very low.

PCR amplifications of mt cyt *b* gene and 12S rRNA gene produced DNA fragments of approximately 360 bp and 387 bp, respectively from the meat samples. However, no amplification product was observed from the bread and biscuit samples. The amplicons from the mt cyt *b* gene amplification were then digested with RE *Bsa*JI resulting in species-specific RFLP profiles. The cyt *b* PCR-RFLP and species-specific PCR identification yielded excellent results for detection of pig derivatives in food products.

Crude protein were successfully extracted from three types of food products and subjected to ELISA. Using this technique, ten samples were shown to be contaminated with pig derivatives. Positive results were confirmed by observing the colour changes in the well of the ELISA plate. This technique highlighted an alternative in detection of pig derivatives in food products.

From these studies, the utilization of PCR-RFLP and the utilization of mt cyt *b* and 12S rRNA gene in detecting pork and lard adulteration in selected food products were demonstrated. The use of ELISA was also shown to be fast and reliable in identification of pork and lard adulterated food products. However, PCR-RFLP and specific PCR techniques were determined to be better detection techniques for pork and lard adulteration in food products compared to ELISA. The findings from this study can serve as a basis of reference for the research in halal food authentication.

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

PENGESANAN PENCEMARAN DAGING DAN LEMAK BABI DI DALAM PRODUK MAKANAN MENGGUNAKAN TEKNIK-TEKNIK BIOLOGI MOLEKUL BAGI PENGESAHAN HALAL

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Pencemaran produk makanan telah menjadi satu fenomena biasa dikebanyakan negara. Pencemaran boleh terjadi dalam bentuk pertukaran satu spesis dengan spesis lain di mana produk makanan daripada spesis tertentu telah dicampur samada dengan bahan ganti yang hampir sama atau spesis yang murah. Di dalam kebanyakan kes, pengeluar makanan selalu memilih lemak babi sebagai bahan ganti bagi minyak kerana harganya yang murah dan mudah diperolehi. Walau bagaimanapun, penggunaan daging dan lemak babi adalah isu yang serius dalam Islam kerana makanan yang mengandungi bahan dari sumber babi adalah haram (melanggar peraturan atau dilarang) bagi orang Islam untuk memakannnya. Oleh itu, teknik yang boleh dipercayai untuk mengesan pencemaran daging dan lemak babi di dalam produk makanan adalah diperlukan bagi melindungi pengguna daripada penipuan yang sengaja atau tidak disengajakan.

Teknik tindakbalas jujukan berantai polymerase (PCR) dan asai imunoserapan berangkai enzim (ELISA) telah direka untuk pengenalpastian spesis di dalam pelbagai jenis produk makanan seperti ikan dalam tin, kekacang dan susu. Teknik-teknik ini terbukti pantas dan spesifik dalam mengesan pencemaran. Dalam kajian ini, kaedah pantas menggunakan PCR dan ELISA telah diguna untuk mengesan pencemaran daging dan lemak babi di dalam produk makanan terpilih.

Pengesanan bahagian gen-gen mitokondria (mt) cytochrome (cyt) *b* dan mt 12S rRNA bagi mengenalpasti spesis dari sampel daging dan lemak mentah serta tiga jenis produk makanan telah direka dan dioptimum menggunakan prinsip asid nukleik (PCR-RFLP) dan PCR spesifik spesis. DNA genomik dari daging dan lemak mentah serta sosej telah berjaya diekstrak dan bermutu tinggi. Tiada DNA genomik dapat dikesan dari proses pengekstrakan DNA dari pembalut sosej dan DNA genomik yang diekstrak dari roti dan biskut adalah rendah hasilnya.

Amplifikasi PCR gen-gen mt cyt *b* dan 12S rRNA dari sampel-sampel daging menghasilkan serpihan DNA lebih kurang pada saiz 360 bp dan 387 bp masing-masing. Walau bagaimanapun, tiada produk amplifikasi kelihatan dari roti dan biskut. Produk PCR dari amplifikasi gen mt cyt *b* telah dipotong dengan enzim penghad (RE) *Bsa*JI menghasilkan profil-profil RFLP spesifik spesis. Identifikasi menggunakan PCR-RFLP cyt *b* dan PCR spesifik spesis menghasilkan keputusan yang unggul bagi pengesanan unsur-unsur babi di dalam produk makanan.

Protein kasar telah berjaya diekstrak dari 3 jenis produk makanan dan diaplikasikan ke teknik ELISA. Dengan menggunakan teknik ini, 10 sampel telah didapati tercemar dengan unsur-unsur babi. Keputusan positif telah dipastikan dengan melihat perubahan warna di dalam telaga plat ELISA. Teknik ini boleh digunakan sebagai alternatif dalam mengesan unsur-unsur babi di dalam produk makanan.

Daripada kajian ini, penggunaan teknik PCR-RFLP serta penggunaan gen mt cyt *b* dan 12S rRNA di dalam mengesan pencemaran daging dan lemak babi di dalam produk makanan terpilih telah ditunjukkan. Penggunaan ELISA juga telah ditunjukkan sebagai teknik yang pantas dan boleh dipercayai di dalam pengesanan produk makanan yang tercemar dengan daging dan lemak babi. Walau bagaimanapun, didapati bahawa teknik PCR-RFLP dan PCR spesifik merupakan teknik pengesanan yang lebih baik bagi mengesan pencemaran daging dan lemak babi di dalam produk makanan berbanding ELISA. Penemuan daripada kajian ini boleh diguna sebagai rujukan asas bagi tujuan penyelidikan pengesahan makanan halal.

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I certify that an Examination Committee met on 8th March 2007 to conduct the final examination of Aida Azrina binti Azmi on her Master of Science thesis entitled “Detection of Pork and Lard Adulteration in Food Products using Molecular Biology Techniques for Halal Authentication” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions

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AIDA AZRINA BINTI AZMI

Date: 13 APRIL 2007

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LIST OF ABBREVIATIONS

ABTS	azino-diethylbenzthiazoline sulfonate
bp	base pair
BSE	bovine spongiform encephalopathy
cDNA	complementary DNA
cyt	cytochrome
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
ds	double-stranded
DSC	Differential Scanning Calorimetry
ELISA	Enzym-linked Immunosorbent Assay
FAME	fatty acid methyl esters
FAO	Food and Agricultural Organization
FTIR	Fourier Transform Infrared Spectroscopy
g	gram
g	*gravity*
GC	Gas Chromatography
GMO	genetically modified organism
HCl	hydrochloric acid
HPLC	High Performance Liquid Chromatography
HRP	horseradish-peroxidase
IEF	isoelectric focusing

IgG	immunoglobulin G
kb	kilobase
KCl	potassium chloride
mg	miligram
$MgCl_2$	magnesium chloride
min	minute
ml	mililiter
mM	milimolar
mt	mitochondrial
NaCl	Sodium chloride
ng	nanogram
NIR	Near infrared
nm	nanometer
PCR	Polymerase Chain Reaction
pmol	pikomol
RE	restriction enzyme
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
s	second
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ss	single-stranded
SSCP	single strand conformational polymorphism

TAG	triacylglycerol
Taq	*Thermus aquaticus*
UV	ultra violet
V	volt
µl	microliter

CHAPTER 1

GENERAL INTRODUCTION

In Islam, a critical factor to the Muslim consumers is the halal or haram status of the food. Nowadays, a wide variety of food exists in the market. Adulteration species in ground and comminute products has been a widespread problem in retail markets whereby the incidence of producing undeclared species appeared to be higher in cooked meat products than in raw products (Hsieh *et. al.,* 1995; Chen *et*. *al*., 1998). Therefore, analytical methods are required for detecting and preventing such adulteration in food (Chen *et*. *al*., 1998).

Methods have been developed based on electrophoresis (Babiker *et. al.,* 1981; Kim & Shelef, 1986), isoelectric focusing (IEF) (King, 1984; Jaussen *et al.,* 1990), chromatography (Ashoor *et al.,* 1988; Saeed *et al.,* 1989), deoxyribonucleic acid (DNA) hybridization (Chikuni *et al.,* 1990; Ebbehøj & Thomsen, 1991), polymerase chain reaction (PCR) (Meyer *et al.,* 1995) and enzyme-linked immunosorbent assay (ELISA) (Hsieh *et al.,* 1996; Chen *et*. *al*., 1998).

Methods for identification of food adulteration based on ELISA have been well documented (Whittaker *et al.,* 1983; Griffiths & Billington, 1984; Patterson *et al.,* 1984; Jones & Patterson, 1986; Dincer *et al.,* 1987; Martin *et al.,* 1988; Ayob *et al.,* 1989; Chen *et al.,* 1998). According to Hübner *et al.* (1999), although ELISA is widely acknowledged to be of practical use at the earliest stages of manufacture,

there is the disadvantage of protein denaturation as a consequence of processing. Thus, the detection of DNA by PCR techniques has a number of advantages, including the survival of DNA in many, albeit not all manufacturing processes. Meanwhile, DNA-based assays involving sophisticated technologies such as the PCR have potential application for food adulteration detection. The main advantage of DNA-based methods over protein-based methods is a combination of a high degree of specificity and the ability to perform reliably with highly processed samples (Jaussen *et al.,* 1998; Colgan *et al.,* 2001). An optimized PCR procedure successfully amplifies the specific target sequence even in a pool of heterogeneous genomic DNAs from complex matrices, such as milk, dairy products and other food commodities (Colgan *et al.,* 2001).

The hypothesis of this study is that molecular biology techniques such as PCR-RFLP, specific PCR and ELISA can be used to detect pork and lard adulteration in food products. Therefore, the objectives of this study were:

1. To utilize a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for pork and lard detection in raw and food products
2. To optimize a species-specific PCR method for the detection of pork and lard adulteration in raw and food products
3. To detect pork and lard in raw and food products using ELISA