COMPARISON OF DNA EXTRACTION METHODS IN THE DETECTION OF GENETICALLY MODIFIED FOODS USING POLYMERASE CHAIN REACTION

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By

NGUYEN CHAU THANH TUNG

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DEDICATION

I wish to dedicate this work to my beloved parents, Mr. Nguyen Chau Boi and Mrs. Nguyen Thi Thanh Lan, who sacrificed their lives for my study

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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Chairman: **Professor Son Radu, PhD**

Faculty: **Food Science and Technology**

Genetically modified organisms (GMOs) can be defined as organisms in which genetic materials have been altered in the way that does not occur naturally by mating or natural combination. According to Novel Food Regulation (EC/258/97, EC/1139/98, EC/49/2000, EC/50/2000 and EC/1829/2003), foods and food ingredients derived from GMOs are strictly regulated and are labeled mandatorily. Polymerase chain reaction (PCR) method is used to detect GM events in foods. The specific objectives of this study are to establish a sensitive, robust and rapid operation method for detection of GM events by using PCR and to conduct a preliminary survey for distribution of animal feeds and foods derived from GM events in both Malaysia and Vietnam.

The two critical factors taken into account to achieve these objectives are applicability of different DNA extraction methods for each kind of samples and PCR amplification conditions.

Five DNA extraction methods (the Wizard method from Switzerland, the modified Wizard method with addition of beta-mercaptoethanol, the combination of Wizard and CTAB method, the CTAB method from Germany and the modified CTAB method with addition of beta-mercaptoethanol method) were optimized. The yield and quality of DNA obtained from raw soyabean, raw maize, animal feed, smooth tofu and soya milk samples were examined to determine the optimum method for each kind of sample.

The results of comparative analysis showed that the CTAB method was the most optimal protocol for extracting total DNA from raw soyabean, raw maize and animal feed samples with 32.7, 28.4, 33.4 ng DNA/mg sample in yield, respectively. In addition, the DNA quality (the ratio of A_{260}/A_{280} ranged from 1.9 to 2) was good enough not only for PCR amplification but also for DNA sequencing. However, the Wizard method was the best candidate for DNA isolation from smooth tofu (13.2 ng DNA/mg sample) and soya milk (3.4 ng DNA/mg sample) with relatively high quality of DNA (A_{260}/A_{280} ratio was 1.7 and 1.9, respectively).

The results of this survey showed that 20 out of 24 animal feed samples contaminated by at least one of three introduced genetic elements as promoter (P35S), terminator (NOS) and structural gene (EPSPS). In particular, all of the 16 animal feed samples from Malaysia and four out of eight animal feeds from Vietnam were GM-contaminant products. In contrast, neither soybean samples (12 samples) nor maize samples (24 samples) were positive with these assays. Therefore they were categorized as non-GM products.

These results revealed that PCR amplification method provides the key advantages of high sensitivity, and robust and rapid operation whilst providing the requisites of careful experimental design that avoids both false-negative and/or false-positive results. Five primer pairs of LEC1/LEC2; ZE03/ZE04; P35S 1-5'/P35S 2-3'; HA-NOS118-F/HA-NOS118-R and EPSPS 1-5'/EPSPS 3-3' chosen in this study produced the amplicons of 164, 277, 101, 118 and 118 base pair, respectively that fulfilled the product-size requirement and completed the whole detection procedure of GM events for raw soybean and raw maize as well animal feed samples. In addition, PCR amplification and DNA sequencing protocols presented in this study should provide a very useful tool for routine GM event detection in foods and feeds with regards to false-negative and/or false-positive results.

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

PERBANDINGAN BAGI KAEDAH PENGEKSTRAKAN DNA DI DALAM PENGESANAN MAKANAN TERUBAHSUAI GENETIK MENGGUNAKAN TINDAKBALAS RANTAIAN POLIMERASE

Oleh

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Organisma terubahsuai genetik (GMO) boleh di definasikan sebagai organisma di mana pengubahsuaian kandungan genetiknya tidak berlaku secara kombinasi semulajadi. Berdasarkan "Novel Food Regulation" (EC/258/97, EC/1139/98, EC/49/2000, EC/50/2000 dan EC/1829/2003), makanan dan kandungan makanan terbitan dari GMOs adalah dikawal rapi dan penglabelan diwajibkan. Kaedah tindakbalas rantaian polimerase (PCR) digunakan untuk mengecam GMOs dalam makanan. Objektif-objektif spesifik dalam kajian ini adalah untuk mengukuhkan kaedah operasi dalam pengecaman GMO dengan PCR yang sensitif, tegap and pantas serta mengendalikan pemeriksaan saringan terhadap pengagihan makanan haiwan dan makanan terbitan dari GMOs dalam Malaysia danVietnam.

Dua faktor kritikal yang diambilkira dalam mengecapi objektif-objektif tersebut adalah aplikasi kaedah ekstraksi DNA yang berlainanan untuk setiap sampel dan keadaan amplifikasi PCR.

Lima kaedah ekstraksi DNA (kaedah Wizard dari Switzerland, kaedah Wizard yang dibahsuai dengan penambahan beta-mercaptoethanol, kombinasi kaedah Wizard dan CTAB, kaedah CTAB dari Germany dan kaedah CTAB yang diubahsuai dengan penambahan beta-mercaptoethanol) telah diperbaikpulihkan. Hasil and ketulenan DNA yang didapati dari sampel seperti kacang soya, jagung mentah, makanan haiwan, tauhu lembut dan susu soya diselidik untuk menentukan kaedah yang paling optimum untuk setiap sampel berkenaan.

Keputusan dari analisis perbandingan memaparkan bahawa kaedah CTAB merupakan protokol yang paling optimam untuk mengekstrak jumlah DNA dari sampel kacang soya mentah, jagung mentah dan makanan dengan nilai yang diperolehi ialah 32.7, 28.4, 33.4 ng DNA/mg masing-masing. Kualiti DNA (nisbah A_{260}/A_{280} antara 1.9 to 2) adalah mencukupi untuk amplifikasi PCR dan jujukan DNA. Akan tetapi, kaedah Wizard merupakan pilihan untuk isolasi DNA dari tauhu lembut (13.2 ng DNA/mg sampel) dan susu soya (3.4 ng DNA/mg sampel) dengan kualiti DNA (nisbah A_{260}/A_{280} adalah 1.7 dan 1.9 masing-masing) yang tinggi perbandingannya.

Keputusan tinjauan menunjukan bahawa 20 daripada 24 sampel makanan haiwan mengandungi kandungan GM sekurang-kurang satu daripada tiga unsur-unsur genetik iaitu “promoter” (P35S), “terminator” (NOS) dan “structural gene” (EPSPS). Kesemua 16 sampel makanan haiwan dari Malaysia dan 4 daripada 8 makanan

haiwan dari Vietnam merupakan produk kontaminasi-GM. Sebaliknya, tiada sampel kacang soya (12 sampel) atau sample jagung (24 sampel) adalah positif dengan analisis tersebut. Oleh yang demikian, sampel tersebut boleh dikategorikan sebagai produk bukan GM.

Keputusan kajian menunjukkan bahawa kaedah amplifikasi PCR membekalkan kunci kelebihan dari segi sensitiviti, ketegapan dan operasi yang pantas sejurus membekalkan keperluan dalam rekabentuk eksperimen yang teliti untuk mengelakkan keputusan negatif-tiruan dan positif-tiruan. Lima pasang primer khasnya LEC1/LEC2; ZE03/ZE04; P35S 1-5'/P35S 2-3'; HA-NOS118-F/HA-NOS118-R dan EPSPS 1-5'/EPSPS 3-3' yang dipilih dalam kajian ini menghasilkan amplikons dengan pasangan bes sebanyak 164, 277, 101, 118 dan 118 masing-masing telah memenuhi syarat saiz-produk dan menyelesaikan keseluruhan kaedah pengesanan GMO untuk kacang soya mentah dan jagung mentah serta sampel haiwan makanan. Tambahan pula, protokol amplikasi PCR dan jujukan DNA yang disampaikan dalam kajian ini boleh dikatakan kaedah yang bersesuainan dalam pengesanan GMO harian khasnya makanan manusia dan haiwan tanpa syak atas keputusan negatif-tiruan dan positif-tiruan.

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Finally, I would like to dedicate my work as a moral gift to my parents and my “tiny” friend from Vietnam for their support and spiritual encouragements.

I certify that an Examination Committee met on 20th December 2004 to conduct the final examination of Nguyen Chau Thanh Tung on his degree thesis entitled "Comparison of DNA Extraction Methods in the Detection of Genetically Modified (GM) Foods using Polymerase Chain Reaction (PCR)" 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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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory 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 is has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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NGUYEN CHAU THANH TUNG

Date: 24 December 2004

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LIST OF ABBREVIATIONS

BLAST	Basic local alignment search tool
CaMV	Cauliflower mosaic virus
CTAB	Cetyl-trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EC	European Council
EDTA	Ethylene-diamine-tetra acetic acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
EtBr	Ethidium bromide
EU	European Union
GM	Genetically modified
GMO	Genetically modified organism
GMOs	Genetically modified organisms
NaOAc	Sodium acetate
NCBI	National Center for Biotechnology Information
P35S	35S promoter
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RR soybean	Roundup Ready soybean
SDS	Sodium dodecyl sulfate
TBE	Tris-Boric acid-EDTA buffer
UV	Ultraviolet

LIST OF SYMBOLS AND UNITS

bp	Base pair
β	Beta
°C	Degree Celsius
U	Enzyme unit
kb	Kilo-base pair
μg	Microgram
μL	Microliter
mg	Milligram
mL	Milliliter
mM	Millimolar
M	Molar
ng	Nanogram
OD	Optical density
%	Percent
pmol	Picomole
rpm	Revolutions per minute
vol	Volume
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Agricultural biotechnology has brought tremendous triumphs in the development of plants for the production of human food, animal feed and other products. In the few years since the commercial introduction of the first genetically modified product (FlavrSarv tomato), the cultivation of several transgenic crop species has increased rapidly by more than 25-fold, from 1.7 million hectares in 1996 (Roush, 2001) to 44.2 hectares worldwide in 2000 (Roush, 2001; Anklam *et al.*, 2002).

Genetically modified organisms (GMOs) contain specific traits which have been added to the organisms to improve their properties that have not occurred by mating or natural combination (Taverniers *et al.*, 2004; Anklam *et al.*, 2002). The addition of foreign genes has often been used in plants to produce novel protein that confer pest and disease tolerance and, more recently, to improve the chemical profile of process product, for example vegetable oils (Hemmer, 1997). Generally, the introduced gene works under the control of a promoter and a terminator. All of them (promoter, introduced gene and terminator) forms a gene construct and is inserted into the plants by means of biological, physical or chemical transformation methods. A genetically modified (GM) plant or product can thus be distinguished from its wild type counterpart by testing for the presence of the introduced DNA (Ahmed, 2002).

In European Union (EU) and other regions, the use of this technology, the consequent release of GMOs in the environment and the marketing of GMO-derived food products are strictly regulated (Huggett and Conzelmann, 1997; Anklam *et al.*, 2002).

The European Parliament and the Council of the EU have demanded in the Novel Food Regulation (EC/258/97, EC/1139/98, EC/49/2000, EC/50/2000 and EC/1829/2003), a clear and mandatory labeling of GMOs in food (Regulation EC, 1997; 1998; 2000; 2003). Because the Roundup-Ready soy bean and the Bt-176 maize were put on the market before Regulation EC/258/97 came into force, labeling of products derived from these GMOs is demanded by the separate Council Regulation EC/1139/98. On April 18th 2004, Regulation EC/1829/2003 on GM-food and feed came into force within the EU countries, regulating the placing on the market of such products and provided mandatory labeling thresholds of 0.9% for authorized GMOs and 0.5% for un-authorized GM events within the EU (Regulation EC, 2003). In addition, methods for specific detection of the most economically important GMOs are already available.

In contrast, Malaysian legislation is finalizing the enactment of food regulation for the presence of GMOs in animal feeds, human foods and ingredients. For this reason, there is a fundamental necessity to carry out a preliminary survey for the distribution of foods and feeds derived from GMOs and to establish an effective, accurate and reliable method for GM-events detection.

The objectives of this study are:

i. to conduct a preliminary survey on distribution of animal feeds and foods derived from GM events in both Malaysia and Vietnam;
ii. to optimize the DNA extraction methods for raw materials, animal feeds and processed foods;
iii. to optimize the Polymerase Chain Reaction (PCR) method for the detection of GM foods.

CHAPTER 2

LITERATURE REVIEW

2.1 Definition of Genetically Modified Organisms (GMO) and Typical Structure of a GMO

Genetically Modified Organisms (GMOs) can be defined as organisms in which the genetic materials (DNA) have been altered in a way that does not occur naturally by mating or natural combination, that is by being genetically modified (GM) or by recombination DNA technology (Anklam *et al.*, 2002). In other words, GM products contain at least an additional trait encoded by an introduced gene(s), which generally produce an additionalprotein(s) that confers the trait of interest (Ahmed, 2002).

A GMO is a living organism, for example a plant, yeast or an organism whose genetic composition has been altered by means of gene technology. The genetic modification usually involves insertion of a piece of DNA (the insert), a synthetic combination of several smaller pieces of DNA, into the genome of the organism to be modified (Gachet *et al.*, 1999; Holst-Jensen, 2001). This process is called transformation (Figure. 2.1).