MATERIALS AND METHODS

1. Materials

1.1 Chemicals

Various chemicals used in this study and their suppliers are summarized as follows :

CHEMICAL	MOLECULAR WEIGHT (Dalton)	COMPANY
- Ammonium persulfate ($(NH_4)_2S_2O_4$	228.2	Sigma Chemical Co.
- Acetone($(CH_3)_2CO$)	58.08	May&Baker Ltd.
- Acetic acid Glacial	60.05	Merck
- Acrylamide(CH_3H_5NO)	71.08	Sigma Chemical Co.
- Artemisinine		Sigma Chemical Co.
- Atovaquone		
- Bovine serum albumin (BSA)	68 k	Calbiochem-Berhing
- Bromphenol Blue	670.02	Sigma Chemical Co.
- Butanol ($C_4H_{10}O$)	74.12	Sigma Chemical Co.
- Chloroquine	515.9	Sigma Chemical Co.

CHEMICAL	MOLECULAR WEIGHT (Dalton)	COMPANY
- Coomassie Brilliant Blue R-250 ($C_{45}H_{44}N_3O_7S_2Na$)	826.0	Sigma Chemical Co.
- Coenzyme Q_0	183.2	Sigma Chemical Co..
- Coenzyme Q_7	658.8	Sigma Chemical Co.
- Coenzyme Q_9	795.2	Sigma Chemical Co.
- Coenzyme Q_{10}	863.4	Sigma Chemical Co.
- Cytochrome c	12,327	Sigma Chemical Co.
- Ethylenediaminetetraacetic acid (disodium salt) (EDTA) ($C_{10}H_{14}N_2Na_2O_82H_2O$)	372.24	Fluka
- Ethanol,absolute		Merck
- Fumaric acid ($C_4H_4O_4$)	116.1	Sigma Chemical Co.
- Gentamycin sulfate		Schering Co.
- Giemsa stain (Azureosin-methylene blue)		Merck.
- Glycin(H_2NCH_2COOH)	75.05	Merck.
- HCl		Merck.
- N-2-Hydroxyl ethyl piperazine-N-2-ethane sulfonic acid(HEPES) ($C_8H_{18}N_2O_4S$)	283.3	Sigma Chemical Co.
- Malonate	148	Sigma Chemical Co.
- 6-Mercaptoethanol	78.13	Merck

CHEMECAL	MOLECULAR WEIGHT (Dalton)	COMPANY
- Methanol absolute		Merck.
- N,N Methylene-bis-acrylamide (BIS)	154.17	Bio-Rad
- Oxaloacetic acid	132.1	Sigma Chemical Co.
- Phenylmethanesulfonyl fluoride (PMSF) ($C_7H_7FO_2S$)	174.2	Sigma Chemical Co.
- Powdered RPMI-1640 Formula No.430-1800		Gibco
- Plumbagin	188.17	Sigma Chemical Co.
- Potassium Chloride	74.55	Sigma Chemical Co.
- Saponin		Sigma Chemical Co.
- Sodium bicarbonate($NaHCO_3$)	84.01	Merck.
- Sodium chloride (NaCl)	58.44	Merck.
- Sodium dodecyl sulfate (SDS)		Sigma Chemical Co.
- Sodium hydroxide	40.01	Merck.
- Sorbitol	182.17	Merck.
- Succinic acid	270	Sigma Chemical Co.
- N,N,N',N'-tetramethyl ethylene diamine (TEMED)	116.21	Bio-Rad
- Triton-X-100		Merck.
- Trizma base ($C_4H_{11}NO_3$)	121.1	Sigma Chemical Co.
- Thenoyltrifluoroacetone	222.18	Aldrich.

1.2 Other Laboratory Supplies

1.2.1 Centricon microconcentrater for small volume concentration (Centricon -10) (Amicon)

1.2.2 Fast Protein Liquid Chromatography (Pharmacia)

1.2.3 Spectrophotometer (Shimadzu UV-160A)

2. Preparation of Stock Material for Culture Medium

2.1 Stock RPMI 1640 Medium

The amount of 10.4 gm RPMI 1640 powder and 5.94 gm HEPES was dissolved in 960 ml double distilled water to give a final concentration of 25 mM HEPES of pH 6.75. The antibiotic, gentamycin sulfate (garamycin), was added to the medium, a final concentration of 40 μg/ml to inhibit bacterial contamination. The medium was sterilized by filtration through a 0.45 μM Millipore filter. It was dispensed aseptically as 200 ml aliquots into steriled bottles. They were stored at $4^{\circ}C$ and used within one month.

2.2 Sodium Bicarbonate, 5% (W/V)

The amount of 5 gm anhydrous sodium bicarbonate was dissolved in 100 ml double distilled water. The solution was sterilized by filtration through a 0.45 μM Millipore filter, dispensed aseptically into 10 ml

aliquots, kept at 4°C and used within one month.

2.3 Serum

Fresh human serum was obtained from clotted blood. It was allowed to clot at room temperature for a few hours and then was incubated at 37°C for one hour for complete clot retraction. It was stored at 4°C overnight to allow for clot retraction. Serum was seperated from clotted blood by centrifugation at 700 x g at 4°C for 20 min. and was transfered into 10 ml aliquots into steriled tubes and kept frozen at -20°C. All steps were performed aseptically. The serum was used within 3 months for obtaining good growth of malarial parasites.

2.4 Culture Medium

For cultivation, RPMI 1640 stock medium (200 ml) was supplemented with 5% sodium bicarbonate (8.4 ml). After mixing, the yellow color of the medium turned to orange indicating that the shift pH from 6.75 to 7.4 . The medium was added with human serum (20 ml) to give a final concentration of 10%. The medium was called complete medium. It was kept at 4°C and used within one week.

For washing of uninfected red blood cells, RPMI 1640 stock medium was made to pH 7.4 with 5% sodium bicarbonate as described above. The medium, called incomplete medium, was freshly

prepared before using.

2.5 Uninfected Red Blood cells

Human blood group O in a steriled bag containing citrate-phosphate dextrose (CPD) as anticoagulant was used. It was dispensed aseptically from bag into 50 ml. steriled bottles, stored at 4°C and used within one month.

3. Cultivation Techniques

3.1 Preparation of Uninfected Red Blood cells.

Ten mililiters of blood was transfered into 50 ml centrifuge tube and centrifuged at 700 x g at 4°C for 10 min. Plasma and buffy coat were discarded. Packed red cells were washed twice with an equal volume of incomplete medium and centrifuged as above. Usually 3 ml washed packed red cells were obtained and these cells were resuspended to 50% (V/V) with complete medium.

3.2 Cultivation of *Plasmodium falciparum*

Infected red blood cell suspension obtained from continuous culture was centrifuged at 400 x g at 4°C for 7 min. and the supernatant fluid was aspirated. Packed red cells were resuspended to 50% (V/V) in

complete medium and diluted with freshly washed uninfected red cells (50% cell suspension), usually to the parasitaemia of 0.3 - 0.5%. To each ml of this suspension, complete medium (5.4 ml) was added to make 8% cells suspension, which was then dispensed into culture dishes 4.5 ml per 60 x 15 mm dish.

3.3 Incubation of Culture in Candle jar

Culture dishes were placed in a candle jar. The cover of the desiccator was sealed with silicone grease and the stopcock open until the flame of a previously lit white candle placed inside was extinguished. The stopcock was then immediately closed. This is an effective way to produce an atmosphere of relatively low O_2 and high CO_2 content (17% O_2, 3% CO_2, 80% N_2) (Scheibel et al., 1979). The desiccator was then placed in an incubator at 37°C.

3.4 Changing of Culture Medium

The culture medium was usually changed every 24 hours. The dish was gently tilted and was aspirated as much medium as possible. The same volume of fresh complete medium was added to each culture dish and the settled red blood cells were resuspended by gentle swirling. The dishes were replaced in the candle jar and incubate at 37°C. After 4 days the parasitaemia was increased to about 8-10 %, and then the fresh red blood cells were added to these culture to form subcultures.

3.5 Parasitaemia Determination

The degree of parasite infection was determined from Giemsa stained thin blood films. Thin films were fixed in acetone-free absolute methanol for 1 min , air dried, stained with 3% Giemsa's stain in 0.06 M phosphate buffer, pH 7.0 for 30 min and washed with tap water, air dried. Using a microscope with an oil immersion lens (100 x magnification), 5,000 - 10,000 total red cells were examined and the percentage of parasite infected cells (parasitaemia) was calculated. A red cell infected by more than one parasite (multiple infection) was counted as one infected cell.

3.5.1 Giemsa's Stain

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

A small amount of dye stain was placed in a mortar and small amount of glycerol added. The dye was grounded 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 hours 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 using.

3.5.2 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* (T_9 isolate) in blood film a phosphate buffer pH 7.0 - 7.2 was used.

3.5.3 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.

3.6 Cryopreservation of Malaria Parasite

3.6.1 Freezing

3.6.1.1 The Freezing Solution

One hundred and eighty ml of 4.2% sorbitol 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.

3.6.1.2 Freezing Procedure

The culture material with parasitemia exceeded 5 % and at mostly ring stage, was centrifuged at 400 x g, at 4°C for 7 min. The supernatant fluid was discarded. The cells were resuspended in an equal volume of freezing solution, and remained at the bottom of the tube. The aliquots of the suspension (0.5 ml) were distributed in small screw capped vials. The vials were quickly frozen by immersion into a liquid nitrogen tank.

3.6.2 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 minnutes. The thawed suspension was transferd to a centrifuge tube and centrifuged at 400 x g 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 fluid 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 % cell suspension. The parasites, adjusted with washed red cells to the desired parasitaemia, were cultivated as described in section 2.

3.7 *P. falciparum* Strain Used in the Experiment

P.falciparum (T_9 isolate) was obtained locally from Tak province, northern Thailand. The human parasite was kindly provided by Professor Sodsri Thaithong, (Department of Biology, Faculty of Science, Chulalongkorn University) and was maintained continously in our laboratory.

4. Procedure for Assay Protein

Protein concentrations were determined by the method of Bradford and the Bio-Rad reagent (Bradford, 1976). Protein assay is a dye-binding assay based on the differential color change of dye is responsed to various concentrations of protein. The principle of the Bio-Rad protein assay is 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.

4.1 Reagent Preparation

The dye reagent is provided as a 5-fold concentrate. It must be

diluted and filtered prior to use in the standard assay procedure. Dilute 1 volume of dye reagent concentrate with 4 volumes of deionized water. Filter equivalent and store diluted reagent in a glass container at room temperature. Discard the diluted reagent after 2 weeks.

4.2 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 yield a concentration of approximately 1.4 mg/ml. Store rehydrated 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 protein will vary. If so, a concentration range should be used that produces an absorbance 595 nm response from 0.1-1.0 absorbance units in the assay. Different proteins may vary in the linearity of their standard curves. In cases where samples contain reagents that alter the dye color development, rehydrate and dilute the standard with the same concentration of these reagents.

4. 3 Sample Preparation

Generally, no sample preparation is required. Dilution with sample buffer may be necessary 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 prior treatment to avoid interference by these chemicals.

4. 4 Standard Assay Procedure

Prepare several dilutions of protein standard containing from 0.2 to about 1.4 mg / ml. Prepare a standard curve each time the assay is performed.

4.4.1 Place 0.1 ml of standards and appropriately diluted samples in clean, dry test tubes. Place 0.1 ml sample buffer in blank test tube.

4.4.2 Add 5.0 ml diluted dye reagent.

4.4.3 Mix several times by gentle inversion of test tube.

4.4.4 After a period of from 5 min to one hr , measure absorbance 595 nm versus reagent blank.

4.4.5 Plot absorbance 595 nm versus concentration of standards. Read unknowns from the standard curve.

5. Procedure for Enzyme Assay of Succinate Dehydrogenase

The activity of succinate dehydrogenase was assayed by following the oxidation of cytochrome c according to the modified method of Krungkrai et al. (1993). Coenzyme Q_0 was used as electron acceptor. The reaction was monitored by the increasing absorbance at 550 nm on a Shimadzu spectrophotometer equiped with 37°C thermostat waterbath. The

assayed mixture contained 50 mM phosphate buffer pH 8.0, succinic acid 1 mM, cytochrome c 0.1 mM, and coenzyme Q_0 0.01 mM. The reaction was initiated by addition of 50 µl of the enzymatic preparation in a final volume of 1 ml of the assayed mixture.

6. Procedure for Enzyme Purification

Enzyme succinate dehydrogenase from *P. falciparum* (T9 isolate) was purified to near homogeneity as follows.

6.1 Preparation of Intact Parasite

The parasites were harvested when the parasitaemia was about 15-20 %. The parasitized cells were obtained by centrifugation at 3,000 rpm for 10 min. The packed parasitized cells were washed 2 times and diluted to make 50 % cell suspension with 0.1 mM 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 waterbath at 37 °C for 20 min. After centrifugation 8,000 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 -196 °C (liquid nitrogen) for enzyme analysis.

6.2 Preparation of Homogenate

Intact parasites, freed from host red cells were broken by freezing in liquid nitrogen and thawing in waterbath 37°C for three times. The mixture was called "Homogenate". The homogenate was determined for enzyme activity and protein concentration.

6.3 Preparation of Supernatant fluid

The homogenate was centrifuged at 14.000 rpm for 60 min at 4°C. The supernatant fluid was saved, dialyzed with 5 mM phosphate, pH 8.0, for 2 hrs at 4°C, assayed for the enzyme activity and used for further purification. The pellets were also saved and resuspended.

6.4 Preparation of Detergent Solubilization

Triton X-100 (a nonionic detergent) was added to the pellets to give a final concentration of 0.15%. The mixture was then stirred for 60 min. on ice. The supernate was then collected after centrifugation at 14,000 rpm for 60 min. The supernate was called "Extract I", and then examined for the enzyme activity and protein concentration.

6.5 FPLC on Mono Q Anion-Exchange Chromatography

The dialyzed supernate (from section 6.2) was applied onto a Mono Q FPLC column equilibrated with 5 mM phosphate pH 8.0 containing 0.1 mM PMSF and 1 mM EDTA. The column was washed with same buffer and proteins were eluted with 40 ml linear gradient from 0 to 600 mM KCl at a flow rate of 1.0 ml/min. The eluates were collected into 38 fractions, each fraction was determined for the enzyme activity and protein concentration. Active fractions containing the enzyme activity were pooled and dialysed against 5 mM phosphate pH 6.0 for two hrs at 4°C.

6.6 FPLC on Mono S Cation-Exchange Chromatography

The dialyzed fractions after the Mono Q chromatography were loaded onto Mono S FPLC column equilibrated with 5 mM phosphate buffer pH 6.0 containing 0.1 mM PMSF and 1 mM EDTA. The column was washed with same buffer and eluted with linear gradient of 5 mM phosphate buffer from pH 6.0 to pH 8.0 at a flow rate of 1 ml/min. The eluates were collected into 38 fractions, each fraction was determined for the enzyme activity and protein concentration.

6.7 Gel filtration Chromatography on Superose 6 FPLC column

Active fractions from the Mono S chromatography were pooled

and concentrated on a Centricon-10 (Amicon) and then applied to a Superose 6 column equilibrated with 5 mM phosphate buffer (pH 8.0) containing 150 mM KCl. The enzyme was eluted with this buffer at a flow rate of 0.5 ml/min, the 0.5 ml fractions were collected and assayed for SDH activity. The Superose 6 column was calibrated with the following molecular weight markers : thyroglobulin 670 KDa, immunoglobulin 158 KDa, ovalbumin 44 KDa, myoglobin 17 KDa and vitamin B_{12} 1.35 KDa. The molecular weight of *P.falciparum* (T_9 isolate) SDH was estimated by gel filtration chromatography.

7. Procedure for Electrophoresis

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Bio-Rad minislab gel apparatus with 5% acrylamide stacking gel and 10% acrylamide running gel in the discontinuous buffer system of Laemmli (1970). The gel was stained with Coomassie Blue R.

7.1 Electrophoresis Buffer and Gel Preparation

7.1.1 SDS-PAGE Reagents

7.1.1.1 Acrylamide / Bis acrylamide was prepared by dissolving 29.2 gm acrylamide and 0.8 gm bis acrylamide in 100 ml double distilled water, stored in brown bottle at $4^{\circ}C$.

7.1.1.2 1 M Tris pH 8.8 was prepared by dissolving 12.1 gm Trizma base in 100 ml double distilled water, then adjusted to pH 8.8 with NaOH.

7.1.1.3 1 M Tris pH 6.8 was prepared by dissolving 12.1 gm Trizma base in 100 ml double distilled water, then adjusted to pH 6.8 with HCl.

7.1.1.4 10% SDS was prepared by dissolving 10 gm SDS in 100 ml double distilled water.

7.1.1.5 10% Ammonium persulfate (APS) was prepared by dissolving 0.5 gm ammonium persulfate in 5 ml double distilled water, then stored in brown bottle at 4°C.

7.1.1.6 0.1% Bromphenol Blue (BPB) was prepared by dissolving 0.1 gm BPB in 100 ml double distilled water.

7.1.1.7 Water saturated in n - butanol was prepared by mixing 50 ml n-butanol and 50 ml double distilled water together.

7.1.2. Seperating Gel (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 with 25 µl 10 % APS and 5 µl TEMED.

7.1.3. Stacking Gel

Acrylamide/Bis acrylamide	0.420	ml
1 M. Tris pH 6.8	0.315	ml
Double distilled water	1.750	ml
10% SDS	0.025	ml

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

7.1.4 Running Buffer

Running buffer was prepared by dissolving 3.03 gm Trizma base and 14.42 gm glycine in 1,000 ml double distilled water, then added with 10 ml 10% SDS and adjusted to pH 8.3 with NaOH.

7.1.5 2 x Sample Buffer

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

The solution was mixed with an equal volume of sample and boiled for 6 min before loading.

7.2 Coomassie Blue R Staining

Coomassie Brilliant Blue R250	0.1	gm
Absolute methanol	50	ml
Glacial acetic acid	10	ml
Double distilled water	50	ml

The dye was first dissolved in methanol and then added with 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.

8. Calculation of Specific activity of Succinate Dehydrogenase (SDH) and Protein Concentration in *P. falciparum*

The supernatant fluid was assayed for the SDH activity (as described in section 4) . The specific enzyme activity was calculated by the following equation.

Specific enzyme activity=Enzyme activity per fraction (nmol / min)

(nmol / min / mg) Protein per fraction (mg)

9. Molecular Weight Determination

Determination of the molecular weight (MW) of enzyme SDH by gel filtration chromatographic technique as described in section 6.7 and by SDS-PAGE as described in section 7.1

10. Study of Kinetic Properties of the Enzyme

Kinetics study of the enzyme SDH purified from *P. falciparum* was determined by varying substrates (succinic acid or CoQ_0) concentration and fixing amount of enzyme. After achieving the values of enzyme activity in each concentration of SDH, these values were plotted as Michealis - Menten kinetics on an Enzfitter computer software program, then calculated the Michealis - Menten constant (Km) and turnover number (kcat) values.

11. Study of Inhibitors of the enzyme

Inhibitory effect to the enzyme SDH by two substrate analog inhibitors, malonate and oxaloacetate; one product inhibitor, fumarate, were studied by varing inhibitor concentrations and fixing the amount of partial purified enzyme SDH. These values were plotted as Michealis - Menten

kinetics and calculated for the inhibition constant (Ki) values.

Some antimalarial drugs, such as atovaquone, chloroquine, were determined for inhibiting the enzyme SDH activity. Percent of inhibition for each antimalarial drugs was calulated.