CHAPTER 2
MATERIALS AND METHODS

2.1 Chemicals and instruments are in appendix A

2.2 Preparation of Stevia extract mixed in Matoom juice

Stevia extract mixed in Matoom juice was prepared at Faculty of Pharmacy, Chiang Mai University, Thailand. (See the preparation method in appendix C)

2.3 Animals

Male Wistar rats (weight 80-100 g) were purchased from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The rats were divided into seven groups of eight animals in each group and were housed two or three rats per cage. The temperature (20-22^{0}C), humidity (50±10%) and lighting (12 h day/night cycle) were constantly controlled. The rats were acclimated one week before the beginning of the experiment.

2.4 Effects of Matoom juice containing Stevia extract as sweetener on Aberrant Crypts Foci (ACF) formation

The protocol of the study of Stevia extract on ACF formation at initiation stage was shown in Figure 8. Male Wistar rats were randomly divided into seven groups of 8 rats each. Group 1 was administrated with water by gavage as a normal control. Group 2 was injected s.c. with azoxymethane (AOM) at 15 mg/kg bw. Once a week for 2 weeks and received water by gavage (positive control). Group 3, 4, 5, 6 and 7 were fed daily with Matoom juice, 0.2%w/v Stevia extract in Matoom juice, 1.0%w/v Stevia extract in Matoom juice, 10.0%w/v Stevia extract in Matoom juice and 10.0%w/v Stevia in water at 3.0 ml/kg bw., respectively. After 35 days of administration, all rats were sacrificed.

The protocol for studying the effect of Stevia extract mixed in Matoom juice at promotion stage was shown in Figure 9. The rats were divided and administered Stevia in Matoom juice as protocol of initiation stage study. Group 2 received AOM at 20 mg/kg bw. s.c. injection once at the first week as positive control to induce ACF formation. All rats were sacrificed after 100 days of the treatment. The colons were expanded by injecting with 10% formalin in PBS, Blood, livers and small intestines were immediately removed and kept at -80°C until used.

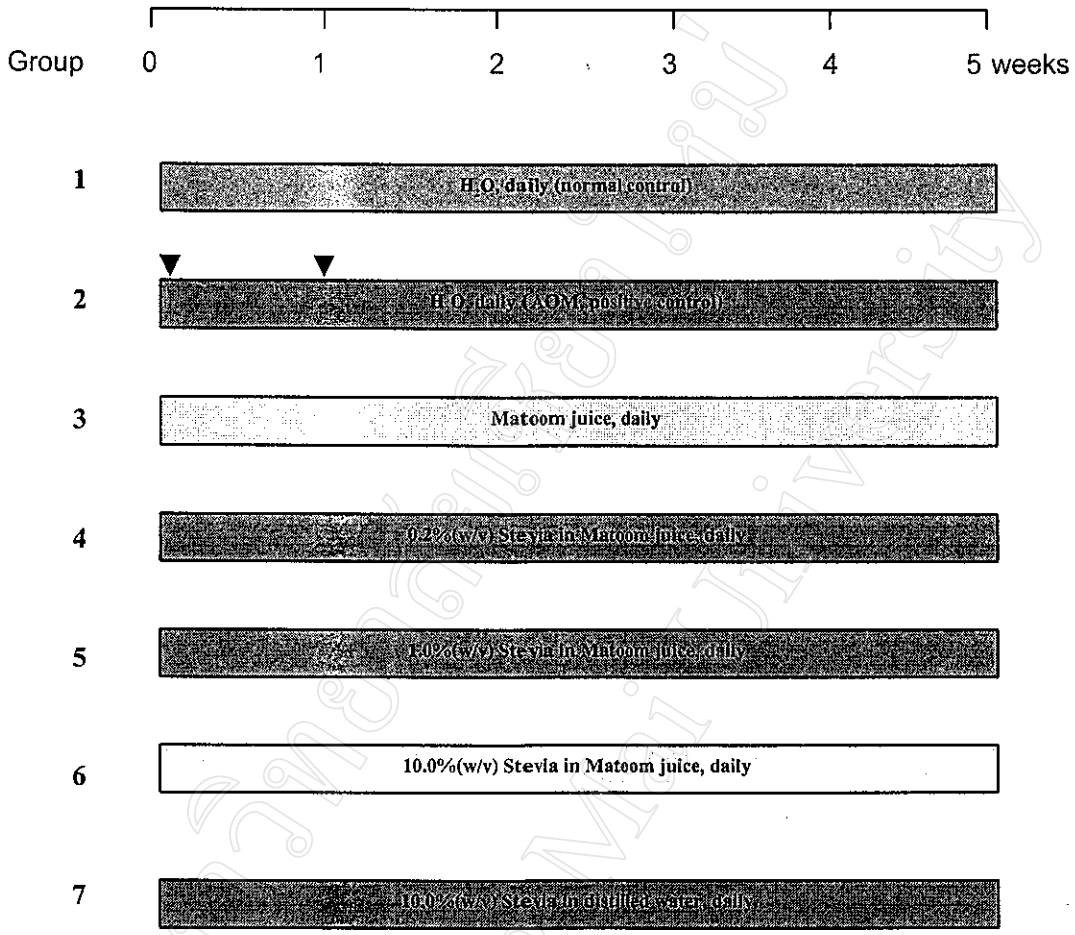


Figure 8. Experiment schedule to study the effects of Mattom juice containing Stevia extract as sweetener on ACF formation in initiation stage

▼ = AOM 15 mg/kg.bw. s.c.

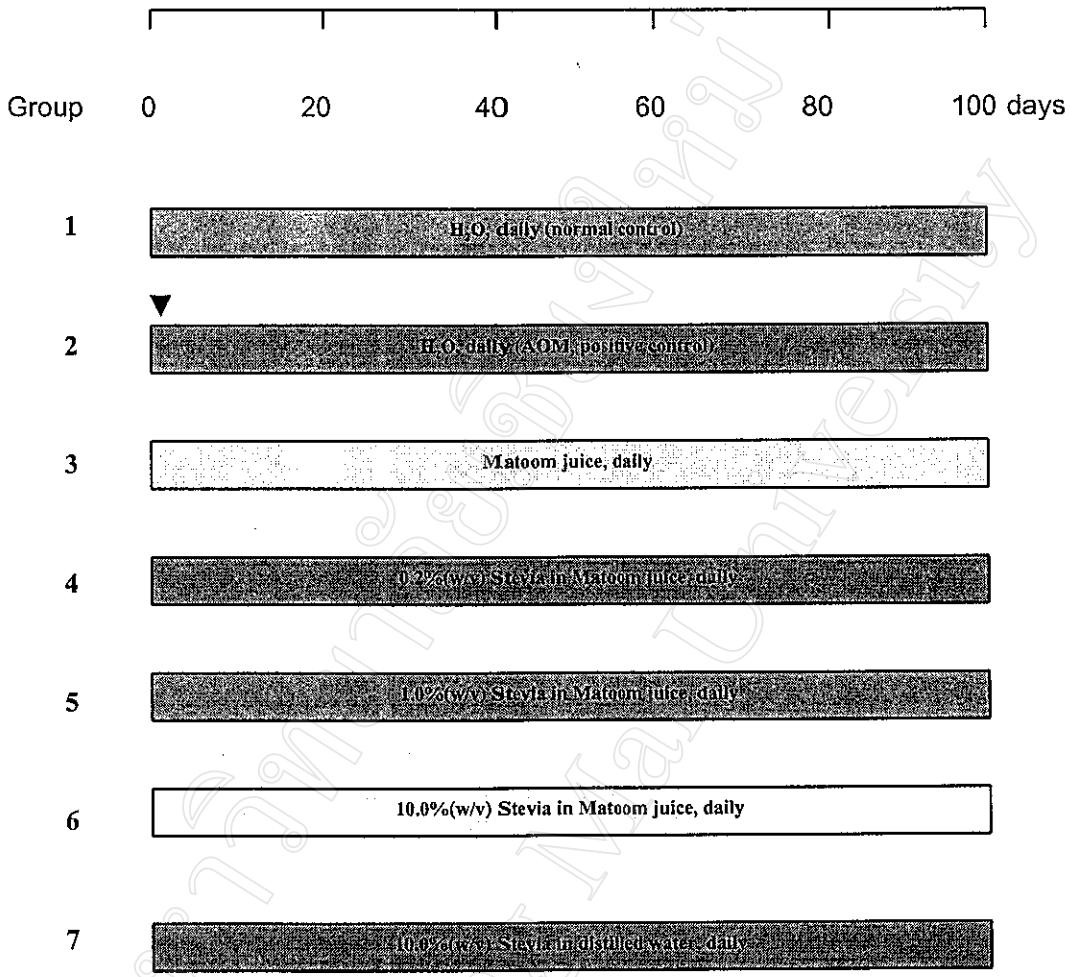


Figure 9. Experiment schedule to study the effects of Mattom juice containing Stevia extract as sweetener on ACF formation in promotion stage

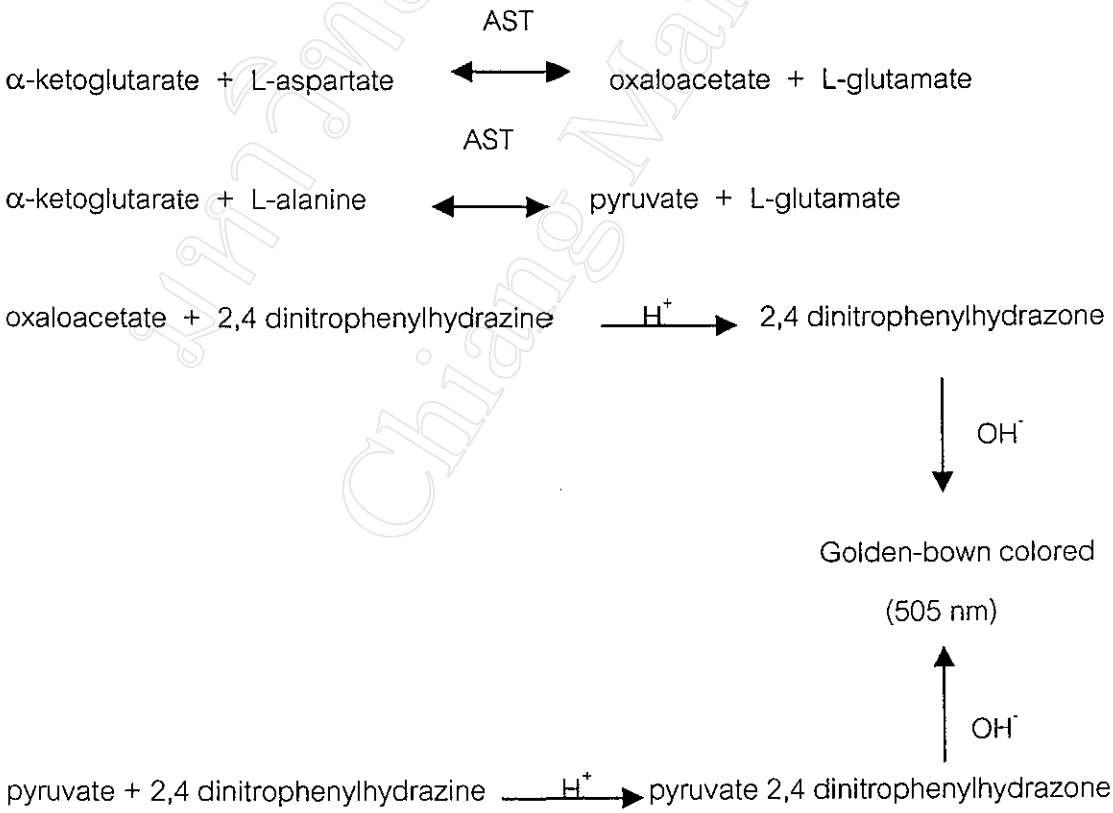
▼ = AOM 20 mg/kg.bw. s.c.

2.5 Aberrant crypt foci (ACF) assay

The colon were evaluated for aberrant crypt foci formation as described by Bird (Bird, 1987). The colons were removed, expanded with 10% formalin in a phosphate buffered saline solution (pH 7.4) on ice for 15 minutes, then split open along the longitudinal axis. The colons were cut into three part of proximal, distal and rectum. Each segment were fixed flat between filter papers and fixed in 10% formalin – PBS pH 7.4 for 24 hours. The ACF was determined after staining with 0.2% methylene blue under a light microscope at a magnification of 10X.

2.6 Determination of serum transaminase activity

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured according the method described by Reitman S and Franlxel SA (Reitman S and Franlxel SA, 1957). The protocol for AST and ALT activities assay are shown in Figure 10, 11. The Serum was first incubated with a substrate solution. The AST substrate contained with α-ketoglutarate and aspartate and the ALT substrate contained with α-ketoglutarate and alanine. The enzymatic reaction was stopped by add with 2,4 dinitrophenylhydrazine in acid and formation of 2,4 dinitrophenyl hydrazone was begun. After sufficient color has been developed, sodium hydroxide is added to convert the 2,4 dinitrophenylhydrazone to a golden brown color. The solution was measured as the increase in absorbance at 505 nm, and the concentration of the enzyme determined from the standard curve. The reaction of the enzyme determination was shown as follows:



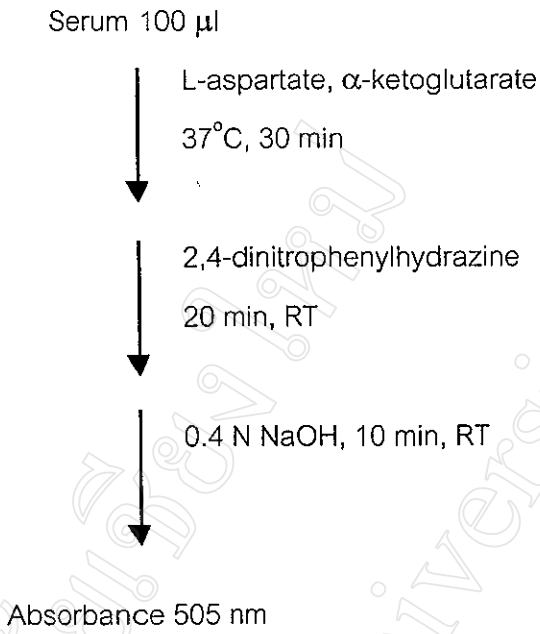


Figure 10. Protocol of aspartate aminotransferase (AST) activity determination

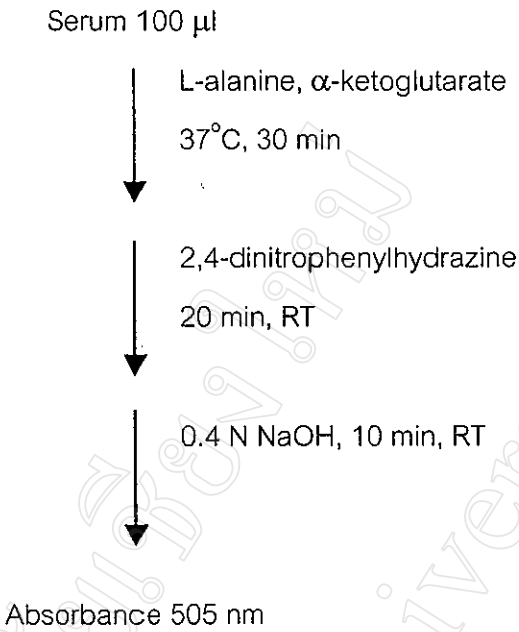


Figure 11. Protocol of alanine aminotransferase (ALT) activity determination

2.7 Preparation of cytosolic fraction.

All procedures were performed at a temperature ranging from 0 to 4°C. The livers were purfused with normal saline and minced. The intestines were slit opened and mucosa layers were collected by scrapings with the edge of a glass slide. The mucosal scrapping and minced livers were homogenized with a Potter homogenizer fitted with a teflon pestle in 3 vol. ice cold 0.05 M Tris buffer (pH 7.4) containing 1.15% w/v KCl. The homogenate was centrifuged at 9,000 g for 30 min. The supernatant was subsequently centrifuged at 105,000 g for 60 min by ultracentrifuge. The supernatant was the cytosolic fraction, aliquoted and stored at -80°C until analyzed for GSH content, GST activity and GST subunit determination. The scheme of the preparation of the cytosolic fraction is shown in Figure 12. The cytosolic protein concentration was determined by Bradford's method (Bradford, 1976) as shown in Figure 13.

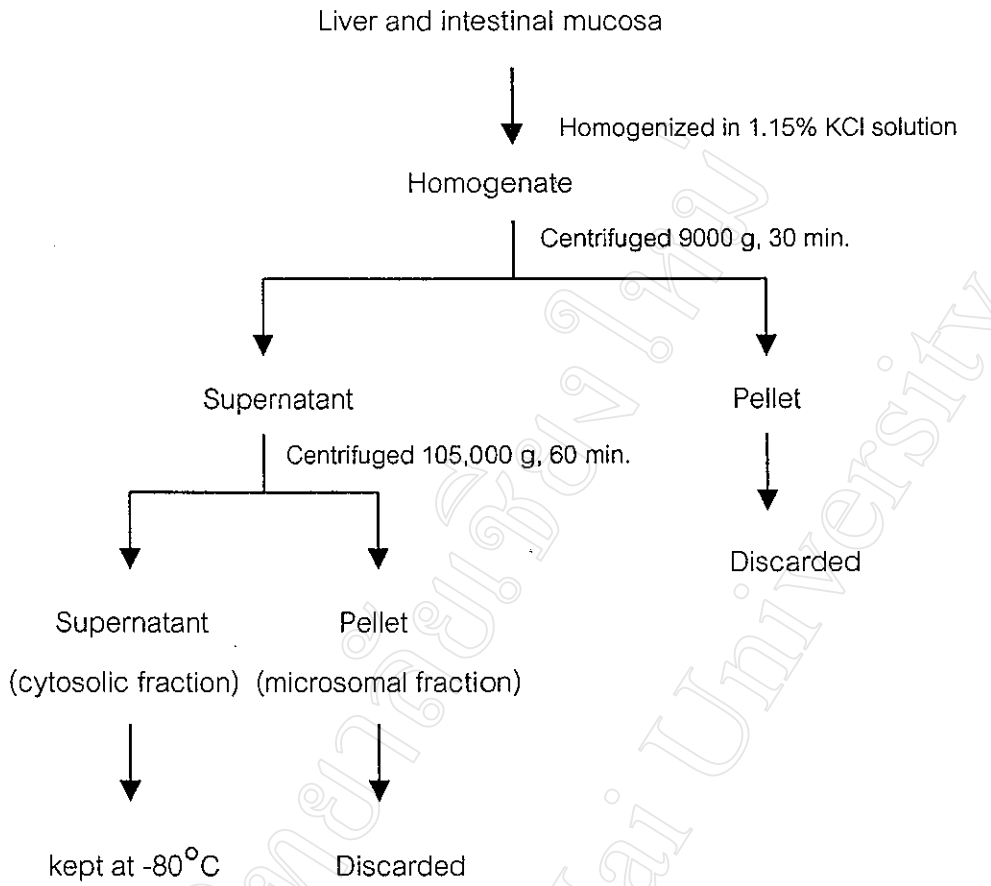


Figure 12. Scheme of cytosolic fraction preparation

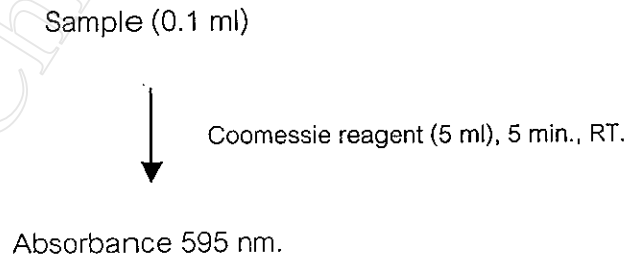


Figure 13. Scheme of cytosolic protein determination

2.8 Reduced glutathione assay

The protocol of sample preparation to determine reduced glutathione is shown in Figure 14. The cytosolic fraction was treated by addition of an equal volume of 2 M $HCLO_4$ and 2 mM EDTA, then centrifuged at 5000 g for 5 min. to remove the protein. The supernatant liquid contained the soluble components (GSH, GSSG and acid-soluble mixed disulfides). The deproteinized extract was neutralized with the solution containing 2 M KOH and 0.5 M N-morpholinopropanesulfonic acid (MOPS) and assayed immediately with method of Theodorus P.M. *et al* (Theodorus P.M. *et al*, 1981). Reduced glutathione is specifically converted by glyoxalase I according the reaction:

$$\text{GSH + methylglyoxal} \xrightarrow{\text{Glyoxalase I}} \text{S-lactoyl-GSH}$$

The reduced glutathione levels were determined by measuring the formation of the reaction product (S-lactoyl-GSH) at 240 nm ($\varepsilon = 3.37 \times 10^6$ cm^2/mol). The scheme of the assay is shown in Figure 15. Reduced glutathione was calculated as follows:

$$A = \varepsilon bc$$

$$C = \frac{A}{3.37 \times 10^6}$$

where:

A = absorbance at 240 nm

ε = 3.37×10^6 = molar extinction coefficient of S–lactoyl-GSH at 240 nm (cm^2/mol)

b = length of cuvette (1 cm)

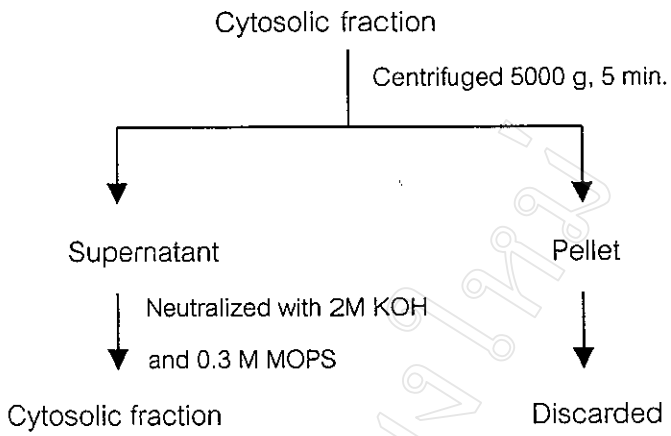


Figure 14. Protocol of sample preparation to determine reduced glutathione

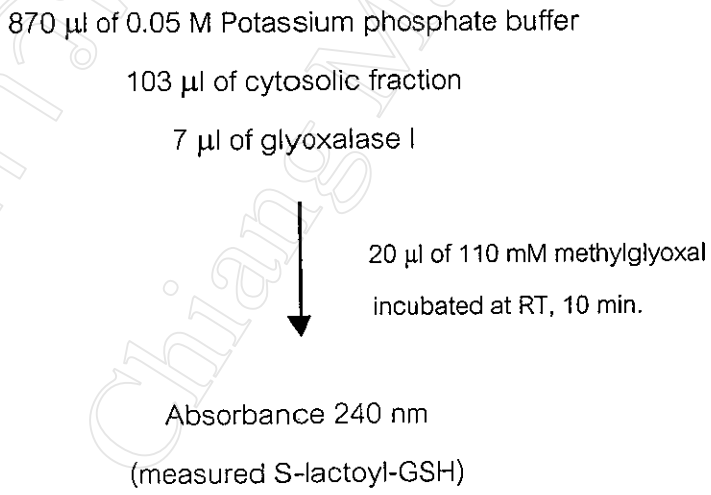


Figure 15. Scheme of reduced glutathione assay

2.9 Glutathione-S-transferase activity assay

Enzyme activity was determined spectrophotometrically at 340 nm according to the method of Habig *et al* (Habig *et al*, 1974), by measuring the formation of 2,4-dinitrophenylglutathione from the conjugation of GSH and 1-chloro-2,4-dinitrobenzene (CDNB). The reaction mixture consisted of 0.1 mM CDNB and 0.1 M potassium phosphate buffer (pH 6.5). The reaction was carried out at 30°C, started by an addition of cytosolic proteins. The increase in absorbance at 340 nm (ε = 9.6 $nM^{-1}cm^{-1}$) was measured. The scheme of assay is shown in Figure 16. GST activity was calculated as the following equation:

$$A = \varepsilon bc$$

$$C = \frac{A}{9.6 \times b}$$

where:

A = absorbance at 340 nm

ε = 9.6 = molar extinction coefficient of 2,4-dinitrophenylglutathione at 340 nm ($nM^{-1}cm^{-1}$)

b = length of cuvette (1 cm)

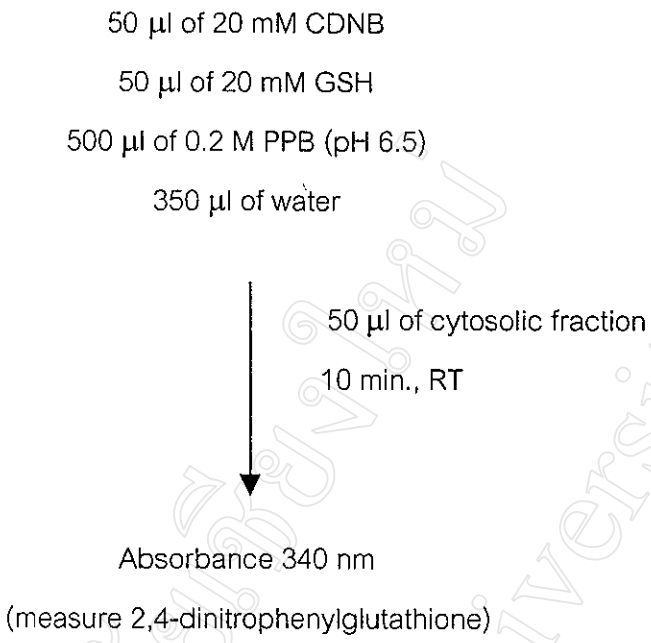


Figure 16. Scheme of glutathione-S-tranferase activity assay

2.10 Characterization of glutathione-S-transferase subunits

The protocol for the characterization of glutathione-S-transferase subunits is shown in Figure 17.

2.10.1 SDS gel electrophoresis

The cytosolic fraction (0.1 mg protein) were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamine gels (SDS-PAGE) as described by Laemmli (Laemmli, 1970). The electrophoresis was carried out at 40 mA until the front of the tracking dye had migrated approximately 0.5 cm from bottom. The resolved proteins were electrotransfered to nitrocellulose membrane and analyzed by Western blot.

2.10.2 Protein Western blot analysis

After transblotting the electrophorically resolved proteins, the blot were blocked at non specific binding sites by immersing the membrane 1% BSA solution at $4^{\circ}C$ overnight. The blot were then rinsed in washing buffer (PBS containing 0.05% tween 20) for 4 min. with three changes of washing buffer. Blot were incubated with monoclonal antibodies against GST Ya, Yb, Yc and T2 of rat cytosol (1:200; Biotrin international Ltd., Ireland) and then dissolved in 0.1% BSA for 1 hour. The membrane was washed extensively in washing buffer and reincubated for 1 hour with peroxidase linked secondary antibody (Antirabbit IgG) that was diluted 1:1000 in 0.1% BSA, then washed with the washing buffer. Immunoreactive proteins were visualized using 4-chloro-1-napthol as a substrate. Visualiable spot were quantitated using a laser densitometer.

2.11 Statistic Evaluation

All of the results between each groups were statistical analyzed by using the ANOVA values of $P<0.05$

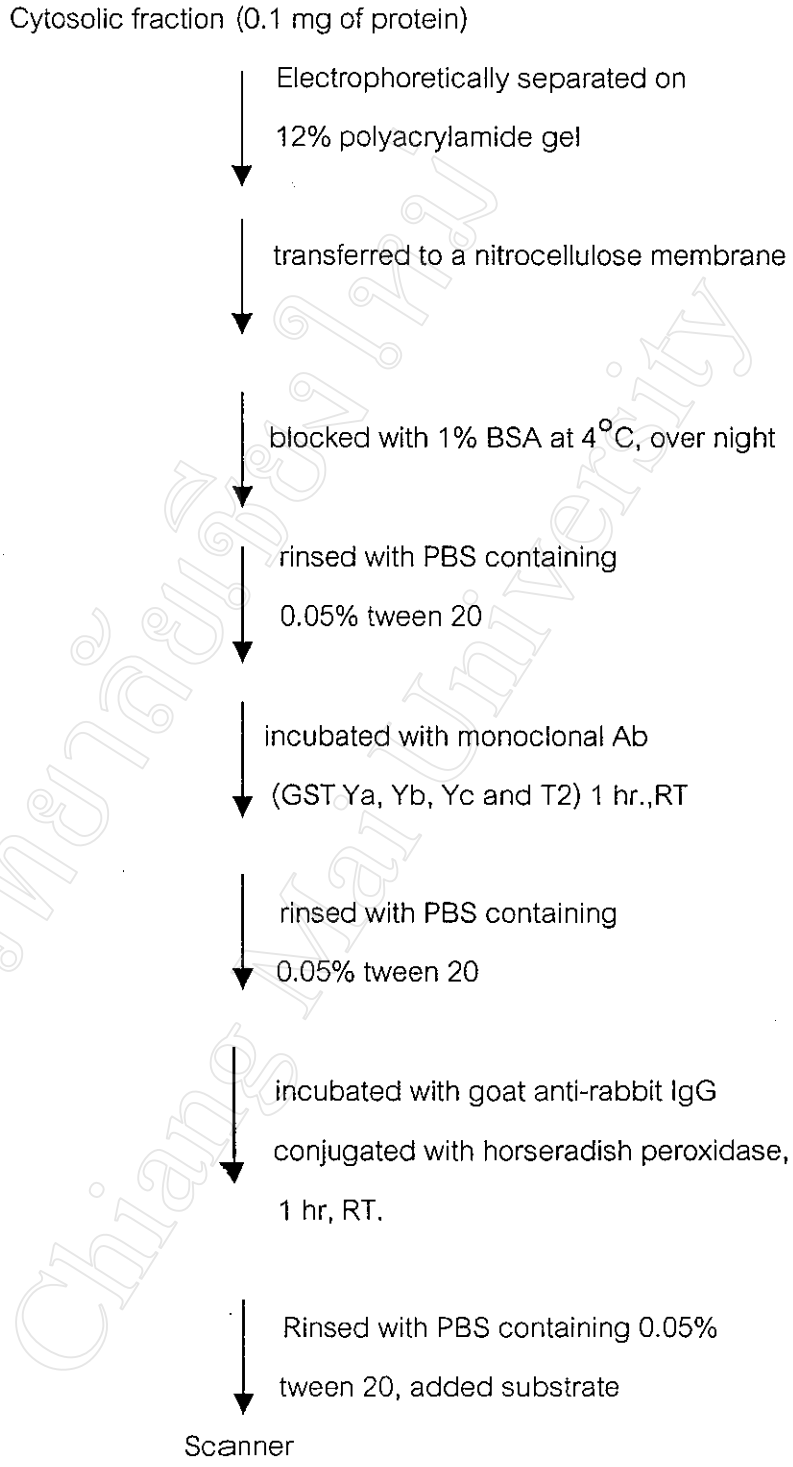


Figure 17. Protocol of glutathione-S-transferase subunits determination