CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Chemicals and reagents were obtained as described in appendix A.

2.1.2 Equipments

Equipments details were described in appendix A.

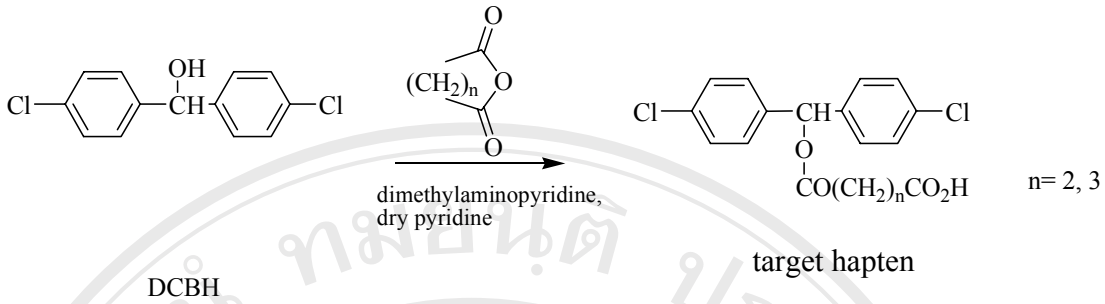
2.2 Methods

2.2.1 Preparation of haptens and immunogens

2.2.1.1 Hapten synthesis

Haptens were designed resembling DDT chemical structure and modified structures were produced by spacer arm attachment. Two kinds of hapten, hapten I and hapten II were synthesized by deriving carboxyl group into DDT derivatives (Figure 2.1and 2.3).

Haptens were synthesized by linking hydroxyl (-OH) group of DCBH with succinic anhydride or glutaric anhydride by esterification in dry pyridine. These substitutes act as spacer arm joining the two aromatic rings through the carbon atom of carboxylic acid. Structures of haptens were confirmed by ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectroscopy (NMR) and gas chromatography/Mass-spectrometry (GC/MS). Two haptens were synthesized as following (Figure 2.1 and 2.3).



DCBH

target hapten

Figure 2.1 Synthesis of targeted haptens using DCBH.

Hapten I: A mixture of 200 mg (0.78 mM) DCBH, 750 mg (7.5 mM) succinic anhydride, and 10 mg (0.08 mM) dimethyl amino pyridine (DMAP) in 10 mL dry pyridine was stirred overnight at room temperature. Twenty milliliters of water was then added and the mixture was evaporated dry. Crude was rinsed twice with 10 mL toluene and dissolved in 10 mL ethyl acetate. Crude solution was washed once with 10 mL of cool HCl, twice with 10 ml water, and twice with 10 mL saturated sodium chloride, respectively. The product was evaporated dry over magnesium sulphate ($MgSO_4$). Purity of the product was confirmed by TLC. Crude product was purified by column chromatography on silica gel using ethyl acetate: hexane (40:60 v/v) solvent. Single-compound fraction was collected and evaporated dry. The structure of the compound was confirmed by ^{1}H and ^{13}C NMR, and GC/MS

Hapten II was synthesized using the same procedure as hapten I but glutaric anhydride was substituted for succinic anhydride.

2.2.1.2 Preparation of immunogens

Immunogens and capture Ag were prepared follow to figure 2.2. and 2.4 Hapten I and II were conjugated to proteins (KLH, BSA, or OVA)**.** In all cases, an active *N*-hydroxyscuccinimide (NHS) ester method was used to couple the carboxylic acid moieties of the haptens to proteins (Langone and Van Vunakis, 1982; Haas and Guardia, 1968). For the preparation of immunogens and capture antigen, 25 μM of the hapten were incubated overnight at RT with stoichiometric amounts of 75 μM NHS and 75 μM dicyclohexylcarbodiimide (DCC) in 0.5 mL of dimethylformamide (DMF). The mixture was then centrifuged at 10,000 rpm for 10 min. Four hundred microliters of supernatant containing the active NHS ester of hapten was collected and then slowly added to 2 mL of 15 mg/mL BSA, KLH or OVA in 50 mM carbonate buffer, pH 9.6. The mixture was allowed to react at RT for 4 h with stirring. The mixture was dialyzed overnight in PBS, pH 7.2. Concentrations of protein were determined by Bradford protein assay.

2.2.1.3 Bradford protein assay

Dye reagent was prepared by diluting 1 part of concentrate dye reagent (Biorad) with 4 parts of water. The working reagent was filtered through Whatman paper #1 to remove particles. Standard BSA range from 0.1 mg/mL to 0.5 mg/mL (0.1, 0.2, 0.3, 0.4, 0.5 mg/mL) was prepared. Protein solutions were analyzed in duplicate. Ten microliters of various dilutions of standard proteins or test samples were added to a microtiter plate in the presence of 300 μL diluted dye reagent. The mixture was mixed and incubated at RT for 5 min. Absorbance at 595 nm was read on the Spectra MR plate reader. Protein concentration was calculated from a standard curve.

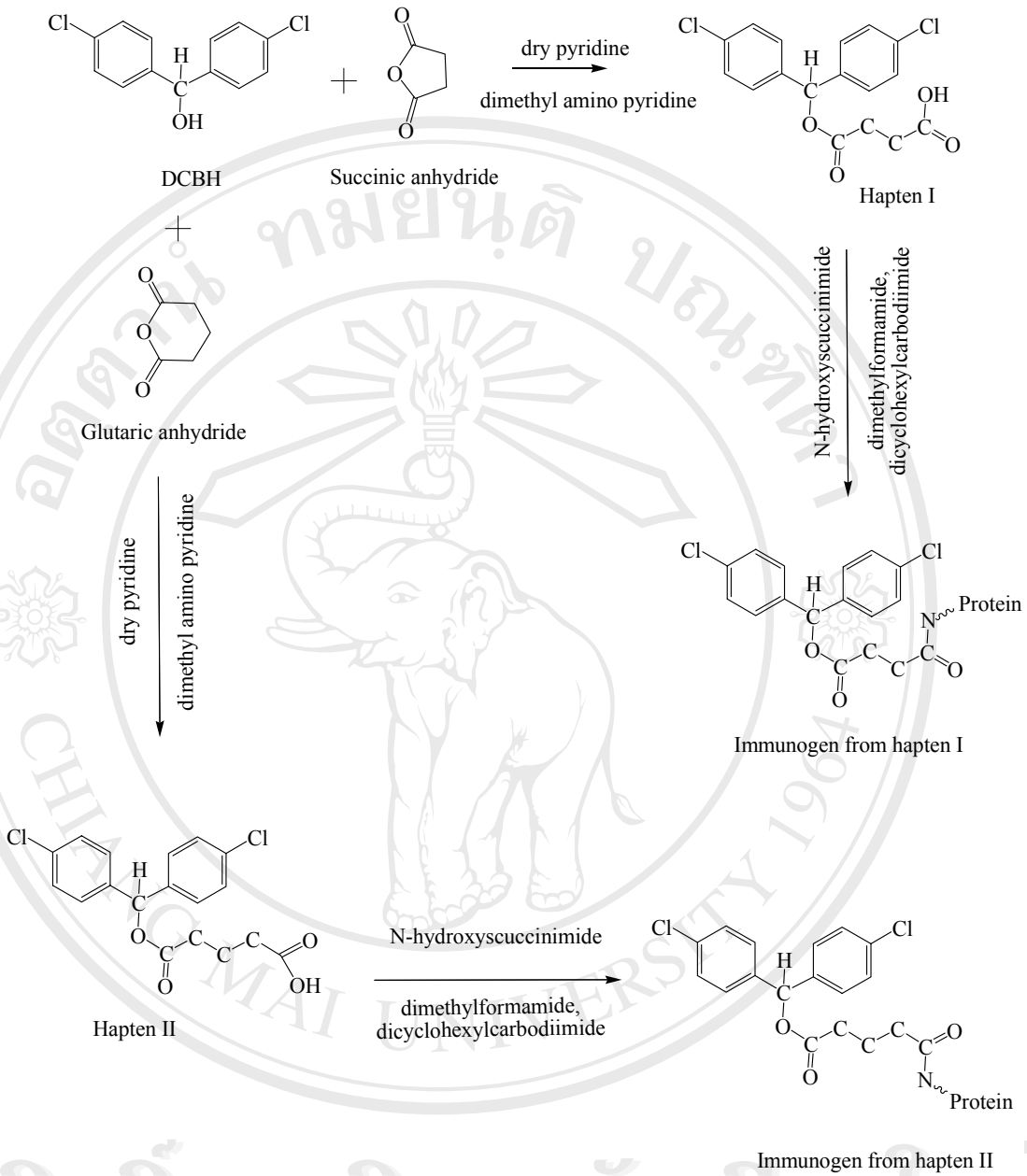


Figure 2.2 Preparation of immunogen from hapten I and II.

2.2.1.4 Hapten density assay

Ten micrograms per milliliter of DCBH and 100 μg/ml of BSA, KLH, OVA, and immunogen were prepared in 1% methanol-PBS, pH 7.5. The hapten-protein conjugates were examined individually by UV/Vis spectrophotometer to confirm coupling. The spectra of conjugates, free hapten, and native proteins were compared.

The absorbance of DCBH was measured at various wavelengths to determine the wavelength of maximum absorbance (λmax). The absorbance of other solutions was read at this wavelength, and the hapten density was calculated according to Beer's law.

$$\mathbf{A = \varepsilon bC}$$

A = Absorbance at λ_{max}

ε = Molar absorbtivity

b = Path length of radiation

C = analyte concentration

Formula for hapten density calculation:

$$\text{Hapten density} = \frac{\text{Hapten amount in hapten protein conjugate}}{\text{Concentration of protein carrier (M)}}$$

DCBH (0.78 mM) + Succinic anhydride (7.5 mM) +

DMAP (0.08 mM) + 10 mL Dry pyridine

stirred overnight at room temperature

↓

Add water 20 mL and evaporated

↓

Rinse with toluene and evaporated

↓

Redissolved with ethyl acetate 10 mL

↓

Wash with 1M HCl, water and saturated NaCl

↓

Evaporated

↓

Dry with $MgSO_4$

↓

Hapten

↓

Confirmation by ^{1}H and ^{13}C NMR, and GC/MS

Figure 2.3 Diagram of hapten synthesis

25 μM hapten + 75 μM NHS and 75 μM DCC in 0.5 mL of DMF

Incubated overnight at RT.

↓

Centrifuged at 10,000 rpm, 10 min

↓

Collected 400 μL supernatant containing the active NHS ester of hapten.

↓

Slowly added to 2 mL 15 mg/mL BSA, KLH or OVA

in 50 mM carbonate buffer, pH 9.6.

↓

Stirred at RT for 4 h

↓

Dialyzed overnight in PBS, pH 7.2

↓

Determined protein concentrations and hapten density.

Figure 2.4 Diagram of immunogen preparation.

2.2.2 Production of antibody

Antibody were produced and characterized by following and step of production was described in figure 2.5.

2.2.2.1 Immunization

To produce anti-DCBH-Ab, five BALB/c female mice were first immunized subcutaneously (s.c.) with 30 µg of immunogen emulsified in complete Freund's adjuvant. Mice were given the subsequent 2 injections s.c. with immunogen emulsified in incomplete Freund's adjuvant at 2 weeks interval. Blood samples were collected from the tail vein at 7 days interval and were allowed to clot. Sera were separated and stored at -20^{o}C.

2.2.2.2 Detection of antibody by non-competitive indirect ELISA

Non-competitive indirect ELISA was performed similarly to previously described protocol (Kramer *et al.*, 2004). One hundred microliters of 2ug/mL DCBH-S-OVA in coating buffer (see appendix A) were added to Maxisorp Immunoplate (Nunc). The assay plate was incubated overnight at room temperature. On the following day, plate was washed with washing buffer (0.05% Tween-PBS) and blocked by incubation with 200 µL/well of 1% gelatin in PBS at 37^{o}C for 1 h. Discarded the blocked buffer and 100 µL of 1:10,000 serum in washing buffer were added in duplicate wells of the assay plate. The plate was incubated at 37^{o}C for 1 h and was then washed 4 times with washing buffer, and 100 µL of 1: 5,000 horseradish peroxidase (HRP) conjugated goat-anti-mouse immunoglobulin G in washing buffer were added to each well. The plate was incubated at 37^{o}C for 1 h, washed 4 times, and 100 µL of the OPD substrate solution (see appendix A) were then added to each well. The plate was incubated in the dark at RT for 30 min and then 50 µL of 2 N H_2SO_4 stop solution were added to each well. Absorbance before and after stopping reaction were measured at 450 nm and 492 nm, respectively.

2.2.2.3 Production of monoclonal antibody by cell fusion method

a. Preparation of spleen cells for fusion

DCBH-S-BSA immunogen was given intravenously to an immunized mouse 3 days before sacrificed, cardiac blood was collected and let stand to clot and then centrifuged at 10,000 rpm for 10 min. Serum was collected and stored at -20^{o}C. Whole spleen was aseptically collected, placed in a petridish containing 3 ml high-glucose Iscove's Modified Dulbecco's Medium (IMDM) and was crushed to make cell suspension with the end of a sterile syringe plunger. Cells were collected and transferred to a sterile centrifuge tube. The tube was let stand for 5 min allowing debris to precipitate and supernatant was transferred to a new sterile tube. After centrifugation at 1,600 rpm (530g) for 7 min, supernatant was discarded and the pellet was dispersed by gently tapping. Seven milliliters of ammonium chloride potassium hydrogen carbonate lysis buffer (ACK) were added to get rid of the red blood cells and the tube was incubated for 5 min at RT. IMDM was added in order to stop the reaction and the tube was then centrifuged at 1,600 rpm for 7 min. After discarding supernatant, the cells were washed twice with IMDM and resuspended in 10% fetal bovine serum (FBS)/IMDM. Viable cells were counted on a hemocytometer with trypan blue and concentration of the cells was calculated as followed: Number of cells (1 mL) = number of cells counted x volume of hemocytometer (10^4 mm^3) x dilution factor. Finally, the cells were adjusted to the desired concentration.

b. Preparation of feeder spleen cells.

Spleen was aseptically collected as 2.3.2.3.a, and then were incubated with mitomycin C for 15 min at 37^{o}C after lysing step.

c. Preparation of myeloma cells.

X63-Ag8.653 murine myeloma cells were kindly given by Associate Professor Dr. Watchara Kasinrerk, Faculty of Associated Medical Sciences, Chiang Mai University and cultured in IMDM supplemented with 100,000 unit/L penicillin and 100,000 µg/L streptomycin and 10% FBS/IMDM. Myeloma cells were collected from culture flasks and transferred to a sterile centrifuge tube. The cells were washed twice by centrifugation at 1,600 rpm for 7 min in IMDM. The cells were counted as described above and resuspended to the desired concentration for fusion.

d. Cells fusion assay

Cell fusion was carried out essentially as described by Nowinski et al. (1979). In brief, mouse spleen lymphocytes were fused with myeloma cells at a 5:1 ratio using 50% polyethylene glycol (PEG) solution as the fusing agent. The fused cells were distributed in 96-well culture plates and hybridomas were selected in HAT (hypoxanthine, aminopterine and thymidine) selection medium (10% FBS/IMDM/2x HAT) in the presence of feeder spleen cells. Half of the medium in each well was replaced by fresh HAT medium on day 7 post fusion. Cells were cultured in HAT medium for another 2 weeks, and then HAT was substituted by HT (hypoxanthine and thymidine) medium. The resulting "hybridomas" are first selected in a HAT medium containing hypoxanthine, aminopterin and thymidine. Aminopterin poisons the parent myeloma cells which lack an enzyme in the nucleic acid pathway. The hybridomas can survive the HAT selection by using a salvage pathway, due to the complementation of the genetic defect by the B-lymphocyte genome.

e. Hybridoma selection and cloning

Three weeks after cell fusion, when expansion of hybridoma cells was observed, culture supernatant was collected and screened for the presence of anti-hapten Ab. non-competitive indirect ELISA was used for antibody-secreting hybridoma screening. The assay was described 2.2.2.2 Absorbance after stopping reaction was measured at 492 nm.

Selected hybridomas were cloned by limiting dilution, and stabled antibody-producing clones were expanded. Antibody produced was determined by competitive inhibition ELISA. Competitive inhibition ELISA was performed as followed. Ninety-six well Maxisorp Immunoplates were coated with 2 µg/mL DCBH-S-OVA in coating buffer at RT overnight. The wells were washed 4 times with washing buffer and then blocked with 200 µL/well of 1% gelatin in PBS. One hundred microliters of a mixture containing DDT and its derivatives at final concentration started at 25 to 0.02 µg/mL (2 fold) and 1:40 diluted antibody was dispensed into each well, and the wells were then incubated at 37^{o}C for 1 h. Non-specific binding and the absorbance of the wells containing no competitors were measured by replacing antibody and the competitor with buffer. The wells were washed and 100 µL of 1:5,000 HRP-conjugated goat anti-mouse IgG in 0.05% Tween20/ PBS were added to each well. After incubation at 37^{o}C for 1 h, the plates were washed and 100 µL of OPD solution were added to the wells. The reaction was allowed to continue for 30 min and was stopped by adding 50 µL of 2N H_2SO_4. The absorbance was read at 492 nm. Selected clones were also cryopreserved in liquid nitrogen.

f. Sensitivity and specificity of antibody

Sensitivity and specificity of produced antibody was evaluated by competitive inhibition ELISA (see 2.2.2.3.e). If culture supernatant is tested, 1:40 dilution will be used and if purified mAb is tested then 10ug/mL antibody is used.

g. Determination of antibody subtype using Zymed's Mouse MonoAb ID/SP Kits

Fifty microliters of 2 μg/mL of DCBH-S-OVA were added to wells of a Maxisorp Immunoplate. The plate was incubated at RT overnight. On the following day, the wells were washed 4 times with washing buffer, and then blocked 200 μL of 1% gelatin in PBS were added. Plate was incubated at 37^{o}C for 1 hour and washed.

Fifty microliters of supernatant were added to each well the plate was incubated at 37^{o}C for 30 min and was then washed 4 times. Fifty microliters of washing buffer were added to each well of the first column to represent blank wells. One drop of biotinylated antibody control was added to each well of the second column, which represented negative control wells. One drop of subclass specific antibodies was added to columns 3 through 10 as the following order; biotinylated anti-mouse IgG1 (γ1 chain-specific), IgG2a (γ2a chain-specific), IgG2b (γ2b chain-specific), IgG3 (γ3 chain-specific), IgA (α chain-specific), IgM (μ chain-specific), κ light chain, and λ light chain, respectively. The plate was incubated at 37^{o}C for 30 min and was then washed 4 times. Fifty microliters of HRP-Streptavidin were added to each well and the plate was incubated at 37^{o}C for 30 min. The wells were washed 4 times and 100 μL of working substrate solution were added. The plate was incubated at RT for 30 min and absorbance was read with a spectrophotometer at 405 nm.

h. Purification of antibody

Five million Dynabeads pan mouse IgG were washed with PBS pH 7.4 twice to get rid of preservative in bead stock solution.

The beads were incubated for 30 min with 100, 500, and 1,000 μL of undiluted culture supernatant, respectively. Bound Abs was eluted with elution buffer (see appendix A) and were tested for protein concentration (see 2.2.1.3).

Note: Purification method of mAb using beads in the present study was not a conventional method for monoclonal antibody purification.

Immunization

30 μg protein of immunogens per mouse (female BALB/c)

↓

Detection of antibody

Non-competitive indirect ELISA

↓

Production of monoclonal antibody

Cell fusion method (Hybridoma technique)

↓

Hybridoma selection and cloning

Limiting dilution (cloning)

Selected stabled antibody-producing clones

↓

Purified antibody

Dynabead/pan mouse IgG

↓

Determination of sensitivity and specificity

Competitive inhibition ELISA

Figure 2.5 Summary diagram of production and characterization of antibody.