APPENDICES

APPENDIX A

1. Enzymatic Colorimetric Test : GOD-PAP Method (Biotech, Bankok, Thailand)

The principle of this method is based on the fact that glucose in sample is oxidized by glucose oxidase to produce D-gluconic acid and hydrogen peroxide. The hydrogen peroxide is then oxidatively coupled with 4-aminoantipyrine and phenol substitute, p-HBS, in thepresence of peroxidase to yield a red quinoneimine dye. The amount of colored complex formed is proportional to glucose concentration and can be photometrically measured at 500 nm against reagent blank.

Reagents

1. Reagent : Enzyme reagent
2. Standard : Glucose standard 100 mg/dl (or 5.55 mmol/l)

Samples

NaF – serum should be separated from the cells as soon as possible in order to prevent glycolysis. Stability of glucose in sample for 24 hours at 4 °C

Procedure

Wavelenght : 500 nm (490 -530 nm)
Optical path : 10 mm
Temperature : 37 °C
Measurment : Read against reagent blank

Pipette Scheme

	Blank	Standard	Sample
Standard	-	10 µl	-
Sample	-	-	10 µl
Reagent	1000 µl	1000 µl	1000 µl

1. Pipette 1.0 ml (1000 µl) of reagent into a 10 mm cuvette and allow to equllibrate to 37 °C.

2. Add 0.010 ml (10 µl) of specimen (serum or standard or control) to reagent and mix gently.

3. Incubate all tube at 37 °C for 5 minutes.

4. Read absorbance against reagent blank at 500 nm.

5. Repeat procedure for each sample.

APPENDIX B

2. Enzymatic Colorimetric Test : Triglycerides (Biotech, Bankok, Thailand)

The principle of this method is based on the fact that triglyceride in sample is hydrolyzed by lipase to glycerol and fatty acids. The glycerol is then phosphorylated by adenosine-5-triphoosphate (ATP) to glycerol-3-phosphate (G3P) and adenosine-5-diphoaphate in a reaction catalyzed by glycerol kinase (GK). Glycerol-3-phosphate is then converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide by glycerophosphate oxidase (GPO). The hydrogen peroxide then reacts with 4-aminoantipyrine (4-AAP) in a reaction catalyzed by peroxidase to yield a red colored quinoneimine dye. The intensity of the color produced is directly propotional to the concentration of triglycerides in the sample when measured at 505 nm.

Reagent

1. Triglyceride Enzyme Powder
2. Triglyceride Enzyme Diluent
3. Triglyceride Standard 200 mg/dl

Preparation Working Reagent

Mix Triglyceride Enzyme Powder and Triglyceride Enzyme Diluent together

Procedure

1. Pipette 1.0 ml of reagent in each cuvette.
2. Add 0.01 ml (10 µl) of specimen (plasma or standard or control) to reagent and mix gently.
3. Incubate all tubes at 37 °C for 5 minutes.
4. Read absorbance against reagent blank at 505 nm.
5. Repeat procedure for each sample.

APPENDIX C

3. Thiobarbituric acid assay (TBAR assay)

3.1. Reagent preparation

3.1.1. Thiobarbituric Acid

3.1.2. TBA Acetic Acid

3.1.3. TBA Sodium Hydroxide

3.1.4. TBA Malondialdehyde Standard

3.1.5. TBA SDS Solution

Weigh 530 mg of TBA and add to ≥ 150 ml beaker containing 50 ml of diluted TBA Acetic Acid Solution. Add 50 ml of diluted TBA Sodium hydroxide and mix until the TBA is completely dissolved. The solution is stable for 24 hours.

3.2. Sample preparation

3.2.1. Weigh out approximately 40 mg of tissue into a 1.5 ml centrifuge tube.

3.2.2. Add 400 µl of Cell lytic buffer with protease inhibitors.

3.2.3. Transfer the sample with 400 µl cell lytic buffer to per-chilled microhomogenizer and homogenization the tissue (~ 20 stroke)

3.2.4. Centrifuge the tube at 1,600 g for 10 minutes at 4 °C. Use the supernatant for analysis.

3.2.5. Store supernatant on ice. If not assaying the same day, freeze at -80°C.

3.2.6. The sample will be stable for one month. Tissue homogenates do not need to be diluted before assaying.

3.3. Standard preparation

Dilute 250 µl of the MDA standard with 750 µl of water to obtain a stock solution of 125 µM. Add the amount of 125 µM MDA stock solution and water to each tube as described in the table.

Tube	MDA (µl)	Water (µl)	MDA Concentration (µM)
A	0	1,000	0
B	5	995	0.625
C	10	990	1.25
D	20	980	2.5
E	40	960	5
F	80	920	10
G	200	800	25
H	400	600	50

3.4. Assay protocol

3.4.1. Add 12.5 µl of sample or standard to appropriately labeled 5 ml vial.

3.4.2. Add 12.5 µl of SDS solution to vial and swirl to mix.

3.4.3. Add 500 µl of the color reagent forcefully down side of each vial.

3.4.4. Cap vials and place vials in foam or some other holder to keep the tubes upright during boiling.

3.4.5. Add vials to vigorously boiling water. Boil vials for one hour.

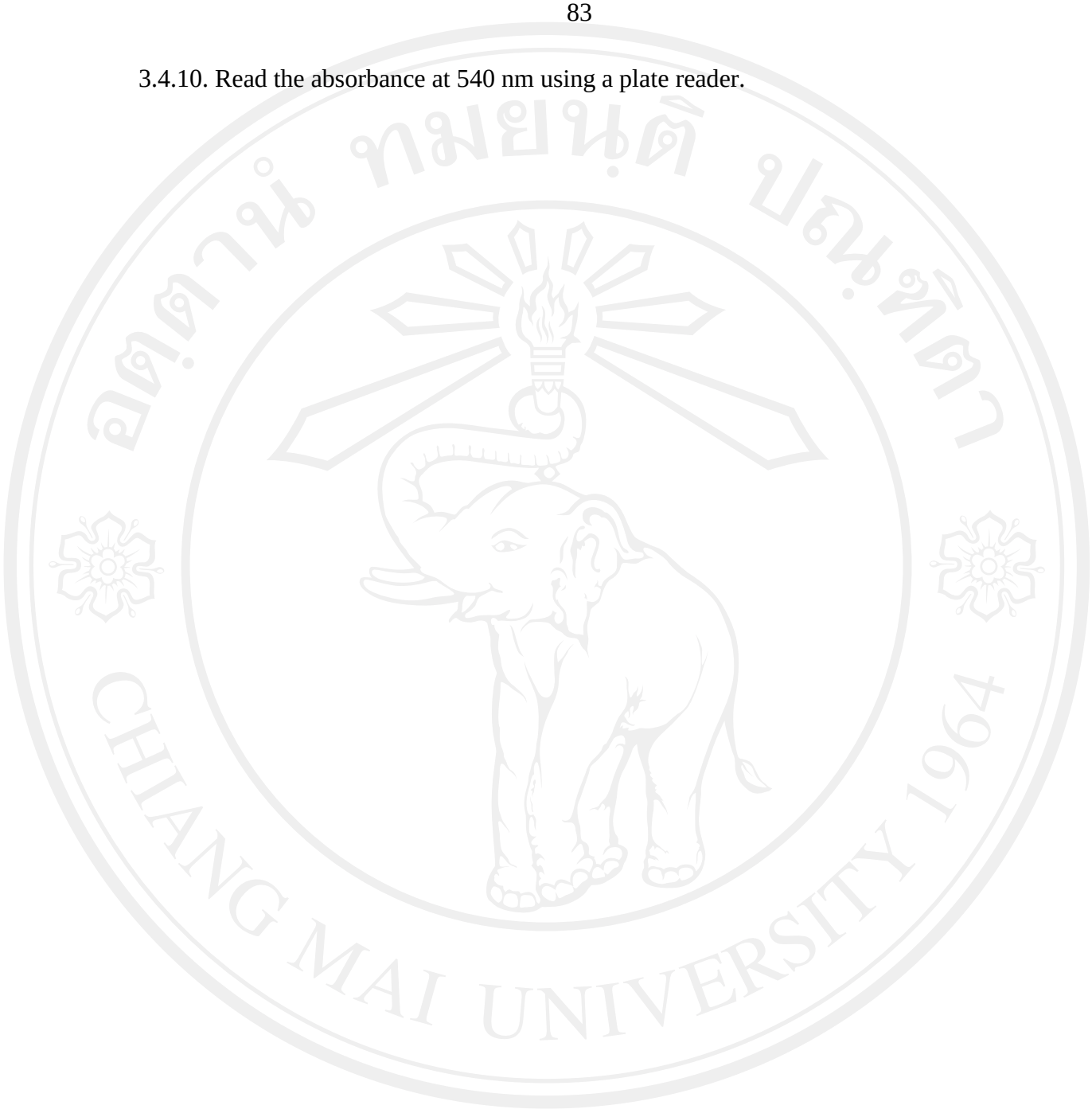
3.4.6. After one hour, immediately remove the vials and place in ice bath to stop reaction. Incubate on ice for 10 minutes.

3.4.7. After 10 minutes, centrifuge the vials for 10 minutes at 1,600 g at 4°C. Vials may appear clear or cloudy. Cloudiness will clear upon warming to room temperature.

3.4.8. Vials are stable at room temperature for 30 minutes.

3.4.9. Load 150 µl (in duplicate) from each vial to plate.

3.4.10. Read the absorbance at 540 nm using a plate reader.



APPENDIX D

4. Solution for uptake experiment

4.1. Cross and Target medium

Component	Final concentration (mM)	Amout (g/L)
NaCl	95	5.500
KCl	5	0.375
$Na_2HPO_4 . 7H_2O$	9.50	2.540
Mannitol	80	14.580
Tris	20	2.420
$CaCl_2 . 2H_2O$	0.74	0.108

pH = 7.4

Note: - Adjusted pH by HEPES

- Added $CaCl_2$ after adjusting pH

4.2. Scintillation buffer

Component	Amout (g/L)
2,5-diphenyloxazole(PPO)	7
1,4-bis(5-phenyloxazole-2-yl)benzene (POPOP)	0.1
Triton-X	500 ml
Toluene	1000 ml

APPENDIX E

5. Bradford's method for protein determination

The principle of this method is based on the fact that an absorbance shifts in the dye Coomassie when the previously red form Coomassie reagent changes and stabilizes into Coomassie blue by the binding of protein. During the formation of this complex, two types of bond interaction take place: the red form of Coomassie dye first donates its free electron to the ionizable groups on the protein, which causes a disruption of the protein's native state, consequently exposing its hydrophobic pockets. These pockets on the protein's tertiary structure bind non-covalently to the non-polar region of the dye via van der Waals forces, positioning the positive amine groups in proximity with the negative charge of the dye. The bond is further strengthened by the ionic interaction between the two. Binding of the protein stabilizes the blue form of coomassie dye, thus the amount of complex present in solution is a measure for the protein concentration by use of an absorbance reading.

Standard procedure

1. Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionized (DDI) water. Filter through Whatman #1filter (or equivalent) to remove particulates. This diluted reagent maybe used for approximately 2 weeks when kept at room temperature.
2. Prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of this microtiter plate assay is 0.05 to 0.5 mg/ml, Protein solution are normally assayed in duplicate or triplicate.
3. Pipet 10 µl of each standard and sample solution into separate microtiter plate wells.
4. Add 140 µl of diluted dye reagent to each well. Mix the sample and reagent thoroughly using a microplate mixer. Alternatively, use amulti-channel pipet to dispense the reagent. Depress the plunger repeatedly to

mix the sample and reagent in the well. Replace withclean tips and add reagent to the next set of wells.

5. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.
6. Measure absorbance at 595 nm.

APPENDIX F

6. Electrophoresis and Western Blotting

6.1 Polyacrylamide gel preparation

The glass plates were cleaned with 70% alcohol and then were set up the gel container by place the short on spacer plate and clipping 2 pieces of the mirrors together. The level of separating gel was marked below the comb about 1 cm. Acrylamide gel and other components were weighed as desire. All components (except APS and TEMED) were de-gassed at least 15 min and then APS and TEMED were added into separating gel with continuous de-gassed by gently mixing. Separating gel was subsequently loaded and let it be set up for 30-45 min and then rinsed by deionized H_2O. . APS and TEMED were added into stacking gel and then mixed continuously and gently. Stacking gel was filled up and the comb was inserted into stacking gel. The gel was set up for 30-45 min and stored at 4 °C prior to use.

6.2 Preparation sample for loading into the gel

All samples and reagents were calculated and used at a desire concentration and volume. Subsequently, the samples wereheated at 70^{o}C for 5-10 min

6.3 Gel electrophroresis

The gel was placed in the electrophroresis chamber. The running buffer (1X) was added into the outer chamber. Subsequently, protein marker (5 µl) and each sample (20 µl) were loaded. The remaining running buffer was added into the inner chamber. The electrophoresis system was then run at 150V for 1.5 hr.

6.4 Membrane transfer

The PVDF membrane was soaked in methyl alcohol 5 min and then incubated in deionized H_2O for 5 min. Fiber pads and filter papers were soaked in 1X Transfer buffer at least 10 min. After gel electrophoresed, the gel was transferred to the sandwich holder along with PVDF membrane, fiber pads and

filter papers. . The sandwich packs (Figure 1) were added into the chamber containing transferred buffer and run at 45 V for 45 min.

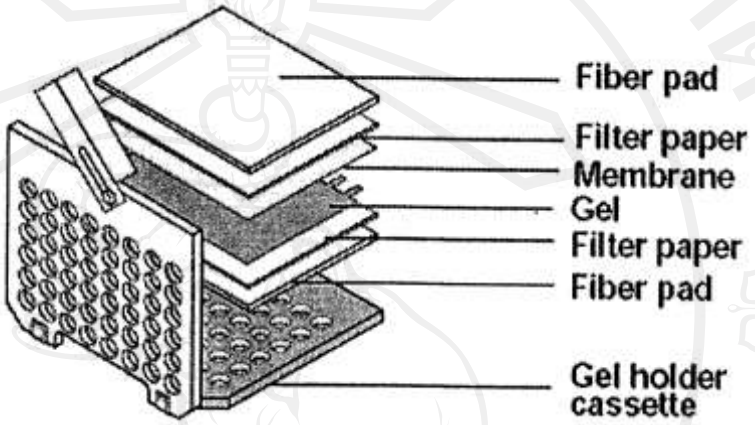


Figure 1 Components of gel holder cassette

6.5 Blocking

After membrane transfer, the sandwich packs was taken off. PVDF membrane was cut as desire and transferred to blocking buffer to eliminate non-specific binding proteins for 1 hr at room temperature. The membrane was then washed with PBS-T for 5 times for 5 minutes each time.

6.6 Immunoblotting

The membrane was incubated in primary antibody solution for 3 to 4 hrs or overnight at 4^0C. The membrane was with PBS-T for 5 times with 5 minutes each time. Subsequently, the membrane was incubated with corresponding secondary antibody solution for 1 hr at room temperature. At the end of this step, the membrane was washed with PBS-T for 5 times with 5 min each time.

6.7 Protein detection

The chemiluminescent solution was added and incubated onto the membrane for 5 min at room temperature. The membrane was then put in an *X*-ray film cassette and autoradiography film (hyper film ECL) was put on the

top of the membrane with 5 min exposure time. The film was then developed until the protein was seen.

APPENDIX G

7. The acrylamide gel components

7.1. Stock solution

7.1.1. 30% Acrylamide/0.8% Bis for 100 ml

Component	Final concentration (mM)	Amout (g or ml /100 ml)
Acrylamide	410	29.2 g
Bisacrylamide	100	1.6 g
Deionized H2O up to		100 ml

* Store in dark at 4 °C

7.1.2. 1.5 M Tris pH 8.8 for 100 ml

Component	Final concentration (mM)	Amout (g or ml /100 ml)
Tris-base	150	18.15 g
Deionized H2O up to		100 ml

* Adjusted pH to 8.8 and store in dark at 4 °C

7.1.3. 0.5 M Tris pH 6.8 for 100 ml

Component	Final concentration (mM)	Amout (g or ml /100 ml)
Tris-base	150	18.15 g
Deionized H2O up to		100 ml

* Adjusted pH to 8.8 and store in dark at 4 °C

7.1.4. Polyarylamide gel preparation

	Final Concentration (mM, or %)		1 gel		2 gels	
	12.5% Separating gel (µl)	4% Stacking gel (µl)	12.5% Separating gel (µl)	4% Stacking gel (µl)	12.5% Separating gel (µl)	4% Stacking gel (µl)
1.5 M Tris, pH 8.8	374.5	-	1,250	-	2,500	-
0.5 M Tris, pH 6.8	-	124.33	-	470	-	940
30% Acrylamide	12.52%	3.96%	2,087	250	4,175	500
10% SDS	0.1%	0.1%	50	18.7	100	37.5
Deionized water	-	-	1,587	1,137	3,175	2,275
10% APS	0.05	0.06	25	12.5	50	25
TEMED	-	-	2.5	1.87	5	3.75
Total volume	-	-	5,001.5	1,890.07	10,005	3,781.25

APPENDIX H

Preparation sample for loading

Example:	Protein sample 50 ug	X	ul
	Sample dye (buffer)	10	ul
	H_2O	X	ul
	Total volume	20	ul

APPENDIX I

8. Solution for western blotting

8.1. 4X Laemmli sample buffer (SDS reducing buffer) for 100 ml

Component	Final concentration (mM)	Amout (g or ml /100 ml)
87% Glycerol	40%	46 ml
1 M Tris	240	24 ml
SDS	10%	8 g
Bromophenol Blue	0.02%	0.02g
Beta-mercaptoethanol		10 ml
Deionized H2O		20 ml

* Adjusted pH to 6.8 and stored in dark at 4 °C

Note: - Adjusted pH by HCl

- Added beta-mercaptoethanol to sample buffer prior to use.

8.2. Electrode buffer (Running buffer) 10X

Component	Final concentration (mM)	Amout (g/L)
Tris	125	30.30
Glycine	1920	144
SDS	1%	10

8.3. Blotting buffer (Transfer buffer) [Freshly prepare]

Component	Final concentration (mM)	Amout (g or ml/L)
Tris	25	3.03
Glycine	192	14.42
Methanol	20%	200 ml

8.4. Phosphate buffered saline with tween (PBS-T)

Component	Final concentration (mM)	Amout (g or ml/L)
$Na_2HPO_4.2H_2O$	161.8	28.8
$NaH_2PO_4.H_2O$	42.9	5.92
NaCl	199.8	11.68
Tween-20	0.2%	2 ml
Deionized H2O		2000 ml

* Adjusted pH to 7.5 and stored in dark at 4 °C

8.5. Tris buffered saline with tween (TBS-T)

Component	Final concentration (mM)	Amout (g or ml/L)
Tris	495.3	60
NaCl	1368.9	80
KCl	26.8	2
Tween-20	0.1%	1 ml
Deionized water		1000 ml

8.6. 5% milk in PBS-T

Component	Amout (g or ml/L)
Non-fat dry milk	2.5
PBS-T	50 ml

8.7. 5% milk in TBS-T

Component	Amout (g or ml/L)
Non-fat dry milk	2.5
TBS-T	50 ml

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March, 2012	Oral presentation in the First ASEAN Plus Three Graduate Research Congress (AGRC) Thailand. Chiang mai, Thailand.

Conference short papers and abstracts

1. **Atcharaporn Ontawong**, Phatchawan Arjinajarn, Anchalee Pongchaidecha, Naruwan Saowakon, Pornpun Vivithanaporn, Anusorn Lungkaphin, Narissara Lailerd, Doungporn Amonlerdpison, and Chutima Srimaroeng (2012) *Spirogyra neglecta* Extract Attenuates Hyperglycemia-induced Oxidative Stress in Type 2 Diabetic Rat Kidneys.The First ASEAN Plus Three Graduate Research Congress (AGRC) Chiang Mai, Thailand.

2. Chutima Srimaroeng, **Atcharaporn Ontawong,** Manunya Arjinacharn**,** Narissara Lailerd, Anchalee Pongchaidecha, Anusorn Lungkaphin and Pornpun Vivithanaporn. (2010) The Beneficial Effects of *Spirogyra neglecta* Extract on Renal

Transport Function in High-fat Diet induced Diabetic Type II Rats. The 4th Asia Pacific ISSX Meeting “APISSX 2011”, Tainan,Taiwan.