CHAPTER 3

EXPERIMENTAL

3.1 Equipments and Chemicals

3.1.1 Equipments

1) Gas chromatograph-mass spectrometer, Agilent 6850 Series GC system coupled to 5973 Network mass selective detector, Agilent technology, USA.

2) UV-Vis Lambda 25, Perkin Elmer, USA.

3) Micropipette, Thermos Scientific, USA.

4) pH Meter, Metrohm, Switzerland.

5) Incubator, Memmert, Germany

3.1.2 Chemicals

1) Hexane, HPLC grade Lab scan, Thailand

2) Ethyl acetate, HPLC grade Lab scan, Thailand

3) Methanol, HPLC grade Lab scan, Thailand

5) Dimethylsulfaoxide (DMSO), Lab scan, Thailand

4) γ- Fe_2O_3 (nanopowder <50 nm), Sigma-Aldrich, Singapore

5) Fe_3O_4 (nanopowder 50-100 nm), Sigma-Aldrich, Singap

6) 3,3,5',5'-Tetramethylbenzidine (TMB), Sigma-Aldrich, Sing

7) Acetylcholinesterase (AChE), Sigma-Aldrich, Singapor

8) Choline Oxidase (CHO), Sigma-Aldrich, Singapore

9) Acetylcholine Chloride, Sigma-Aldrich, Singapore

10) Hydrogen peroxide, H_2O_2(30 wt. %),NCG, Thailand

11) Potassium dihydrogen phosphate KH_2PO_4, Sigma-Aldrich, Singapore

12) Sodium acetatetrihydrate ($CH_3COONa.3H_2O$), Carlo Erba, Germany.

13) Di- sodium hydrogen phosphate (Na_2HPO_4), Carlo Erba, Germany.

14) Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), Carlo Erba, Germany.

15) Potassium chloride (KCl), Ajax, Australia

16) Sodium carbonate (Na_2CO_3), Ajax. Australia

17) Sodium chloride (NaCl), Carlo Erba, Germany.

18) Trizma hydrochloride ($C_4H_{11}NO_3.HCl$), Sigma-Aldrich, Singapore

19) Hydrochloric acid (HCL), Sigma-Aldrich, Singapore

20) Tris-Sodium citrate ($C_6H_5Na_3O_7.2H_2O$), Merck, USA

21) Citric Acid ($C_6H_8O_7.2H_2O$), Merck, USA

22) Standard dimethoate and profonefos, Sigma-Aldrich,Singapore

3.2 Experiments

3.2.1 Preparation of iron oxide NPs

5 mg of each iron oxide NPs (γ-Fe_2O_3, Fe_3O_4 and mixed γ-Fe_2O_3:Fe_3O_4 in the ratio of 1:1, 1:2, 1:3, 1:4, 1:5) were prepared in 1 mL of acetate buffer at pH 3.0 to have the final concentration of 500 µg/ml. The mixture was then stirred vigorously for 20 min to form a homogenous suspension.

3.2.2 Study of peroxidase activity of the iron oxide NPs

Peroxidase like activities of pure and mixed iron oxide NPs were examined spectrophotometrically based on the catalytic oxidation of peroxidase TMB in the presence of H_2O_2. The study was performed as follows: 50 µL of each pure iron oxide NPs and mixed iron oxide NPs was first mixed with 100µL of 0.1 M of H_2O_2 and 10 µL of TMB in 840 µL acetate buffer solutions (0.2 M, pH 3.0). Subsequently, the mixture was then incubated for 20 minutes at room temperature. Finally, the absorption spectrum (300-800 nm) was recorded after the iron oxide NPs were removed from the reaction solution by centrifugation. The reaction was shown in Figure 3.1 The catalytic properties of the mixed iron oxide NPs of γ-Fe_2O_3 and Fe_3O_4 in the ratios of 1:2, 1:3, 1:4 and 1:5 were also studied.

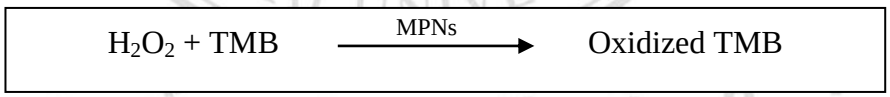

Figure 3.1 Oxidation reaction of TMB

3.2.3 Catalytic performance of mixed iron NPs under optimum conditions.

Catalytic performance of the mixed iron oxide NPs was studied by optimizing the parameters such as pH of buffer solution, concentration of NPs, concentration of H_2O_2, concentration of TMB and the different ratios of mixed mixed iron NPs (γ-Fe_2O_3:Fe_3O_4) at the ratio of 1:1, 1:2, 1:3, 1:4, 1:5).

1) pH of buffer solution

The different pH from 3.0 – 6.0 of acetate buffer solution were was studied.

2) Concentration of mixed iron NPs

The different concentrations of nanozymes in the ratio of 1:1 (25, 50, 75, 100, 250, 300, 350 and 400 µg/mL) and the control were prepared from the stock of 500 µg/ml and their catalytic performance was studied.

3) Concentration of TMB

The different concentrations of TMB (0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL) were prepared and their effects on the catalytic performance of the mixed nanozymes were studied. Each time prior to absorbance measurements iron oxide NPs were removed from the solutions by centrifugation for 5 min.

3.3. Detection of OPs by mixed iron oxide NPs based on colorimetric assay

3.3.1 Validation of colorimetric assay

The colorimetric assay was validated as follows : in the first step, 100 µL of phosphate buffered saline solution (PBS, 10 mM, pH 7.74) containing 20 µL of 20 mM acetylcholine chloride (Ach) in DI water and mixed with 10 µL of 10 U/ml of acetylcholinesterase enzyme (AChE) and choline oxidase enzyme (ChOx), respectively which were incubated at 37 °C for 15 min (solution A); in the second step, 900 µL of acetate buffer solution (ABS, 0.2 M, pH 3.0) containing 30 µL of 10 mg/ml TMB and 100 µL of 5 mg/ml mix iron oxide NPs was added into solution A. After that, the mixed solution was immediately incubated for 20 min at room temperature. The

absorbance of the solution was measured at 654 nm after removal of nanozymes by centrifugation. The reaction of this assay shown in Figure 3.2.

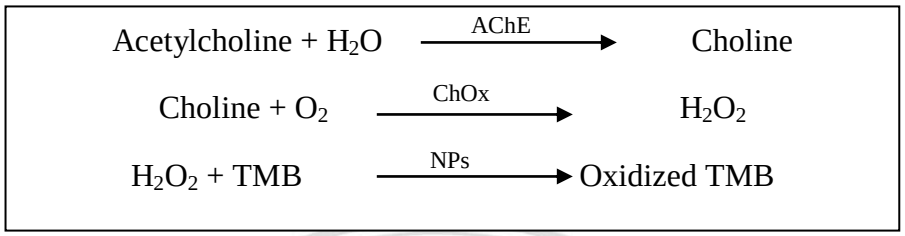

Figure 3.2 The formations of H_2O_2 from catalyses of AchE and ChOx and oxidized TMB catalyzed by NPs.

3.3.2 Detection of OPs in spiked real sample using developed mixed iron oxide NPs based on colorimetric assay.

OPs at different concentrations (1.0, 1.5, 2.0, 2.5 and 3.0 µM), were first mixed with 10 µL of 10 U/ml of acetylcholinesterase enzyme (AChE), and followed by adding 20 µL of 20 mM acetylcholine chloride (Ach) in DI water and 10 µL of 10 U/ml of choline oxidase enzyme (ChOx), respectively in 100 µL of phosphate buffered saline solution (PBS, 10 mM, pH 7.74). The mixture solution was incubated at 37 °C for 15 min. After that, the reaction solution was then added 30 µL of 10 mg/ml TMB and 100 µL of 500 µg/ml mix nanozymes in 900 µL of acetate buffer solution (ABS, 0.2 M, pH 3). Then, the resulting solution was immediately incubated for 20 min at room temperature and the absorbance of the resulting solution was measured at 654 nm after removal iron oxide NPs by centrifugation.

3.3.3 % Recovery determination of OPs using mixed iron oxide NPs based on colorimetric assay.

To determine the validity of assay with real sample, colorimetric assay was evaluated by determining OP standard (dimethoate and profenofos) in orange samples. The sample was spiked with two concentrations (2.0 and 2.5 µM) for each pesticide. The % recovery was calculated as O/E*100, where O is observed concentration and E is the expected OP standard concentration.

3.4 Detection of OPs in the spiked real sample by Gas Chrometography-Mass Spectrometry (GC-MS)

3.4.1 Preparation of OP standard solutions

Dimethoate and profenofos were used as OP standards. These OP were prepared by dissolving in methanol from the stock solution (10 µM) to get the working solutions at difference concentrations as 1.0, 1.5, 2.0, 2.5 and 3.0 µM, respectively.

3.4.2 Real sample preparation

Orange samples were purchased from local supermarket (Chiang Mai Province, Thailand). 10 g of the oranges peels were chopped into small pieces and then extracted with 50 mL of hexane and ethyl acetate (1:1) using sonication at room temperature for 30 minutes. After that, the sample mixture was filtered using 0.22 µM polytetrafluoroethylene before used.

3.4.3 GC-MS analysis

OPs were analyzed using an Agilent model 6890-A series gas chromatograph equipped with a HP-5MS capillary fused silica column (30 m, 0.25 mm. I.D.; 0.25 µm film thickness) coupled with an Agilent model 5973 mass selective detector. The oven temperature was initially programmed at 150 °C, then increased at a rate of 10 °C/min to 250 °C and maintained for another 5 min. Helium was used as a carrier gas in constant flow mode at 1 ml/min. An injection volume of 1 µl with a split ratio of 1:250 was used. The injector and detector temperatures were 250 °C and 280 °C, respectively. Mass spectra were taken at 70 eV, ranging from m/z 50 to 550 amu. The relative percentage amounts of the separated compounds were calculated from the total ion chromatography (TIC) by a computerized integrator.