RESULTS

1. Demonstration of Enzyme Dihydroorotate Dehydrogenase in P. falciparum

The results of the study of DHODase activity in crude homogenate and solubilization fraction of P. falciparum are shown in Table 1. The specific DHODase activity in crude homogenate was 8.31 ± 6.26 nmol/min/mg (n= 13) and in detergent solubilization fraction is 3.30 ± 2.61 nmol/min/mg (n =13).

And after synchronization the culture, according to the method of Lambros and Vanderberg (1979) as described in section 2.9 of 'Materials and Methods', the results of the study of specific DHODase activity and protein concentration in ring, trophozoite, and schizont stages of P.falciparum are shown in Table 2 and Fig.4. The highest specific activity was found in trophozoite stage (2.59 nmol/min/mg), lower in ring stage (2.06 nmol/min/mg), and lowest in schizont stage (1.63 nmol/min/mg).

For protein concentration, the highest level was found in trophozoite stage (3.84 mg/ml) start to decline in schizont stage (3.26 mg/ml) and further decline until reaching the lowest stage at ring stage (1.76 mg/ml).

By using the immunoblotting technique to determine the amount of DHODase protein, it is apparent that the DHODase in trophozoite and schizont stage are much more than when they were at righ statge parasites. This is due to the fact that the DHODase activity band can be clearly seen in trophozoite and schizont stages but not in ring stage parasite, after staining with mouse antibody against the malarial DHODase (Fig. 5).

TABLE 1 : Specific DHODase activity in crude extracts and detergent solubilizations of P.falciparum.

Experiment No.	Specific Activity (nmol/min/mg)		Storage Days
	Crude Homogenate	Triton Extract	
1	16.23	2.95	10
2	4.04	0.75	90
3	0.69	0.14	270
4	3.55	1.31	20
5	3.83	2.56	15
6	14.27	7.01	0
7	9.51	5.37	7
8	2.49	2.49	14
9	2.95	1.53	20
10	20.33	9.11	0
11	16.67	5.89	0
12	4.62	1.04	30
13	8.85	2.73	7

TABLE 2 : Specific DHODase activity and protein concentration in ring, trophozoite, and schizont stage of P.falciparum.

Stage	prot. conc. (mg/ml)	activity (nmol/min)	Specific activity (nmol/min/mg)
Ring Form	1.76	0.18	2.06
Trophozoite	3.84	0.49	2.59
Schizont	3.26	0.27	1.63

FIGURE 4: : Specific DHODase activity and protein concentration in ring, trophozoite and schizont stage of P.falciparum.

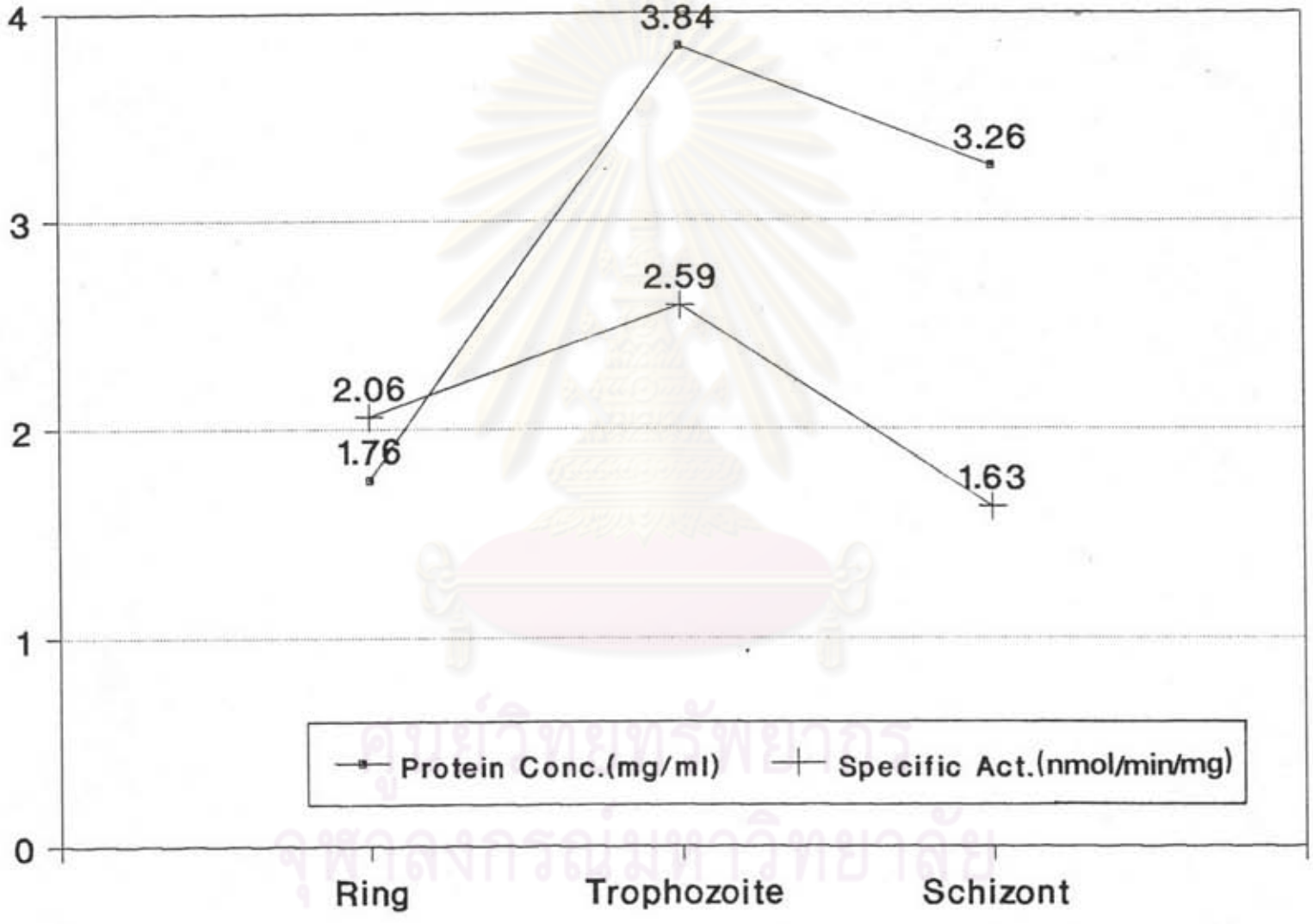

4
3
2
1
0
3.84
3.26
2.59
2.06
1.76
1.63
Protein Conc.(mg/ml)
Specific Act.(nmol/min/mg)
Ring
Trophozoite
Schizont

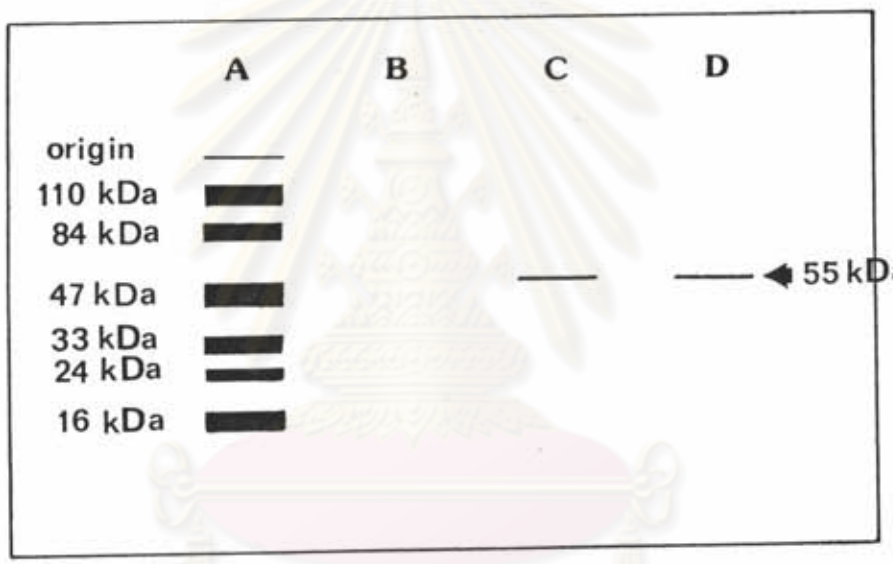
FIGURE 5 : Western analysis pattern of Triton X-100 solubilized extract of P.falciparum probed with mouse-anti-DHODase antiserum.

lane A = Standard molecular weight marker proteins, composed of phosphorylase b (MW=110 kDa), bovine serum albumin (MW=84 kDa), ovalbumin (MW=47 kDa), carbonic anhydrase (MW = 33 kDa), soybean trypsin inhibitor (MW=24 kDa) and lysozyme (MW = 16 kDa).

lane B = Detergent solubilization of ring stage of P.falciparum

lane C = Detergent solubilization of trophozoite stage of P.falciparum

lane D = Detergent solubilization of schizont stage of P.falciparum



2. Purification of P. falciparum Dihydroorotate Dehydrogenase

The results of the study of DHODase purification are shown in Table 3. The detergent solubilization fraction (prepared as described in 'Materials and Methods' section 6.2.2) has specific DHODase activity of 2.94 nmol/min/mg and protein concentration of 1.28 mg/ml.

The detergent solubilized DHODase was directly applied to an anion-exchange Mono Q 5/5 column and assayed enzyme activity as described in section 5 of 'Materials and Methods'. The peak of DHODase activity was found in fractions number 4 and 5,as specific activity of 4.64 nmol/min/mg and protein peaks were found in fraction numbers 3, 4, 5 as a first peak and 10,11,12 as a second peak, as protein concentration of 0.21 mg/ml. (Fig. 6).

The active fractions from Mono Q 5/5 column were pooled and concentrated on a Centricon-10 (Amicon),and then applied to the Cibacron Blue F3GA-agarose affinity column as described in section 6.2.4 of 'Materials and Methods'. The peak of DHODase activity was found in fraction numbers 7 and 8 as specific activity of 21.67 nmol/min/mg and protein peaks were found in fraction numbers 5,6 as a first peak and fraction numbers 7,8,9 as a second peak, as protein concentration of 0.09 mg/ml. (Fig. 7.2)

After each step of purification,the small amount of pooled active fractions was checked for the purity of enzyme by SDS-PAGE technique. The result are shown in Fig. 8.

The active fractions from affinity column were pooled and concentrated on Centricon-10 (Amicon). They were then applied to the Gel filtration Superose 12 column as described in section 6.2.5 of 'Materials and Methods'. It was found that the purification fold of this step is lower than the affinity column's as the enzyme lost its activity tremendously due to long running time (40-45 minutes) at room temperature of Superose 12. However, the result of this step can still be used in determining molecular weight (Fig. 9). As shown in Fig. 9, the enzyme obtained showed heterogeneity, containing at least 2 bands on the SDS-PAGE analysis.

TABLE 3 : Purification of DHODase from P.falciparum.

Steps	Volume (ml)	Total Protein (mg)	Total Activity (nmol/min)	Specific Activity (nmol/min/mg)	Purification Fold	% Yield
1. Detergent Extraction	2.40	3.07	9.04	2.94	1.00	100.00
2. Mono Q	2.00	0.42	1.95	4.64	1.58	21.57
3. Affinity (PIERCE)	1.00	0.09	1.95	21.67	7.37	21.57

FIGURE 6 : Profile of DHODase activity of P.falciparum, eluted from a anion-exchange Mono Q column of FPLC system.

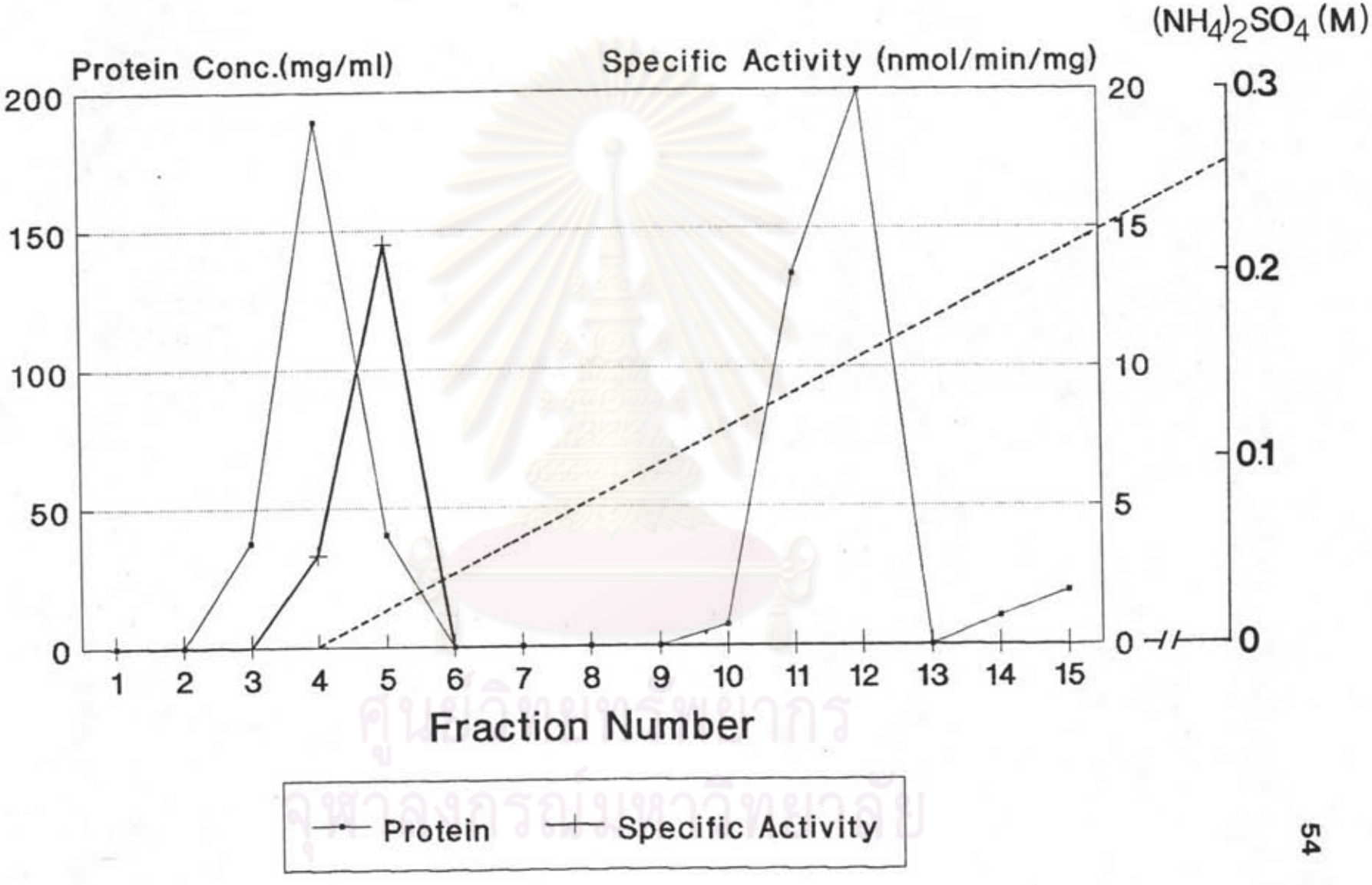

Protein Conc.(mg/ml)
Specific Activity (nmol/min/mg)
$(NH_4)_2SO_4$ (M)
200
150
100
50
0
20
15
10
5
0
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Fraction Number
Protein
Specific Activity

FIGURE 7 : Profile of DHODase activity of P.falciparum after Mono Q column (Fig. 6), eluted from a Cibacron Blue F3GA–Agarose affinity column of FPLC system.

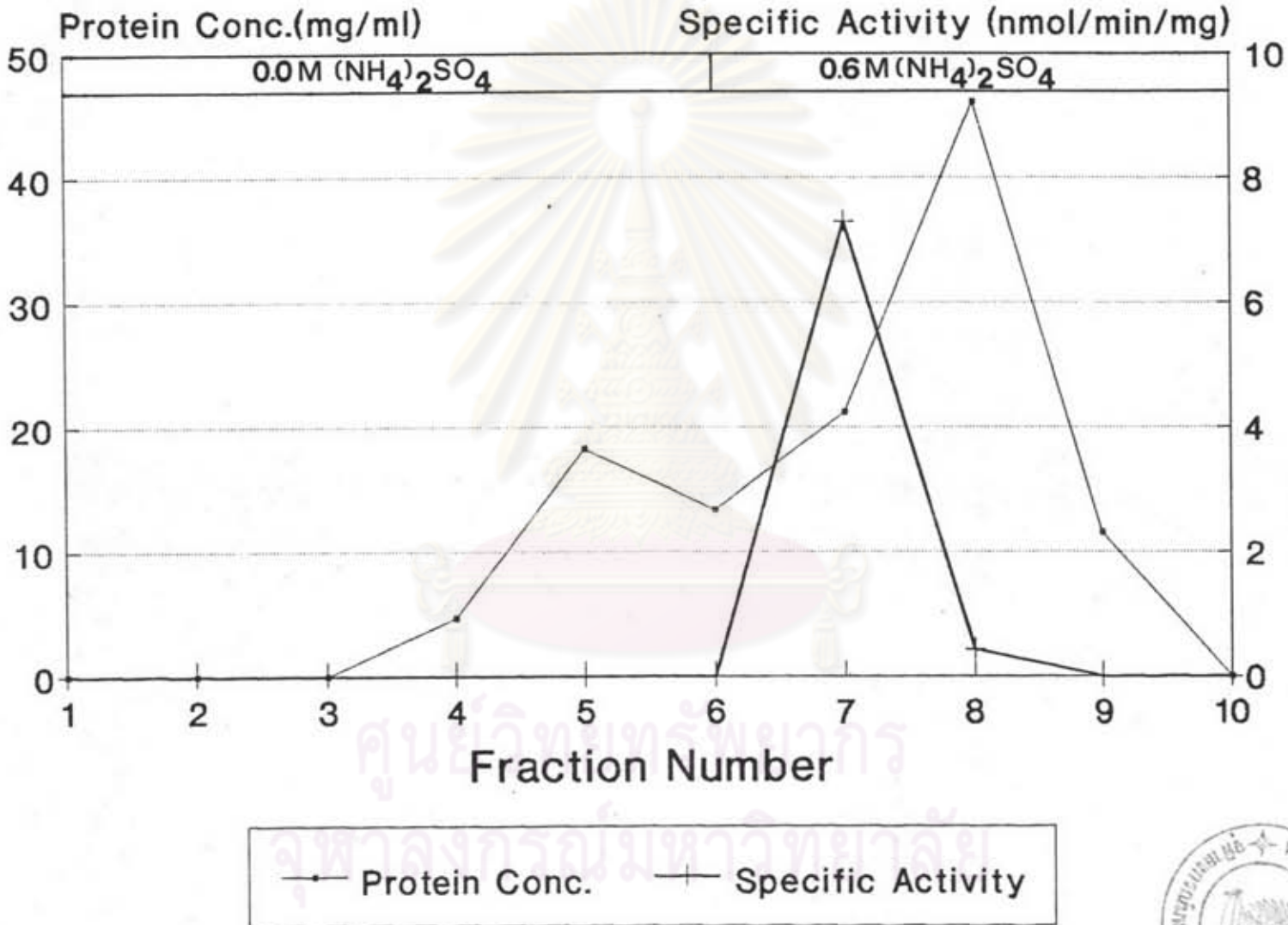


FIGURE 8 : SDS–PAGE pattern of purification steps of P.falciparum DHODase.

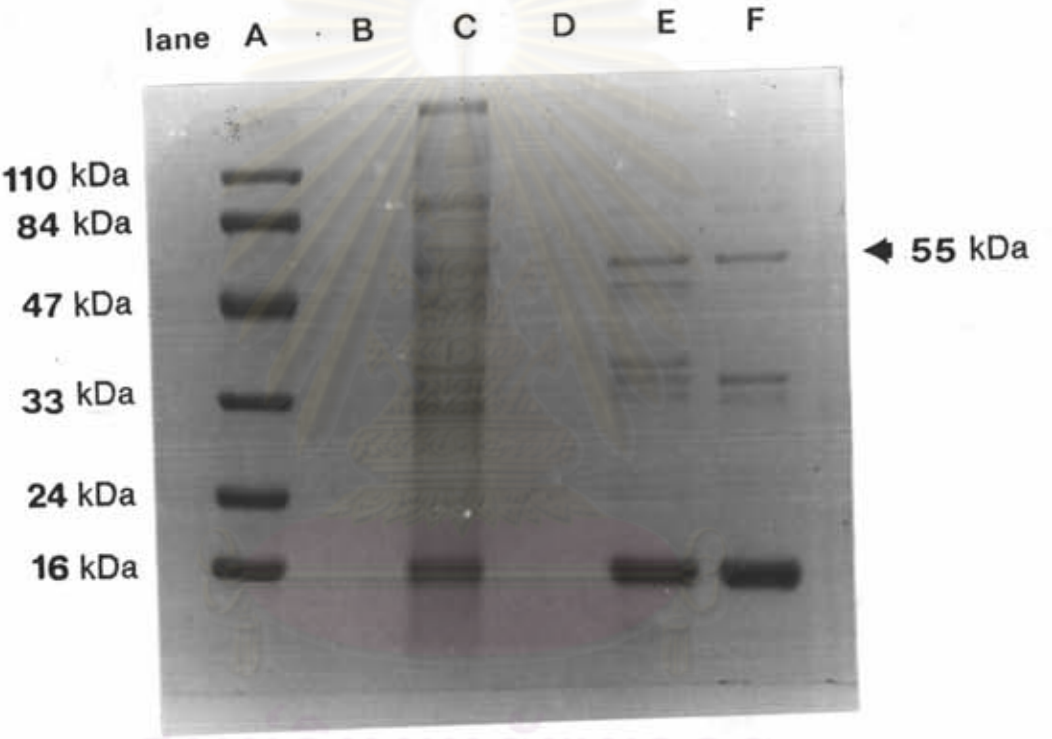
lane A = Standard molecular weight marker proteins,composed of phosphorelase b(MW=110 kDa), bovine serum albumin (MW = 84 kDa), ovalbumin (MW=47 kDa), carbonic anhydrase (MW = 33 kDa), soybean trypsin inhibitor (MW=24 kDa) lysozyme (MW = 16 kDa)

lane C = Triton X–100 extraction part of P.falciparum

lane E = Pooled active fraction from Anion–exchange Mono Q column

lane F = Pooled active fraction from Cibacron Blue F 3 GA–agarose Affinity column

lane B , D = Reagent blank



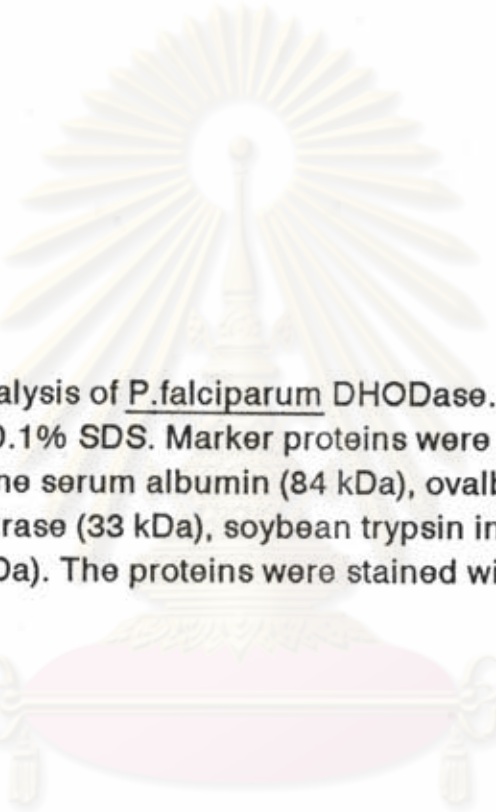
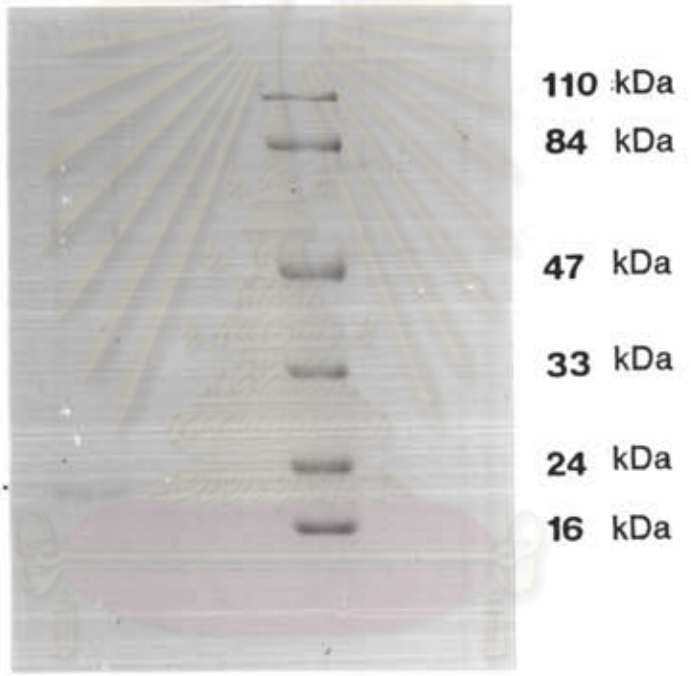


FIGURE 9 : SDS–PAGE analysis of P.falciparum DHODase. A 10% polyacrylamide gel was run in 0.1% SDS. Marker proteins were phosphorylase B (110 kDa), bovine serum albumin (84 kDa), ovalbumin (47 kDa), carbonic anhydrase (33 kDa), soybean trypsin inhibitor (24 kDa), lysozyme (16 kDa). The proteins were stained with Coomassie Blue.



3.Properties of Purified Dihydroorotate Dehydrogenase

3.1 Stability of Dihydroorotate Dehydrogenase

The result of stability studying of P.falciparum DHODase at −20°C (freezer) and −196°C (liquid N_2),by considering the first day of laboratory experiment as day 0 and has 100% activity,is shown in Table 4 and Fig. 10. Cell-free extracts of the malarial DHODase were found to be extremely labile. Even in the presence of protease inhibitors at −20°C, the activity was decreased by more than 80% overnight.And this enzyme was more stable at −196°C than −20°C.

3.2 Kinetic Parameters Determination

The result of kinetic parameters, studying by varying substrate (DHO) concentration and fixing enzyme (DHODase) concentration are shown in Table 5 and Fig.11 . The apparent Michaelis constant (Km) for L-DHO and catalytic constant (Kcat) were estimated from calculations. The Km value for L-DHO was 88.7 ± 24.1 μM and Kcat value was 0.36 ± 0.04 min^{-1}.

3.3 Inhibitor Studies

The study of inhibitor were done by studying the inhibition ability of 2 chemicals; 5- fluoroorotic acid (FOA) and 5-methylorotic acid (CH_3OA). The results are shown in Table 6 that the inhibitory ability of FOA (with 50% inhibition at 0.16 mM) is much more than CH_3OA (with 50% inhibition at 5.41 mM) (the inhibitor concentration that has 50 % inhibition of the two chemicals were calculated by extrapolation).

TABLE 4 : The result of the study of DHODase's stability at –20°C and –196°C. In this study, the first day of laboratory experiment was considered as day 0 and has 100% activity.

Day	Per Cent Activity at	
	- 20 °C	- 196 °C
0	100.0	100.0
1	19.5	98.0
2	7.5	97.0
3	7.2	94.0
4	7.0	92.0
5	6.7	90.5
6	6.5	85.5
7	4.0	80.5
8	3.0	78.0
9	2.8	77.0
10	2.7	75.5
11	2.5	75.0
12	2.3	73.0
13	2.1	71.0
14	2.0	70.5
15	1.8	70.0

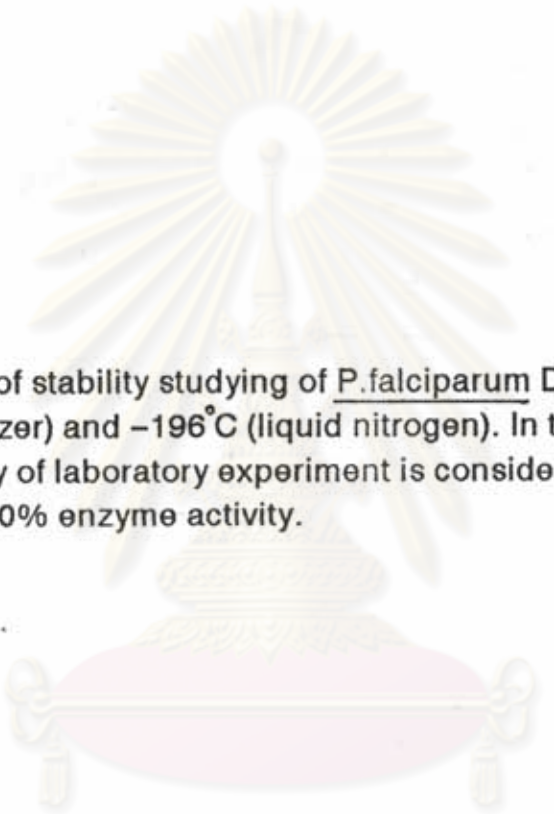


FIGURE 10 : The result of stability studying of P.falciparum DHODase at −20°C (freezer) and −196°C (liquid nitrogen). In this study, the first day of laboratory experiment is considered as day'0' and has 100% enzyme activity.

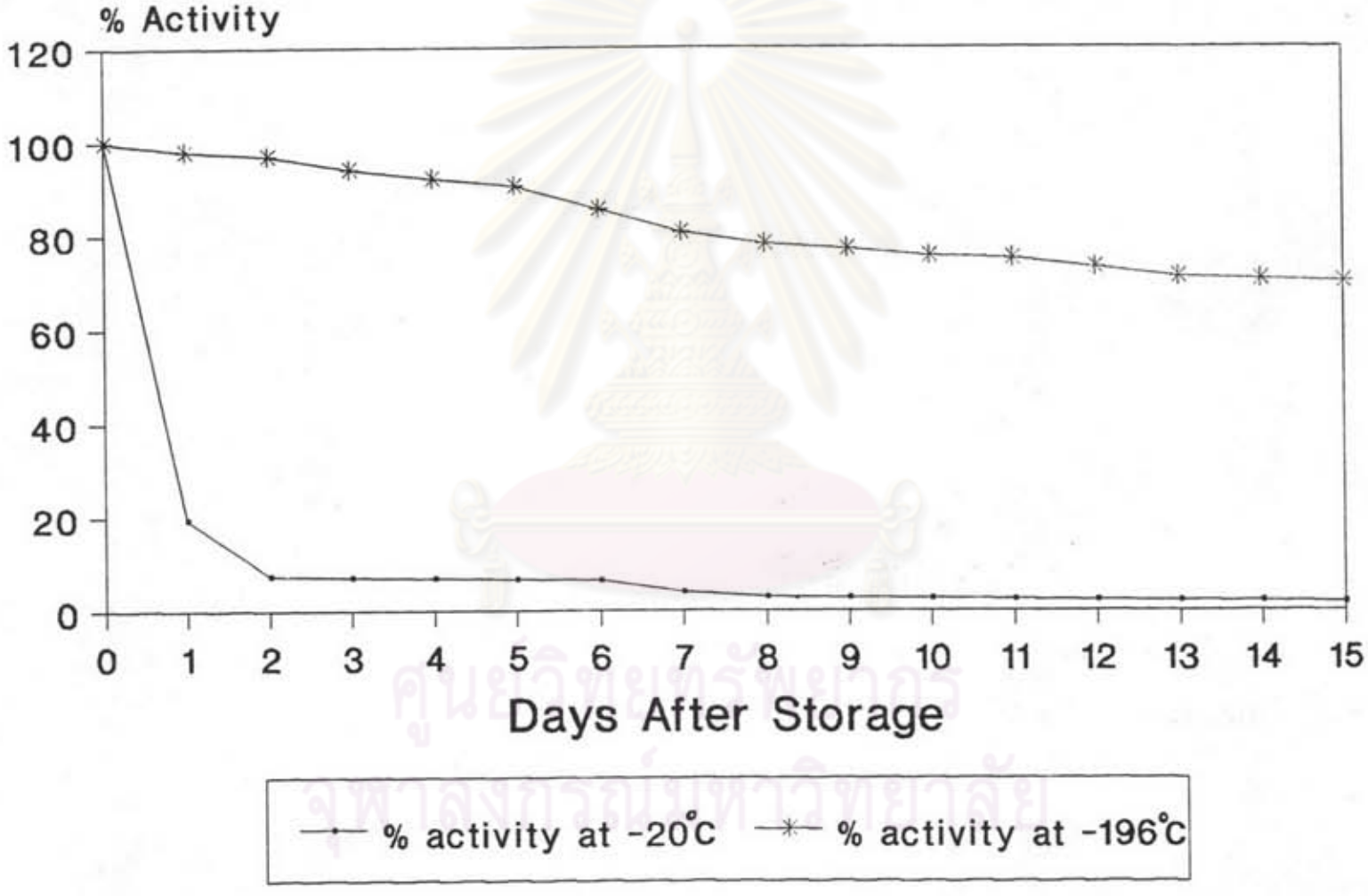

% Activity
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days After Storage
% activity at -20°C
% activity at -196°C

TABLE 5 : Kinetic parameter studies of P.falciparum DHODase by varying L–DHO concentrations at fixed concentration of DHODase .

[L-DHO] (mM)	RATE (v.nmol/min)
50	0.126
100	0.195
150	0.242
200	0.233
250	0.274

FIGURE 11 : Michaelis Menten kinetics and Lineweaver – Burk plot (inset) of P.falciparum DHODase.

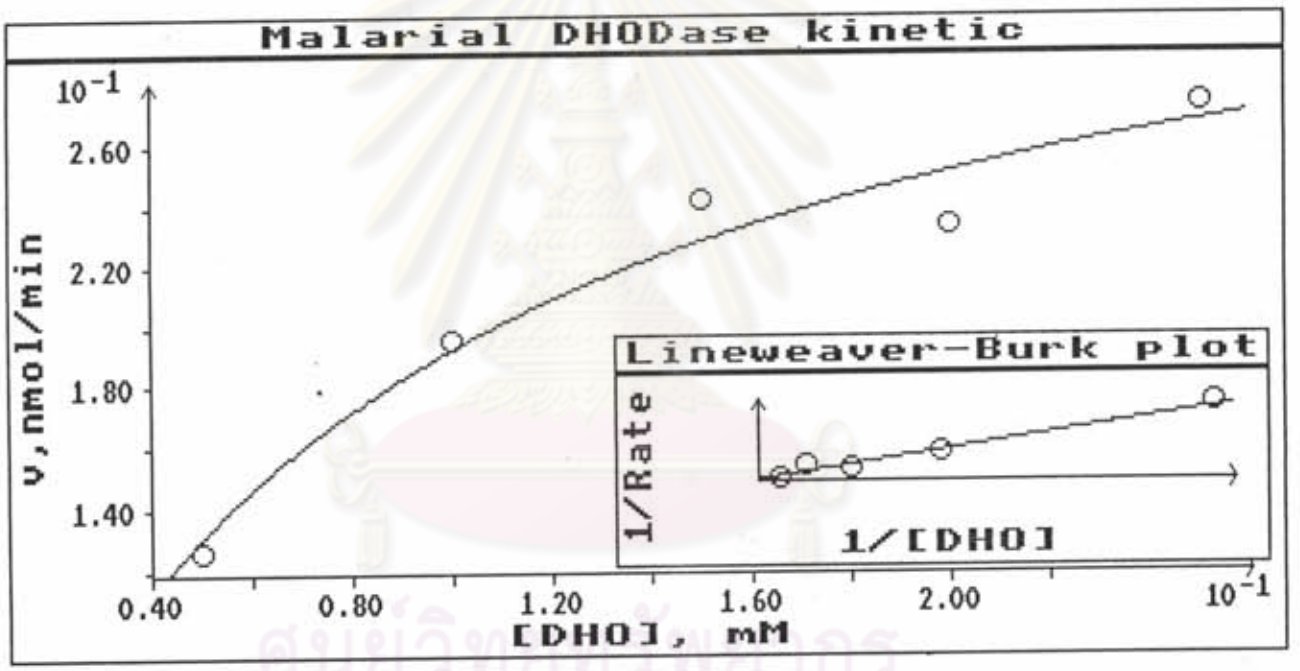


TABLE 6 : The results of the study of inhibition ability of two compounds;

TABLE 6a : 5–Fluoroorotic acid (FOA)

TABLE 6b : 5– Methylorotic acid (CH_3OA)

Table 6a.

FOA (mM)	% INHIBITION
0.00	0.00
0.05	26.73
0.10	31.47

Table 6b.

CH_3OA. (mM)	% INHIBITION
0.00	0.00
0.10	0.00
0.20	11.52
0.30	30.78

3.4 Molecular Weight Determination

The molecular weight determination were performed by 2 techniques; immunoblotting and gel-filtration chromatographic technique.

By the immunoblotting techniques,standard molecular weight marker proteins, composed of Phosphorylase B(MW=110 kDa), Bovine Serum Albumin (MW=84 kDa), Ovalbumin (MW=47 kDa), Carbonic Anhydrase (MW=33 kDa), Soybean Trypsin Inhibitor (MW=24 kDa) and Lyzozyme (MW=16 kDa), were loaded into the precast 10% SDS-Polyacrylamide Gel. The electrophoresis was carried out according to the method described in 'Materials and Methods'. The values of relative mobility (the ratio of the distance of the tracking dye) were plotted against log molecular weight of the protein (Fig. 12). Then the molecular weight of DHODase was calculated from standard curve. The result shown that the molecular weight of DHODase is 55 ± 5 kDa (Fig. 9).

Attempt has been made to determine molecular weight of purified enzyme by immunoblotting technique but DHODase protein band can not be seen. This may due to the low protein concentration of purified enzyme. The loaded protein was believed to be insufficient to make the band apparent.

By using gel filtration chromatographic technique, standard molecular weight marker proteins, consisting of Thyroglobulin (MW=670 kDa), Immunoglobulin (IgG; MW=158 kDa), Ovalbumin (MW=44 kDa), Myoglobin (MW = 17 kDa) and Vitamin B (MW=1350 Da) (Bio-Rad), were applied to calibrate the Superose 12 column before use (Fig. 13). Standard curve was then constructed from the calibration (Fig. 14). The enzyme from the affinity step was eluted at a position which corresponds to 53 ± 6 kDa (Fig. 15).

3.5 NH_2-Terminal Sequence Analysis

Attempt has been made to determine NH_2-terminal sequence analysis for aiming at molecular cloning of the malarial DHODase. It was found that the malarial DHODase had NH_2-terminal blocked. Therefore, no sequence data was obtained.

FIGURE 12 : Standard curve for molecular weight determination on immunoblotting technique (SDS–PAGE).
*, indicate position of the malarial DHODase.

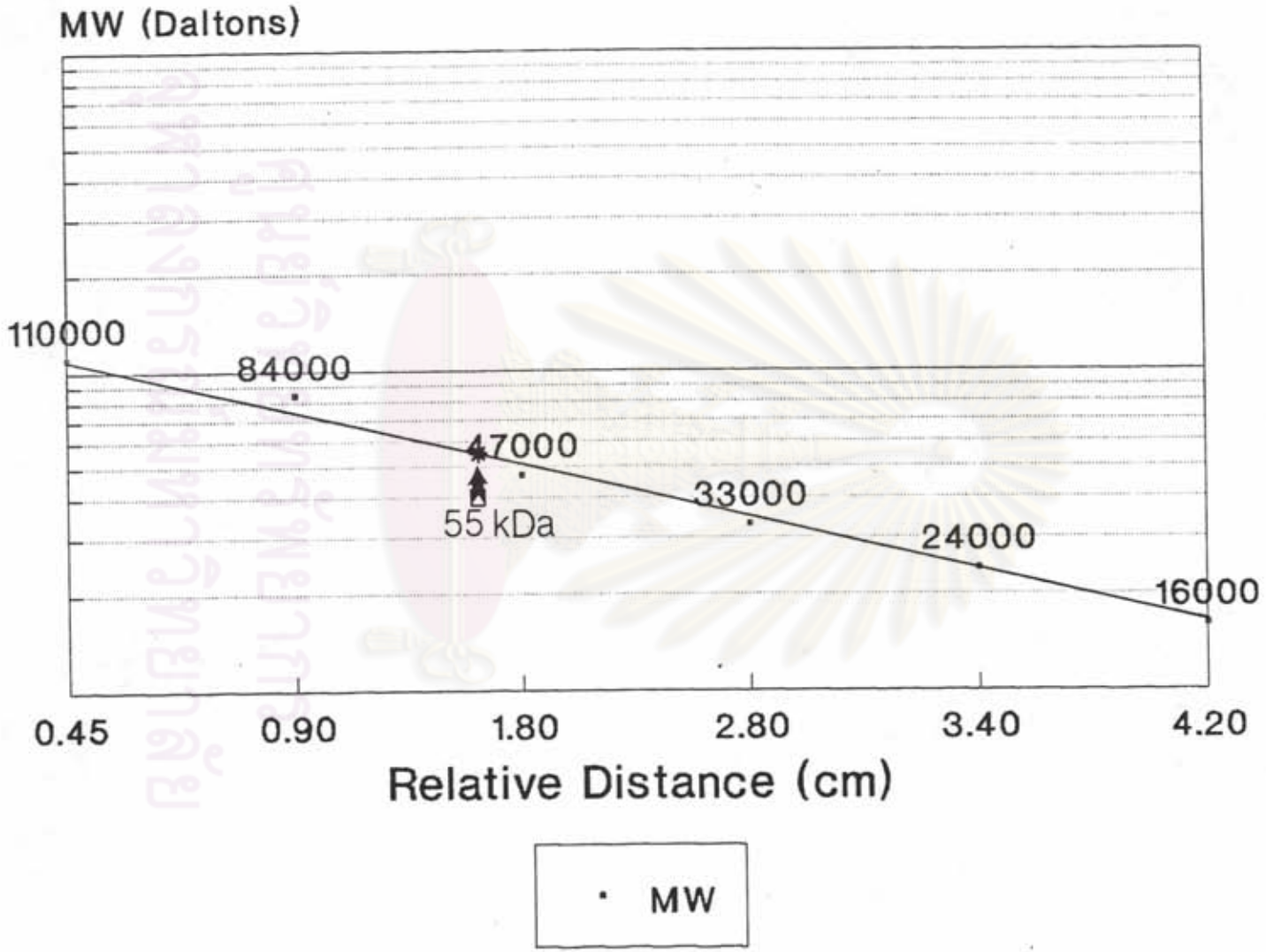
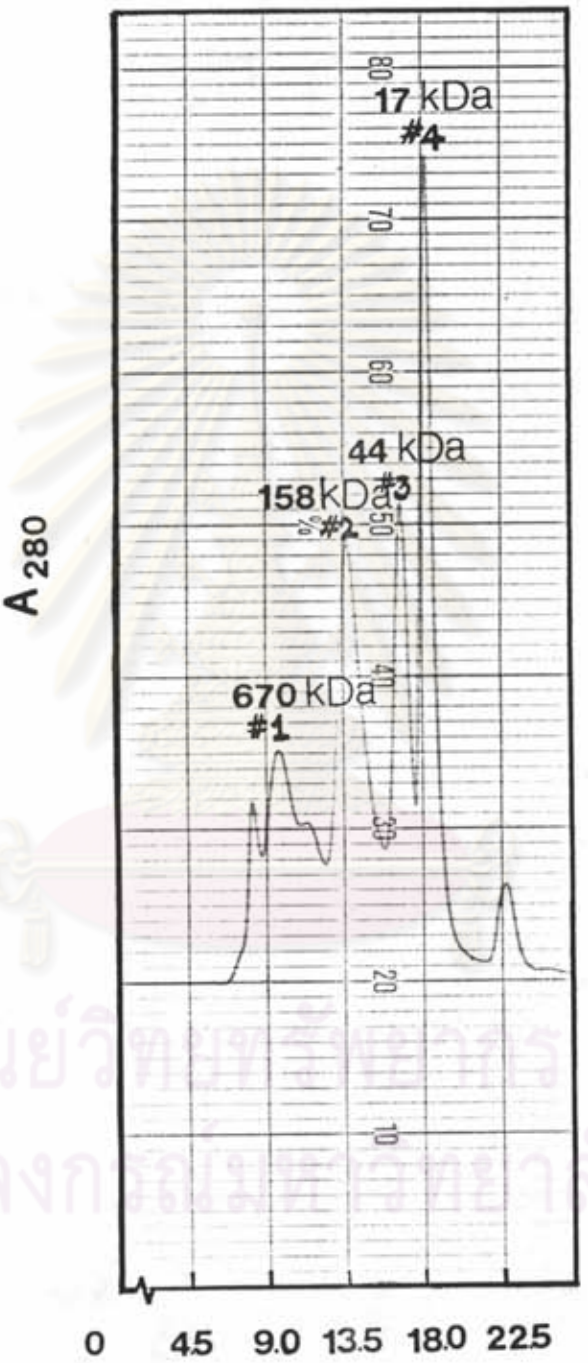

MW (Daltons)
110000
84000
47000
55 kDa
33000
24000
16000
0.45
0.90
1.80
2.80
3.40
4.20
Relative Distance (cm)
MW

FIGURE 13 : Gel-Filtration Chromatography (Superose 12) pattern of standard molecular weight markers, composed of thyroglobulin (MW=670 kDa), immunoglobulin (IgG; MW=158 kDa), ovalbumin (MW=44kDa), myoglobin (MW=17 kDa) (Bio-Rad).



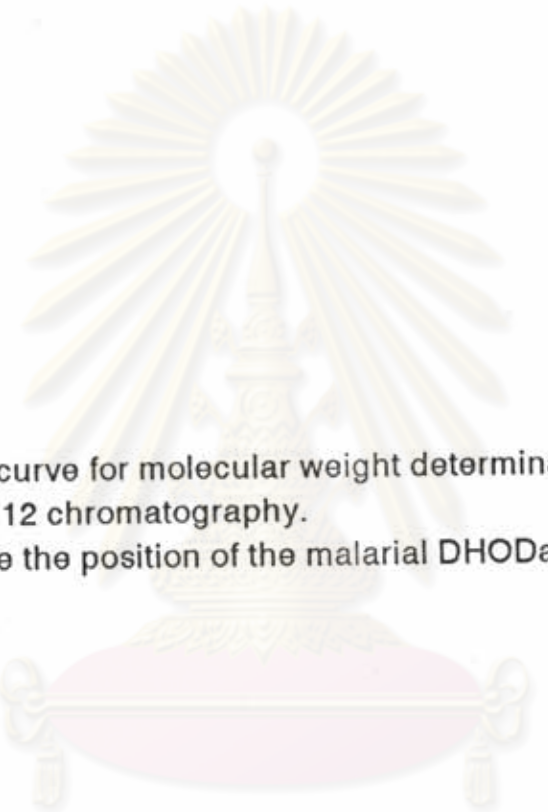


FIGURE 14 : Standard curve for molecular weight determination on gel filtration Superose 12 chromatography.
*, indicate the position of the malarial DHODase.

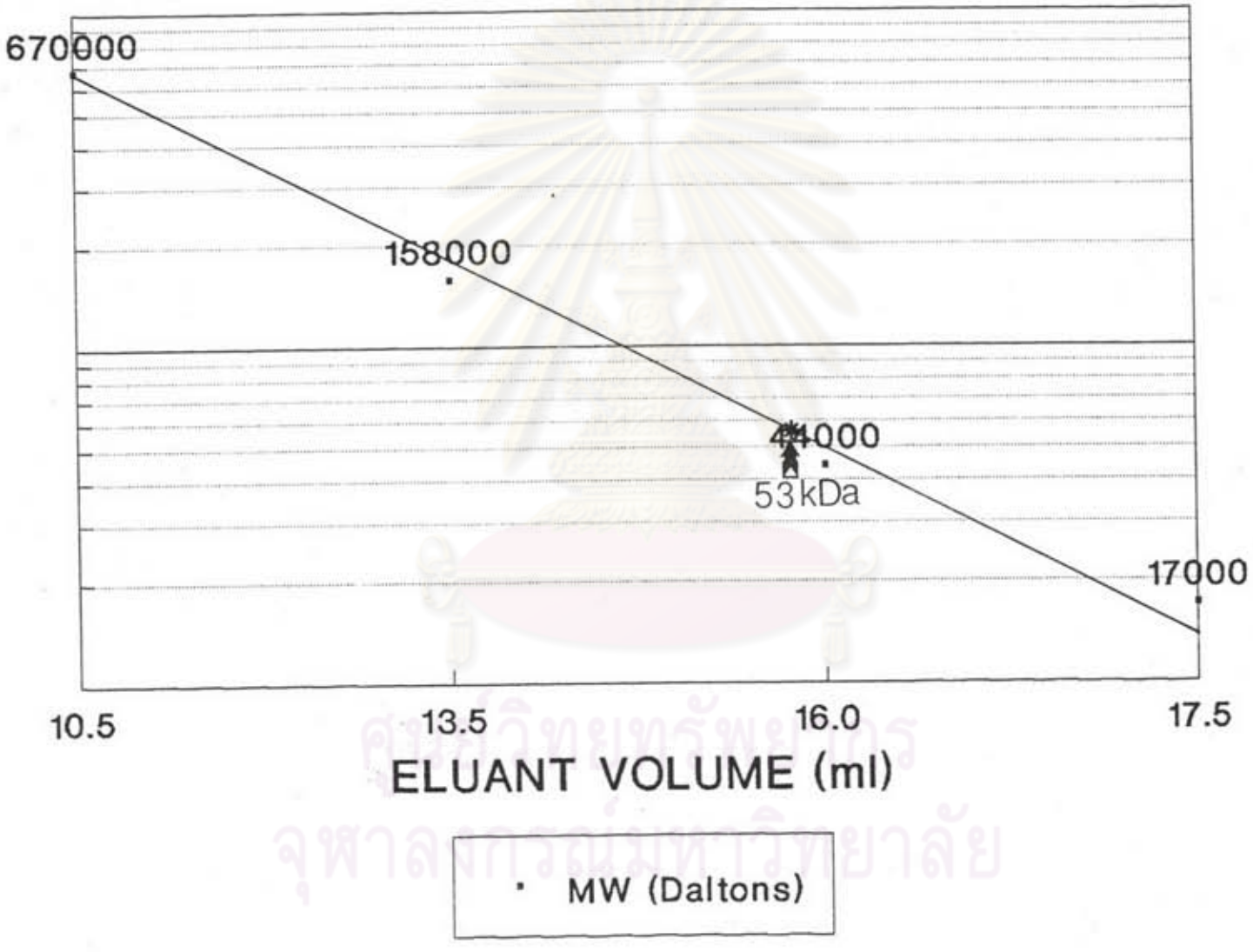
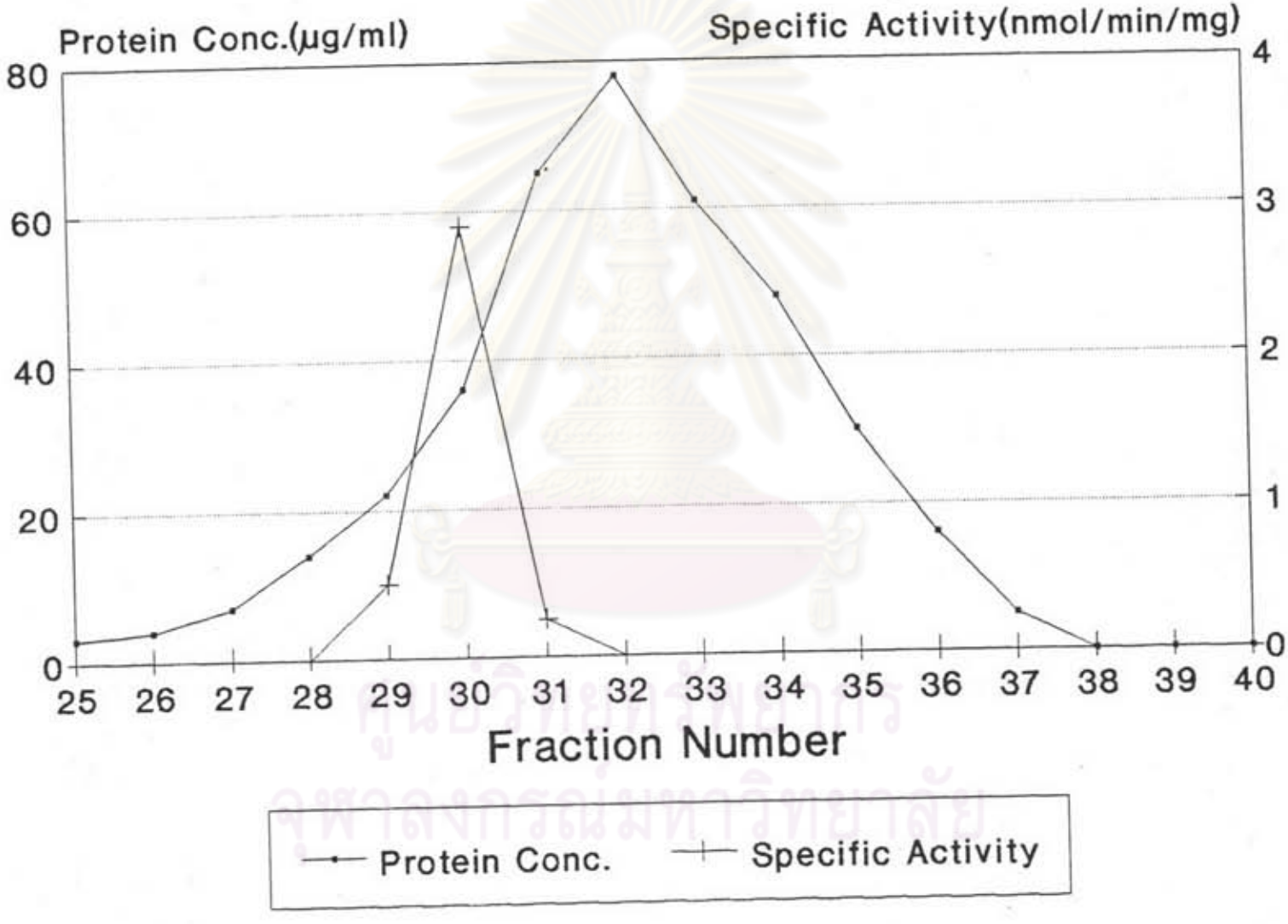


FIGURE 15 : Profile of DHODase activity of P.falciparum after affinity column (Fig.7) eluted from a gel filtration Superose 12 column of FPLC system.


Protein Conc.(µg/ml)
Specific Activity(nmol/min/mg)
80
60
40
20
0
4
3
2
1
0
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Fraction Number
Protein Conc.
Specific Activity