DETECTION OF THE IDENTITY OF PROCESSED MEAT BY USING POLYMERASE CHAIN REACTION – RESTRICTION FRAGMENT LENGTH POLYMORPHISM MARKER

CAROLINE LIEW EE CHI

THIS THESIS IS SUBMITTED TO FULFILL THE REQUIREMENT TO OBTAIN A BACHELOR OF BIOTECHNOLOGY DEGREE WITH HONOURS

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Alamat Tetap: 17, LRG 1, TMN RIDGEVIEW, PHS 9, 88200 KOTA KINABALU, SABAH

Dr. Michael Wong
Nama Penyelia

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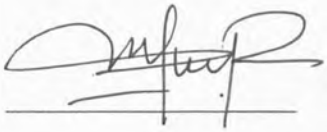
1. SUPERVISOR

   (Dr.Clemente Michael Wong)

2. CO-SUPERVISOR

   (Dr. Vijay Kumar)



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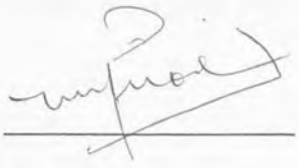
   (Ms. Teoh Peik Lin)



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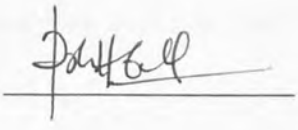

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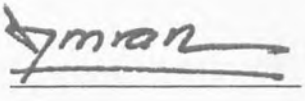

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ABSTRAK

Pengesanan identiti makanan-makanan yang diproses dengan menggunakan penanda "Polymerase chain reaction – Restriction fragment length polymorphism"

Identiti makanan-makanan yang diproses yang terdapat di pasaran perlu dikesan untuk mengelakkan penipuan yang terjadi semasa makanan-makanan itu diproses. Dengan itu, satu cara untuk mengesan makanan-makanan yang diproses itu adalah penting. Lapan produk yang terdapat di pasaran seperti sosej daging ayam, burger daging ayam, nugget daging ayam, kepingan "toast" ayam, sosej daging lembu, burger daging lembu, nugget daging lembu dan kepingan "toast" daging ayam belanda telah digunakan untuk memeriksa identiti kepada makanan-makanan yang diproses tersebut. DNA telah diekstrak daripada sampel-sampel tersebut dan amplifikasi PCR telah dijalankan dengan menumpukan gen bahagian cytokrom b yang terdapat di DNA mitokondria. Primer universal CYTb1 dan CYTb2 telah digunakan untuk mengamplifikasi satu produk bersaiz 360 bp. Produk yang telah diamplifikasi itu telah dipotong dengan menggunakan tujuh enzim pembatasan yang berlainan (*Rsa I, BsaJ I, BstN I, Taq I, Alu I, BstU I and Nsi I*) untuk tujuan pengenalpastian. Analisis kepada corak pembatasan telah dilakukan. Fragmen RFLP telah didapati daripada produk PCR yang telah dipotong. Pemeriksaan kepada setiap spesis daging adalah mungkin. Analisis RFLP menunjukkan produk-produk yang diperbuat daripada daging lembu telah dicemari dengan produk darpada daging ayam. Selain daripada itu, produk-produk daripada daging lembu juga telah ditukar kepada daging kerbau. Manakala produk daging ayam belanda pula telah di perbuat daripada daging ayam. Dengan itu, identifikasi spesis menggunakan gene cytokrom b boleh digunakan dan didapati berkesan. Selain itu, analisis ini juga boleh digunakan dalam masa yang pendek.

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ABBREVIATION

bp	base pair
g	gram
kb	kilo base
mmole	micromole
M	molar
mg	milligram
mM	millimolar
µl	microlitre
pmole	picomole
r.p.m	revolution per minute
%	Percent
°C	degree Celsius

ATP	Adenosine triphosphate
bp	base pairs
cyt b	cytochrome b
DNA	Deoxyribonucleic
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
$MgCl_2$	Magnesium chloride
min	minutes
mtDNA	mitochondrial DNA
NaCl	Natrium chloride
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction- Restriction Fragment Length Polymorphism
RAPD	Random Amplified Polymorphic DNA Analysis
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism

RNase	Ribonuclease
rRNA	ribosomal Ribonucleic acid
SDS	Sodium dodecyl sulphate
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TE	Tris-HCl EDTA
Tris	Tris (hydroxymethyl) aminomethane
Tris HCl	Tris (hydroxymethyl) aminomethane hydrochloride
tRNA	transfer ribonucleic acid
UV	ultra violet
V	volt

CHAPTER 1

INTRODUCTION

1.1 Introduction

Many food products in the market do not have proper labeling system especially in the small scale sundry shop. Although the government has set the law to provide proper labeling, some producer ignore the ruling. Labeling is very important to inform consumers the ingredients of the food. The meat type food is the most important food to put label as some people are allergy to some of the meats. For example, Malays do not eat pork as it is non 'halal'. Besides that, those who practices Buddhism and Hinduism do not eat beef. Thus, labeling is very important to prevent accidental consumption of forbidden food.

The problem with the labeling nowadays is that it is not reliable. Mislabeling is still an issue. The example of mislabeling is that the cow meat maybe is substituted with cheaper buffalo meat. Besides that, we also not sure of the processed food manufacturer that the foods that are manufactured are not contaminated with other type of meat. This is

because the manufacturers usually produce various kinds of meat in their factory. Thus, contamination rate are high.

Other than that, the authority such as Jabatan Kimia, Ministry of Health or Ministry of Trade and Consumer Affairs need to carry out routine check on the food that are sold in the supermarket to ensure it is not contaminated with other food products.

Besides that, the goal of establishing a Global Halal Hub in Malaysia has been the aim of the government. They aim is to export the 'halal" food to other Islamic countries. Thus, we must have a scientific mean to ensure that our food is 'halal' as claimed. Therefore, a reliable and practical method needs to be developed to authenticate 'halal' food to ensure that they are not contaminated by other meat especially pork. Besides that, this reliable and practical method also can be used to identify the meat content in a product.

Previously, some methods of detection of meat based on protein identification have been developed. The protein identification method works but it is not reliable enough because most proteins of the meat will be denatured after it is subjected to high temperature treatment. Alternatively, the molecular detection is believed to be more sensitive and reliable method (Dooley *et al.*, 2004). The molecular detection use DNA as their target and it is more heat tolerant.

DNA sequence of some genes in every species of meat is different. One gene in the mitochondrial DNA that has been shown to be useful in identifying meat identity is the cytochrome b gene. Certain portion of the cytochrome b gene is different between one species to another due to polymorphism. Thus, we are developing a method of identification based on the cytochrome b gene. The amplified cytochrome b gene is species specific; and when it is cut by different restriction enzymes, it will produce different sizes of amplified product which is unique to an animal.

Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) has shown to be a technique for identification of meat product. This method had been tested and successfully identified raw meat. This can be seen in Bellagamba *et al.*(2001). They have successfully identified the species in meat meal and animal feedstuff. Thus, now we would like to expand the studies on the processed meat based food.

1.2 Objectives

Therefore, the objectives of this project are:

i. to detect the identity of raw and processed meat by using PCR-RFLP and

ii. to test the method of identifying the identity of the raw and processed meat.

CHAPTER 2

LITERATURE REVIEW

2.1 Protein Based Analysis For Food Authentication

Protein based analysis for identification of foods or meat species had been developed before the findings of PCR. The methods include electrophoresis, immunoassays, liquid chromatography (Pfeiffer *et al.*, 2004) and enzyme linked immuno-sorbent assay (ELISA) (Cheng *et al.*, 2003).

These methods rely upon proteins that are heat labile and easily lose their biological activities (Sanjuan *et al.*, 2002). Muscle protein degrades easily if exposed under high temperature and prevents the identification, as these techniques require large amount of high quality protein (Cheng *et al.*, 2003). Another disadvantage of protein-based analysis is that the methods failed in detecting all the amino acid differences that may occur between different proteins (Sanjuan *et al.*, 2002). Therefore, DNA based methods for identification of species origin in food products is preferred over protein analysis (Pfeiffer *et al.*, 2004). This can be seen, as there are some highly conserved regions on the mitochondrial cyt b gene that are suitable for species identification.

2.2 DNA-based method For Food Authentication

Species identification methods based on DNA molecule has been widely used in food authentication (Horstkotte and Rehbein, 2003). The initial studies using DNA is through DNA hybridization. The labeled DNA probes were hybridized to the samples of genomic DNA. Probes containing the labeled DNA from a given species will hybridize to the DNA from the same species with little cross-activity (Lockley and Bardsley, 2000). The binding of complementary repetitive sequences was the cause of species-specific binding of the probes to their target (Lockley and Bardsley, 2000).

Besides that, newer DNA-based species detection method using PCR based had been introduced. PCR-based method was used together with several methods such as the sequencing of PCR products based on mitochondrial DNA, Polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP), species-specific PCR primers, single strand conformational polymorphism analysis and random amplified polymorphic DNA analysis (RAPD) (Lockley and Bardsley, 2000).

Food authentication using DNA hybridization was time consuming and difficult in handling it as it involved in radioactive material whereas PCR-based method manage to obtain large amount of DNA eventhough the source DNA is degraded (Lockley and Bardsley, 2000).

2.3 Meats

Meats are the part of flesh that is taken from animals that are used as food. It can be obtained from any parts of the flesh. Generally, meats are eaten for nourishment. Peoples have been eating meats since the pre-history time. The types of meat that one generally consumed are chicken, beef, pork and lamb. These meats have been the source of energy as they contain protein for everyday life. Raw meats do not retain their freshness long after a few days, the meat will rotten. Thus, processed meats are introduced to the market.

Processed meat had been introduced to the market for long time. Processed meat is known as the meat product, which contained 300g/kg of meat that had undergone a method of processing and combine with other ingredients or additives (http://www.foodstandards.gov.au/_srcfiles/Standard_2_2_1_Meat_v67.doc). Processed foods are now very convenient for peoples, as it only needs short time to prepared and come in various types. Some examples of the processed foods are sausage, burger, nugget, sandwich toast, meatballs and others. Contrary to raw meats, processed meats last longer and easy to deliver to other places or country.

2.4 Mitochondria

Mitochondria are known as the respiratory organelle of eukaryotic organisms (Madigan *et al.*, 2003). It is one of the cells that have an important role in the cells. Besides that, its main function is to produce energy where it is known as the site of energy production in the cell (Weaver, 2003). Mitochondria are known as the powerhouse of the cell as they produce ATP molecules for the cells to use (Scheffler, 1999). The size of mitochondria is similar with the size of prokaryotic organisms and the shape can be spherical or rod-shaped (Madigan *et al.*, 2003). The general structure of a mitochondrion is it consists of an outer membrane, an inner membrane, matrix, cristae and the intermembrane space (Cooper and Hausman, 2004). The membrane of a mitochondrion does not contain sterols which act to give the firmness to the membrane. Sterol can be found in the membrane of eukaryotic cell's cytoplasmic membrane. A mitochondrion is very special among the other organelles in the cell as it has its own DNA that is known as the mitochondrial DNA (mtDNA). It will encode tRNAs, rRNAs and some of the mitochondrial protein (Cooper and Hausman, 2004). Typical animal cells can have more than 1000 number of mitochondria. Thus, the number of mitochondria in each cell differs among species. There are also a number of nonchromosomal genetics elements inside the mitochondria (Madigan *et al.*, 2003) (Figure 2.1).

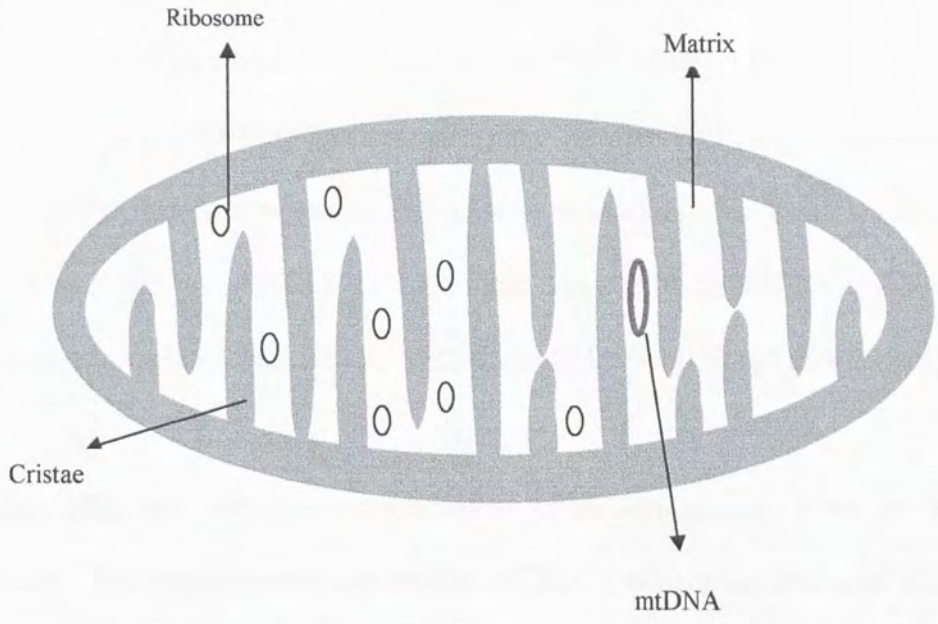

Figure 2.1 Mitochondrial DNA in Mitochondria

2.5 Mitochondrial DNA

There are a few organelle components in the mitochondria that encodes by the genome which exists inside the organelles itself. The mitochondrial DNA is located inside the mitochondria (Hartwell *et al.*, 2004). The number of mtDNA inside cells varies because the number of mtDNA in each cells are not specific. Mitochondrial DNA is easier to recover from a degraded biological sample than the nuclear DNA. It happens as the nuclear DNA only has two copies per cell whereas the mtDNA has hundreds to thousands copies of cells (Cheng *et al.*, 2003). Besides that, the size of mitochondrial DNA is much smaller than nuclear DNA which is between 16 to 17 kb only (Borgo *et al.*, 1996).

The difference between mitochondrial DNA and nuclear DNA is their base composition. Their density also different as mtDNA and nuclear DNA can be separated by using the density-gradient centrifugation on cesium chloride gradients (Tyler, 1992).

Mitochondrial DNA structure present in a covalently closed circular form (Madigan *et al.*, 2003) (Figure 2.2). This type of DNA can be found in prokaryotes. Thus, it is said that the bacteria or prokaryotes is the ancestor of mitochondria. Bacteria's DNA exists in plasmid form. Therefore, we can see the similarity between mitochondrial DNA and plasmid found in bacteria.

The size and gene content of mitochondrial varies between different species. For example, yeast are 75,000 base pair long compare to avian or human's mitochondrial

DNA which range only between 16,569 base pairs length and carry the information of 37 genes which is 22 tRNA genes, 2 rRNA genes and 13 protein coding regions (Hartwell *et al.*, 2004). Mitochondrial DNA has very high rate of mutation. Thus, the variation that occurs provides a valuable tool in studying the organisms with slightly similar organisms (Sanjuan *et al.*, 2002).

The function of mitochondrial is the same as the DNA of prokaryotic cells and the nuclear DNA of eukaryotic cells. Three classes of RNA are yield to obtain the expression of genetic information that is ribosomal, transfer and messenger RNA. However, mitochondrial DNA has their unique feature. For example, the codon UGA was use to encode tryptophan codon instead of the stop codon in the standard code.

Mitochondrial DNA had been proven to be the better tool in identifying the identity of the cell as the rate of evolution in the mtDNA are greater than the nuclear DNA. Besides that, the mtDNA genome had also been sequence out and the recombination during sexual reproduction ensures that there will be no scrambling of information (Scheffer, 1999).

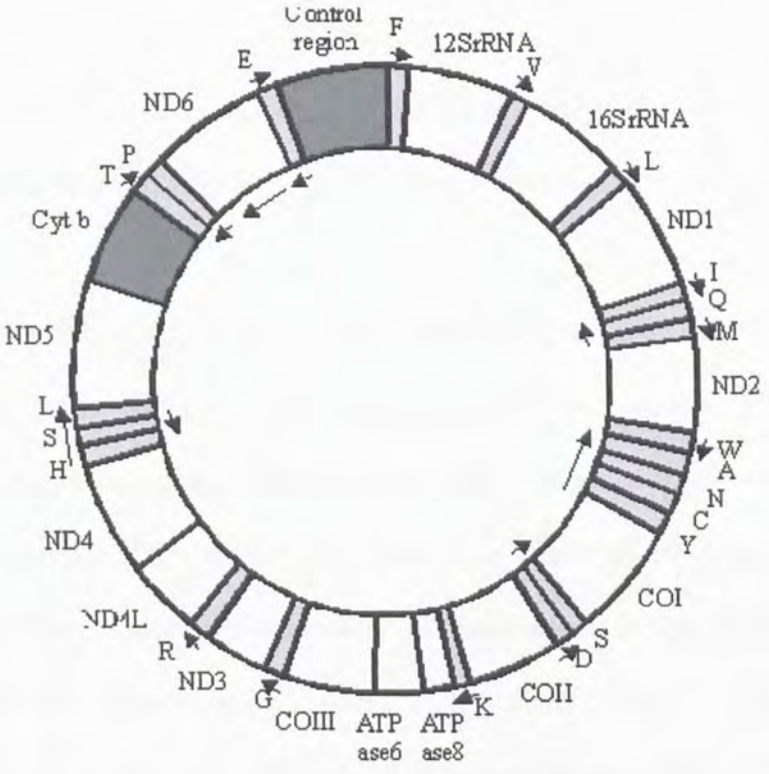

Figure 2.2 Mitochondrial DNA locus (http://herkules.oulo.fi/isbn9514255364/html/)

2.6 Cytochrome b gene

Cytochrome b (cyt b) gene is found inside the mitochondrial genome. It involved in the electron transport in the mitochondria's respiratory chain. Besides that, cyt b specificity is that it is the only cytochrome that will be coded by mitochondrial DNA. One of the coding regions that will be discussed here is the cytochrome b gene which has a region with DNA sequence that is highly polymorphic flanks by two well conserved regions.

Cytochrome b gene is very useful in identifying meat species. This can be concluded by the facts that one allele exists per individual because of the maternal inheritance of the mitochondria. Other than that, the number of mitochondrial DNA is more than the nuclear DNA in the cell, thus it is easier to be amplified in PCR. Furthermore, the mutation rate in mitochondrial genes are high that there will be even small different in the closely related species (Chao *et al.*, 2001). This can be seen between chicken and turkey. In vertebrates, cyt b gene is among the widest sequenced genes in comparing the intra-species and inter-species difference of animals includes amphibians, birds, fishes and mammals (Wang *et al.*, 2004). Therefore, cytochrome b analysis is a best method to detect the identity of the meats.

2.7 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method of copying the DNA template and producing more of the same template to enable the analysis. PCR was invented by Kary Mullis in 1987. DNA polymerase will copy the DNA molecules by undergoing changes of temperature (Madigan *et al.*, 2003). The early invention of Mullis was not efficient. This is because he was using the in vitro enzyme which will be destroyed when subjected to high temperature. Few years later, the technique was improved by using the DNA polymerase that derived from thermophilic bacteria. The bacterium which was taken is *Thermus aquaticus*. The DNA polymerase that derived from it was known as the Taq polymerase (Weaver, 2003).

Taq polymerase is an ideal choice for PCR as it has the advantages of heat stability and has high optimum temperature (McPherson *et al.*, 1990). Taq polymerase can withstand repeated exposure to the high temperatures that are needed for the strands separation (Innis *et al.*, 1990). It is important in PCR to initiate the synthesis of DNA in a 5' to 3' direction (Manson *et al.*, 2002). At the suitable temperature, it will anneal to the ssDNA and starts to elongate the ssDNA forming a new dsDNA. It is important to ensure that only 1 unit of Taq polymerase is used in each reaction.

Besides the Taq polymerase, other components need to be included in a mixture of DNA template. There are the deoxynucleoside triphosphate (dNTPs), the divalent cations

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