CHAPTER 3

METHODOLOGY

3.1 Site geography

The study site, Sa-Meung district, is located in the western part of Chiang Mai province. Many crops and orchards are growing in this part due to its temperature. Cool moist nights coupled with cool, sunny days are suitable to grow many orchards in the most appropriate climate in northern of Thailand. Geologically this area is mostly hills and valleys. Only twenty percent of 626,250 rais is used for agricultural growing crops (Chiang Mai, 2004).

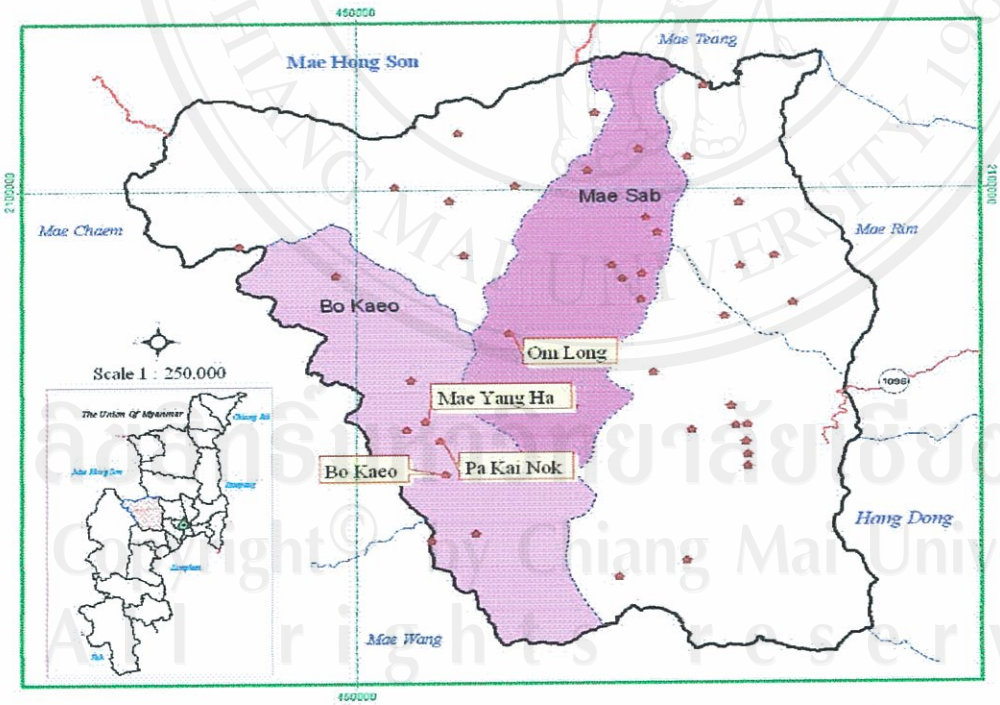


Figure 11 Map of villages and subdistricts in Sa-Mueng district, Chiang Mai province

There are 5 sub-districts; Bo-Kaeo, Mae-Sab, Yan Murn, Sa-Meung Nuer and Sa-Meung Tai subdistrict includes 44 villages. Population in this area is 22,981 people (11,831 males and 11,143 females), most of them are ethnic Karen, a hill tribe in northern Thailand. Only appoximately 4,000 Thais live in this area, especially in Bo-Kaeo and Om-Long subdistricts (National Statistic Organization, 2004).

One major source of income is from the export of strawberry, about two hundred million baht a year. Strawberries are generally grown in Sa-Meung district due to geographical area, climate and temperature. However, strawberries are extremely vulnerable to diseases and insect pests. The major diseases and biggest problems of strawberries growing are anthracnose, leaf spot, and phytoptera. Government officers noted that most farmers lack adequate knowledge, but still heavily load pesticide in their farms. However, the governor tried to use crop protection policy as a tool to attempt to reduce of highly toxic pesticide use.

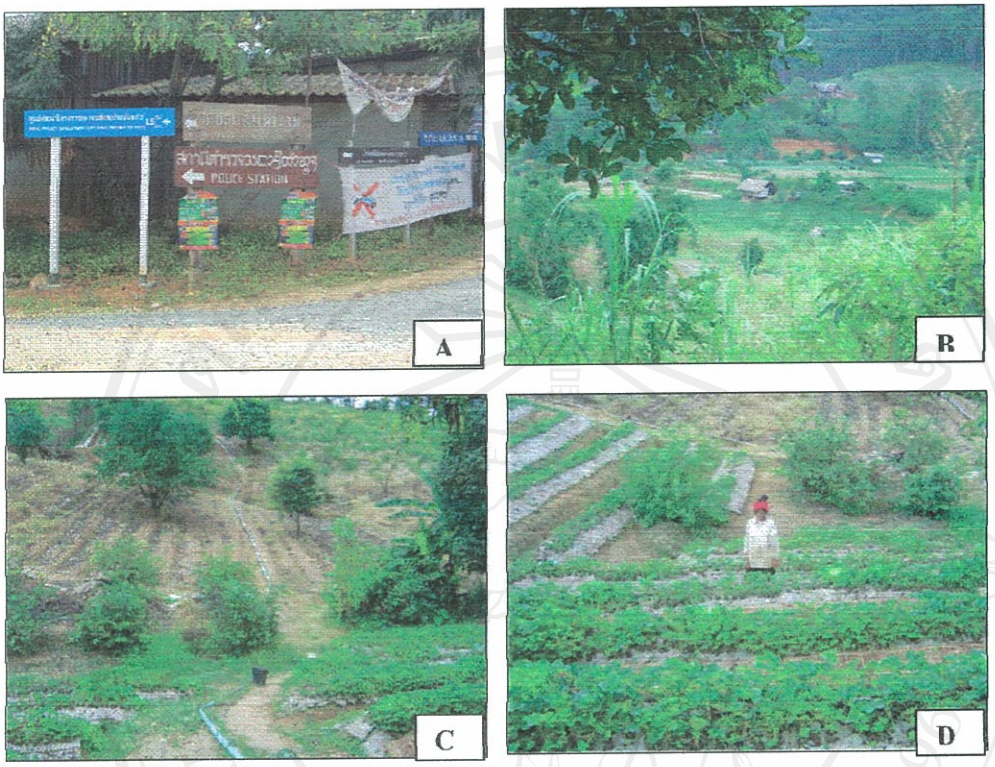


Figure12 Study site, Bo-Keaw subdistrict, July 17, 2003

A: Pesticides advertising posters are commonly found in this village.

B: Geological feature of this area is hills and valleys.

C: Soil preparation for strawberry cultivation

D: Cutivating strawberry in small area

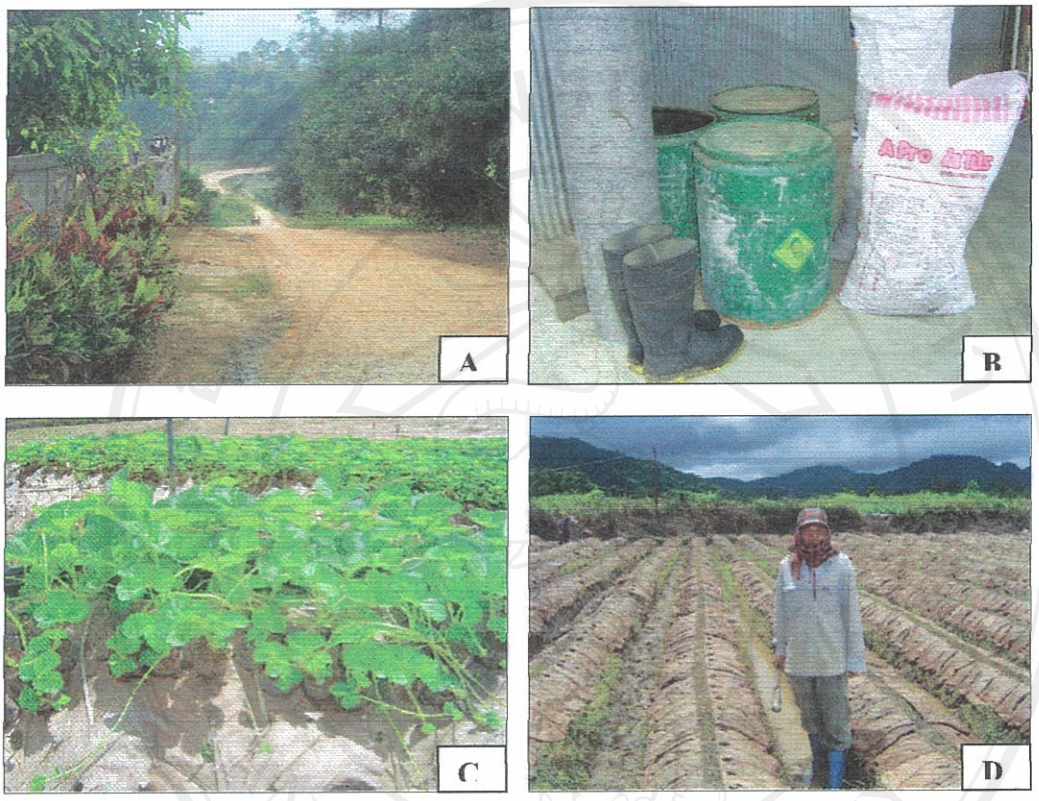


Figure 13 Study site, Bo-Keaw subdistrict, August 15, 2003

A: Geological feature of this area is hills and valleys

B: Pesticides and agrochemical storage in farmer's house.

C: Strawberry cultivation

D: Soil preparation for strawberry cultivation.

3.2 Study design

This study was prospective follow up study which considered genetic variants and cholinesterase and paraoxonase enzyme activity due to pesticide exposure. The study was started in November 2003 and conducted in Thai strawberry farmers who live in Sa-Meong district. The ethnic Thai strawberry farmers were invited to be volunteers. All subjects' behavior of pesticide use were investigated during growing and harvesting seasons. Paraoxonase and cholinesterase levels in their blood samples were considered as biomarker of pesticide exposure.

The first investigation was carried out on farming and pesticide spraying in growing season during November 2003. The second investigation was carried out in harvesting season in April 2004.

3.2.1 Sample size definition and calculation

Number of randomized subjects was calculated according to the formula:

$$n = \frac{Z^2\sigma^2}{E^2}$$ with confidence interval at 95%

Paraoxonase level and its genetic variants have not been reported in the Thai population. However, serum cholinesterase level is generally measured in several different Thai populations as pesticide exposure indicator.

By measurement of serum cholinesterase level in normal Thai people it was shown that the median of 503 Thai persons was 1,247 unit/L. Estimate of the standard deviation between individual Thai was 300 unit/L (Pongraveevongsa, 2000).

$$n = \frac{Z^2\sigma^2}{E^2}$$ with confidence interval at 95%

$$n = \frac{(1.96)^2(1247)^2}{(300)^2} = 66$$

n = number of subject

Z^2 = value of normal distribution at confidence interval at 95%

σ = variation in the population

E = estimation of variation, $E = |\bar{x} - \mu|$

The MM phenotype is rarely found in oriental populations. The study of PON1 gene has not been done in a Thai population, and this study is a pilot study of PON1 gene distribution. Sample size is calculated from the phenotype represented in the Chinese population. LL phenotype was found 92.8% (Sanghera, 1998).

$$n = \frac{Z^2 pq}{d^2} \quad \text{with confidence interval at 95\%}$$

$$n = \frac{(1.96)^2(0.928)(0.072)}{(0.05)^2} = 104$$

n = number of subject

Z^2 = value of normal distribution at confidence interval at 95%

p = Proportion of subjects who have LL phenotype

q = (1-p)

d = Acceptable range of error

However, the study design is a follow up study and some of subject will be lost during follow up. The suitable of sample size should be over 104 volunteers.

3.2.2 Subject inclusion criteria

The subjects must meet these criteria to be eligible for study enrollment :

1. Subjects must be strawberry farmers.
2. Subjects are willing to give their agricultural information as following
    a. Pesticide information, amounts of pesticide used and storage.
    b. Exposure during working in field and protective equipment
3. Subjects are willing to give information on health impacts of exposure.
4. Subjects permit the investigators to collect their blood samples 2 times for a total of 1.7-2.0 ml.
    a. The first time, a blood sample of 1.5 ml will be collected.
    b. The second time, a blood sample of 0.2 – 0.5 ml will be collected.
5. Subjects are capable of giving informed consent or have an acceptable surrogate capable of giving consent on the subject's behalf.

3.2.3 Duration of study

1 year from August 2003 –June 2004

3.3 Materials and Methods

3.3.1 Data collection of pesticide used in the farm

Information of pesticide used in the farm was collected from each individual subject. This section included pesticide exposure data (pesticide used, amounts, storage, disposal), use of protective clothing, spraying exposure, avoidance of fields during periods prescribed by the pesticide label. Following topics were collected from all subjects.

a. Pesticide information:

i. Brand name and Common name

ii. Type and WHO classification

iii. Chemical family

b. Amounts of pesticide used, storage and disposal

i. Quantify amount of pesticides used per unit of time

ii. Spraying frequency

iii. Contamination in water, food through their storage and disposal place.

c. Exposure during working in field and protective equipment

i. Route of pesticide entrance into the human body

ii. Protective clothing and equipment

iii. Practices and knowledge about handling during work.

iv. Health information: Signs and symptom before, during, after spraying.

Figure 14 Subject's data and blood collection on the study site

Fig. A, B: The process of informing volunteers about this research project. Fig C: Subjects signed agreement in consent form. Fig D: Document preparation and consent forms collection. Fig E: Blood collection for laboratory analysis. Fig F: Interviewing for farmer's pesticide use and cultivation information.

3.3.2 Measurement of Cholinesterase enzyme activity

A. Principle

The method for cholinesterase measurement is Ellman's method which is sensitive and simple. Choline and acetic acid are released and they are proportional to cholinesterase activity (Ellman, 1957). Cholinesterase was measured 2 times (at both the high and low pesticide use season). The method monitors the increase formation of 5-thio-2-nitrobenzoic acid (yellow reaction product). The method has been approved as an interim official first action by AOAC (Harlin 1990). Repeatability standard deviations (RSDr) ranged from 4.30 to 14.2%; reproducibility standard deviations (RSDR) ranged from 6.99 to 19.3%. The lower limit of detection was estimated to be 0.10 mumole/mL/min.

B. Equipments and Materials

1. 0.5 ml Microcentrifuge tubes, Oxygen company, USA.
2. 10 ml Pyrex glass test tubes, Union Science company, Thailand
3. Ice box timer and thermometer
4. pH meter, Beckman model 40, USA
5. Shaking incubator, GCA Precision model I7AE4, USA
6. Automatic pipette volume 100 ul and 200 ul, Pipetman model P100 and P200, Gilson company inc., USA
7. Centrifuge Machine, Sorvall, USA
8. Serological pyrex glass pipette volume 10 ml, Pyrex company, USA
9. Spectrophotometer, UV-PC model 2100, England
10. Refrigerator and Freezer -20 degree Celsius
11. Magnetic stirrer, Fisher , USA

C. Chemical Reagents

1. 500 mM Sodium dihydrogen phosphate (NaH_2PO_4), Merck, USA
2. 500 mM Disodium hydrogen phosphate (Na_2HPO_4), Merck, USA
3. 0.5 mM 5,5'- dithiobis-2-nitrobenzoic acid (DTNB), Sigma, USA prepared in 5 mM Phosphate buffer pH 7.4
4. 156 mM Acetylthiocholine iodide ($C_7H_{16}NO_2I$), Sigma, USA
5. 156 mM Butylrylthiocholine iodide ($C_9H_{20}NO_2I$), Sigma, USA
6. 12 mM Eserine ($C_{15}H_{21}N_3O_2$), Sigma, USA

D. Assay methods

1. Sample preparation

Blood samples were separated for red blood cell (Rbc) and plasma for measurement of acetylcholinesterase and butylrylcholinesterase enzyme activities by using refrigerated (4°C) centrifugation. Packed red blood cell and plasma samples were kept in microcentifuge tubes sealed with parafilm at -20°C until analysis.

Red blood cell was diluted 1:100 (red cell 10 ul in dH_2O 990 ul) in deionized water, while plasma was diluted 1:50 (plasma 20 ul in dH_2O 980 ul) in deionized water before analysis.

2. Chemical preparation

5 mM Phosphate buffer pH 7.4 was prepared by titration of 500 mM di-sodium hydrogen phosphate with 500 mM Sodium dihydrogen phosphate. DTNB concentration 0.5 mM (0.198 g) was prepared in 5 mM Phosphate buffer, pH 7.4 (10 ml) and made up to volume with deionized water (990 ml). Acetylthiocholine iodide (0.4512 g), butylrylthiocholine iodide (0.4948 g) and eserine(0.0778 g) were weighed and prepared in deionized water (10 ml).

3. Cholinesterase Analysis

Each sample was assayed for cholinesterase activist in duplicate test tubes and calculated for mean of activity. 3ml of DTNB were added in 3 test tubes as Blank tube, Test-1 and Test-2. Acetylthiocholine iodide (10 ul) or Butylrylthiocholine iodide (40 ul) was added in each tube for red blood cell and plasma analysis, respectively. Only 12 mM Eserine (50 ul) was added in Blank tube before assay as shown in Table 8 and 9. Three test tubes were incubated at 25°C in a shaking water bath for 10 minutes. Then 100 ul of diluted sample was added in each tube and incubated for 15 minutes. Reactions were stopped with 12mM Eserine. The absorbance was measured at 405 n.m. and calculated for enzyme activity as below.

$$\Delta Abs = AbsTest - AbsBlank$$

ΔAbs	=	*Absolute absorbance*
AbsTest	=	*Absorbance in Test tube*
AbsBlank	=	*Absorbance in Blank tube*

$$Activity = \frac{\Delta Abs}{13600} x \frac{TV}{SD} x \frac{1}{time}$$

Activity	=	*Cholinesterase activity (μmole/min)*
ΔAbs	=	*Absolute absorbance*
TV	=	*Total volume of enzyme analysis (ml)*
SD	=	*Sample dilution (ml)*
Time	=	*Incubation time (min)*

Table 8 Procedure of acetylcholinesterase activity measurement in red blood cell

Reagent	Blank tube	Test -1	Test-2
1. 0.5mM DTNB	3.0 ml	3.0 ml	3.0 ml
2. 156 mM AChI	10 ul	10 ul	10 ul
3. 12 mM Eserine	50 ul	-	-
Mixed and incubated at 25°C for 10 minutes			
4. 1:100 diluted red cell	100 ul	100 ul	100 ul
Mixed and incubated at 25°C for 15 minutes			
5. 12 mM Eserine	-	50 ul	50 ul
Mixed and measured absorbance at 405 n.m.			

Table 9 Procedure of butylrylcholinesterase activity measurement analysis in plasma

Reagent	Blank tube	Test -1	Test-2
1. 0.5mM DTNB	3.0 ml	3.0 ml	3.0 ml
2. 156 mM BuchI	10 ul	10 ul	10 ul
3. 12 mM Eserine	50 ul	-	-
Mixed and incubated at 25°C for 10 minutes			
4. 1:50 diluted plasma	100 ul	100 ul	100 ul
Mixed and incubated at 25°C for 15 minutes			
5. 12 mM Eserine	-	50 ul	50 ul
Mixed and measured absorbance at 405 n.m.			

2.0 ml blood sample collected from the venous blood vessel in heparinized tube

↓

Centrifuged at 1,500 rpm for 15 minutes

↓

Kept pack red blood cell and plasma at -20°C until analysis

↓

Thawed and diluted in deionized water

(1:100 for red blood cell, 1: 50 for plasma)

↓

Added 100 ul of diluted sample in 3 ml 0.5mM DTNB

+

Substrate

(10 ul of 156 mM AChI or 40 ul of 156mM BuChI)

↓

Incubated for 15 minutes in water bath at 25°C

↓

Stopped reaction with 50ul of 12mM Eserine

↓

Measured the absorbance at 405 n.m.

↓

Calculated enzyme activity

Figure 15 Flow chart of processes of acetylcholinesterase and butylrylcholinesterase enzymes activities measurement

3.3.3 Determination of cholinesterase genetic variants

A. Principle

Dibucaine and sodium fluoride are cholinesterase inhibitors. They can inhibit cholinesterase enzyme. Dibucaine and sodium fluoride number could be calculated and identify enzyme phenotype. The heterozygous of UU, UA and AA cholinesterase contain dibucaine number (DN) roughly 71-90, less than 20 and 40 -70, respectively (Kalow, 1957).

Sodium fluoride is another inhibitor used to delineate the FF phenotype, the resistant homozygous gene. FF cholinesterase activity is substantially low approaching zero. Howerever, determination of dibucaine number is still the best, most accurate, simplest and most widely used method for cholinesterase phenotype. Therefore, each sample was measured in triplicate of dibucaine and fluoride numbers. The intra-individual variation of these measurements should not more than 3% (Kalow, 1957; Goodall, 2004).

Table 10 Dibucaine and fluoride number in difference cholinesterase genetic variants (Kalow, 1957; Cattozzo, 1993)

ChE Genetic variants	Dibucaine number (DN)	Fluoride number (FN)
UU	≥77	≥55
UF	72-76	≥53
UA	48-72	≥44
AF	45-59	<44
FF	64-69	<44
AA	>35	-

Source: http://www.medal.org/adocs/docs_ch13/doc_ch13.13.html

B. Equipments and Materials

1. 0.5 ml Microcentrifuge tube size 0.5 ml, Oxygen company, USA
2. pH meter, Beckman model 40, USA
3. Ice box, timer and thermometer
4. Shaking incubator, GCA Precision model I7AE4, USA
5. Automatic pipette volume 100 ul and 200 ul, Pipetteman model P100 and P200, Gilson company inc, USA
6. Centrifuge Machine, Sorvall model , USA
7. Serological pyrex glass pipette volume 10 ml, Pyrex company, USA
8. Spectrophotometer, UV-PC model 2100, England
9. Refrigerator and Freezer -20 degree Celsius
10. Magnetic stirrer, Fisher , USA

C. Chemical reagents

1. 500 mM Sodium dihydrogen phosphate (NaH_2PO_4), Merck, USA
2. 500 mM Disodium hydrogen phosphate (Na_2HPO_4), Merck, USA
3. 0.5 mM 5,5'- dithiobis-2-nitrobenzoic acid (DTNB), Sigma, USA prepared in 5 mM Phosphate buffer pH 7.4
4. 156 mM Butylrylthiocholine iodide ($C_9H_{20}NO_2I$), Sigma, USA
5. 12 mM Eserine ($C_{15}H_{21}N_3O_2$), Sigma, USA
6. 0.3 mM Dibucaine ($C_{20}H_{29}N_3O_2$), Sigma, USA
7. 40 mM Sodium Fluoride (NaF), Sigma, USA

D. Assay method

1 Sample preparation

Plasma was separated from whole blood sample by refrigerated centrifugation and used to determine cholinesterase genetic variants. Plasma samples were kept in microcentrifuge tube sealed with parafilm at -20°C until analysis. Plasma was diluted 1: 50 (plasma 20 ul in dH_2O 980 ul) in deionized water before analysis.

2. Chemical preparation

100 ml of 500 mM di-sodium hydrogen phosphate was titrated with 500 mM sodium dihydrogen phosphate to obtain pH 7.4, then used as 10x phosphate buffer. DTNB concentration 0.5 mM (0.198 g) was prepared in 5 mM phosphate buffer, pH 7.4 (10 ml) and made up to volume with deionized water (990 ml).

Butylrylthiocholine iodide (0.4948 g) and eserine (0.0778 g) were weighed and prepared in deionized water (10 ml).

Cholinesterase inhibitors, 0.3mM dibucaine (0.687 g) and 40mM sodium fluoride (0.617 g) were also prepared in deionized water (100ml).

3. Genetic variants determination using dibucaine (DN) and fluoride numbers (FN)

3.1 Dibucaine number (DN)

Dibucaine number was determined by inhibition method and compared the inhibited enzyme level to uninhibited enzyme level. Three milliliter of 0.5mM DTNB and 50ul of butylrylthiocholine iodide were added into 5 test tubes which labeled as Blank, test-1, test-2, DN-1 and DN-2. 100ul of 0.3mM dibucaine was added to DN-1 and DN-2. Only 12 mM Eserine (50 ul) was added in Blank tube before analysis, then incubated at 25°C for 10 minutes in a shaking water bath. Then 100 ul of diluted sample were added. DN-1 and DN-2 were incubated for 3 minutes and the reaction was stopped, while Test-1 and Test-2 were incubated for 15 minutes and stopped with 12 mM Eserine as shown in Table 11. The absorbance was measured at 405 n.m. and calculated for enzyme activity as below (Kalow, 1957; Cattozzo, 1993).

$$DN = 1 - \left(\frac{\bar{x}AbsIn}{\bar{x}AbsUn} \right) x100$$

DN = *Dibucaine Number*

$\bar{x}AbsIn$ = *The average of absorbance in DN-1 and DN-2*

$\bar{x}AbsUn$ = *The average of absorbance in Test-1 andTest-2*

3.2 Fluoride number (FN)

Fluoride number was determined by inhibition method and the inhibited enzyme level was compared to uninhibited enzyme level. Three milliliter of 0.5mM DTNB and 50ul of Butylrylthiocholine Iodide were added into 5 test tubes labeled as Blank, test-1, test-2, FN-1 and FN-2. 100ul of 20mM sodium fluoride was added in FN-1 and FN-2. Only 12 mM Eserine (50 ul) was added in Blank tube before analysis, then incubated at 25°C for 10 minutes in a shaking water bath. 100 ul of diluted sample were added. FN-1 and FN-2 were incubated for 3 minutes and the reaction was stopped, while Test-1 and Test-2 were incubated for 15 minutes and stopped with 12 mM Eserine as shown in Table 12. The absorbance was measured at 405 n.m. and calculated for enzyme activity as below (Kalow, 1957; Cattozzo, 1993).

$$FN = 1 - \left(\frac{\bar{x}AbsIn}{\bar{x}AbsUn} \right) x100$$

FN = *Fluoride Number*

$\bar{x}AbsIn$ = *The average of absorbance in FN-1 and FN-2*

$\bar{x}AbsUn$ = *The average of absorbance in Test-1 andTest-2*

Table 11 Determination of Dibucaine Number (DN) in plasma

Reagent	Blank tube	Test-1, 2	DN-1,2
1. 0.5mM DTNB	3.0 ml	3.0 ml	3.0 ml
2. 156 mM BuchI	10 ul	10 ul	10 ul
3. 12 mM Eserine	50 ul	-	-
4. 0.3 mM Dibucaine	-	-	100 ul
Mixed and incubated at 25°C for 10 minutes			
4. 1:50 diluted plasma	100 ul	100 ul	100 ul
Mixed and incubate at 25°C	15 minutes		3 minutes
5. 12 mM Eserine	-	50 ul	50 ul
Mixed and measured absorbance at 405 n.m.			

Table 12 Determination Method of Fluoride Number (FN) in plasma

Reagent	Blank tube	Test-1, 2	FN-1,2
1. 0.5mM DTNB	3.0 ml	3.0 ml	3.0 ml
2. 156 mM BuchI	10 ul	10 ul	10 ul
3. 12 mM Eserine	50 ul	-	-
4. 0.3 mM Dibucaine	-	-	100 ul
Mixed and incubated at 25°C for 10 minutes			
4. 1:50 diluted plasma	100 ul	100 ul	100 ul
Mixed and incubate at 25°C	15 minutes		3 minutes
5. 12 mM Eserine	-	50 ul	50 ul
Mixed and measured absorbance at 405 n.m.			

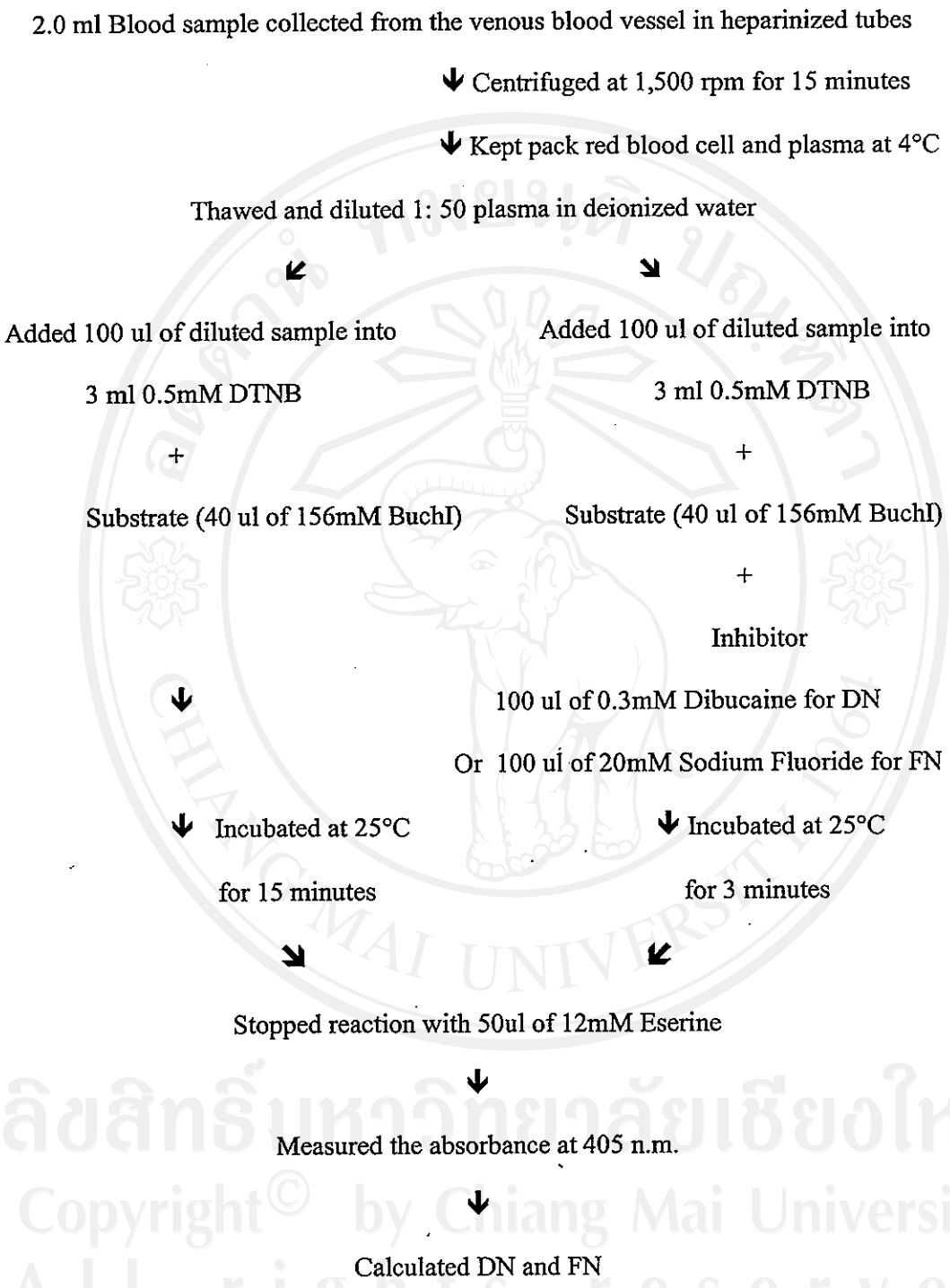


Figure 16 Flow chart of processes of Dibucaine number (DN) and Fluoride number (FN) determination.

3.3.4 Measurement of paraoxonase enzyme activity

A. Principle

The method of paraoxonase enzyme activity determination is measuring of p-nitrophenol as product of paraoxon hydrolysis. The rate of paraoxon hydrolysis was monitored for p-nitrophenol formation by its absorbance at 405 n.m. at 37°C.

B. Equipments and Materials

1. 0.5 ml Microcentrifuge tubes, Oxygen company, USA
2. Ice box, timer and thermometer
3. pH meter, Beckman model 40, USA
4. Shaking incubator, GCA Precision model I7AE4, USA
5. Automatic pipette volume 100 ul and 200 ul, pipetman model P100 and P200, Gilson company inc., USA
6. Centrifuge Machine, Sorvall, USA
7. Serological pyrex glass pipette volume 10 ml, Pyrex company, USA
8. Spectrophotometer, UV-PC model 2100, England
9. Refrigerator and freezer -20 degree Celsius
10. Magnetic stirrer, Fisher, USA

C. Chemical reagents

1. 0.132M Tris-hydrochloric acid (Tris-HCl), Gibco, USA
2. 1.32 mM Calcium chloride ($CaCl_2$), Merck, USA
3. 1M Sodium chloride (NaCl), Merck, USA
4. 120mM diethyl-p-nitrophenyl phosphate or Paraoxon ($C_{10}H_{14}NO_6P$), Sigma, USA

D. Assay method

Assay buffer was 0.132 M Tris–HCl pH 8.5, 1.32 mM $CaCl_2$ and 1 M NaCl. For each set of assays, 6 mM freshly prepared paraoxon substrate solution of 120 mM paraoxon in acetone diluted with 0.132 mM Tris–HCl was used. The assay tube contained 760 ml Tris buffer, 40 ml serum (1:2 diluted with water) and 200 ml 6 mM paraoxon. The reaction was initiated at 37°C by the addition of the substrate solution, and absorbance was continuously monitored at 405 nm. A molar extinction coefficient of 14050 was obtained and used for calculations of activity, and units were expressed as micromoles of paraoxon hydrolysed per minute. Paraoxonase enzyme activity was calculated as follows (Akgur, 2003).

$$PON = \varepsilon(Abs_{t60} - Abs_{t0})$$

PON = *Paraoxonase enzyme activity*

ε = *Extinction Coefficient (14050)*

Abs_{t60} = *Absorbance monitored at 60 second as test*

Abs_{t0} = *Absorbance monitored at 0 second as blank*

1. Sample preparation

Plasma was separated from whole blood sample by centrifugation and used to determine cholinesterase genetic variants. Plasma samples were kept in microcentrifuge tube sealed with parafilm at -20°C until analysis. Plasma was diluted 1: 2 (plasma 100 ul in dH2O 100 ul) in deionized water before analysis.

2. Chemical preparation

Tris buffer contained 0.132 M Tris HCl (5.202 g), 1.32mM CaCl2 (0.036g) and 1M NaCl (14.5 g), weighed and prepared in deionized water (250ml). 6 mM Paraoxon , the substrate of paraoxonase, was freshly prepared in each assay set by diluted stock paraoxon (120mM prepared in acetone) in 0.132mM Tris-HCl.

3. Paraoxonase activity measurement

Assay tube was mixture of 760 ul of Tris-buffer, 40 ul of 1:2 diluted serums and 200 ul of 6mM freshly prepared paraoxon. The mixture was incubated in the cuvette chamber at 37°C for 1min and absorbance was monitored at 60 second as Abs_{t0} and 120 second as Abs_{t60} . Paraoxonase activity was calculated.

2.0 ml Blood sample collected from the venous blood vessel

↓ Centrifuged at 1,500 rpm for 15 minutes

Kept pack red blood cell and plasma at 4°C until analysis

↓

Thawed and diluted 1: 2 plasma in deionized water

↓

40 ul of diluted plasma

+

760 ul of Tris HCl

+

200 ul of 6mM freshly prepared Paraoxon

↓

Mixed by inverting tube

↓

Incubated in cuvette chamber at 37°C for 1 minute

↓

Monitored absorbance at 405 n.m. with in 1 minute

↓

Calculated for paraoxonase activity

Figure 17 Flow chart of the processes of paraoxonase activity measurement

3.3.5 Determination of paraoxonase genetic polymorphism

A. Principle

The genetic polymorphims of the inter-individual variability of paraoxonase enzyme activity has recently been attributed to the presence of a glutamine (Q) and arginine (R) substitution at amino acid position 192 and a leucine (L) and methionine (M) substitution at amino acid on position 55 of the PON1 protein (Mackness 1998). The genetic variants of PON1 gene were determined by using Polymerase Chain Reaction (PCR) technique.

There are three major steps, denaturing, annealing and extension steps, in PCR technique, which are repeated for 30 or 40 cycles. This is done on an automated thermocycle machine, which can heat and cool the tubes with the reaction mixture in a very short time (Coyne 2001).

1. Denaturing step, during the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop.
2. Annealing step, the ionic bonds are formed between the single stranded primer and the single stranded template. Polymerase can attach and starts copying the template.
3. Extension step, the bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' sides, bases are added complementary to the template).

The standard PCR protocol recommended reagent concentrations as following:

1. Primers : 0.2 - 1.0 uM
2. Nucleotides : 50 - 200 uM each dNTP
3. Tag polymerase : 0.5 - 1.0 Units/50ul of reaction volume
4. Target DNA (sample) : 1 ng - 1 ug
5. 10x Tag Buffer : use proprietary or home made. Buffer should contain the minimum of 1.5mM Mg^{2+}.

The recommended reaction conditions

1. Initial conditions: initial denaturation at start: 92 - 97°C for 3 - 5 min.
2. Initial annealing temperature: as high as feasible for 3 min (50 - 75°C).
3. Initial elongation temperature: 72°C for 3 - 5 min. This allows complete elongation of product on rare templates.

Temperature Cycling: Appropriate cycles are 25-35 cycles for these three steps.

92 - 94°C for 30 - 60 sec (denature)

37 - 72°C for 30 - 60 sec (anneal)

72°C for 30 - 60 sec (elongate)

Then, initial elongation temperature to 72oC for 5 min at end to allow for complete elongation of all DNA products.

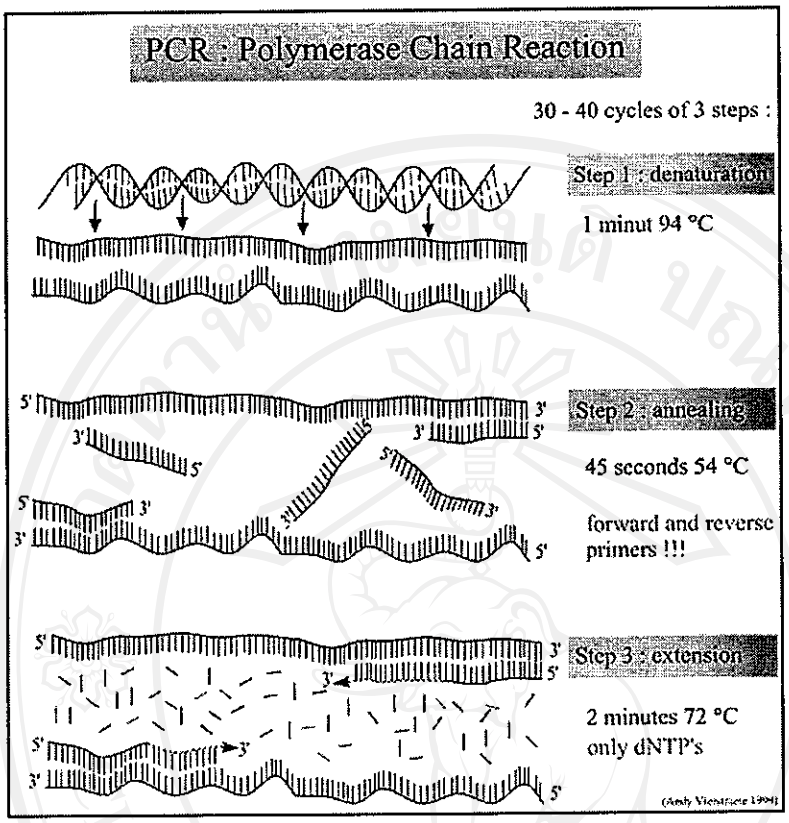


Figure 18 The importance 3 steps in Polymerase Chain Reaction (Andy 1999)

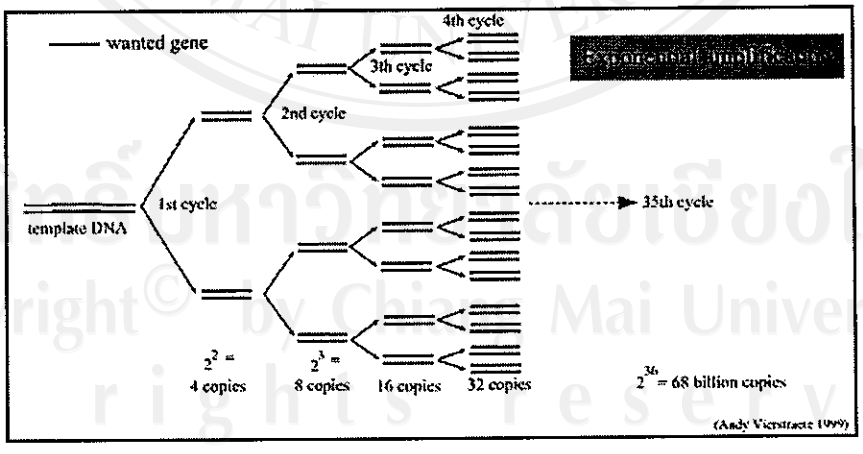


Figure 19 The exponential amplification of PCR (Andy 1999)

Because both strands are copied during PCR, there is an exponential increase of the number of gene copies. The number of copied gene will be 2^n (n is the number of cycle). There will be 2, 4, 8 and 16 copies of wanted gene after 1, 2, 3 and 4 cycle of PCR, respectively (Andy 1999).

Although, the interested gene on PON1 are copied, but the genetic variants still be not presented in this step. Restriction enzyme will be used as a scissor to cut the specific sequences of DNA bases and breaks in sugar-phosphate on the recognized DNA region. The Restriction Fragment Length Polymorphism (RFLP) will be present on acrylamide gel, the RFLP could be said to be polymorphism in the population.

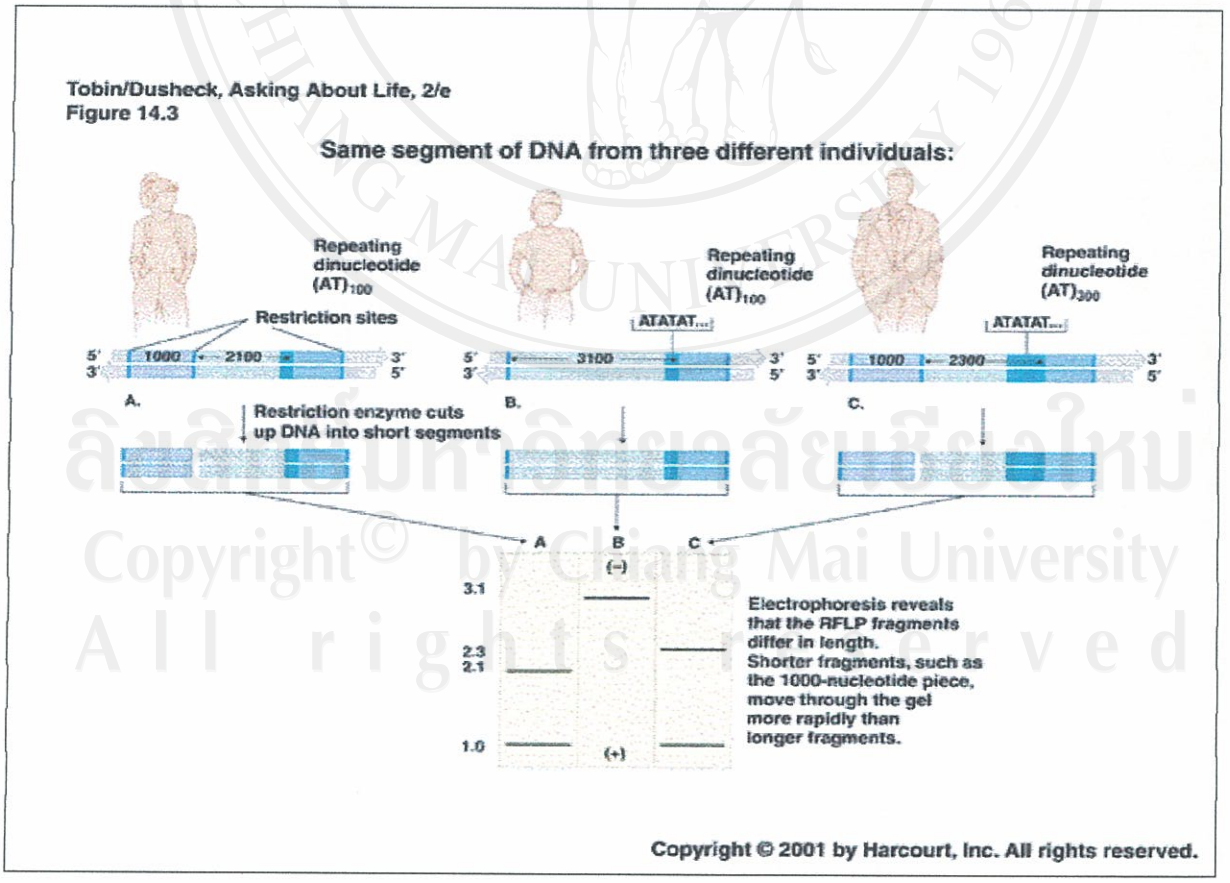


Figure 20 The RFLP map of the different individuals

B. Equipments and Materials

1. Analytical balance, Metler, USA
2. Automatic pipette volume 10, 20 and 100 ul, pipetman model P10, P20 and P100, Gilson company inc., USA
3. 0.2 ml Domed PCR tubes, Oxygen Company, USA
4. 1.5 ml Microcentrifuge tubes, Oxygen Company, USA
5. Centrifuge Machine, Sorvall, USA
6. Microcentrifuge Machine, Eppendorf Company, USA
7. Freezer and Refrigerator
8. Thermocycle, GeneAmp 9700, USA
9. Glove and Mask
10. Water bath with timer, Precision, USA
11. Mini gel electrophoresis, Qiagen Company, USA
12. Acrylamide Gel Electrophoresis set with power supply, Biorad, USA
13. Gel doc Camera,

C. Chemical reagents

1. Agarose gel, Molecular grade, Invitrogen Company, USA
2. 29:1% Acrylamide gel for molecular laboratory, Biorad company, USA
3. Ultrapure Water
4. Trizma base, Analytical grade, Fisher, USA
5. EDTA
6. Boric acid (H_3BO_3), Analytical grade, Sigma, USA
7. Xylene cyanol, Analytical grade, Sigma, USA
8. Ethidium Bromide (), Sigma, USA
9. Designed primer for PON192 and PON55
10. dNTPs set, Invitrogen company, USA
11. Restriction enzyme MboI from New England Laboratory (NEL), Qiagen company, Japan
12. Restriction enzyme Nla III from New England Laboratory (NEL), Qiagen company, Japan
13. Tag polymerase enzyme with 10x Tag buffer, Hot Start Tag, Qiagen company, Japan
14. 100% Glycerol
15. Flexi gene DNA kit, Qiagen company, Japan

D. Assay method

1. Sample preparation

50 ul of heparinized blood sample was collected into 1.5 ml microcentrifuge tube. DNA was isolated and purified by Flexigene DNA kit from Qiagen company, then 50 ul of individual DNA was obtained. Extracted DNA samples were kept in refrigerator at 4°C until analysis.

2. Chemical preparation

2.1 Reagent for master mix preparation

The required mixtures for amplification of interested gene are primer, dNTPs, Tag polymerase, buffer and water.

(1) 1mM dNTPs : The stock dNTPs, 100mM of dNTPs (10ul per each of ATP, TTP, CTP and GTP) were diluted to 1 mM in ultrapure water (Type I) and kept in 1.5 ml sterilized microcentrifuge tube at -20°C.

(2) 5uM Primer Mix: Primers were designed and synthesized as the following sequence primer below.

PON192F sequence (5 to 3')

TAA TCC TGT AAT GTT CAA TAC CTT CAC C

PON192R sequence (5 to 3')

TTT CAG AGA GTT CAC ATA CTT GCC ATC G

PON55 F sequence (5' to 3')

ACC TAT TAA AGA AGA GGT ATG TAT AGC C

PON55 R sequence (5' to 3')

CAA TGT AGA CCG AAG AAC ACA AAT ATG C

50uM Synthesized Primers (forward and reverse primer) were diluted as 5 uM Primer Mix in ultrapure water (Type I) and stored in sterile tube at -20°C.

(3) 5x TBE buffer: TBE buffer was used in Acrylamide, Agarose gel preparation and used for electrophoresis running. The 5x TBE buffer was contained 8.9M Trizma (26.94g), 8.9M H_3BO_3 (13.76g) and 2M EDTA (1.86g) in deionized water (500 ml).

2.2 Reagent for gel electrophoresis

(1) 0.2%Agarose gel preparation for electrophoresis: The DNA fragments were approximately 300-400 bp, which 2% agarose gel will resolve well. 2 g of agarose gel were prepared in 100 ml ultrapure water, and mixed well and microwaved for about 1 minute to dissolve the agarose. It was left to cool on the bench for 5 minutes down to about 60°C to minimize production of ethidium bromide vapour. Then 1μL of ethidium bromide (10mg/mL) was added and swirled to mix. Pour the gel slowly into the tank and insert the comb and double check that it is correctly positioned. Leave to set for at least 30 minutes, preferably 1 hour, with the lid on if possible. Pour 0.5x TBE buffer into the electrophoresis gel tank as the running buffer to submerge the gel to 2–5mm depth.

(2) 8% Acrylamide gel : The digested DNA fragments were approximate 100-300 bp, which acrylamide gel will show the good resolution of DNA fragment with the.8% Acrylamide gel was prepared before loading on gel template. 4ml of 29:1 acrylamide-bis solution were mixed well with 4 ml of dH2O, 2ml of 5x TBE, 3.5 ul of TEMED and 70 ul of 10%APS. Leave to set for at least 30 minutes, rinse the gel wells with 1x TBE for 3 times. Pour 1x TBE buffer into the electrophoresis gel tank as the running buffer to submerge the gel to 5mm depth.

2.3 Loading dye for electrophoresis gel running

The loading buffer gives color and density to the sample to make it easy to load into the wells. It also allows you to monitor the progress of the gel. The most common and generally used dyes in PCR lab are bromophenol blue and xylene cyanol. Both of dyes were mixed with glycerol. 0.25%xylene cyanol (700ul) were mixed with 30% glycerol (300ul) and stored at 4°C.

2.4 Markers and DNA ladders

The expected sizes of amplicons in this study were 280 (codon 192) and 384 bp (codon 55), while the restriction fragment length polymorphism was approximately 100 – 261 bp. The appropriate marker should be the low molecular weight DNA ladder and 100 bp DNA ladder. Each marker (10ul) was mixed with loading dye (30ul) and stored at 4°C.

3. Assay method for genetic polymorphism of PON192

The DNA fragment was amplified by a PCR method by using a sense primer (5' TAA TCC TGT AAT GTT CAA TAC CTT CAC C 3') and an antisense primer (5' TTT CAG AGA GTT CAC ATA CTT GCC ATC G 3') with genomic DNA as a template. The amplified reactions were performed in a 20 ul volume containing 100 uM of dNTPs, 0.25uM of primer mix, 3mM MgCl2 and 0.25 unit of Tag polymerase and 2 ul of DNA sample. Amplification was carried out in a Gene Amp 9700, the DNA thermal cycle was designed as following

Denaturation at 94°C for 5 min

Annealing at 94°C for 30 sec

55°C for 45 sec

72°C for 2 min

40 cycles of annealing

Extension at 72°C for 5 min

4 ul of amplicon (280 bp) was digested with 1Unit of ***Mbo I***, restriction enzyme for overnight and detected on 8% acrylamide gel with ethidium bromide staining. The DNA fragments were visualized under the UV transilluminator. Bands were estimated with 50 bp of DNA ladder.

4. Assay method for genetic polymorphism of PON55

The DNA fragment was amplified by a PCR method by using a sense primer (5' ACC TAT TAA AGA AGA GGT ATG TAT AGC C 3') and an antisense primer (5' CAA TGT AGA CCG AAG AAC ACA AAT ATG C 3') with genomic DNA as a template. The amplified reactions were performed in a 20 ul volume containing 100 uM of dNTPs, 0.25uM of primer mix, 3mM MgCl2 and 0.25 unit of Tag polymerase and 2 ul of DNA sample. Amplification was carried out in a Gene Amp 9700, the DNA thermal cycle was designed as following

Denaturation at 94°C for 5 min

Annealing at 94°C for 30 sec

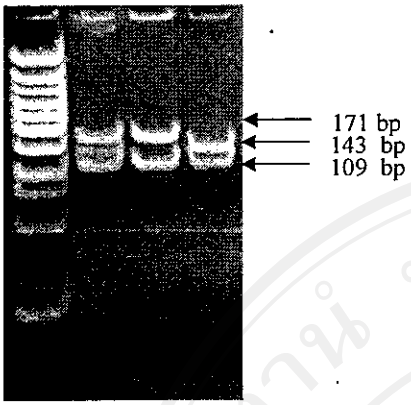
55°C for 45 sec

72°C for 2 min

40 cycles of annealing

Extension at 72°C for 5 min

4 ul of amplicon (384 bp) was digested with 1Unit of ***Nla III***, restriction enzyme for overnight and detected on 8% acrylamide gel with ethidium bromide staining. The DNA fragments were visualized under the UV transilluminator. Bands were estimated with 100 bp of DNA ladder.



RFLP map for PON 192

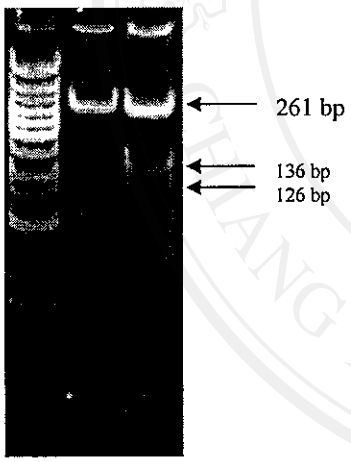
Lane

1. 50bp DNA ladder

2. QR phenotype (171, 143 and 109 bp)

3. QQ phenotype (171 and 109 bp)

4. RR phenotype (143 and 109 bp)



RFLP map for PON 55

Lane

1. 50bp DNA ladder

2. LL phenotype (261 bp)

3. LM phenotype (261, 136 and 126 bp)

Figure 21 The RFLP map for PON1 loci 192 and loci 55 determination