CHAPTER 4

DISCUSSION AND CONCLUTIONS

The present study was proposed to produce monoclonal antibody that could bind with DDT and its derivatives and use for detecting them in biological matrices.

4.1 Hapten synthesis and immunogen preparation

DDT is a small and simple organic molecule non immunogenic by itself and lacking a functional group for coupling to proteins (Aamand *et al*., 2004). Therefore, the synthesis of haptens resembling as much as possible the structure and steric distribution of DDT is a necessary and critical step in the production of high affinity antibodies. Accordingly, two haptens were synthesized (DCBH-S, and DCBH-G) by the introduction of a spacer arm of different lengths through the central carbon atom of DCBH and deriving carboxyl group into DCBH. The molecular structure of DDT seems not be directly coupled to proteins. DCBH were therefore synthesized by replacing OH group in DCBH as spacer arm through the carbon atom that joins the two aromatic rings by C-4 and C-5 carboxylic acid. ^{1}H-, ^{13}C-NMR spectrum and GC/MS of each DDT hapten was determined to confirm the structure and molecular weight of derivatization. Banerjee *et al*. (1987) was the first group who synthesized a hapten for DDT with a spacer arm at the central carbon atom of DDA that joins the two aromatic rings.. DDA was used by Hong *et al*. (2002) and obtained DDT antibody while dicofol was used by Burgisser *et al*. (1990) and Abad *et al*. (1997) were also obtained DDT antibody. DCBH could be viewed as having two aromatic rings. Succinic anhydride and glutaric anhydride were used as linkers, acting as a spacer between the carrier proteins (BSA, KLH, OVA). Of 6 immunogens produced, 5 immunogens used in mice immunization were DCBH-S-BSA,

DCBH-S-KLH, DCBH-G-BSA, DCBH-G-OVA, and DCBH-G-KLH, and an immunogen used as capture antigen was DCBH-S-OVA. In considering the functional groups of the hapten molecules for hapten–protein conjugation, hydroxyl group were thought them desirable to utilize, the single functional group that is common to all hapten. This approach has been successfully applied to prepare of immunogens. Direct conjugation to protein amino groups, however, was shown to result in insufficient hapten density of the conjugate. The free amine groups on lysine residues leads to spontaneous formation of covalent linkage with the stoichiometric moiety under basic conditions. The amount of proteins in hapten-protein conjugates were quantitatively determined using UV absorbance values due to the overlapping of UV spectra at 215 nm between hapten and protein.

After linked with a spacer arm, haptens were coupled to carrier proteins by diazotization following reduction of the nitro group. Other research groups had used similar approaches to make highly selective assay for each hapten (Burgisser *et al*., 1990; Abad *et al*., 1997; Hong *et al*., 2002, 2003).

Generally, the techiniques for confirmation of hapten-protein conjugates are UV/Vis spectrophotometry, enzyme immunoassays (EIAs), and Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF). Conjugation of the present prepared haptens to protein was confirmed by UV/Vis spectrophotometry. The UV/Vis spectrophotometer could be found in general laboratory and it is rather simple, and inexpensive than the others. The EIA is an indirect proof of conjugation, but results obtained are often too late to know the unsuccessful conjugation. Also, the EIA alone may not be able to specifically indicate the failure of hapten-protein conjugation. However, EIA will enhance the spectrophotometric technique to confirm the

conjugation. MALDI–TOF/MS is an ultimate technique for examining the conjugates. The technique requires rather simple sample preparation, speed, and capable to quantify hapten density as well as confirms the coupling (Ranulfo, 2001). However, it is an expensive instrument and of course not common laboratory instrument.

The present study employed UV/Vis spectrophotometry for the first confirmation of all prepared immunogens. After that, EIA was used for confirmation of conjugation. Hapten densities of 6 immunogens namely DCBH-S-BSA, DCBH-S-KLH, DCBH-G-BSA, DCBH-G-OVA, DCBH-G-KLH, and DCBH-S-OVA are 11, 378, 17, 13, 1,470, and 17 hapten/molecule proteins, respectively. KLH conjugated gave the highest number of hapten on molecule proteins because KLH has a big molecule so it could be attached by hapten at high number.

4.2 Production of antibody

The assay systems (combinations of antibody and ELISA antigen) tested are summarized in figure 3.5 and 3.6. In general, the ELISA strategy of Harrison *et al*. (1990) was applied to antibody screening. The titer of each antibody was monitored over time by using ELISA to measure binding to hapten conjugates compared with a control antigen as been performed, an example, by Goodrow *et al*. (1990). All of the immunized mice demonstrated significant titers on homologous ELISA antigens, and most of these sera also exhibited moderate to high titers on at DCBH-S-BSA. The antisera from mice immunized with all immunogens exhibiting the highest early titers by non-competitive indirect ELISA. In general, the hapten I (DCBH-S; structures in figure 3.1) produced better antibodies than the hapten II (DCBH-G; structure in figure 3.2). This different response seems unlikely to be due entirely to the observed solubility differences among haptens. Differences in antibody levels were also observed among the carrier proteins

used for immunization. Most of the BSA and KLH conjugates produced acceptable Ab responses, while the OVA conjugates were less successful.

In addition to the immunizing hapten, DCBH-S-OVA was used for coating agent (Prapamontol et. al., 2003; Burgisser *et al*., 1990) since all prepared immunogens would present the DCBH. The DCBH-S-BSA was used in the Ab assay which were detected DCBH as hapten. Once the Abs were produced and drawn from mice, series of screening steps designed to identify the best combination of Ab and Ag for selective detection of DCBH in serum was performed. The serum screening assays, using all mice that were immunized against different immunogens and available coating Ags, yielded a rather large number of potentially useful Ab-Ag combinations with good assay. Many sera had high absorbance with few days after immunization.

The development of antibody response in mice immunized with 6 immunogens was evaluated by ELISA. Sera were collected weekly and it was found that the level of Ab to the immunogens gradually increased and reached maximum levels at week 6 post immunization. The Ab levels for all groups were increased as early as day 7 post immunization. DCBH-S-BSA found to be good immunogen and gave the highest Ab level although had the lowest of hapten density (Abad *et al*., 1997). Shorter spacer arm showed lower affinity than the longer one and the length of space arm plays an important role for steric hindrance for binding affinity between antibody and coating ligand. Similar results were also obtained in the report from Burgisser *et al*. (1990). Therefore, it is important to screen a best matching pair of antibody and coating ligand for affinity and displacement with target analytes i.e. DDT and its derivatives.

The culture supernatants from hybridoma positive wells were screened for antibodies against hapten by indirect ELISA. Fourteen wells gave the highest absorbance

of more than 3. Therefore, they were selected and the titration of anti-hapten antibody was tested. Hybridomas from 4 wells were selected because they gave high titer Ab, that for inhibition assay with DDT and its derivatives. By examining the results with DDT and its derivatives in different concentration, hybridomas from 3B8 well gave cross-reactivity better than the others. The different inhibition curves for hybridoma wells the fitting of the curve with the logistic equation gave the DDT and its derivatives mAb from hybridoma wells. Concentrations at 50 % inhibition (IC_{50}) of DCBH, dicofol, *p,p'*-DDD, and *p,p'*-DDA were 0.42, 2.30, 4.13, and 6.09 µg/ml, respectively and the other gave inhibition less than 10%.

Hybridomas were cloned by limiting dilution in order to obtain a single cell clone. The healthy single clone was selected for further studies. The hybridomas were expanded and supernatant was collected. The supernatant was tested for anti-hapten antibody activity using DDT and its derivatives as competitors. Monoclone 3B8.10D9 well showed IC_{50} of DCBH, dicofol, *p,p'*-DDD, and *p,p'*-DDA at 0.33, 1.55, 2.29, and 2.97 µg/ml, respectively.

The isotype of anti-hapten monoclonal antibody was found IgG1 (kappa chain) from that the obtain Ab was the most relied for immunoassay as the major Ig.

The ability of the mAb to recognize DDT and its derivatives was determined using the DCBH-S-OVA coated 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 (Table 4.1).

Table 4.1 Comparison of IC_{50} of inhibitors in supernatants.

Supernatants	IC_{50} (µg/ml)			
	DCBH	dicofol	*p,p'*-DDD	*p,p'*-DDA
Hybridoma 3B8	0.42	2.30	4.13	6.09
Monoclone 3B8.10D9	0.33	1.55	2.29	2.97
Purified monoclonal antibody	0.30	0.36	3.19	2.89

The specificity was evaluated by using compounds that are structurally related to the target analyte as antibody inhibition. The specificity of the mAbs was evaluated by performing inhibition assays with DDT and its derivatives as inhibitors, 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. The Ab could detect DCBH at 100 % while dicofol had specific cross-reactivity at the best (83.33%), the second was *p,p'*-DDD (10.38%) and *p,p'*-DDA (9.40%), respectively. Abed *et al*. reported in 1997, the best of produced Ab could detected p,p'-DDT IC_{50} at 0.74-4.08 µg/m and gave % cross-reactivity to p,p'-DDD at 56.2 % , and dicofol at 8.2 %. Hong *et al*. (2002) found DDT analysts can be detected simultaneously with the detection limit of 0.0003 µg/mL for DDT and 0.0034 µg/mL for DDA and DDE by an indirect enzyme immunoassay. The relative reactivity values of DDT analytes when using the pair of mAb 1A1 and DDHHAP-BSA were

calculated as 100, 4.2, 3.3, and 6.0%. With mAb 1A3 and DDA-OVA, they were 100, 3.3, 201, and 172%; with mAb 1A3 and DDHP-OVA, they were 100, 122, 146, 129%, and with mAb 1A4 and DDHP-OVA, they were 100, 107, 134, and 121%. Monoclonal Ab 1A4 and DDHP-OVA coating ligand exhibited high sensitivity values for the DDT analytes (DDT, DDA, DDE and DDD), but the combination of mAb 1A3 and DDA-OVA exhibited the best response, covering a wide range of DDT levels.

The experimental data of cross-reactivity, antibodies were present but could not bind the o,p'-DDE, p,p'-DDE, o,p'-DDT, p,p'-DDT although hapten heterology has been demonstrated as a good approach to improve the sensitivity of pesticide immunoassays (Wie and Hammock, 1984; Marco *et al*., 1995). Suitable heterologous haptens are those prepared by introducing slight changes in the hapten structure (change or elimination of a substituent), by changing the type and length of the spacer arm, or by changing the attachment site to a position proximal to that successfully used in the immunogen (Manclu and Montoya, 1996; Manclu *et al*., 1996; Abad *et al*., 1997).

The matrix effect experiment revealed that high quantity of human serum showed interference on the DCBH standard curve. Serum is a complex matrix for many assays compared the effect between human serum and fetal bovine serum (as the clean matrix). Human serum has varieties of chemicals, and some of them have potential cross-reactants, into assay, even when diluted with PBS at dilution 1:5. Eight human serum samples were added with DCBH at the different concentration (low to high; 5, 2.5, 1.25, 0.62, 0.31 µg/ml). The results were compared with standard DCBH in PBS. The serum dilution at 1:5 was selected and used to test for recovery at the same concentration. Mean percentage recovery of 5, 2.5, 1.25, 0.62, 0.31 µg/ml were 91.01, 109.57, 118.61, 118.24, and 131.42, respectively. Therefore the present produced monoclonal antibody

showed good recovery detection of DCBH, a DDT derivative. Other DDT derivatives would warrant further study.

4.3 Conclusion and future study

This study has tried to produce antibody that could bind to DDT and its derivatives by using DCBH which has similar structure as one approach. Monoclonal antibody obtained can bind to its antigen (DCBH) at very high specificity at the same time it can bind to dicifol at good specificity (88%). Even though not much cross reactivity can be observed here but one should not expect mAb to elicit high cross reactivity since it has such high specificity. At least the mAb produced can bind to dicofol which is one of DDT derivative and it is also one of the pesticides used in the field that need monitoring. This mAb can be used as reagent for immunoassay to detect dicofol biological samples, the assay needs to optimize and determine the limit of detection. The test can be developing either for simple field work or laboratory test assay. Antibody to DDT and its derivatives are the need for test kit development in order to strengthen capacity for Thai researcher and general users in screening of biological and environmental samples such as serum, soil, vegetables, and water.