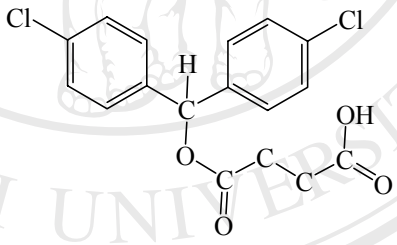
CHAPTER 3

RESULTS

3.1. Haptens and immunogens

3.1.1 Haptens

Hapten I was white powder compound. The ^{1}H NMR showed peaks at (400 MHz, $CDCl_3$) δ 7.268 (dd, J=8.4, 28.8 Hz, 8H), 6.812 (δ, 1H), 2.722 (t, *J*: 3.2, 4H). The peak of ^{13}C NMR was (100 MHz, $CDCl_3$) δ 178.3, 171.3, 138.3, 134.5, 129.2, 128.4, 76.4, 29.4, 29.2. MS was used for confirmation that homogeneous peak, t_R =11.31 min (30% in 12 min); MSD 5973 (EI) Hewlett Packard calculated for $C_{17}H_{10}Cl_2O_4$ ($M+H^+$) 251.6 was 252. (Figure 3.1)

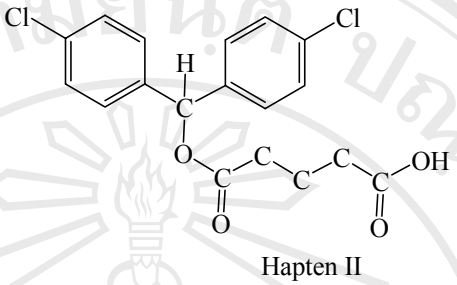


Hapten I

Figure 3.1 The structure of hapten I resulted from the conjugation reaction of DCBH and succinic anhydride linker.

Hapten II was smoky-white powder compound. ^{1}H NMR showed peaks at (400 MHz, $CDCl_3$) δ 7.290 (dd, J = 2.1, 29.6 Hz, 8H), 6.823 (s, 1H), 2.529 (t, *J*: 7.2, 2H), 2.442(t,*J*: 7.6, 2H), 1.993 (m, 2H). The peak of ^{13}C NMR was (100 MHz, $CDCl_3$) δ 138.2, 134.1, 128.9, 128.4, 75.6, 33.3, 32.8. MS was used for confirmation

that homogeneous peak, t_R =11.32 min (30% in 12 min); MSD 5973(EI) Hewlett Packard calculated for $C_{18}H_{10}Cl_2O_4$ ($M+H^+$) 250.18 was 252 (Figure 3.2).



Hapten II

Figure 3.2 The structure of hapten II resulted from the conjugation reaction of DCBH and glutaric anhydride linker.

3.1.2 Immunogens

Five immunogens were used for immunization and one immunogen were used in ELISA assay. Immunogens I and II were prepared from hapten I conjugating with BSA and KLH using succinic anhydride as the linker (Figure 3.3). Immunogens III, IV, and V were prepared from hapten II conjugating with BSA, OVA, and KLH using glutaric anhydride as the linker (Figure 3.3). Capture antigen in ELISA assay was prepared from hapten I conjugating with OVA (Figure 3.3).

3.2 Hapten density assay

UV/Vis spectra showed the maximum absorbance of hapten (Figure 3.4) at 215.15 nm. The molar ratio of hapten to carrier protein of conjugates was then estimated from the spectral data of hapten, carrier proteins, and the corresponding conjugates at wave length 215 nm.

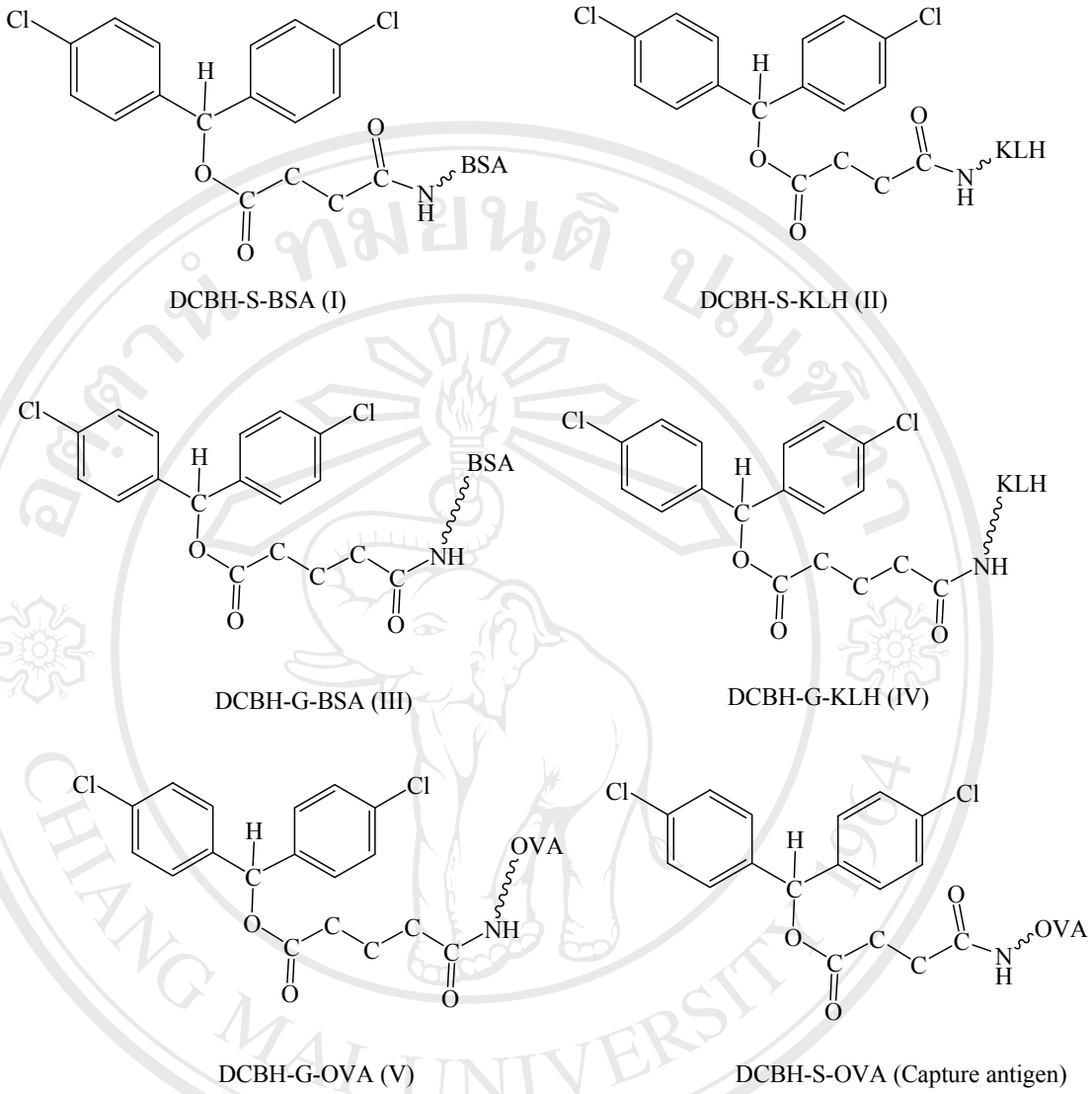


Figure 3.3 Structures of immunogen I-V and capture antigen. Immunogen I, II, and capture Ag were prepared from hapten I. Immunogen III- V were prepared from hapten II.

By assuming that the molar absorbtivity of hapten was the same for the free and conjugated forms, estimated molar ratios of hapten to carrier proteins were 11, 378, 17, 13 and 1,470 for immunogens DCBH-S-BSA, DCBH-S-KLH, DCBH-G-BSA, DCBH-G-OVA, and DCBH-G-KLH, respectively. The estimated molar ratio of capture antigen, DCBH-S-OVA, was 17(Table 3.1).

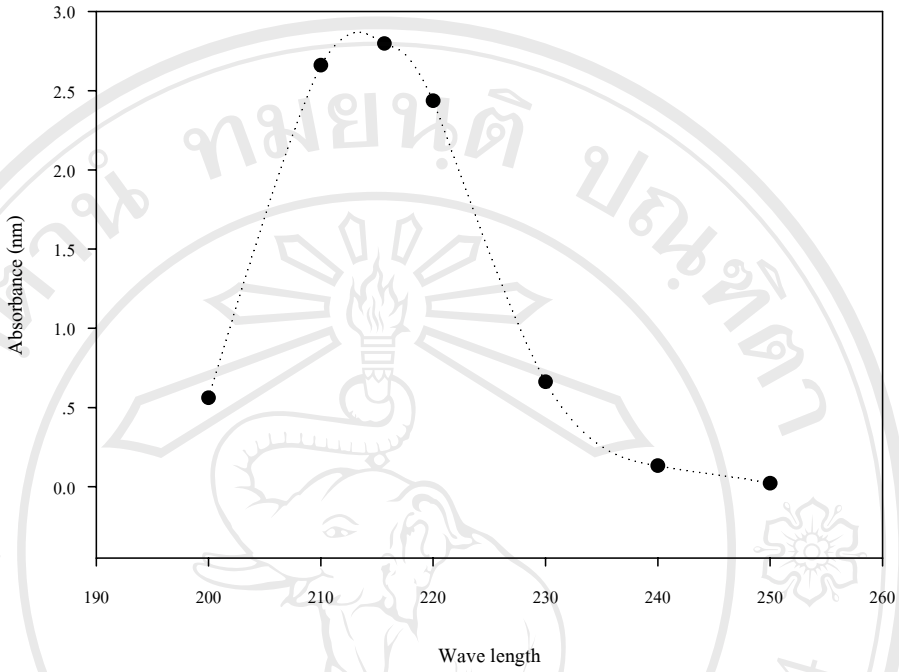


Figure 3.4 Wave length scanned for maximum absorbance of DCBH.

3.3 Protein concentration of immunogens

The immunogens and capture Ag were assessed for protein concentration after removal of unconjugated proteins. The concentration of immunogens DCBH-S-BSA, DCBH-S-KLH, DCBH-G-BSA, DCBH-G-KLH, DCBH-S-OVA, and DCBH-G-OVA were calculated from a standard curve, which are shown in table 3.2.

Table 3.1 Absorbance of carrier proteins and hapten-protein conjugates at 215 nm.

Solutions	Absorbance	Hapten density (hapten/molecule protein)
BSA	1.153	-
OVA	3.026	-
KLH	0.356	-
DCBH-S-BSA	2.241	11
DCBH-S-KLH	4.785	378
DCBH-G-BSA	2.853	17
DCBH-G-KLH	3.026	1470
DCBH-S-OVA	3.670	17
DCBH-G-OVA	4.038	13

3.4 Determination of antibody titer in mouse sera

The development of antibody response in mice immunized with 5 immunogens was evaluated by ELISA. It was found that the level of antibody to the immunogens gradually increased and reached maximum levels at week 6 post immunization. The antibody levels of all mice were increased as early as day 7 post immunization (Figures 3.5). Antibody from mice immunized with BSA did not bind to OVA (data not shown).

To check titers of the antibody by indirect ELISA, sera collected from each mouse after 3rd immunization were two-fold serially diluted. The data showed that antibody titer from one mouse was more than 640,000 and the others were equal 640,000 (Figure 3.6).

Table 3.2 Protein concentration of immunogens determined by BioRad dye assay as described in 2.2.1.3.

Immunogens	Protein concentration (mg/ml)
DCBH-S-BSA	2.28
DCBH-S-KLH	0.50
DCBH-G-BSA	6.5
DCBH-G-KLH	0.45
DCBH-G-OVA	3.66
DCBH-S-OVA	3.40

3.5 Antibody screening of hybridoma

The culture supernatant from 602 hybridoma positive wells was screened for antibodies to hapten by indirect ELISA. It was found that supernatant from 196 (32.56 %) hybridoma containing wells gave absorbance readings of greater than 2 and 14 wells gave the high absorbance of greater than 3. Hybridomas from 5G9, 1F9, 3B8, and 2G2 wells gave good antibody titer. Therefore, the hybridomas from 4 wells were selected for inhibition assay with DDT and its derivatives (8 compounds) results were shown in figure 3.7.

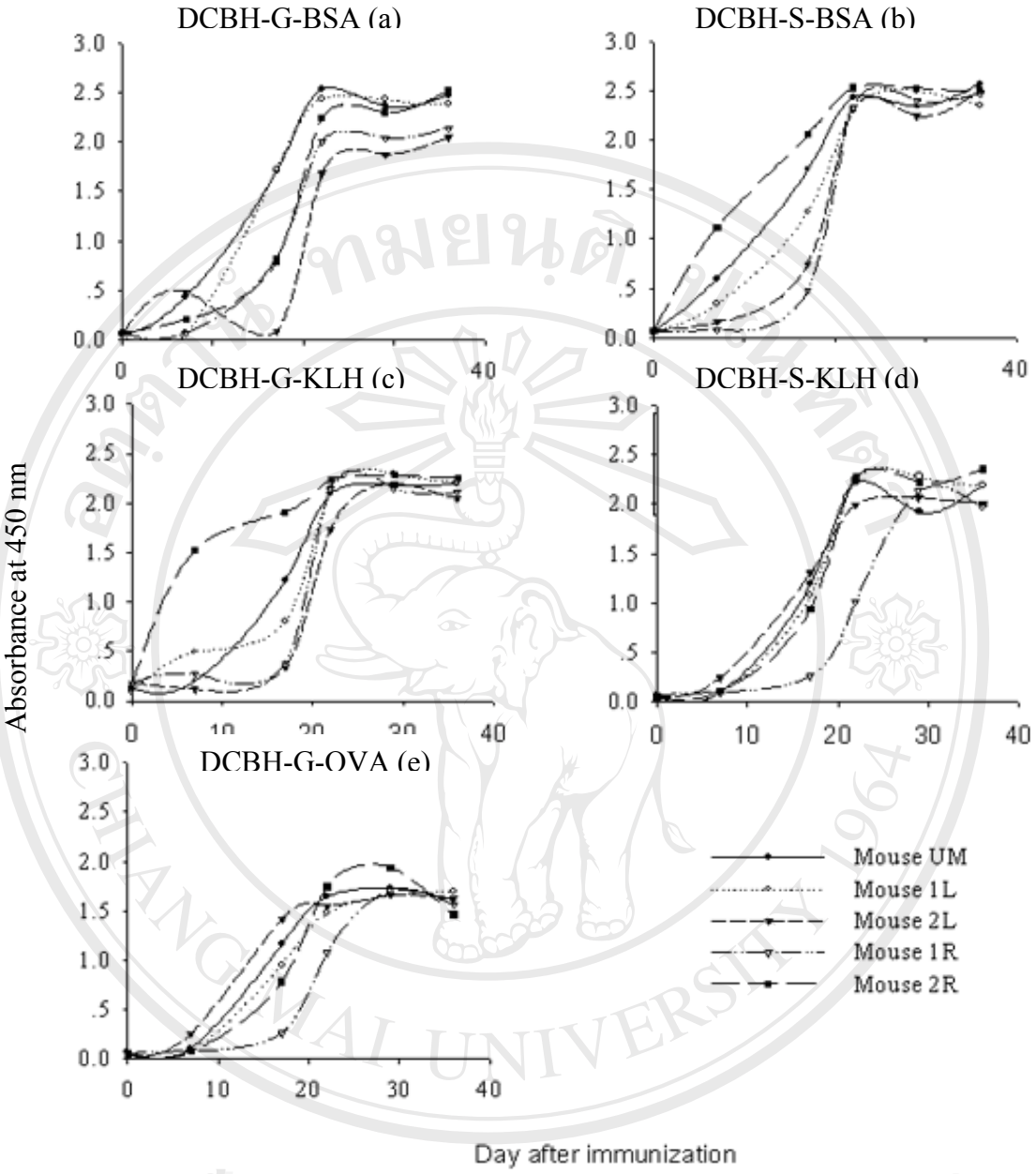


Figure 3.5 Antibody responses against 5 immunogens. (Non-competitive inhibition ELISA, see 2.2.2.2. (a-d were coated with DCBH-S-OVA, e. was coated with DCBH-S-BSA.)

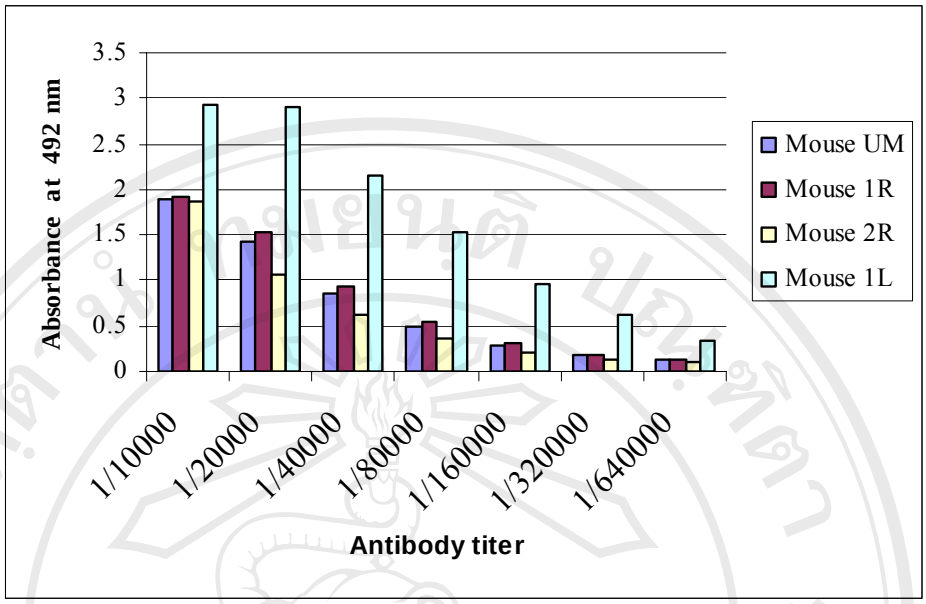


Figure 3.6 Antibody titers in the sera of mice after 3rd immunization with DCBH-S-BSA as described in 2.2.2.2. Absorbance of nonimmune mouse sera is 0.042.

Antibody from selected wells was tested for inhibition with DDT and its derivatives. The result is shown in figure 3.9, hybridomas from 3B8 well could detect DDT and its derivatives such as DCBH, dicofol, *p,p'*-DDD, and *p,p'*-DDA better than the other wells and gave IC_{50} at concentration 0.42, 2.30, 4.13, and 6.09 μg/mL, respectively. 3B8 well gave a good inhibition curve and was selected for cloning.

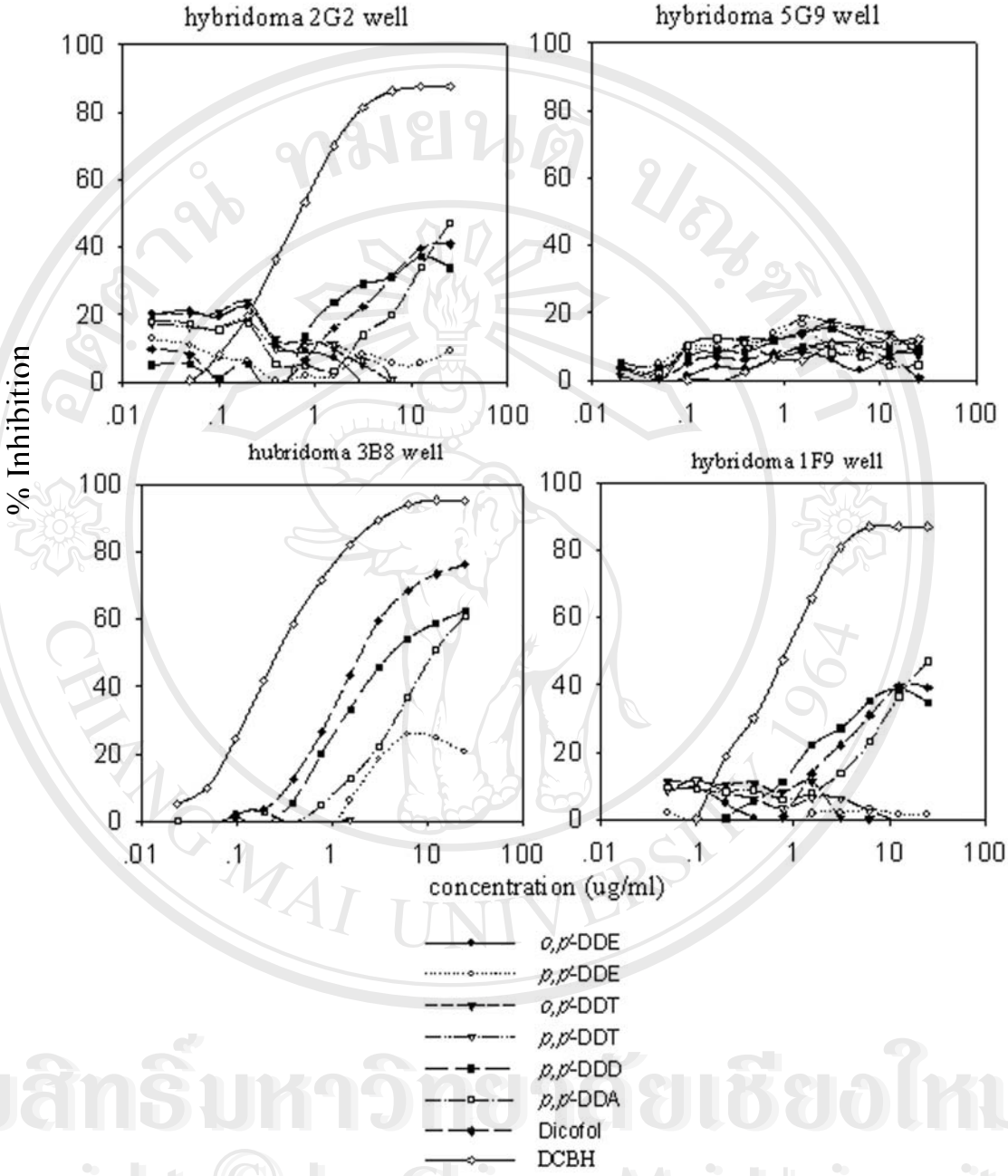


Figure 3.7 Supernatant from 4 selected hybridomas were tested for inhibition activity with DDT and its derivatives. Competitive inhibition ELISA were performed as described in 2.2.2.3 e. Supernatant at 1:40 dilution were used in the assay.

3.6 The inhibition by DDT and its derivatives

Cross reactivity of the monoclonal Ab was assessed by non – competitive inhibition indirect ELISA. The different inhibition curves for hybridoma wells after limiting are shown in figure 3.8. The dilution of supernatant was used at 1:40. The fitting of the curve with the logistic regression gave inhibition at concentration 50 % (IC_{50}) for sensitivity of the DDT and its derivatives such as *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDA, dicofol, and DCBH .

Logistic transformation of data was performed according to the expression (Healy, 1988).

$$\ln (x) = \frac{\ln (y/1 - y) - a}{b}$$

x = concentration of inhibitor

y = absorbance of test well/absorbance of negative well

a = constant of fitting curve

b = slope

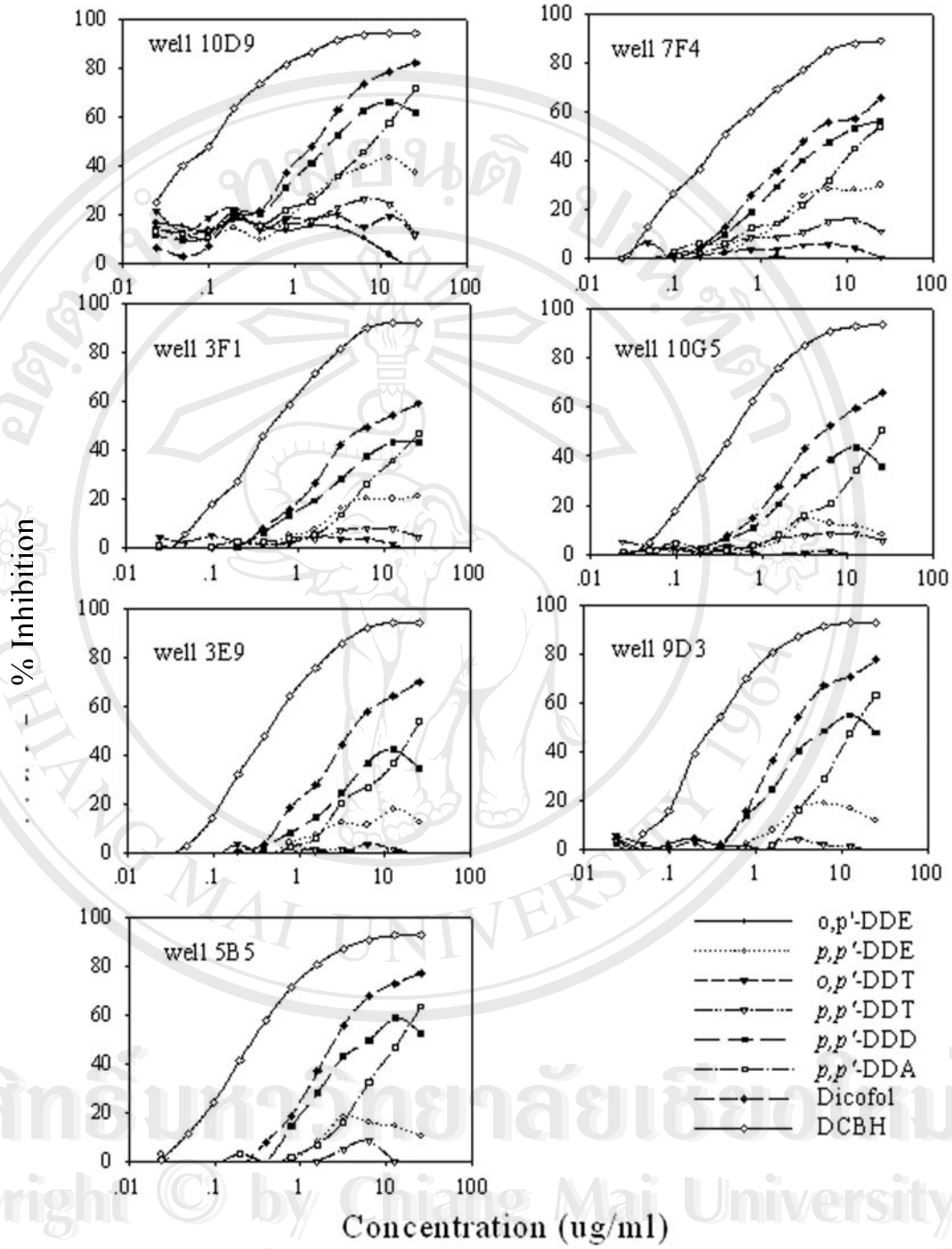


Figure 3.8 Inhibition of antibody by DDT and its derivatives as determined by competitive inhibition ELISA. Clone 10D9 had sensitivity better than the other, was selected for production of monoclonal antibody.

3.7 Cloning of antibody-secreting hybridoma cells by limiting dilution

Hybridomas from 3B8 well were cloned by limiting dilution in order to obtain a single cell clone. The healthy single clone, named 3B8.10D9, was selected for further studies. 3B8.10D9 hybridomas were expanded and supernatants were collected. The supernatant was tested for anti-hapten antibody activity using DDT and its derivatives as competitors (Figure 3.9).

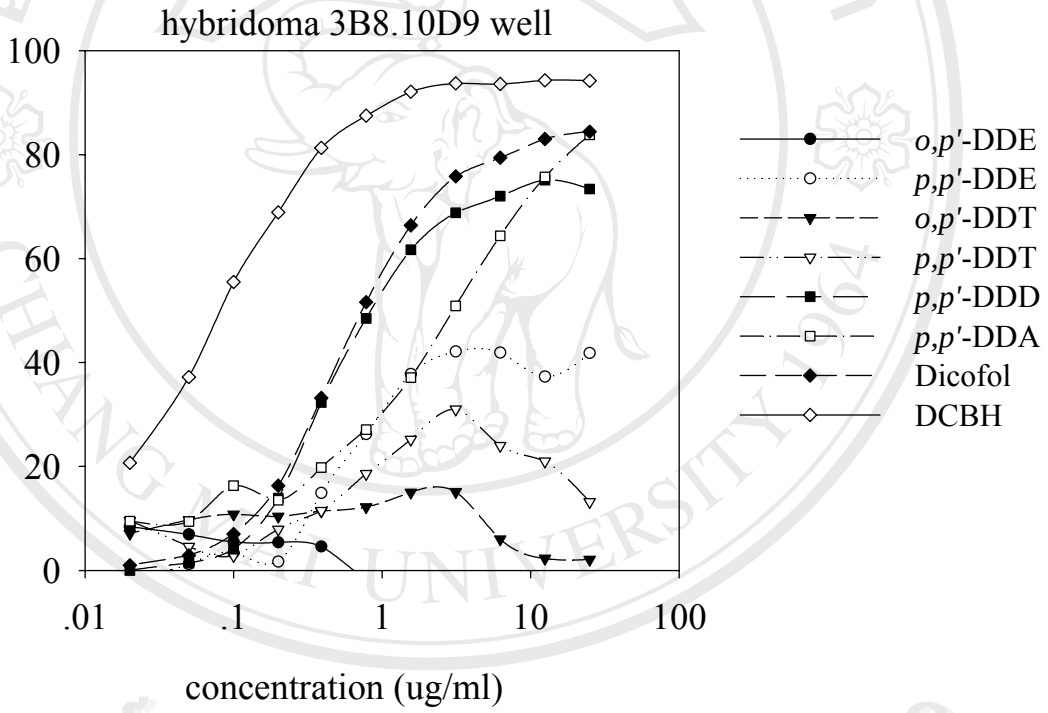


Figure 3.9 ELISA inhibition curves for DDT and its derivatives at difference concentration by competitive inhibition ELISA assay of 1:40 diluted supernatant from clone 3B8.10D9.

The culture supernatant from 300 wells was screened for antibodies to hapten by indirect ELISA. It was found that supernatant from 32 (10.67 %) hybridoma containing wells gave absorbance readings greater than 3. Hybridoma clone 3B8.10D9 gave a good inhibition, as shown in figure 3.11 gave 50 %inhibition of

DCBH, dicofol, *p,p'*-DDD, and *p,p'*-DDA at the concentration 0.33, 1.55, 2.29, and 2.97 μg/mL, respectively and the others gave less than 10% inhibition.

3.8 Determination of the isotype of monoclonal antibodies

The isotypes of anti-hapten monoclonal antibody produced by 3B8.10D9 clone were determined by capture ELISA. It was found that the isotype was IgG1 (kappa chain) (Figure 3.10). It is the same as when tested in immune mice.

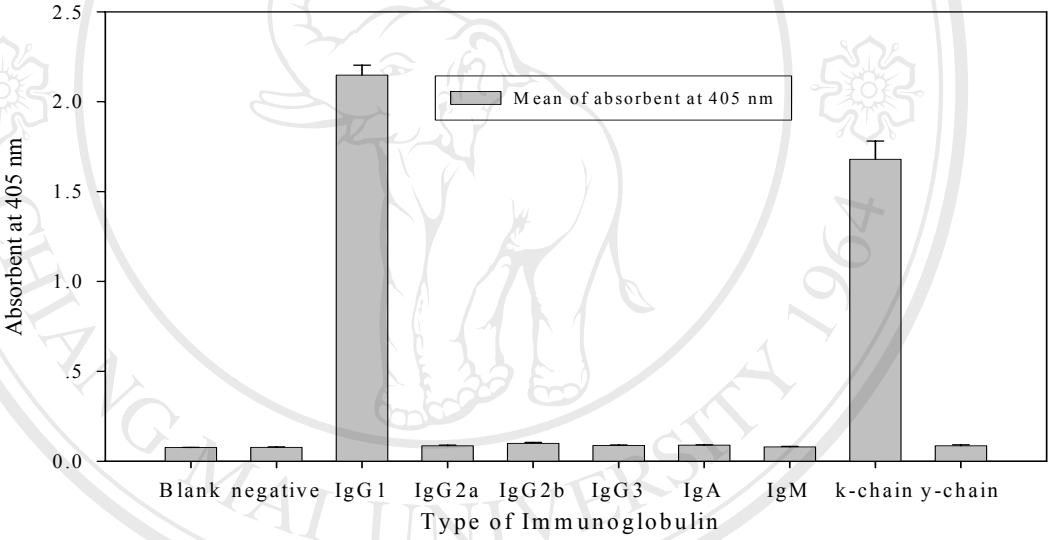


Figure 3.10 Isotypes of anti- hapten monoclonal antibody produced by 3B8.10D9 hybridomas.

3.9 Preparation of antibody for application

3.9.1 Purification of Antibody

Antibody was purified by incubating with 5 x 10^6 beads and 100, 250, 500, and 1,000 μL of cultured supernatant, respectively. The protein concentrations in eluted buffer were 0.144, 0.111, 0.238, 0.326 mg/mL, respectively. The Ab was tested for appropriated absorbance at 492 nm by two-fold dilution. The concentration

at 10 µg protein/mL was found to be most appropriate and was used for inhibition assay by using DDT and its derivatives as competitors.

3.9.2 Sensitivity of antibody

The ability of the mAb to recognize DDT and its derivatives was determined using the DCBH-S-OVA coating ELISA format. The purified Ab gave IC_{50} of DCBH, dicofol, *p,p'*-DDD, and *p,p'*-DDA at the concentration 0.30, 0.36, 3.19, and 2.89 µg/ml, respectively. Inhibition curve is shown in figure 3.11

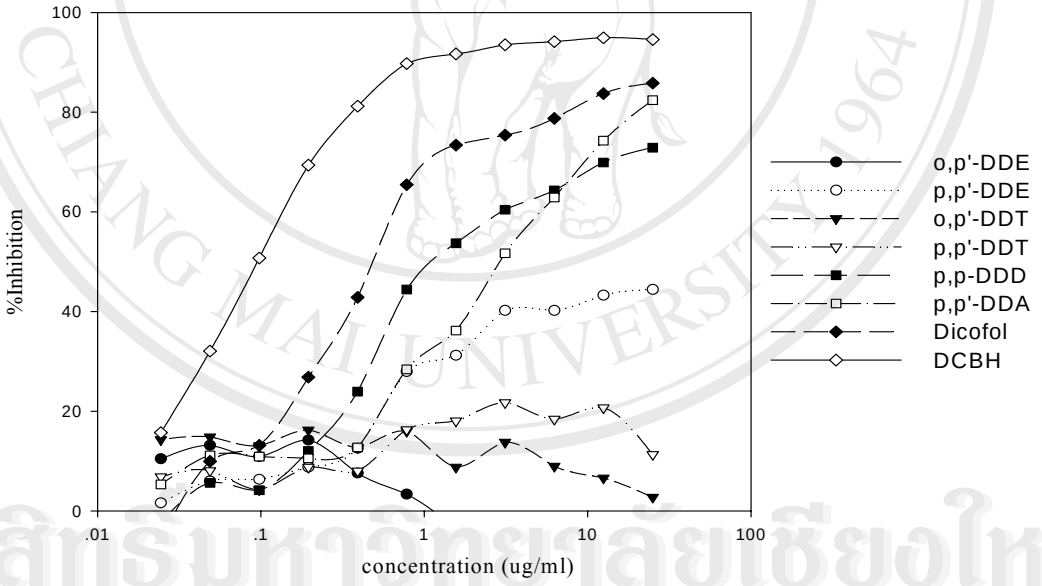


Figure 3.11 Purified Ab (10µg/ml) from clone 3B8.10D9 was tested by inhibition ELISA assay against DDT and its derivatives.

3.9.3 Specificity and cross-reactivity of antibody

The specificity was evaluated by using compounds that are structurally related to the target analyte as antibody inhibitor, and the obtained IC_{50} values were used to calculate cross-reactivity. The specific cross-reactivity is calculated after determination of the concentrations of DCBH (test substances: TS), and the cross-reacting substances (*o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDA, dicofol: CS) required for 50% reduction of the absorbance reading compared to that of the zero control standard using the following equation.

Relative cross-reactivity, % = TS ÷ CS x 100

Table 3.3 Relatives cross-reactivity of monoclonal antibody.

Substances	% related cross-reactivity
DCBH	100
o,p'-DDE	ni
p,p'-DDE	ni
o,p'-DDT	ni
p,p'-DDT	ni
p,p'-DDD	10.38
p,p'-DDA	9.40
dicofol	83.33

ni = non-inhibition (<10% inhibition was measured at highest concentration, 25 μg/ml).

Anti-DDT antibody exhibited high cross-reactivity with dicofol (83.33 %) and *p,p'*-DDD (10.38%) in the studied range of concentrations. Weak interactions were observed with *p,p'*-DDA (9.40%) (Table 3.3). The tested Ab did not exhibit cross-reactivity with *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT.

3.10 Recovery determination of DCBH in human serum samples

Eight human sera (individual) were diluted 1: 5 with 0.05%Tween-PBS, pH 7.2. DCBH at the final concentrations of 5.00, 2.50, 1.25, 0.62, and 0.31 µg/mL were added, and was then assay by inhibition indirect ELISA. Mean concentration range, SD, and % recovery are shown in table 3.4.

The conditions for the spiked DCBH into human serum samples (individual samples) were as described above and the obtained sample homogenates were analyzed without clean-up process. The mean recovery of DCBH measured by the non-competitive inhibition indirect ELISA agreed with the concentration of the spiked DCBH into human serum samples. The measured recovery and variation of coefficient (CV) are shown in table 3.4.

The mean of DCBH concentration of all tested samples in table 3.5 were shown within range 4.57 ± 0.65, 2.75 ± 0.41, 1.53 ± 0.39, 0.79 ± 0.24, and 0.45 ± 0.18 µg/ml for standard additions of 5.00, 2.50, 1.25, 0.62, and 0.31 µg/ml, respectively. Mean± SD recoveries (%) of 5 added concentrations were 91.01±13.34, 109.57±16.35, 118.61±23.72, 118.24±23.01 and 118.24±23.01, respectively.

Table 3.4 Recoveries of DCBH added to human serum compared with a standard curve.

Sample No.	Standard addition (µg/ml)	Mean±SD of DCBH Conc. (µg/ml) (n=3)	% Recovery	%CV
1	5.00	4.11 ± 0.49	82.16	12.03
	2.50	2.63 ± 0.14	114.39	8.96
	1.25	1.50 ± 0.39	128.53	30.34
	0.62	0.66 ± 0.05	105.95	7.89
	0.31	0.34 ± 0.13	108.66	39.53
2	5.00	4.31 ± 0.15	86.128	3.58
	2.50	2.89 ± 0.24	115.72	8.16
	1.25	1.28 ± 0.16	102.33	12.60
	0.62	0.71 ± 0.03	114.15	3.88
	0.31	0.39 ± 0.20	126.09	50.7
3	5.00	4.48 ± 0.08	88.16	4.48
	2.50	2.57 ± 0.19	101.77	7.86
	1.25	1.90 ± 0.68	124.52	6.46
	0.62	1.13 ± 0.48	123.26	20.40
	0.31	0.69 ± 0.34	138.02	50.61
4	5.00	4.05 ± 0.12	84.32	5.90
	2.50	2.72 ± 0.44	119.55	5.46
	1.25	1.26 ± 0.04	104.59	5.03
	0.62	0.77 ± 0.16	129.45	13.90
	0.31	0.45 ± 0.10	111.48	46.87
5	5.00	3.99 ± 0.26	80.30	6.17
	2.50	2.80 ± 0.25	102.50	13.83
	1.25	1.74 ± 0.10	127.40	19.84
	0.62	0.81 ± 0.15	123.93	24.07
	0.31	0.55 ± 0.11	157.66	5.54

Table 3.4 Recoveries of DCBH added to human serum compared with a standard curve (continued).

Sample No.	Standard addition (µg/ml)	Mean±SD of DCBH Conc. (µg/ml) (n=3)	% Recovery	%CV
6	5.00	5.31 ± 0.51	106.17	9.68
	2.50	3.09 ± 0.57	123.63	18.38
	1.25	1.36 ± 0.11	109.00	8.20
	0.62	0.66 ± 0.13	106.04	19.35
	0.31	0.40 ± 0.07	127.52	17.47
7	5.00	5.51 ± 0.75	110.21	13.70
	2.50	2.17 ± 0.44	87.00	20.39
	1.25	1.32 ± 0.51	105.33	38.96
	0.62	0.67 ± 0.27	107.69	39.61
	0.31	0.44 ± 0.15	139.84	33.50
8	5.00	4.82 ± 0.29	101.28	12.08
	2.50	3.15 ± 0.17	119.08	13.56
	1.25	1.89 ± 0.09	138.37	14.09
	0.62	0.87 ± 0.18	129.78	19.64
	0.31	0.33 ± 0.12	115.80	25.30

* Each sample has no inhibitor as 100% activity of antibody.

Table 3.5 The recoveries of DCBH ± SD, Min, and Max and mean recovery± SD.

Standard addition (µg/ml)	DCBH Conc. (µg/ml)			% Recovery Mean±SD
	Mean±SD	Min	Max	
5.00	4.57±0.65	3.58	6.35	91.01±13.34
2.50	2.75±0.41	1.88	3.68	109.57±16.35
1.25	1.53±0.39	0.97	2.68	118.61±23.72
0.62	0.79±0.24	0.52	1.68	118.24±23.01
0.31	0.45±0.18	0.19	1.04	131.42±40.48