RESULTS

1. Detection of the Enzyme Succinate Dehydrogenase in *P. falciparum* (T_9 isolate)

P. falciparum (T_9 isolate) parasites from in vitro cultivation were isolated from host red cells by saponin lysis. Host cell-free parasite were then lysed and called crude homogenate. The pellet, after centrifugation of the crude homogenate, was extracted as detergent solubilization fraction. The supernatant fluid was also saved.These three fractions were subjected to enzyme assay of SDH activity. The results of SDH activities in crude homogenate, supernatant fluid and detergent solubilization fraction of *P. falciparum* (T_9 isolate) are shown in Table 2. The specific SDH activity in the crude homogenate was 91.11±59.25 nmol/min/mg protein (n=7), in the supernatant fluid was 85.02 ± 58.73 nmol/min/mg protein (n=7) and in detergent solubilization fraction of the pellet was 5.12 ± 4.04 nmol/min/mg protein (n=7). The specific SDH activity in crude homogenate was calculated to 100%, the enzyme activities in the supernatant fluid and detergent solubilization fraction (pellet) were found to be 93 % and 7 % respectively.

	SDH Activity (nmol/min/mg protein)		
Experiment No.	crude homogenate	supernate	detergent extract
1	91.11	110.56	1.61
2	72.42	56.28	7.12
3	67.87	56.63	0.87
4	165.60	174.76	10.88
5	176.16	142.14	9.04
6	16.86	12.31	1.24
7	47.77	42.50	5.08
mean±SD	91.11±59.25	85.02±58.73	5.12±4.04
%	100	93	7

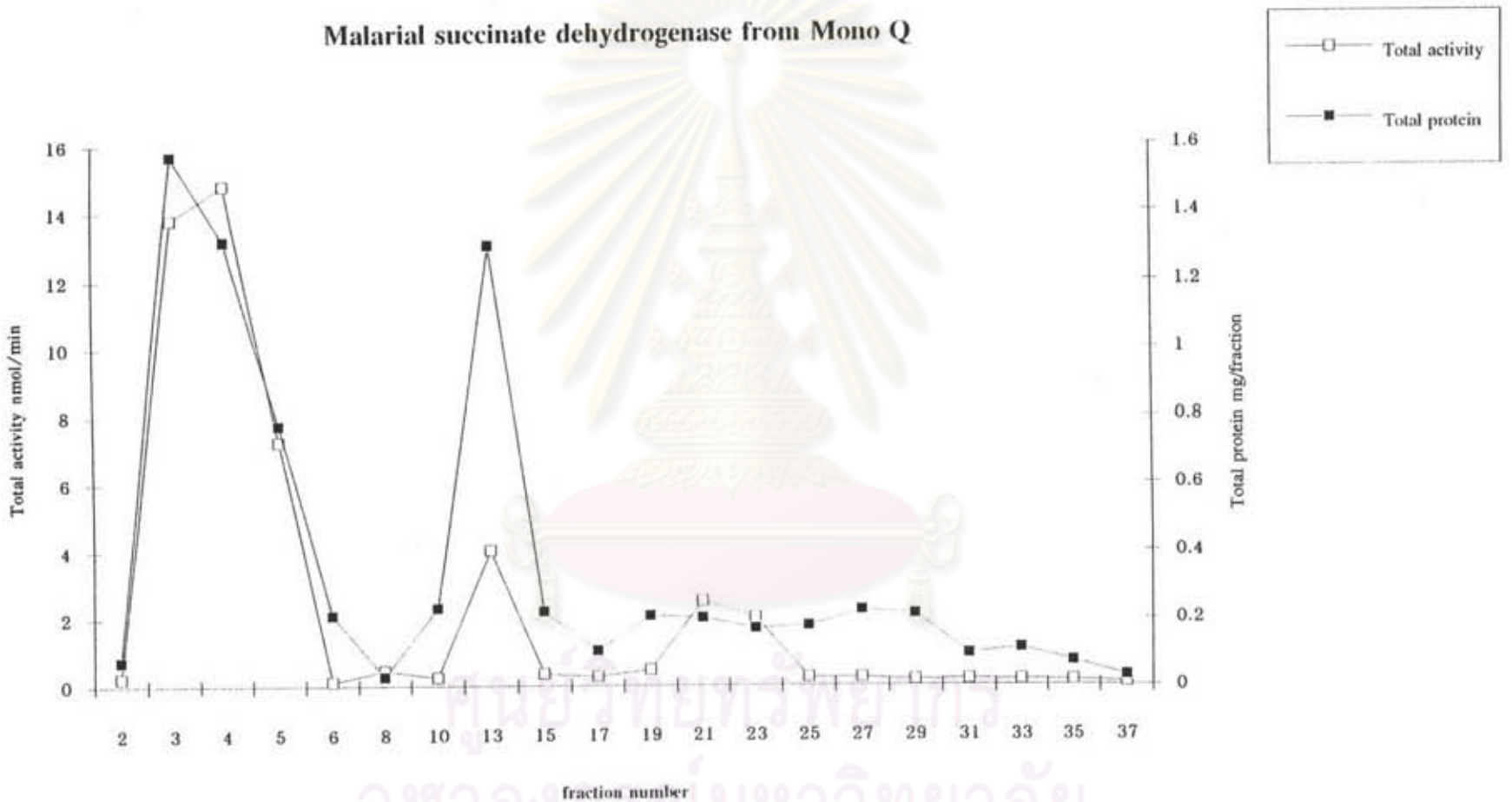
2. Purification of *P. falciparum* (T_9 isolate) Succinate Dehydrogenase

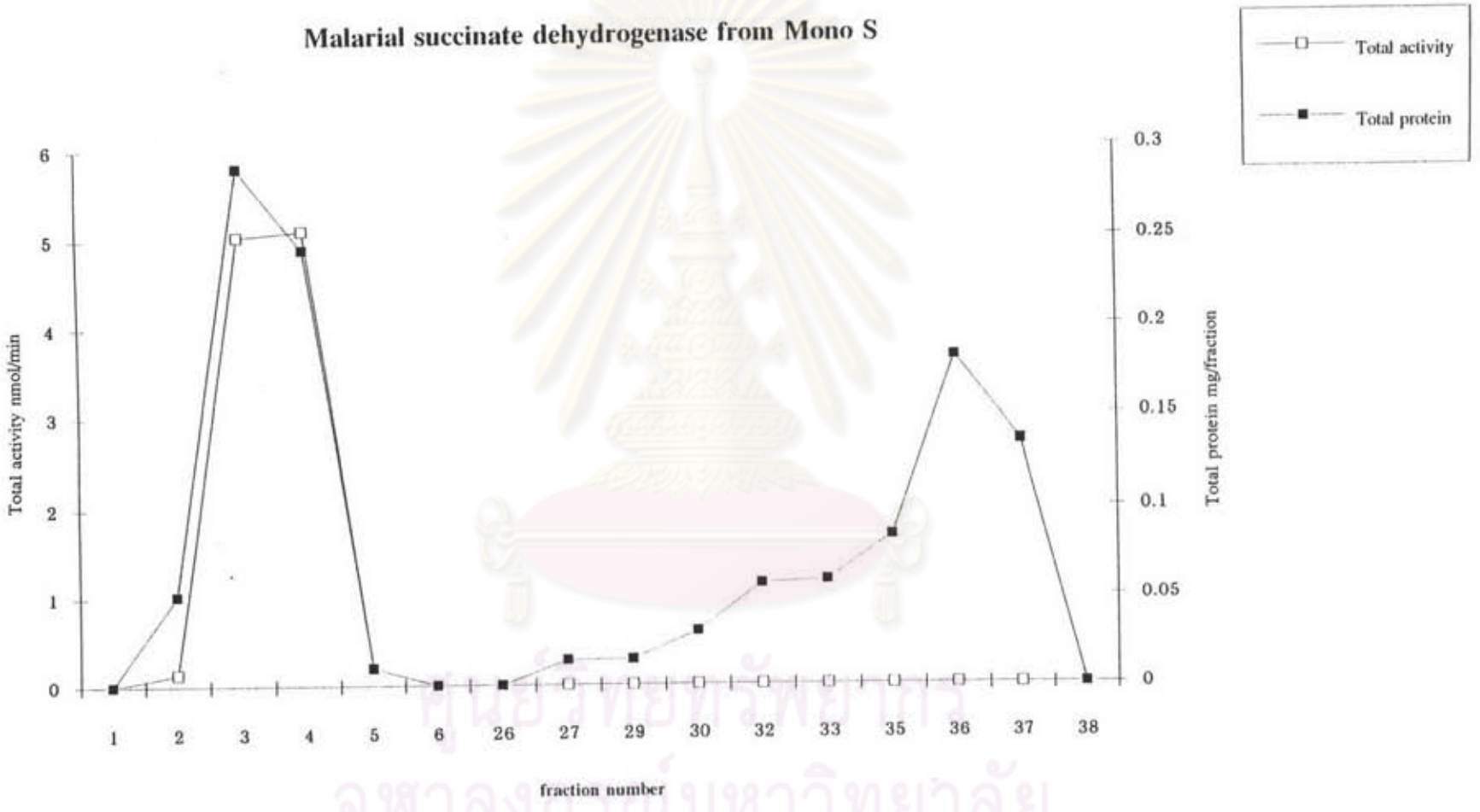
The supernatant fluid containing more than 90 % of enzyme SDH activity was used as a source of starting material for purification. It was directly applied to anion-exchange Mono Q chromatography column and assayed for enzyme activity (as described in materials and methods, section 6.5). The major SDH activity was found in the fraction numbers 3, 4 and 5 with specific activity of 5.38 nmol/min/mg protein and protein concentration of 3.76 mg/ml (Fig. 4).

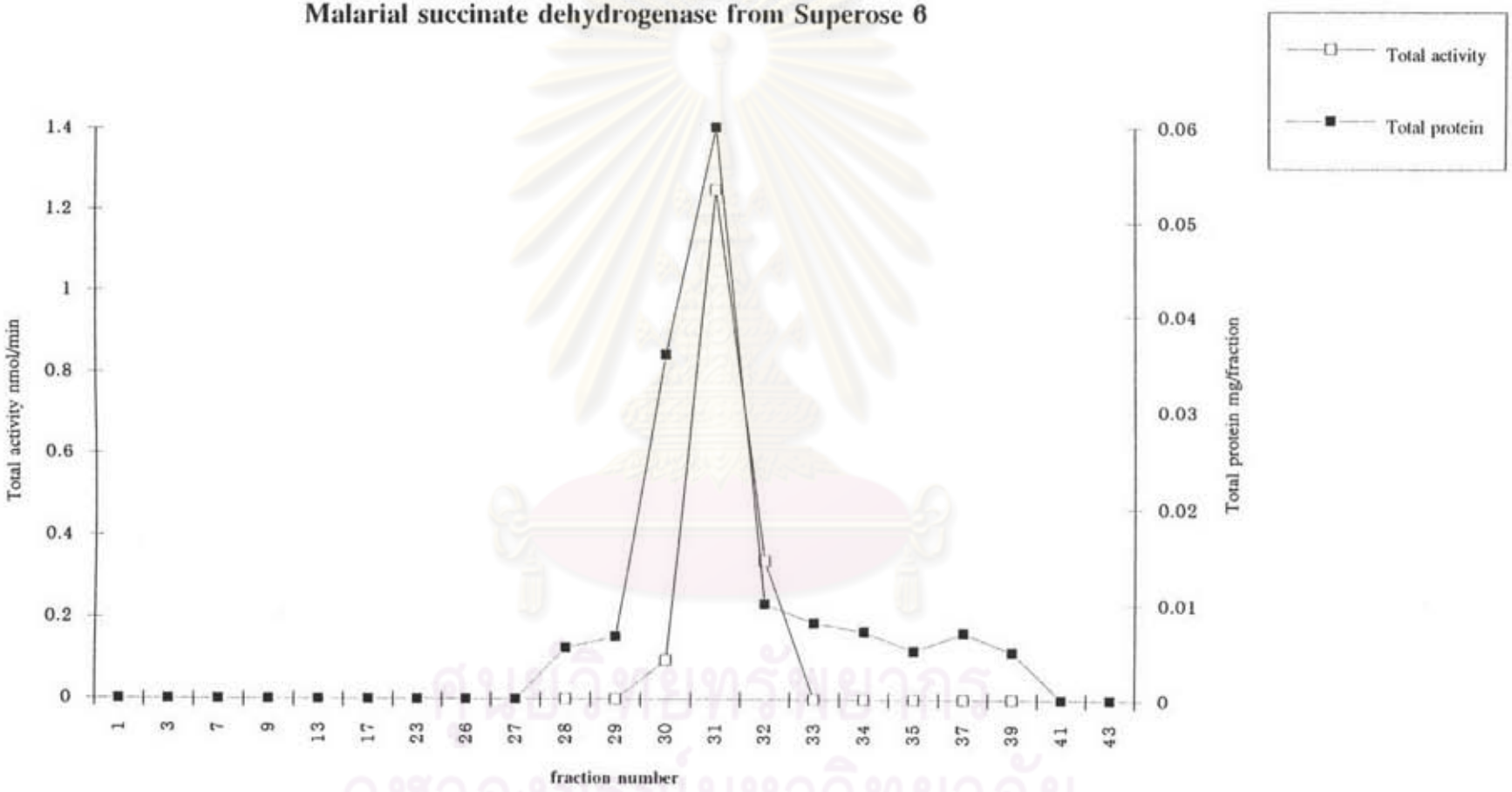
The active fractions containing SDH activity eluted from the Mono Q column were pooled and then applied to the Mono S column (as described in materials and methods, section 6.6). The major SDH activity was found in the fraction numbers 3 and 4 with specific activity of 6.72 nmol/min/mg protein and protein concentration of 0.33 mg/ml (Fig.5).

The active fractions containing SDH activity eluted from Mono S column were pooled and then applied to the Superose 6 gel filtration FPLC column (as described in materials and methods, section 6.7). The major SDH activity was found in fraction numbers 30 and 31 with specific activity of 9.0 nmol/min/mg protein and protein concentration of 0.1 mg/ml (Fig. 6).

It was found that the purification fold of each step was relatively low, as the enzyme tremendously lost its activity due to the long running time at room







temperature, especially in the Superose 6 gel filtration chromatography. However, the results on the Superose 6 gel filtration column was used to determine molecular weight of the native enzyme. Table 3 shows a representative experiment on SDH purification from *P. falciparum* (T_9 isolate). It was noted here that very low % yield and fold purified was obtained.

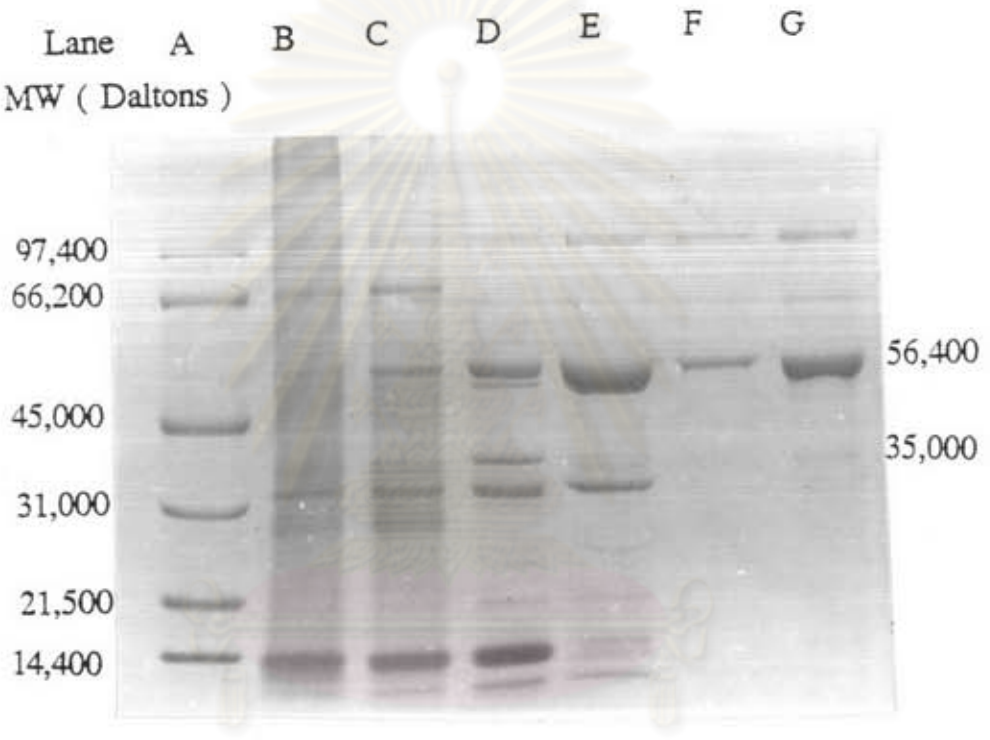
After each step of purification, the small amount of pooled active fractions was checked for the purity of enzyme preparation by nondenaturing and SDS - PAGE techniques. Based on SDS - PAGE analysis of the enzyme obtained from each step purification, it was demonstrated that the purified enzyme after the Superose 6 column (lane F, G, Fig. 7) contained 2 major protein bands, indicating the subunits of the enzyme purified from the parasite. However, the low molecular weight protein (35,000 Da) was apparently shown only after loading the enzyme with 20 μg. As shown in Fig. 8, the purified enzyme obtained from the Superose 6 column (see Table 3) was then subjected to nondenaturing PAGE analysis and found to be one protein band after Coomassie staining.

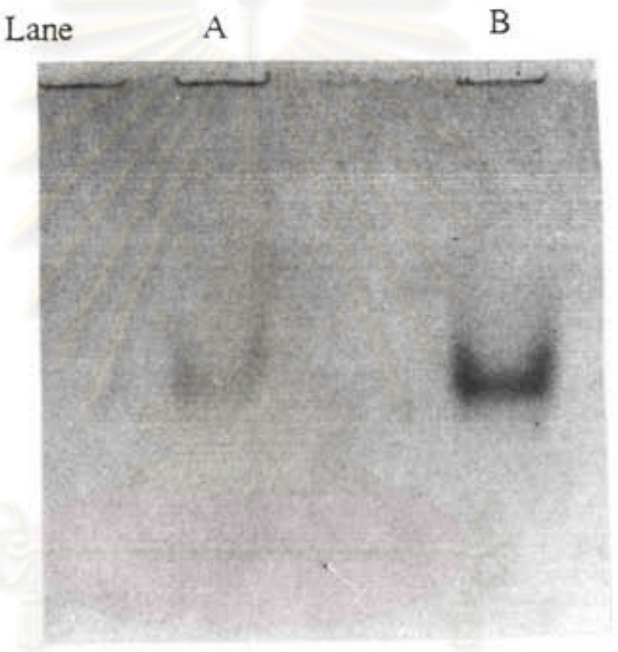
3. Properties of Purified Malarial Succinate Dehydrogenase

3.1 Stability of Malarial Succinate Dehydrogenase

Stability of *P. falciparum* (T_9 isolate) SDH was performed by keeping at 4°C, -20°C (freezer) and -196°C (liquid N_2) and considering the first day of laboratory experiment as day 0 having 100 % activity. The result is

purification step	total protein (mg)	total activity (nmol/min)	specific activity (nmol/min/mg)	purification fold	% yield
1. Homogenate	34.89	47.77	1.37	1.00	100
2. Supernate	14.22	42.50	2.99	2.18	88.96
3. Mono Q	3.76	20.22	5.38	3.92	42.33
4. Mono S	0.33	2.22	6.72	4.91	4.65
5. Superose 6	0.01	0.09	9.00	6.57	0.18





Days	Percent Activity at		
	4°C	-20°C	-196°C
0	100.0	100.0	100.0
1	18.0	62.0	90.0
2	1.2	12.2	79.0
3	0	0.1	17.0
4	0	0	3.5
5	0	0	0

shown in Table 4. The enzyme SDH activity was found to be extremely labile. The activity was decreased by more than 80 % overnight at 4°C. And this enzyme was more stable when stored at -196°C than that of -20°C.

3.2 Kinetics Parameters Determinations

Kinetics parameters were determined by varying concentrations of substrates of the enzyme SDH, succinate and CoQ_0 , and fixing enzyme SDH concentration. The results are shown in Tables 5, 6, 7 and Fig. 9. The apparent Michaelis - Menten constants (Km) and catalytic constant (kcat) for succinate and CoQ_0 were calculated from plots of Enzfitter software program (Fig. 9). The Km values for succinate and CoQ_0 were 3.01 and 0.20 μM, and kcat values were 0.11 and 0.06 min^{-1}, respectively (n=2)(Table 7).

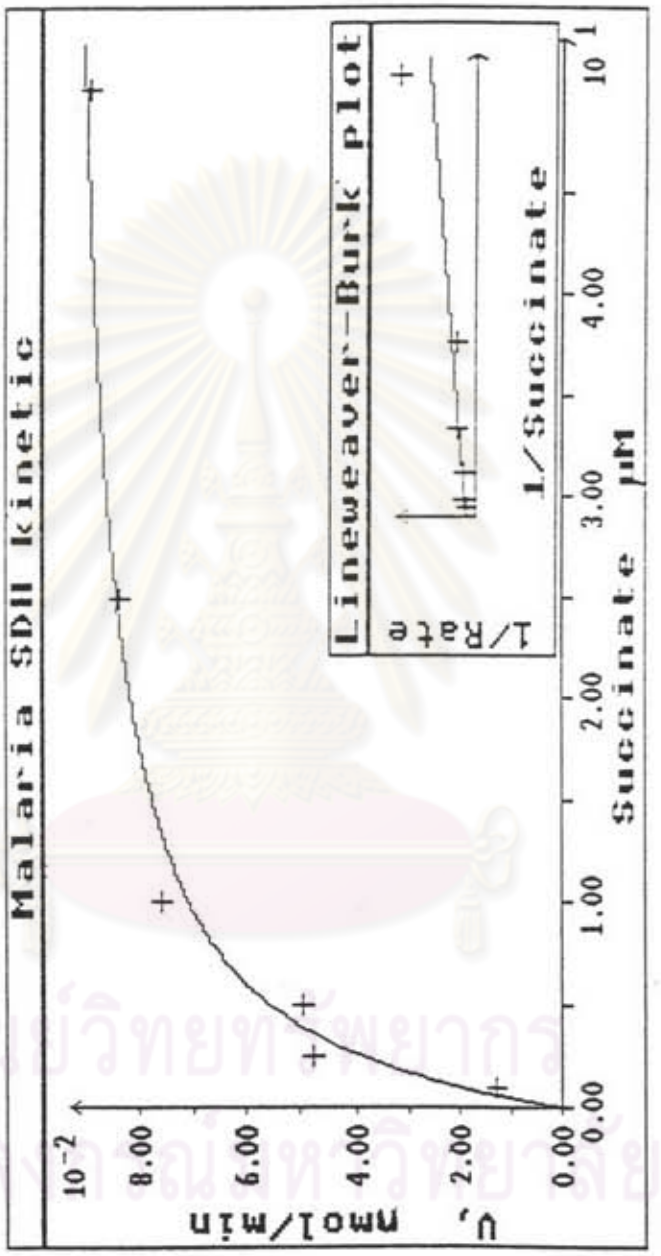
3.3 Inhibitor studies

Inhibitory effects of various compounds on the purified malarial SDH were performed. It was demonstrated that fumarate, the product of the enzymatic reaction, was a competitive inhibitor with Ki value of 80.99 μM. Malonate and oxaloacetate were also competitive inhibitors with Ki values of 13.02 and 12.06 μM. (n=2). Plumbagin was found to inhibit more than 50% at a concentration of 5 μM. Other antimalarial drugs, such as chloroquine, artemisinine and atovaquone were found to have no effect on the malarial enzyme, as what found with 2-thenoyltrifluoroacetone, a wellknown inhibitor of mammalian SDH (Table 8). The structures of some antimalarial drugs are also shown in Fig. 10.

Succinate (μM)	rate (nmol/min)
1.0	.0128
2.5	.0477
5.0	.0497
10.0	.0758
25.0	.0837
50.0	.0893

CoQ_0 (μM)	rate (nmol/min)
0.2	.0310
0.5	.0487
0.7	.0500

Substrates	Km (μM)	kcat (min^{-1})
Succinate	3.01	0.11
CoQ_0	0.20	0.06


Malaria SDH kinetic
Lineweaver-Burk plot
1/Rate
1/Succinate
10^{-2}
8.00
6.00
4.00
2.00
0.00
V_o nmol/min
0.00
1.00
2.00
3.00
4.00
Succinate μM
1

Compound	Ki (μM)	(% inhibition) SDH
Antimalarial agents:		
Chloroquine		0[a]
Artemisinine		0[b]
Atovaquone		0[c]
Plumbagin		55[d]
Inhibitors:		
Malonate	13.02	
Oxaloacetate	12.06	
Fumarate	80.99	
Thenoyltrifluoroacetone (TFA)		0[e]

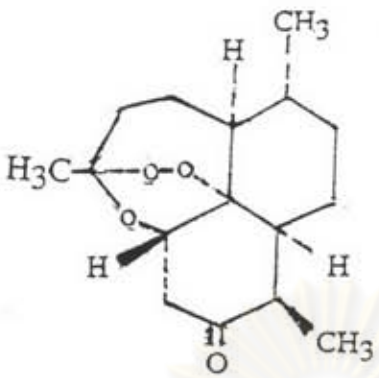
a = concentration of chloroquinine 100 μM

b = " artimisinine 5 "

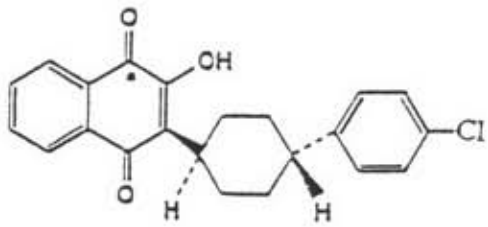
c = " atovaquone 5 "

d = " plumbagin 5 "

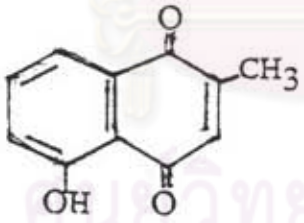
e = " TFA 50 "



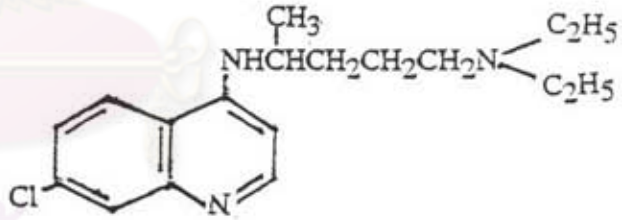
Artemisinine



Atovaquone



Plumbagin



Chloroquinine

3.4 Molecular Weight Determination

Determination of the molecular weight of the purified malarial SDH was performed by 2 analytical techniques : sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS-PAGE) and gel filtration chromatographic techniques.

By using SDS-PAGE analysis various molecular weight marker proteine were loaded on a 10 % gel of SDS-PAGE . These included phosphorylase b (MW = 97.4 KDa) , bovine serum albumin (MW = 66.2 KDa) , ovalbumin (MW = 45 KDa) , carbonic anhydrase (MW = 31 KDa), soybean trypsin inhibitor (MW = 21.5 KDa) and lysozyme (MW = 14.4 KDa). The relative mobility of proteins were plotted against molecular weights on a semi-log graph (Fig. 11). The molecular weight of malarial SDH was calculated from the standard curve. It was shown that the molecular weight of the subunits of purified malarial SDH were 56.4 ± 3.4 and 35.0 ± 1.7 KDa (n=6) (Fig. 11).

By using gel filtration chromatographic technique, molecular weight marker proteins : thyroglobulin (MW = 670 KDa) , immunoglobulin (MW = 158 KDa) , ovalbumin (MW = 44 KDa) , myoglobin (MW = 17 KDa) and vitamin B_{12} (MW = 1350 Da) were applied to the Superose 6 FPLC column for calibration (Fig. 12) . Standard curve was then constructed. The malarial SDH was eluted as a native form at a position which corresponded to 86.0 ± 2.6 KDa (n=6) (Fig. 13).