MATERIALS AND METHODS

1. Materials

CHEMICALS	MOLECULAR WEIGHT	COMPANIES
Acetone ($(CH_3)_2CO$)	58.08	May & Baker Ltd.
Acetic acid Glacial, 100%	60.05	E Merck
Acrylamide (C_3H_5NO)	71.08	Sigma Chemical Co.
Ammonium persulfate ($(NH_4)_2S_2O_4$)	228.20	Bio-Rad
Ammonium sulfate ($(NH_4)_2SO_4$)	132.14	Fluka
Bovine serum albumin (BSA)	68K	Calbiochem-Behring Corp.
Coomassie Brilliant Blue R-250 ($C_{45}H_{44}N_3O_7S_2Na$)	826.0	Sigma Chemical Co.
2,6-Dichlorophenol-indophenol(sodium salt) (DCIP)($C_{12}H_6Cl_2NO_2Na$)	290.1	Sigma Chemical Co.
Dihydro-L-orotic acid (DHO)($C_5H_6N_2O_4$)	158.1	Sigma Chemical Co.
Ethylenediamine tetra-acetic acid (disodium salt)(EDTA)($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	372.24	Fluka
Ethanol,absolute		
Formic acid,99%(HCOOH)	46.026	Farmitalia Carlo Erba
Gelatin	–	Bio-Rad
Gentamycin sulfate (Garamycin) injection	–	Schering Co.
Giemsa stain (Azur-eosin- methylene blue)	–	E Merck
Glycerin ($C_3H_8O_3$)	92.10	E Merck
Glycin (H_2NCH_2COOH)	75.07	E Merck

1. Materials (cont.)

CHEMICALS	MOLECULAR WEIGHT	COMPANIES
N-2-Hydroxyl ethyl-piperazine-N-2-ethane sulfonic acid (HEPES) ($C_8H_{18}N_2O_4S$)	283.3	Sigma Chemical Co.
ß-Mercaptoethanol	78.13	E Merck
Methanol,absolute		
N,N-Methylene-bis-acrylamide (BIS)	154.17	Bio-Rad
Orotic acid methyl ester ($S-CH_3-OA$)	170.1	Sigma Chemical Co.
Phenylmethanesulfonyl fluoride (PMSF) ($C_7H_7FO_2S$)	174.2	Sigma Chemical Co.
Powdered RPMI-1640 Formula No. 430-1800	-	GIBCO
Soponin	-	Sigma Chemical Co.
Silver nitrate ($AgNO_3$)	169.87	Kodak
Sodium azide (NaN_3)	65.01	Sigma Chemical Co.
Sodium bicarbonate ($NaHCO_3$)	84.01	E Merck
Sodium chloride (NaCl)	58.44	E Merck
Sodium dodecyl sulfate (SDS)	-	Sigma Chemical Co.
Sodium hydroxide(NaOH)	40.00	E Merck
D-Sorbitol(D-Gluticol), anhydrous	182.2	Sigma Chemical Co.
N,N,N',N'-tetramethyl ethylene diamine (TEMED)	116.21	Bio-Rad
Trichloroacetic acid (CCl_3COOH)	163.39	Farmitalia Carlo Erba
Triton-X-100	-	E Merck
Trizma base ($C_4H_{11}NO_3$)	121.1	Sigma Chemical Co.
Tween-20	-	Bio-Rad

2.Procedure for Handling P.falciparum Culture (Trager & Jensen, 1976)

2.1. Preparation of Stock RPMI 1640 Medium

A package of 10.4 g of RPMI 1640 powder and 5.94 g of HEPES (N-[2-hydroxyethylpiperazine] - N'-[2-ethanesulphonic acid]) buffer were dissolved together in 960 ml of double-distilled water to give a final concentration of 25 mM HEPES at pH of 6.75. For preventing bactierial contamination an antibiotic of 40 mg garamycin was added to the medium. The medium was then dispensed aseptically as 100 ml aliquots into sterile bottles. They were stored at 4°C and used within one month.

2.2. Preparation of Sodium Bicarbonate, 5% (W/V)

Five grams of sodium bicarbonate (anhydrous) was dissolved in 100 ml double distilled water sterilized by filtration through a 0.45 μM Millipore filter, dispensed aseptically into 9 ml aliquots and kept a 4°C with a shelf-life for one month.

2.3. Preparation of Human Serum

Type O and AB human serum were obtained by collecting fresh whole blood without anticoagulant. They were allowed to clot at room temperature for a few hours and then were incubated at 37°C for one hour for complete clot retraction. Serum was separated by centrifugation at 700xg at 4°C for 20 min and was transferred into 11.5 ml aliquots. The serum was

stored at –20°C and used within three months for obtaining good growth of malaria parasites. All steps were performed aseptically.

2.4. Preparation of Culture Medium

An aliquot of 100 ml RPMI 1640 stock medium was supplemented 4.2 ml of 5% sodium bicarbonate. After mixing, the yellow color of medium turned to orange indicating that the pH had been shifted from 6.75 to 7.40. Approximately 10 ml of human serum was then added to give final concentrations of 10% human serum, 2 mg/ml sodium bicarbonate and 6 mg/ml (25 mM) HEPES buffer. The medium, called complete medium, was kept at 4°C and used within one week for culture.

For washing of uninfected red cells, stock medium of RPMI 1640 was adjusted to pH 7.40 by adding 5% sodium bicarbonate as described above. The medium, called incomplete medium, must be freshly prepared before use.

2.5. Preparation of Red Cells for Culture

Type O human blood in a sterile bag containing citrate phosphate dextrose (CPD) was used. Whole blood was aseptically dispensed into aliquots of 50 ml in sterile bottles and stored at 4°C. It must be used within 4 weeks.

About 10 ml of packed red cells at the bottle was washed twice by using equal volume of incomplete medium, and centrifuged at 700 x g,

4°C for 10 min. The supernatant fluid and buffy coat were discarded for elimination of platelets and leucocytes. The washed red cells were then resuspended in complete medium to obtain 50% cell suspension.

2.6. Cultivation of P.falciparum

P.falciparum in culture may come from 2 sources : continuous culture and from frozen samples.

For continuous culture source, the red cell suspension was centrifuged at 400 xg at 4°C for 7 min. The supernatant fluid was discarded and packed red cells were resuspended in an equal volume of complete medium. The degree of parasitemia was then determined (see section 2.8). The appropriated parasitemia, usually 0.3 – 0.5 % was adjusted by adding freshly washed erythrocytes. To each ml of this cell suspension, 5.4 ml of complete medium was added to make an 8% cell suspension, and 4.5 ml of this diluted blood was dispensed into a 60 x 15 mm or 100 x 15 mm dish. If the higher parasitemia was needed, 10 ml of 3% cell suspension was used. The dished were placed in a candle jar, a desiccator with cover and a white candle inside it. The cover was tightly sealed with silicone grease. The open stopcock was closed as soon as the flame of a previously lit white candle placed inside was extinquished. The culture atmostphere then was relatively low O_2 and high CO_2 content (17% O_2, 3% CO_2 80% N_2) (Scheibel et al., 1979). The dessicator was then placed in an incubator at 37°C.

The method of preparation of the parasitized erythrocytes from frozen samples is mentioned in section 2.10 .

2.7. Changing Culture Medium

Usually, the culture medium had to be changed every 24 hours, by removing the old medium and adding the same volume of fresh complete medium. The cells were resuspended by gentle swirling. The dishes were then placed in the candle jar and incubated at 37°C. If the parasitemia is higher than 10% the medium change had to be frequently performed, or larger volume of medium was needed to be used.

2.8. Parasitemia Determination

The level of parasitemia was determined from Giemsa's stain thin blood films from the cultures. The thin blood films were fixed in acetone – free absolute methanol for 1 min and air – dried. The slides were then stained with 3% Giemsa's stain in 0.067 M phosphate buffer, pH 7.0 –7.2 for 30 min and washed with tap water, air–dried. Using a microscope with an oil immersion lens (1,000 X magnification), 10,000 total erythrocytes were examined and calculated for the percentage of parasite infected cells. An erythrocyte infected by more than one parasite as multiple infection was counted as one infected cell.

2.8.1. Giemsa's Stain

Giemsa's powder	0.6 g
Glycerol(99% purity)	50 ml
Absolute methanol, acetone free	50 ml

A small amount of dry stain was placed in a mortar and a small amount of glycerol added. The dye was ground thoroughly, and then poured off into a clean bottle. The grinding was repeated in this manner until all the stain had been mixed with glycerol. The glycerol–dye mixture was incubated in a water bath at 55–60 °C for 6–8 h with periodic shaking and then cooled to room temperature. Fifty ml methanol was added, and the dye mixture kept stoppered at 37°C for 2 weeks. The dye mixture was then filtered and stored in a dark bottle until use.

2.8.2. Phosphate Buffer

0.067 M Na_2HPO_4	9.47 g/l
0.067 M KH_2PO_4	9.38 g/l

To give a fine picture of nucleus (red color) and cytoplasm (blue color) of P.falciparum in blood film a phosphate buffer pH 7.0–7.2 was used.

2.8.3. Staining

A solution containing 3% (V/V) Giemsa's stain in phosphate buffer (pH 7.0–7.2) was used to stain the blood film about 30 min. after fixation in absolute methanol (1–5 min) and the stained film was then washed with tap water.

2.9. Synchronization of Culture

In the culture, all stages of asexual cycle were readily seen at any particular time, since the cultures had lost the synchrony. This was a characteristic of P.falciparum seen in vivo. Ring stage of P.falciparum was used for synchronization the culture according to the method of Lambros and Vanderberg (1979). The cultures were centrifuged at 400 X g at 4°C for 7 min and the supernatant fluid was discarded. The packed red cells were thoroughly mixed with 5 times of its volume of sterile aqueous 5% D-Sorbitol and allowed to stand at room temperature for 5 min. During this treatment, selective destruction of red cells infected with late stage of parasites (late trophozoite and schizont) occured, leaving only red cells with ring stage. Then it was centrifuged at 400 X g for 7 min and the supernatant fluid was discarded, and the packed red cells were diluted with washed uninfected red cells to an appropriate percent parasitemia, and subsequently cultivated in the candle jar.

2.10. Cryopreservation of Malarial Parasites

2.10.1. Freezing

a) The Freezing Solution

180 ml of 4.2% sorbitol (or mannitol) in 0.9% NaCl solution was added to 70 ml glycerol (99% purity) to give a 28% glycerol, 3% sorbitol and 0.65% NaCl.The solution was then sterilized by 0.45 μM membrane filtration.

b) Freezing Procedure

The culture material with parasitemia exceeded 5% and at mostly ring stage, was centrifuged at 400 Xg, at 4°C for 7 min. The supernatant was discarded. The cells were resuspended in an equal volume of freezing solution, and mixed well before use because glycerol is dense and would remain at the bottom of the tube. 0.5 ml aliquots of the suspension were distributed in a small screw – capped vials. The vials were quickly frozen by immersion into a liquid nitrogen tank.

2.10.2. Thawing

The vial was removed from liquid nitrogen tank. The cap of the vial was slightly loosened and the vial was set upright in water at room temperature for few minutes. The thawed suspension was transferred to a centrifuge tube and centrifuged at 400 xg for 7 min and the supernatant was removed. The cells of about 0.2 ml were resuspended in an equal volume of hypertonic saline, sterile 3.5% NaCl, and gently mixed before centrifugation as above. The supernatant was removed, the cells (now about 0.15 ml) were then washed twice in an equal volume of the incomplete medium. Finally, the cells were resuspended in an equal volume of the complete medium to give 50% cells suspension. The parasites, adjusted with washed red cells to the desired parasitemia, were cultivated as described in section 2.6.

2.11. P. falciparum Strain Used in The Experiment

P. falciparum (K_1 isolate) was obtain locally from an infected patient in Kanchanaburi province, west of Thailand and kept in culture since 1979. T_9 isolate was obtained locally from Tak province, northern Thailand. These human parasites were kindly provided by Professor Sodsri Thaithong, department of biology, faculty of Science, Chulalongkorn University, Bangkok, Thailand, and were maintained in our labolatory.

3.Procedure for Preparation of P.berghei Infected Mouse Red Cells

P. berghei was maintained by serial intraperitoneal passage of infected red cells. Mice were inoculated with $10^7 - 10^8$ infected red cells. Infected mice on the fifth day of infection (approximately 40 – 60% parasitemia) were sacrificed by cardiac puncture to obtain whole blood, using acid–citrate–dextrose (ACD) as anticoagulant, made up of 1.46 g citric acid (anh. $H_3C_6H_5O_7$), 4.4 g sodium citrate ($C_3H_4(OH)(COONa).2H_2O$), 4.6 g glucose in 200 ml of distillted water ; 0.15 ml/ml blood. Plasma and buffy coat were removed carefully after centrifugation at 2000 Xg for 10 min at 4°C. The cells were washed twice in PBS, pH 7.4, to remove ACD and the packed cells were collected after centrifugation as above.

4.Procedure for Quantitation of Protein

The Bio-Rad Protein Assay was used for protein quantitation. The principle of the assay was based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.

Assay Procedure

4.1. Reagent Preparation

4.1.1. Dye Reagent

The dye reagent is provided as a five-fold concentrate. It must be diluted and filtered prior to use in the standard assay procedure. Diluted 1 volume of Dye Reagent Concentrate with 4 volumes of high quality distilled or deionized water. Filter through Whatman No.1 paper or equivalent and stored diluted reagent in a glass container at room temperature. Discard the diluted reagent after 2 weeks.

In time, diluted reagent slowly forms a precipitate and it is therefore best diluted in quantities which will be utilized within 2 weeks. While aged diluted reagent may be refiltered and used, a loss of linear response at high protein levels may occur.

4.1.2. Protein Standard

The Bio-Rad Protein Standard supplied in the kit consists of lyophilized bovine gamma globulin or bovine albumin sealed under nitrogen. To reconstitute, add 20.0 ml of distilled water which will yield a concentration of approximately 1.4 mg/ml (for exact concentration see protein standard level). Stored rehydrate protein up to 60 days at 4°C; for longer periods, aliquot and store at -20°C. Because the specific response of the reagent to individual purified proteins will vary, the user may wish to produce his own protein standards from a purified preparation or mixture that most closely approximates the samples to be run. If so, a concentration range should be used that produces and OD 595 response from 0.10-1.00 units in the assay (vs. a reagent blank which is based on a 100 ml aliquot of the dilution buffer). Different proteins may vary in the linearity of their standard curves. In case where samples contain reagents that after the dye color development, rehydrate and dilute the standard with the same concentrations of these reagents.

4.2. Sample Preparation

Generally no samples preparation is required. Dilution with sample buffer may be nescessary to reduce the concentration of protein in the sample. Particulate matter in the sample should be removed by centrifugation or filtration. Samples containing strongly alkaline reagents or detergent may require treatment to avoid interference by these chemicals.

5. Procedure for Enzyme Assay

The spectrophotometric method, based on 2,6–dichlorophenolindophenol (DCIP) reduction , was used according to Miller et al (1968), DCIP was used as final electron acceptor. The reaction was monitored by the loss of DCIP absorbance at 610 nm (ϵ = 21500 $M^{-1}cm^{-1}$). The reaction (1.0 ml) contained 0.10 mM L–DHO, 0.043 mM DCIP and enzyme in 50 mM Tris–HCl (pH 8.0). This method was used for routine activity assay during enzyme purification

6. Procedure for Enzyme Purification

6.1. Parasite Preparation

The parasites were harvested when the parasitemia was about 20–40%. The parasitized cells were obtained by centrifugation at 3000 rpm for 10 min (HERMLE).The packed parasitized cells were washed 2 times and diluted to make 50% cell suspension with 1.0 mM Phenylmethylsulfonyl fluoride (PMSF) in phosphate buffer saline (PBS).Then the packed parasitized cells were lysed by addition one part of 0.15% Saponin (in 0.1 mM PMSF in PBS) and mixed gently. The solution was incubated in a shaking water bath at 37 °C for 20 min. After centrifugation at 8000 rpm for 10 min, the dark–brown pellet was washed 3 times with excess amount of ice–cold 0.1 mM PMSF in PBS. The intact parasite was then kept in –20°C for Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) or –196°C (liquid nitrogen) for enzyme analysis.

6.2. Enzyme Purification

Each step was carried out a 4°C unless otherwise stated. All buffer solutions contained 2.5 mM L-DHO, which stabilized the enzyme activity during purification. The DHODase from P. berghei and P. falciparum were purified to apparent homogeneity as follows .

6.2.1. Membrane Extraction

The isolated parasite were mixed with 10 mM Tris-HCl (pH8.0) and protease inhibitors : 1.0 mM phenylmethane sulfonyl fluoride (PMSF) and 1.0 mM ethylenediaminetetraacetic acid (EDTA). After three cycles of freezing (liquid nitrogen) and thawing (37°C) the homogenate was centrifuged (39000xg for 30 min). The pellet fraction was resuspended with 10 mM Tris-HCl (pH 8.0) containing 1.0 mM EDTA ,1.0 mM PMSF and 2.5 mM L-DHO and designated the "crude membrane extract".

6.2.2. Detergent Solubilization

Triton X-100 was added to the crude extracts to give a final concentration of 0.15%. The mixture was then stirred for 30 min at 4°C. The supernatant fluid collected after centrifugation (39000xg for 30 min) contained solubilized DHODase.

6.2.3. FPLC on Anion–Exchange Mono Q column

The Triton X–100 solubilized DHODase was directly applied to a Mono Q 5/5 column at a flow rate 1.0 ml/min. The column was washed with 10 mM Tris–HCl (pH 8.0) containing 0.15% Triton X–100, 1.0 mM EDTA, 1.0 mM PMSF and 2.5 mM L–DHO (buffer A) and eluted with linear gradient of 0–0.6 M$(NH_4)_2SO_4$.Active fractions were pooled and concentrated on a centricon – 10 (Amicon).

6.2.4. Cibacron Blue F3GA – Agarose Affinity Column Chromatography

Active fraction from Mono Q chromatography were applied to a Cibacron Blue F3GA–agarose affinity column which had previously been equilibrated with buffer A. The column was washed with the buffer A and then eluted with buffer A containing 0.6 M $(NH_4)_2SO_4$.The active 1.0 ml fractions were pooled and concentrated on a centricon–10 (Amicon).

6.2.5. FPLC on Gel Filtration Superose 12 Column

Final purification was carried out by gel filtration on a Superose 12 column by elution with buffer A containing 0.6 M $(NH_4)_2SO_4$ at a flow rate of 0.5 ml/min. The column was calibrated with the following molecular weight markers : Thyroglobulin, 670 kDa ; Immunoglobulin (IgG), 158 kDa ; Ovalbumin 44 kDa ; Myoglobin, 17 kDa ; Vitamin B_{12} , 1350 Da (Bio–Rad). The purified enzyme was stored at –196°C.

7. Procedure for Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a Bio–Rad minislab gel apparatus with a 5% acrylamide stacking gel and a 10 % acrylamide running gel in the discontinuous buffer system of Laemmli (1970). The gels were stained with both Coomasie Blue R and Silver (Wray et al, 1981).

7.1. Electrophoretic Buffer and Gel

7.1.1. SDS–PAGE Reagents

1. Acrylamide / Bis acrylamide was prepared by dissolving 29.2 g acrylamide and 0.8 g bis acrylamide in 100 ml double distilled water, stored in brown bottle at 4°C.
2. 1M Tris pH 8.8 was prepared by dissolving 12.1 g Trizma base in 100 ml double distilled water, then adjusted to pH 8.8 with HCl.
3. 1M Tris pH 6.8 was prepared by dissolving 12.1 g Trizma base in 100 ml double distilled water, then adjusted to PH 6.8 with HCl.
4. 10% SDS was prepared by dissolving 10 g SDS in 100 ml double distilled water.
5. 10% Ammonium persulfate (APS) was prepared by dissolving 0.5g ammonium persulfate in 5 ml double distilled water, then stored in brown bottle at 4°C.
6. 0.1 % Bromphenol Blue (BPB) was prepared by dissolve 0.1 g BPB in 100 ml double distilled water.
7. Water–saturated n–Butanol was prepared by mixing 50 ml n–butanol and 50 ml double distilled water together.

7.1.2. Seperating (Running Gel)

Acrylamide / Bis acrylamide	2.500	ml
1 M Tris pH 8.8	2.800	ml
Double distilled water	2.170	ml
10 % SDS	0.075	ml

The solution was mixed well, then added 25 µl 10% APS and 5 µl TEMED

7.1.3. Stacking Gel

Acrylamide / Bis acrylamide	0.420	ml
1 M Tris pH 8.8	0.315	ml
Double distilled water	1.750	ml
10 % SDS	0.025	ml

The solution was mixed well, then added 12.5 µl 10% APS and 2.5 µl TEMED

7.1.4. Running Buffer

Running Buffer was prepared by dissolving 3.03 g Trizma base and 14.42 g glycine in double distilled water 1000 ml, then added 10 ml 10% SDS and adjusted to pH 8.3 with NaOH

7.1.5 2X Sample Buffer (SDS reducing buffer)

1 M Tris pH 6.8	0.5 ml
Glycerol	0.8 ml
10 % SDS	1.6 ml
ß–Mercaptoethanol	0.4 ml
0.1% BPB	0.2 ml

The solution was mixed equal volume with sample and boiled for 5 min before loading.

7.2 Coomassie Blue R Staining

Coomassie Brilliant Blue R 250	0.1 g
Absolute methanol	50 ml
Glacial acetic acid	10 ml
Double distilled water	50 ml

The dye was first dissolved in methanol and then in acid and water. After electrophoresis, the gel was stained with Coomassie Blue R for 30 min.

7.3 Destaining Solution

Absolute methanol	50 ml
Glacial acetic acid	50 ml
Double distilled water	400 ml

The stained gel was subsequently destained with the destaining solution until the gel was clear.

7.4 Silver Staining

The silver staining technique was modified from the method of Wray et al. (1981) as follows:

a) After running gel, the gel was washed with double distilled water 2–3 times (about 30 min).

b) The gel was washed in 50% methanol for 30 min.

c) The gel was washed in double distilled water 2 times, 5 min each.

d) Silver stain solution was made up as follows:

i) In a clean tube, add 0.4 g silver nitrate to 2 ml double distilled water.

ii) In a clean 100 ml graduated cylinder, add
11 ml double distilled water
0.125 ml 10 N NaOH
0.7 ml ammonium hydroxide
(add the latter in the hood)

iii) Slowly (drop by drop and with rapid stirring) add the silver to the NaOH–NH_4OH solution. A brown precipitate will appear and rapidly dissolved with each

drop. The solution should be cleared after completely dissolution, if there is a brown precipitate then start over again and add more slowly and/or with more rapid stirring.

iv) Add double distilled water to make 100 ml.

e) The gel was then stained with silver stain and rocked vigorously for 3–5 min.

f) The gel was washed in double distilled water several times,5 min each.

g) The gel was then developed with developing solution containing.

50	ml	double distilled water
0.025	ml	40 % formalin
0.25	ml	1 % citric acid

h) The gel was shaken vigorously until bands developed as dark against a clear background, depending upon the amount of protein loaded. This could take 5–15 min.

i) The gel was washed in double distilled water several times, 5 min each.

j) The gel was soaked in 50% methanol.

8. Procedure for Goat Antimouse – Gold (GAM–Gold) Conjugated Immunoblot Assay

8.1. Solutions used for the immunoblot assay

Solutions used for immunoblot assay were prepared as follows.

8.1.1. Blotting Buffer

Trizma base	3.03	g
Glycine	14.42	g
Absolute Methanol	200	ml

Mixed the above chemicals then diluted with double distilled water to achieve a total of 1000 ml then adjust to pH 8.3.

8.1.2. Tris Buffered Saline (TBS)

Tris buffered saline (TBS) was prepared by adding 2.42 g.Trizma base and 29.22 g. of NaCl in 1000 ml double distilled water, then adjusted to pH 7.5 with HCl.

8.1.3. Washing Solution (TTBS)

Washing solution (TTBS) was prepared by adding 0.1 ml Tween–20 in 200 ml TBS.

8.1.4. Blocking Solution (3% Gelatin–TBS)

Blocking solution (3% Gelatin–TBS) was prepared by dissolving 1.5 g. gelatin in 50 ml TBS (microwave 30 sec).

8.1.5. 1% Gelatin TTBS

1% gelatin TTBS was prepared by dissolving 0.2 g gelatin in 20 ml TTBS (microwave 20 sec).

8.1.6. Secondary Antibody Buffer

Secondary antibody buffer was prepared by dissolving 0.04 g gelatin in 10 ml TTBS (microwave 10 sec) then adding 0.01g Bovine Serum Albumin (BSA).

8.2. Electroblotting

8.2.1. After running gel, the gel was soaked in the blotting buffer for 5 min.

8.2.2 The protein bands on gel were electroblotted to nitro–cellulose membrane (150 volts, 1 h 15 min).

8.3 Antibody Staining

8.3.1. The nitrocellulose membrane was soaked in TBS (10 ml) for 15 min.

8.3.2 The membrane was immersed at a 45 angle into the blocking solution and then shaken for 45 min.

8.3.3. The membrane was transferred to a dish containing TTBS and then washed for 5 min, for 3 times.

8.3.4. The membrane was transferred to a dish containing primary antibody (0.2 ml antiserum in 9.8 ml antibody buffer) and incubated at room temperature (at least 1.5 hr) with gentle agitation.

8.3.5. The membrane was then washed with TTBS for 5 min, for at least 3 times.

8.3.6 Gold conjugated GAM-Gold solution was prepared by taking 0.4 ml Bio-Rad's reagent into 9.6 ml dilution buffer.

8.3.7. The membrane was immersed to GAM-Gold solution and then incubated for 4 h.

8.3.8. The stained membrane was washed twice with TTBS, 5 min each, and then 1 time with TBS.

8.3.9. The membrane was air-dried and sealed in cellophane bag.

9. Study of Specific Activity of Dihydroorotate Dehydrogenase (DHODase) and Protein Concentration in Ring, Trophozoite and Schizont Stages of P. falciparum

After synchronization of the culture of P.falciparum ,according to the method of Lambros and Vanderberg(1979) as described in section 2.9, only red cells with ring stage parasites will remain in the culture as shown in Fig. 3a.

The dishes were then placed in the candle jar and incubated at 37°C for 18–24 h. The culture will have only red cells with trophozoite stage parasites as shown in Fig. 3b

And if the dishes that have only red cells with trophozoite stage parasites were incubated in the candle jar at 37°C for 8–12 h. The culture will have only red cells with schizont stage parasites as shown in Fig. 3c.

Each stage of parasitized erythocytes was harvested and prepared as described in section 6.1 then the intact parasite were further processed in two steps as follows:

Step 1 :Membrane extraction (as explained in section 6.2.1)
Step 2 :Detergent solubilization (as described in section 6.2.2)

The detergent solubilization fraction was assayed for the DHODase activity (as described in section 5) and protein concentration (as described in section 4). The specific enzyme activity was then calculated by the

FIGURE 3a : Ring stage parasite of P.falciparum.

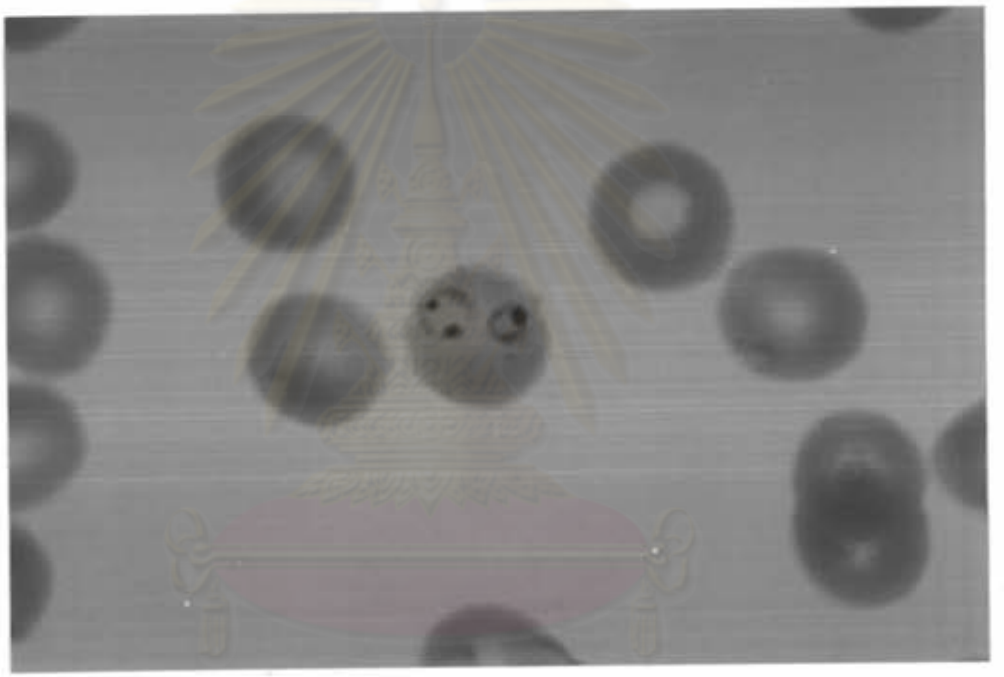


FIGURE 3b : Trophozoite stage parasite of P.falciparum.

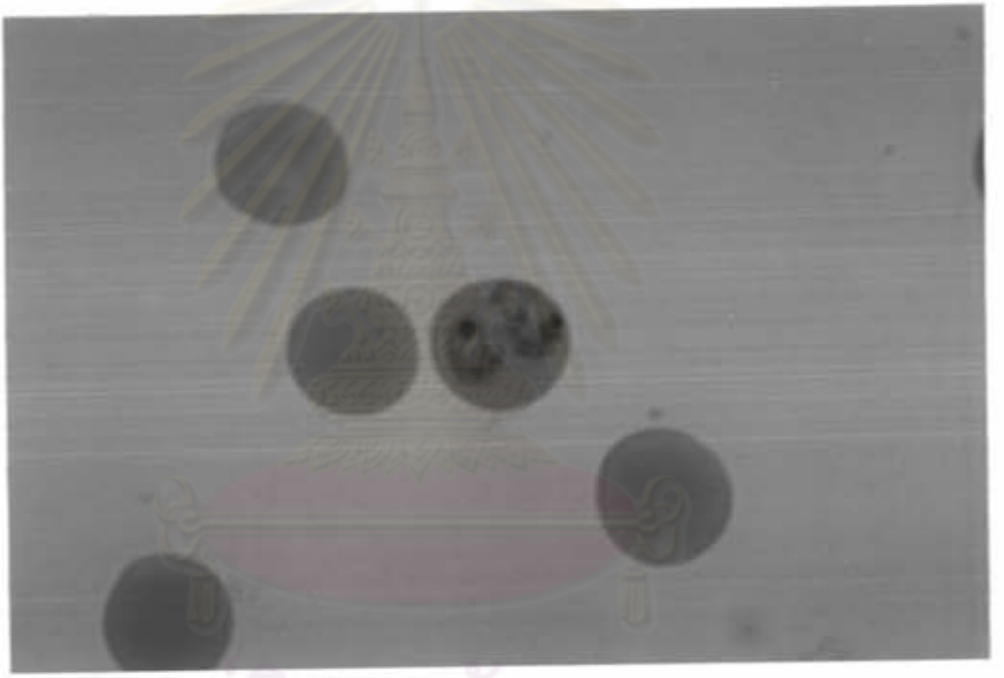
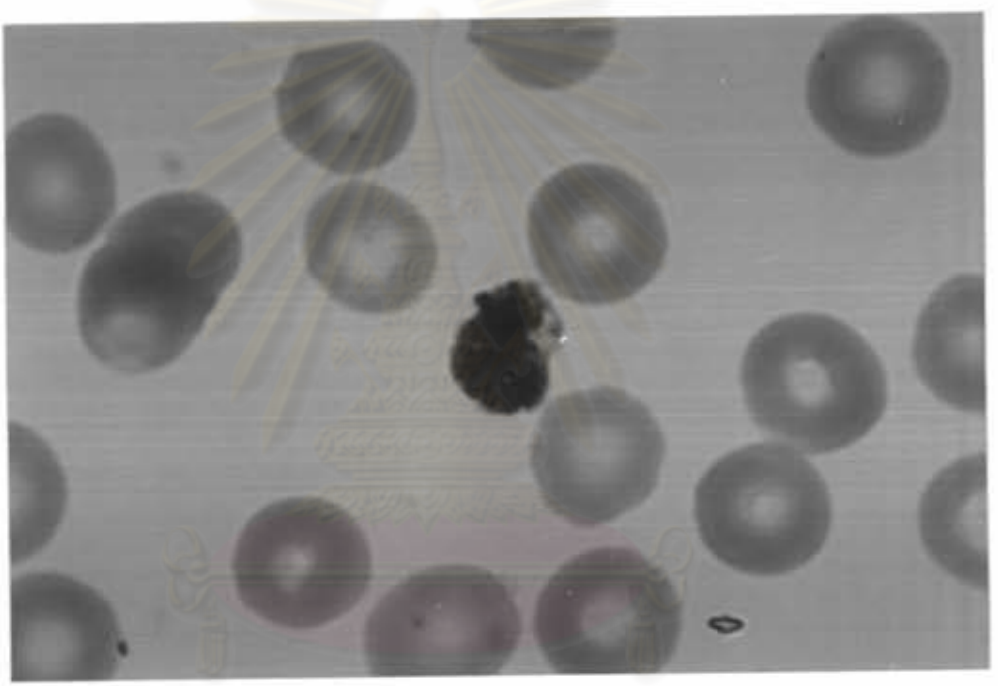


FIGURE 3c : Schizont stage parasite of P.falciparum.



following equation.

$$\text{Specific Enzyme Activity (nmol/min/mg)} = \frac{\text{Enzyme Activity per Fraction (nmol/min)}}{\text{Protein per Fraction (mg)}}$$

After achieving the calculated values of specific enzyme activity, these values were plotted in graph in relation to all the three stages of parasites i.e. ring form, trophozoite and schizont.

10. Study of Physical and Kinetic Properties of the Enzyme

10.1. Molecular Weight Determination

Study of molecular weight (MW) of enzyme DHODase by immuno-blotting technique (as described in section 8), the detergent solubilization fraction of ring, trophozoite and schizont stages of P.falciparum were loaded into the gel to determine the molecular weight of this enzyme. And gel filtration chromatographic technique as described in section 6.2.5.

10.2. Study of Stability of The Enzyme

Study of the stability of DHODase by store detergent solubiliza-tion fraction of P.falciparum at $-20^{\circ}C$ (freezer) and $-196^{\circ}C$ (liquid N_2). The first day of labolatory experiment is being considered as day"0"which has 100% enzyme activity.

11. Study of Kinetic Property of The Enzyme

Study of kinetic property of enzyme DHODase by varying substrate (DHO) concentration when using fixed amount of purified enzyme.

After achieving the values of enzyme activity in each concentration of DHO,these values were plotted Michealis- Menten kinetics and Lineweaver-Burk, then calculated the Km and Kcat values.

12. Study of Inhibitors of The Enzyme

Study of inhibition ability to DHODase of two chemicals; 5-fluoro-orotic acid (FOA) and 5-methylorotic acid (CH_3OA) by varying inhibitor concentration and fixing the amount of purified enzyme then assaying for the DHODase activity and calculating for percent of inhibition (% inhibition) in each inhibitor concentration.