รายงานการวิจัยฉบับสมบูรณ์

การผลิตโมโนโคลนอลแอนติบอดีต่อออกซีเตตราซัยคลิน เพื่อพัฒนาชุดตรวจสอบ โดยวิธีเอนไซม์ลิงค์อิมมูโนซอร์เบนท์เอสเสย์ ปีที่ 2

Production of monoclonal antibody against oxytetracycline for developing enzyme-linked immunosorbent assay (ELISA) test kit, 2nd year

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บทคัดย่อ

ในประเทศไทยออกซีเตตราไซคลินถูกนำมาใช้ในการป้องกัน และรักษาการติดเชื้อในกุ้ง ซึ่งการใช้ยาออกซีเตตราไซคลินอย่างไม่ถูกวิธีอาจทำให้เกิดการตกค้างขึ้นในเนื้อกุ้งที่นำมาบริโภค ดังนั้นเพื่อป้องกันผู้บริโภคจากการได้รับยาปฏิชีวนะที่ตกค้างในอาหาร และป้องกันการเกิดการ ดื้อ ยาของเชื้อก่อโรคในคน หน่วยงานที่ควบคุมความปลอดภัยของอาหารในหลายประเทศจึงได้ กำหนด และบังคับใช้ค่าปริมาณออกซีเตตราไซคลินสูงสุดที่อนุญาตให้ตรวจพบได้ (MRLs) ใน อาหารขึ้น ดังนั้นจึงจำเป็นต้องมีวิธีการตรวจคัดกรองออกซีเตตราไซคลินที่ตกค้างในอาหารที่มี ประสิทธิภาพ โดยวิธีเอนไซม์ลิงค์อิมมูโนซอร์เบนต์แอสเสย์ (ELISA) เป็นวิธีที่เหมาะสมที่จะนำมา ตรวจคัดกรองตัวอย่างจำนวนมากพร้อมกัน เนื่องจากวิธีดังกล่าวสามารถตรวจวัดได้รวดเร็ว ง่าย และคุ้มค่าต้นทุนการตรวจ ดังนั้นจุดประสงค์ของงานวิจัยนี้คือการพัฒนาการตรวจสอบข้างต้นเพื่อ ตรวจวัดออกซีเตตราไซคลินที่ตกค้างในเนื้อกุ้ง จากการทดลองพบว่าการตรวจแบบ indirect competitive ELISA โดยใช้ goat anti mouse IgG-hosreradish peroxidase (GAM-HRP) มีความ เหมาะสมในการตรวจออกซีเตตราไซคลิน โดยมีค่าความเข้มข้นของสารแข่งขันที่ทำให้ค่าการ ดูดกลืนแสงลดลง 50 เปอร์เซ็นต์ (IC_{50}) ที่ 5.5 นาโนกรัมต่อมิลลิลิตร และค่าความเข้มข้นต่ำสุดที่ ตรวจวัดได้ (LOD) ที่ 0.9 นาโนกรัมต่อมิลลิลิตร และ ELISA ที่พัฒนาขึ้นสามารถตรวจวัดออกซีเต ตราไซคลินได้ในช่วงความเข้มข้น 0.5 ถึง 32 นาโนกรัมต่อมิลลิลิตรซึ่งครอบคลุมค่า MRLs ใน ปัจจุบัน นอกจากนี้ยังพบว่าการตรวจสอบจะทำปฏิกิริยาข้ามกับสารโรลิเตตราไซคลินสูง (142%) แต่ไม่ทำปฏิกิริยาข้ามกับยาปฏิชีวนะอื่นที่เกี่ยวข้อง และเมื่อนำชุดตรวจสอบแบบ ELISA นี้ไปทำ การตรวจวัดปริมาณออกซีเตตราไซคลินที่ถูกเติมลงในตัวอย่างเนื้อกุ้ง พบว่าสำหรับ Intra-assay จะ มีอัตราการได้กลับคืน (%recovery) อยู่ในช่วง 82%-118% และสำหรับ Inter-assay จะเท่ากับ 96%- 113% โดยมีค่าสัมประสิทธิ์การความแปรปรวน (%CV) อยู่ที่ 5.5%-13.9% และ 5.5%-14.9% ตามลำดับ

Abstract

In Thailand, oxytetracyline (OTC) has been used in the protection and treatment of infected shrimps. The misuse of OTC could lead to OTC residues in shrimps destined for consumption. To prevent consumers from exposure to drug residues and increase of drug resistance pathogens, several food safety authorities in many countries have set the maximum residue limits (MRLs) for OTC and enforced the surveillance detection programs. Therefore, an effective screening method is essential to detect OTC residue in food. Enzyme-linked immunosorbent assay (ELISA) is the suitable methods for screening a large number of samples due to its simplicity, rapidity and cost effectiveness. Consequently, the aim of this study was to develop the methods in order to detect OTC residue in shrimp samples. The results showed that the indirect competitive ELISA using goat anti mouse IgG-hosreradish peroxidase (GAM-HRP) was suitable for OTC detection with the 50% inhibition concentration (IC_{50}) of 5.5 ng/ml and the limit of detection (LOD) of 0.9 ng/ml. The developed ELISA could detect OTC at the concentration between 0.5 ng/ml and 32 ng/ml which covered the current MRLs. In addition, the detection showed strong cross-reactivity to rolitetracycline (RTC) (142%) but did not cross-react with other related antibiotics. When the ELISA was used to detect OTC in fortified shrimp samples, the %recoveries were in the range of 82%-118% for an intra-assay and 96%-113% for an inter-assay. The coefficients of variation of ELISA were 5.5%-13.9% and 5.5%-14.9%, respectively.

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CHAPTER I

INTRODUCTION

1.1 Rationale

Oxytetracycline (OTC) is a broad spectrum antibiotic in the tetracycline (TC) group. In Thailand, it is allowed for use in medicated feed in aquaculture and is usually administered through the feed during the growing period with a recommended withdrawal period of 4-16 days before harvesting, depending on the concentration, water temperature and shrimp species. Overuse and insufficient lengths of withdrawal periods lead to OTC residues in aquaculture products, causing a serious threat to human health. TCs including OTC have an impact on human health in two different ways. At high concentration, they inhibit mammalian protein synthesis, while at low concentration, they cause acquired resistance in microorganisms

To prevent harmful health effects in consumers due to OTC residues, maximum residual limits (MRLs) have been established for OTC. The European Union (EU) proposed MRLs of 100 ng/g for muscle, 300 ng/g for liver and 600 ng/g for kidney for all animals used for human consumption (Commission Regulation No. 2377/90, 1990). These limits require sensitive and specific methods for the detection of OTC in food-producing animals. Therefore, various methods have been reported, most of which were based on chemical methods such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectroscopy (LC-MS) and capillary electrophoresis. Although these methods offer high precision and accuracy, they require expensive equipment and well-trained professionals. Moreover, most chemical methods require a complicated sample preparation step. Commonly, cleanup methods are based on solid-phase extraction or metal chelate affinity chromatography methods that are time consuming, costly and require large sample volumes. Considering the disadvantages of chemical method, it is not suitable for use in screening a large number of samples.

On the contrary, immunological method has been generally used for screening samples before performing confirmation test by chemical methods because of its simplicity, cost effectiveness and ability to screen large numbers of samples in a short period. Importantly, an immunoassay does not require a complicated step to clean up samples. The most widely used

immunoassay is enzyme-linked immunosorbent assay (ELISA), which is a semi-quantitative method based on the specific binding between antibody and antigen on the solid-phase. Because of its high sensitivity and specificity, ELISAs have been commercially developed available as assay kits. Although ELISA is easier to be used than the chemical methods, In 2011, the success of the production of monoclonal antibody specific to OTC by conventional somatic cell fusion technique was developed (Wirongrong Natakuathung, 2011). This monoclonal antibody has high sensitivity of OTC; therefore, it was used to develop ELISA for OTC residue detection in this research.

1.2 Objective of the Study

- To develop a prototype ELISA test kit
- To evaluate the effectiveness of the prototype ELISA test kit and ICA test strip

1.3 Scope of the Study

- Literature review
- Production and purification of monoclonal antibody
- Preparation of coating agent and enzyme-labeled tracer agent
- Development of ELISA test kit
- Validation of prototype ELISA test kit
- Report writing

1.4 Advantages of the Study

The development of a domestic ELISA test kit will be able to replace those imported kits. Since the cost of detection would be reduced, more samples could be screened, resulting in a safer food for consumers.

2.1 Tetracyclines Antibiotics

Tetracycline antibiotics (TCs) were first discovered from the group of fungus *Streptomyces* in the late 1940s. The main structure of TCs molecules is hydronapthacene skeleton consisting of a linear fused tetracyclic nucleus (rings A, B, C, and D) to which a variety of functional groups are attached (Chopra and Roberts, 2001). The structure of interested members of TCs is shown in Table 2.1.

Table 2.1 Structure of the interested members of TCs.
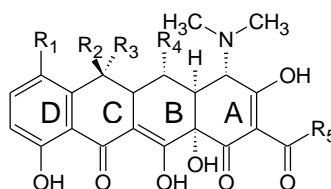
Compound	R_1	R_2	R_3	R_4	R_5	structure
Tetracycline (TC)	H	OH	CH_3	H	NH_2	
Chlortetracycline (CTC)	Cl	OH	CH_3	H	NH_2	
Oxytetracycline (OTC)	H	OH	CH_3	OH	NH_2	
Doxycycline (DC)	H	H	CH_3	OH	NH_2	
Rolitetracycline (RTC)	H	OH	CH_3	H		

The TCs are usually bacteriostatic in action, but may be bactericidal in high concentration. They are broad-spectrum antimicrobial agent, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, including some anaerobes. The activity range of the TCs also includes *Escherichia coli*, *Klebsiella* sp., *Pasteurella* sp., *Salmonella* sp., *Staphylococcus* sp., and *Streptococcus* sp. They are also active against chlamydia, mycoplasmas, several rickettsiae, and some protozoa (USP veterinary pharmaceutical information monographs-antibiotics, 2003).

The wide range antibacterial properties of TCs are from the inhibition of bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome. Therefore, to interact with their targets, TCs need to traverse one or more membrane systems depending on passive diffusion and active transport. When TCs transfer across the cytoplasm membrane, TCs can inhibit protein synthesis by binding reversibly to receptors of the 30S ribosomal subunit of susceptible bacteria (Figure 2.1). The binding of a tetracycline to the subunit blocks the binding of the aminoacyl-tRNA to the acceptor site on the mRNA-ribosomal complex and prevents the addition of new amino acids to the peptide chain, inhibiting protein synthesis (Schnappinger and Hillen, 1996; Chopra and Roberts, 2001).
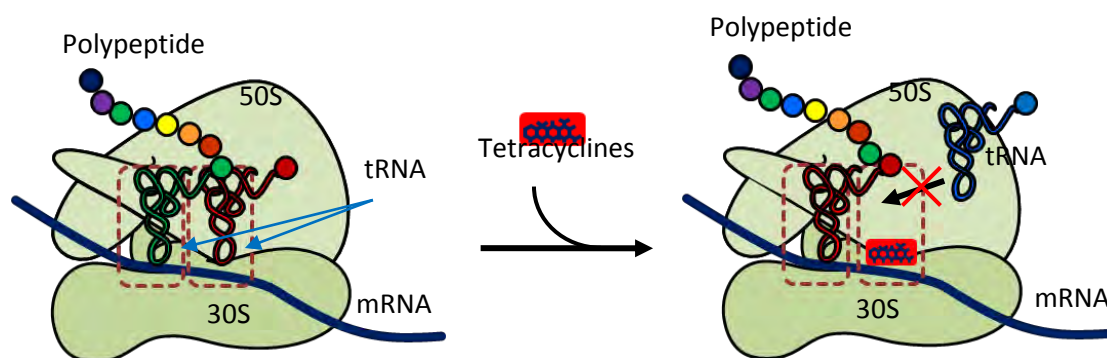
Figure 2.1 Mechanism of TCs when transfer into the cell of susceptible bacteria.

TCs are usually subdivided into short-acting (tetracycline, oxytetracycline), intermediate-acting (demeclocycline) and long-acting (doxycycline, minocycline) analogues. According to several good properties of TCs, for example, they are active against most common pathogens, they show good oral absorption, they exhibit low toxicity, they cause only few allergic reactions, and they are relatively inexpensive, this has led to an intensive use of TCs for human and veterinary medicine in prevention and treatment of bacterial infections over the last 50 years.

Subtherapeutic levels of TCs are also used as food additives for growth promotion in animal husbandry. As a result of this extensive application, TCs have been produced on the largest scale of all antibiotics (Schnappinger and Hillen, 1996).

2.2 Oxytetracycline

Oxytetracycline (OTC) is an odorless, bitter taste and yellow to tan-colored crystalline powder produced by the growth of certain strains of *Streptomyces rimosus*. It is only slightly soluble in water but dissolves well in dilute acids and alkalis. Oxytetracycline hydrochloride is more water soluble. Both forms deteriorate in solutions having a pH of less than 2 and are rapidly destroyed by alkali hydroxide solution. Their selective toxicity for bacteria appears to depend, in part, on energy-dependent uptake of antibiotic by bacteria, but not mammalian cell. This results in a greater accumulation of TCs by bacterial cells (Chopra and Roberts, 2001).

OTC can be illegally added to foods and preservatives. Their residues are carried over to food chain from animal husbandry and aquaculture practices. OTC is used in farmed animals primarily for the treatment and control of infectious diseases caused by bacterial pathogens as well as in aquaculture including shrimp farming. It is usually recommended to Thai shrimp farmers as administering through medicated feed during the growing period with a recommended withdrawal period of 4-16 days before harvesting depending on the OTC concentration and shrimp species (Gómez-Jimenez et al, 2008; Nogueir-Lima et al., 2006; Wang et al., 2004 and Uno et al., 2006). The usual daily dose of OTC as drug is 250 mg administered once every 24 hours or 300 mg given in divided doses at 8 to 12 hour intervals for adults. Overuse and insufficient lengths of withdrawal periods lead to OTC residues in aquaculture products, causing a serious threat to human health. TCs including OTC have an impact on human health in two different ways. At high concentration, they inhibit mammalian protein synthesis, while at low concentration, they cause acquired resistance in microorganisms (Srisomboon, 1995).

The problem concerning drug residues in cultured shrimp was remarkable. In 1992, the Ministry of Health and Welfare of Japan had banned a few consignments of frozen shrimp from Thailand because of the presence of antimicrobial residues. The rest had to go through strict sampling procedures and laboratory examination where contaminated consignments were rejected. During June 1992 to April 1994, 30 shipments of frozen cultured shrimp from Thailand

were found to contain antimicrobial agents by Japanese quarantine stations. Nine out of the 30 cases were due to OTC residues, the rest were because of oxolinic acid residues. This event has caused economic loss to shrimp farmers, frozen cultured shrimp processors and to exported shrimp business of Thailand. Claims had been made to Thai frozen seafood processors and responsible authority in Thailand mentioning that it has been prescribed in Japanese Food Sanitation Law that food shall contain no antibiotic (Srisomboon, 1995).

In order to prevent harmful health effects to consumers due to OTC residues, the maximum residual limits (MRLs) for animals for consumption have been established. The European Commission (EC) proposed MRLs of 100 ng/g (ppb) in muscle for all animals for human consumption (Commission Regulation No. 2377/90, 1990). The Codex Alimentarius Commission established the MRLs in muscle of giant prawn (*Paeneus monodon*) at 200 ng/g (ppb) (Codex Alimentarius Commission, 2011). Whereas, the United State Food and Drug Administration (US FDA) established the sum of residues of the tetracyclines anitbiotics including chlortetracycline, oxytetracycline, and tetracycline, in muscle at MRLs of 2 μg/g (ppm). These limits require the development of sensitive and specific methods for the detection of OTC in food-producing animal.

Table 2.2 Maximum residual limits (MRLs) of OTC.

Target tissues	EC* (ppm)	US FDA** (ppm)	Codex*** (ppm)
Muscle	0.1	2	0.2
Liver	0.3	6	0.6
Kidney	0.6	12	1.2
Milk	0.1	0.3	0.1
Eggs	0.2	-	0.4

(*Commission Regulation No. 2377/90, 1990; **U.S. Food and Drug Administration, 2003; ***Codex Alimentarius Commission, 2011)

2.3 Analytical Methods for OTC Detection

Analytical methods for OTC residues detection can be classified in two groups: confirmatory and screening methods (Cháfer-Pericás et al., 2010). The confirmatory methods are

mostly based on chromatographic techniques including thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS) to quantify OTC concentrations. And other methods based on capillary electrophoresis (CE) are also used for OTC analysis. These confirmatory methods show high recovery, sensitivity, specificity and reproducibility of OTC detection as reviewed by Yuwono and Indrayanto in 2005. However, all these methods are time-consuming, expensive, and require complex laboratory equipment and trained personnel. Also, they require complicated sample-preparation procedures based on solid-phase extraction (SPE) and multi-step clean-up.

Screening methods can detect an analyte or a family at the level of interest, and usually provide semi-quantitative results. Most of them are based on microbiological assays, immunoassays and, more recently, biosensors. The ideal characteristics of a screening method are low rate of false-positive samples, high throughput, ease of use, short analysis time, good selectivity and low cost.

In general, the reported methods are grouped depending on the type of analytical technique and the corresponding percentages for each one are depicted in Figure 2.2. As can be seen, LC-MS is the most employed analytical method 38%), followed by LC-UV (18%) and ELISA (18%). However, the development of other screening methods (12%) and biosensors (8%) is increasing considerably (Cháfer-Pericás et al., 2010).
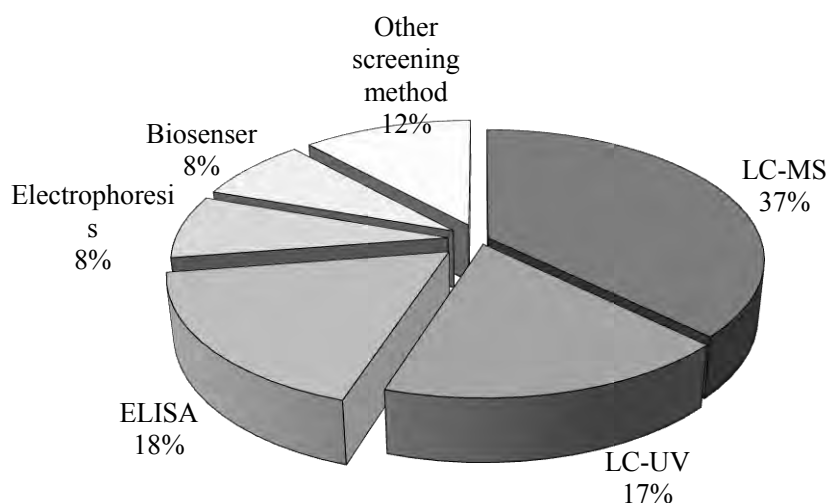
Figure 2.2 Distribution of the analytical methods used for antibiotic determination in food. (Cháfer-Pericás et al., 2010)

2.4 Antibody

Antibody is an essential composition for all immunological assays because it could direct the sensitivity and specificity of the methods. The antibody is a glycoprotein that belongs to the group of immunoglobulin. Primarily, they are secreted by plasma cell in response to an immunogen. Each plasma cell secretes about 2000 antibody molecules per second, amounting to about 20% (w/w) of blood. Because blood can be collected easily, antibodies provide for a powerful analytical tool as a result of their specific interaction with antigens. All immunoassay are an analytical technique, quantitative or qualitative, that depend on the specificity and affinity of the interaction between antibody and antigen. Most of immunoassays are semi-quantitative methods characterized by their high specificity, high sensitivity, simplicity and cost effectiveness, which make them particularly useful in routine work. Immunoassays applied to antibiotic determination have been classified depending on the detection label such as enzyme-linked immunosorbent assay (ELISA) labeled with enzymatic system, fluoroimmunoassay (FIA) labeled with fluorescent molecules and immunochromato- graphic assay (ICA) labeled with gold nanoparticle.

2.4.1 Polyclonal Antibody

Polyclonal antibodies (PAbs) are frequently obtained from the antisera of immunized animals and comprised of a heterogenous mixture of antibodies of varying binding affinities and different specificities. Because they are produced in response to any immunogenic epitope in the injected material which includes any impurities in the preparation and any carrier protein or linker attaching them, there will also be background antibodies of unknown specificity that were present before the active immunization started. Therefore, it is advisable to inject as pure a material as possible. For PAbs production on a research scale, rabbits are the most common species used. Which balances the low maintenance costs with useful volumes of serum (expected yields can be up to several hundred ml of serum per animal with repeated bleeds). For commercial scale production, larger domestic animals such as sheep, goats, horses and donkeys are used (Wild, 2005).

Major advantages of PAbs are relatively quick and inexpensive to produce but their specificity is dependent on the purity of the immunogen and on the purification of the

antiserum. PAbs are also the formation of large insoluble immune complexes with polyvalent antigens. The multivalent antigens and the bivalent antibodies form a three-dimensional network, which is opaque and can be determined quantitatively in immuneaggregation technique. Additionally, PAbs are usually well-suited for cell straining, immunoblotting, and immunoassays with labeled antigens, but it is difficult to perform immunoassays with labled antibodies. When using antibodies as a reagent for immunoassay, it should be considered that there is a batch-to-batch variation of polyclonal antibodies, even those deriving from the same animal (Schalkhammer, 2002).

2.4.2 Monoclonal Antibody

In 1984 G. Kohler and C. Milstein were awarded with the Nobel Prize for development of the hybridoma technique, which yields monoclonal and, consequently, monospecific or monoclonal antibodies (MAbs) (Schalkhammer, 2002). The overall process for the production of MAb is shown in Error! Reference source not found.. The immunized animal becomes the source of sensitized antibody-producing cells (B lymphocyte). These cells are then mixed with a specially selected immortal cell line (myeloma cell) and the mixture exposed to an agent which promotes the fusion of cells such as polyethylene glycol (PEG). Under these conditions the fusion between cells takes place in a random manner, and the cell suspension containing fused cells and non-fused lymphocytes and myeloma cells are the products. Whereas lymphocytes are mortal and lose their viability during future propagations, the myeloma cells are immortal. In order to eliminate the myeloma cell, the cell suspension is cultured in a selective culture medium; therefore, the only cells to persist in culture from the fusion mixture are the hybrid cells between lymphocyte derived from the immunized donor and myeloma cells, which are hybridomas. The other possible combinations of fused cells are not able to survive the selection pressure that is applied (Liddell and Cryer, 1991). At this stage, the hybridomas that survive will still be a very mixed population, not all of which will be able to synthesize specific antibody. Furthermore, single antibody-secreting hybridoma cell can be isolated in individual tissue culture wells from which large colonies (monoclones) and develop through mitotic division, each secreting identical antibodies (Wild, 2005). These MAbs can be made available in limitless quantities since the hybridoma cell can be grown in tissue culture virtually indefinitely

and at industrial scales. There is also the added advantage that the cells can be frozen for storage, and recovered when required without the need for recharacterization as would be necessary for a new batch of polyclonal antiserum (Wild, 2005). However, the selection of a good MAbs is still expensive and time consuming; PAbs are therefore more widely used in immunoassays.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is an immunoassay in which one reactant is immobilized on a solid phase and the signal generator or reporter is an enzyme. It is developed from the classical radioimmunoassay (RIA) to simplified antigen quantitation and antibody detection (Butler, 2000). The only major difference between ELISA and RIA is the use of an enzyme to label the antigen of antibody, rather than a radioactive isotope. The enzymatic activity of non-labeled and labeled enzyme occurring on the solid-phase is quantitated by the enzyme-catalyzed conversion of a relatively non-chromatic substrate to a highly chromatic product that is determined and calculated back to the amount of analyte (Maggio, 1980).

ELISA is a fundamental tool of modern biology market in diagnostics and analysis. This powerful technique combines the sensitivity and specificity of antibodies and other binding partners in a format that is cost effectiveness and easily automated for high-throughput assays. ELISAs are extraordinary adaptable and take advantage of many combinations of ligand pairs, methods of measuring signal (fluorescent, chemiluminescence or enzymatic) and experimental strategies. The presence or concentration of an antigen, antibody, or other target can be determined in solution with excellent limits of detection, generally in the ng/ml to pg/ml (Howard and Kaser, 2007).

2.5.1 Formats of ELISA

ELISA may be classified under four major formats: direct, indirect, sandwich and competitive format (Crowther, 1995).

2.5.1.1 Direct ELISA

This format is used to detect analytes which can directly immobilize on a solid phase. Then the analytes are detected directly with the enzyme labeled antigen or antibody. The format has disadvantage if used to detect analytes from crude sample, which contain a high

concentration of contaminating substances, since low levels of analytes attach to the surface of solid-phase owing to competition by such contaminants. Thus, this format has a poor applicability to diagnostic test. This format can be divided into 2 types following the analytes (antigen or antibody) that are shown in Figure 2.3.

Direct ELISA for antigen detection

The analyte (Ag) is attached on solid-phase.	The enzyme-labeled Ab is added to detect the analyte.	The substrate is added to develop the color by enzymatic
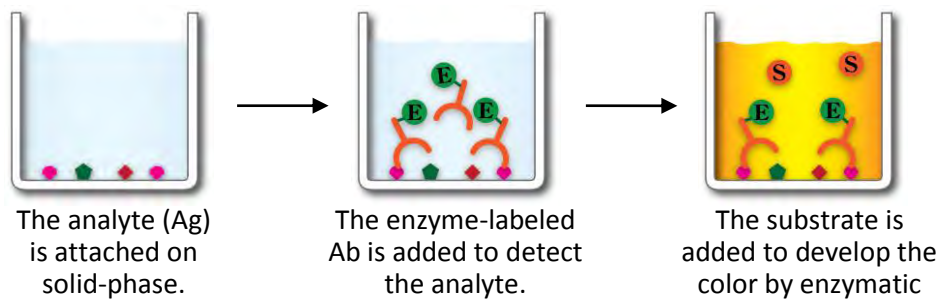
Direct ELISA for antibody detection

The analyte (Ab) is attached on solid-phase.	The enzyme-labeled Ag is added to detect the analyte.	The substrate is added to develop the color by enzymatic
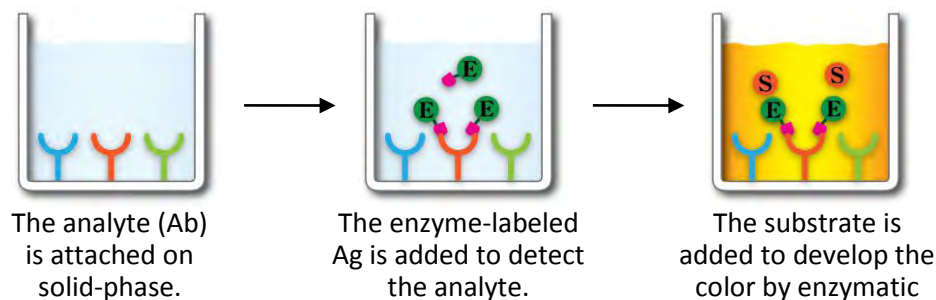
Figure 2.3 The diagram of direct ELISA format for antigen detection (above) and for antibody

detection (bottom).

2.5.1.2 Indirect ELISA

This format is extensively used for the detection of specific antibodies from serum sample or culture media. The specificity of the assay is directed by the antigen attached on the solid-phase. After immobilization of antigen by incubation, the wells are washed to get rid of unbound antigen. Serum or culture media containing antibodies against immobilized antigen can

then be added into the wells. After incubation and washing, the bound specific antibodies are then detected by enzyme-labeled anti-species antibody. The diagram is shown in Figure 2.4.

| The excess Ag is attached on solid-phase. | The analyte (Ab) is added to specifically bind with the Ag. | The enzyme-labeled anti-species Ab is added to detect the analyte. | The substrate is added to develop the color by enzymatic |
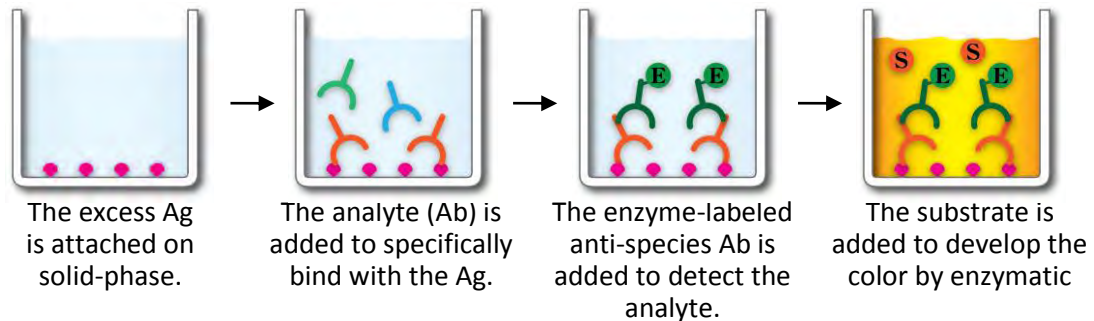
Figure 2.4 The diagram of indirect ELISA format for detecting the specific antibody in serum.

2.5.1.3 Sandwich ELISA

This format is popularly used to detect analytes that are macromolecule or contain at least two epitopes; by the trapping between two different specific antibodies. The process of this format is shown in Figure 2.5. The first specific antibodies are attached on the solid-phase. The sample containing analytes are added into the wells, and then the analytes can be bound with the attached antibodies. Amount of bound analytes are then detected by the addition of enzyme-labeled second antibody which can specific on another epitope. This format can also be divided to indirect and direct sandwich according to the use and nonuse of enzyme-labeled anti-species antibody, respectively. Moreover, sandwich formats are useful in the quantitation of macromolecule such as protein and hormone.

Direct Sandwich ELISA

| The excess Ab is attached on solid-phase. | The analyte is added and then is bound specifically with Ab. | The enzyme-labeled Ab which is specific on another epitope is added. | The substrate is added to develop the color by enzymatic reaction. |

Indirect Sandwich ELISA

| The analyte is added and then is bound specifically with Ab. | The second Ab which is specific on another epitope is added. | The enzyme-labeled anti-species Ab is added to detect the second Ab. | The substrate is added to develop the color by enzymatic reaction. |

Figure 2.5 The diagram of direct and indirect sandwich ELISA format for macromolecule analyte

detection.

2.5.1.4 Competitive ELISA

Competitive format imply that two reactants (analytical antigen and enzyme-labeled antibody/antigen) are trying to bind to a third (antigen/antibody) by the simultaneous addition of the two competitors. This format is significant ELISA for the determination of small molecules with single antigenic determinant or hapten. This format can be divided following the type of enzyme-labeled: enzyme-labeled antibody and enzyme-labeled antigen format.

The antibody-labeled competitive format is defined as the detection of analyte in the sample by the competition between the enzyme-labeled antibodies and the analyte to bind the immobilized antigen on solid-phase. The analyte in the sample can be detected ether in direct and indirect format (Figure 2.6).

Antibody-labeled Direct Competitive ELISA

The enzyme-labeled Ab and analyte are added simultaneously to competition.

The enzymatic reaction is developed the color.

Antibody-labeled Indirect Competitive ELISA

The Ab and analyte are added simultaneously to competition.

The enzyme-labeled anti-species Ab is added to detect the Ab.

The enzymatic reaction is developed the color.

The excess Ag is attached on solid-phase.

Figure 2.6 The diagram of antibody-labeled direct and indirect competitive ELISA format for hapten detection.

The second one is the antigen-labeled direct competitive format. In the case, the enzyme-labeled antigen and the sample are added simultaneously. The amount of target analyte and the labeled antigen are competed to bind the immobilized antibody on solid-phase (Figure 2.7).

The excess Ab is attached on solid-phase.

The enzyme-labeled Ag and analyte are added simultaneously to competition.

The enzymatic reaction is developed the
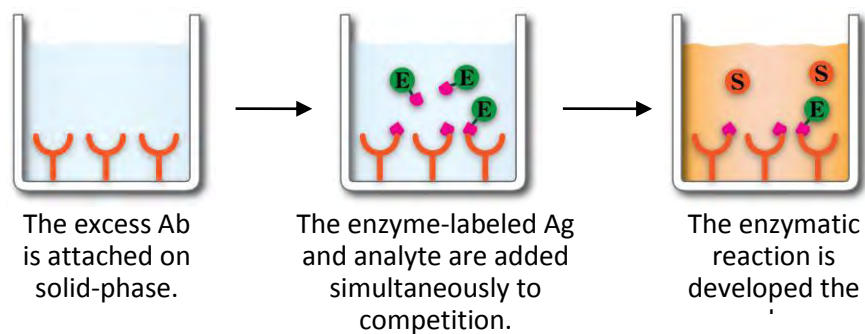
Figure 2.7 The diagram of antigen-labeled direct competitive ELISA format for hapten detection.

The color result of competitive format is decreased when the amount of analyted antigen in the sample are increased.

2.5.2 Biotin-Streptavidin System

The strong interaction between avidin and biotin was discovered as early as 1941. Avidin is a glycoprotein commonly purified from chicken egg white with molecular weight of 67 kDa, containing four binding sites while biotin, also know vitamin H or coenzyme R, is a vitamin found in all cells. Streptavidin, a bacterial homologous protein to avidin, isolated from the actinobacterium *Streptomyces avidinii*, is more frequently used than avidin and is commercially available also in a number of engineered forms. The structure of the biotin-streptavidin complex (Figure 2.8) shows a β-barrel structure of streptavidin binding biotin into its interior. The binding between avidin or streptavidin and biotin has long been regarded as the strongest, noncovalent, biological interaction known, having a dissociation constant, Kd, in the order of 4×10^{-14} M. The bond forms very rapidly and is stable in wide ranges of pH and temperature (Holmberg et al., 2005).
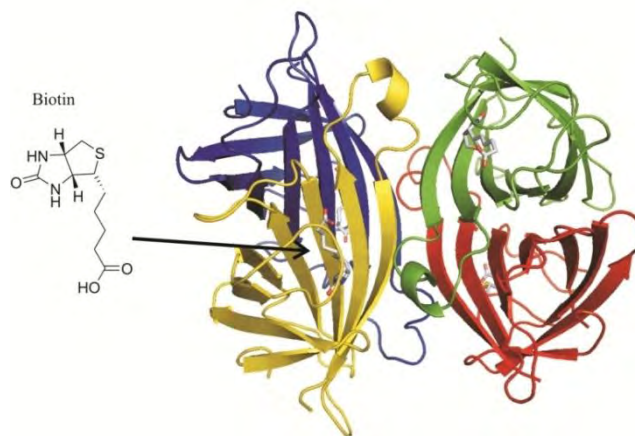
Figure 2.8 The tetrameric structure of streptavidin with 4 bound biotins.

(http://www.luschtinetz.com/html/images/stories/biotin-streptavidin.jpg)

Because of its high specificity and strong affinity, the biotin–streptavidin system (BSAS) has been widely used in immunohistochemistry and immunoassay. Being attractive for researchers, excellent sensitivity could be achieved through signal amplification introduced by BSAS. In such a case, numbers of active biotins and enzymes could be conjugated to per antibody

and per streptavidin, respectively, enabling more enzyme molecules catalyzing the substrate than the non-BSAS system. Obviously, signal amplification could be implemented (Lin et al., 2008).

2.5.3 Enzyme Labeling and Colorimetric Substrates

An attractive alternative to radioactivity is to introduce and enzymatic reaction by attaching an enzyme to the antibody or antigen. The most universal enzyme to be applied in immunoassay is horseradish peroxidase (HRP), for which there is a wide variety of suitable substrates, yielding soluble or in soluble products. HRP reacts with hydrogen peroxide to release an oxygen free radical, which in turn reacts with a colorimetric substrate. The substrates for producing soluble products suitable for ELISA are ABTS, OPD and TMB as shown in Table 2.3.

Table 2.3 The Substrate and its color yield of detection in ELISA (Howard and Kaser, 2007).

Substrate of HRP	Color	Absorbance (nm)
ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid))	Green	410 or 650
OPD (o-phenylenediamine)	Orange	492
TMB (3,3',5,5'-tetramethylbenzidine)		
Before stopping by acid	Blue	650
After stopping by acid	Yellow	450

ABTS or 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) is a water-soluble substrate that yields a green end product upon reaction. The green product has two major absorbance peaks, 410nm and 650nm. ABTS is less sensitive than other substrates. It is less readily oxidized, and its color development is slower (approximately 20 minutes).

Another HRP substrate is OPD (o-phenylenediamine) that has an orange oxidation product which can be measured at low concentration at 492 nm. This is probably the most sensitive activity indicator but is light sensitive and possibly mutagenic.

One of commonly used substrate is TMB (3,3',5,5'-tetramethylbenzidine). The product appears not to be as hazardous and can be as sensitive as OPD. The reaction give a

soluble blue detecting at 650 nm, while after stopping the enzymatic reaction with acid, it can be read yellow product at 450 nm.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals, Antibodies and Kits

• Acetic acid	Sigma-Aldrich, St. Louis, Missouri
• Acetonitrile	RCI Labscan, Bangkok, Thailand
• 40% acrylamide	Bio-Rad, Hercules, California
• Albumin, from bovie serum (BSA)	Sigma-Aldrich, St. Louis, Missouri
• Albumin, from chicken egg white (OVA)	Sigma-Aldrich, St. Louis, Missouri
• Ammonium persulfate	Merck, Darmstadt, Germany
• β-mercaptoethanol	Sigma-Aldrich, St. Louis, Missouri
• BCA Protein Assay Kit	Thermo Scientific, Rockford, Illinois
• Biotinamidocapate N-hydroxysuccinimide ester	Sigma-Aldrich, St. Louis, Missouri
• Brompphenol blue	Merck, Darmstadt, Germany
• Chlotetracyclin hydrocholine	Fluka, Luoyang, China
• Citric acid monohydrate	Sigma-Aldrich, Wien, Austria
• Coomassie brilliant blue G	Pierce, Rockford, Illinois
• N,N-Dimethylacetamide (DMA)	Fluka, Steinheim, Germany
• Dimethylformamide (DMF)	Merck, Darmstadt, Germany
• Dimethylsulfoxide (DMSO)	Merck, Darmstadt, Germany
• Di-sodium hydrogen phosphate (Na_2HPO_4)	Merck, Darmstadt, Germany
• Doxycycline	Sigma-Aldrich, St. Louis, Missouri
• Ethanol	Merck, Darmstadt, Germany
• Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Barcelona, Spain
• Fetal calf serum (FCA)	PAA lab, Pasching, Austria
• 40% formaldehyde	Carlo Erba, Milan, Italy
• Glucose	Sigma-Aldrich, St. Louis, Missouri
• L-Glutamine	Sigma-Aldrich, St. Louis, Missouri

- Glycerol   Merck, Darmstadt, Germany
- Glycine   Sigma-Aldrich, St. Louis, Missouri
- Goat anti mouse IgG (GAM)   Jackson Immuno, West Grove,
                                                         Pennsylvania
- Goat anti mouse IgG-Horseradish peroxidase   Jackson Immuno, West Grove,
  conjugate (GAM-HRP)   Pennsylvania
- Hybridoma serum free media   Invitrogen, Carlsbad, California
- Hydrochoric acid (HCl)   Merck, Darmstadt, Germany
- 30% Hydrogen peroxide (H_2O_2)   Merck, Darmstadt, Germany
- Methanol   Merck, Darmstadt, Germany
- Oxytetracycline dehydrate   Sigma-Aldrich, St. Louis, Missouri
- Peroxidase from horseradish   Sigma-Aldrich, St. Louis, Missouri
- Potassium citrate   Sigma-Aldrich, St. Louis, Missouri
- Rolitetracyclin   Sigma-Aldrich, St. Louis, Missouri
- RPMI 1640 medium   Biochrom, Berlin, Germany
- Skim milk   Anline, Bangkok, Thailand
- Sodium chloride (NaCl)   Merck, Darmstadt, Germany
- Sodium citrate   Sigma-Aldrich, St. Louis, Missouri
- Sodium dihydrogen phosphate (NaH_2PO_4)   Carlo Erba, Milan, Italy
- Sodium dodecyl sulfae (SDS)   Sigma-Aldrich, St. Louis, Missouri
- Sodium hydroxide (NaOH)   Sigma-Aldrich, St. Louis, Missouri
- Sodium pyruvate ($C_3H_3O_3Na$)   Sigma-Aldrich, St. Louis, Missouri
- Streptavidin hoseradish peroxidase conjugate   Invitrogen, Carlsbad, California
  (SV-HRP)
- Sucrose   Ajax Finechem, New south wales,
                                                         Australia
- Sulfuric acid (H_2SO_4)   Merck, Darmstadt, Germany
- Tetracycline hydrocholine   Sigma-Aldrich, St. Louis, Missouri
- 3,3',5,5'-Tetramethylbenzidine (TMB)   Sigma-Aldrich, St. Louis, Missouri
- N, N, N', N'-tetramethylethylenediamine (TEMED)   Pierce, Rockford, Illinois

- Thimerosal   Sigma-Aldrich, St. Louis, Missouri
- Tris(hydroxymethyl)aminomethane (Tris base)   Merck, Darmstadt, Germany
- Tween-20   Sigma-Aldrich, Gllinyham, UK

3.2 Equipments

- -20°C Freezer   Sanyo, Chachoeng Sao, Thailand
- -70°C Freezer   Sanyo, Osaka, Japan
- 37°C 5%CO_2 Incubator   Yamato, Tokyo, Japan
- 4°C Refrigerator   Toshiba, Nonthaburi, Thailand
- 96-well ELISA plate   Corning, New York, New York
- Autoclave (high pressure steam sterilizer)   Udono, Tokyo, Japan
- Autopipette tip, 10, 100, 300 and 1000   Axygen, Union City, California
- Autopipette, P2.5, P10, P20, P200, P1000 and P5000   Eppendorf, Hamburg, Germany
- Biological Stirrer, 2 Position   Techne, Staffordshire, UK
- Centrifuge tube, 15 and 50 ml   Axygen, Union City, California
- Centrifuge, model: universal 320,   Hettich, Tuttlingen, Germany
  swing out rotor 1619
- Cryotube, 2 ml   Nunc, Roskilde, Denmark
- Dialysis membrane, Cellu Sep,   Membrane Filtrotion Products, Seguin,
  MWCO: 12,000-14,000   Texas
- Disposable syringe, 5 ml and 10 ml   Nipro, Autthaya, Thailand
- Examination gloves   Magaglove, Chon Buri, Thailand
- Filter paper #1   Whatman, Kent, UK
- High speed refrigerator centrifuge,   Kubota, Tokyo, Japan
  model: 6500, rotor AG-508CA
- Hot air oven   Memmert, Schwabach, Germany
- Hot plate stirrer, model: C-MAGHS7   IKA Work, Wilmington, North Carolina
- Industrial N_2 gas   Linde, Samut Prakan, Thailand
- Inverted microscope, model TMS   Nikon, Tokyo, Japan
- Laminar flow   Lab Survice Ltd., Bangkok, Thailand

- Liquid Nitrogen Tank   Harsco Corp., Camp Hill,
                                                               Pennsylvania

- Microcentrifuge tube, 1.5 ml   Axygen, Union City, California

- Microplate reader, model: MCC/340   Titertek multiskan, Helsinki, Finland

- Multichannel autopipette   HTL, Warsaw, Poland

- Multi-detection microplate reader, Synergy HT   BIO-TEK, Richmond, Virginia

- Orbital shaker   Fisher Scientific,
                                                               Illkirch-Graffenstadam, France

- Petri dish, 90 mm   Sterilin Ltd., Newport, UK

- pH Meter, model: AB15   Fisher Scientific, UE Tech Park,
                                                               Singapore

- Pipettes, 10 ml   HBG, Luetzelinden, Germany

- Precision weighting balance,   Mettler Toledo, Greifensee,
  model: AG204 and PG402S   Switzerland

- Protein G sepharose 4 fast flow   GE Healthcare, Cardiff, UK

- Protein III System for SDS-PAGE   Bio-Rad, Hercules, California

- Scanner, model ColorPage-HR6X Slim   Genius, Beijing, China

- Spinner Flask, 500 ml   Techne, Staffordshire, UK

- Syringe filter, Nylon membrane, 0.45 µm, 13 mm   Whatman, Kent, UK

- Vacuum pump   Iwaki pump, Fukushima, Japan

- Vortex mixer   Scientific Industries, Boulder,
                                                              Colorado

- Water bath   Memmert, Schwabach, Germany

3.3 Experimental Procedures

3.3.1 Preparation of OTC-OVA Conjugates

3.3.1.1 Conjugation of OTC-OVA

OTC was conjugated to OVA by a modified method of Mannich reaction from Faraj and Ali (1981). Twenty mg of OVA were dissolved in 1.0 ml of DDI water and mixed with

60 µl of 100 mg/ml OTC in DMF. Three molar of sodium acetate buffer, pH 5.5 (0.4 ml) and 7.5% (v/v) formaldehyde (0.4 ml) were added into the OVA solution and stirred 2 h at room temperature. The conjugation of OTC-OVA is shown in Figure 3.. The mixture was dialyzed against PBS for 5 times. The protein concentration was determined by BCA protein assay. Finally, the OTC-OVA solution was filtrate with 0.2 µm cellulose acetate membrane and kept at -20ºC until use. The UV‑VIS spectrums of OVA, OTC and OTC-OVA were scanned at wavelength between 200 and 500 nm to assess the level of the conjugation by calculating the molar ratio of OTC and OVA from the absorbance coefficient (ε). In addition, the molar ratio OVA and OTC-OVA was also analyzed by MALDI-TOF-MS.
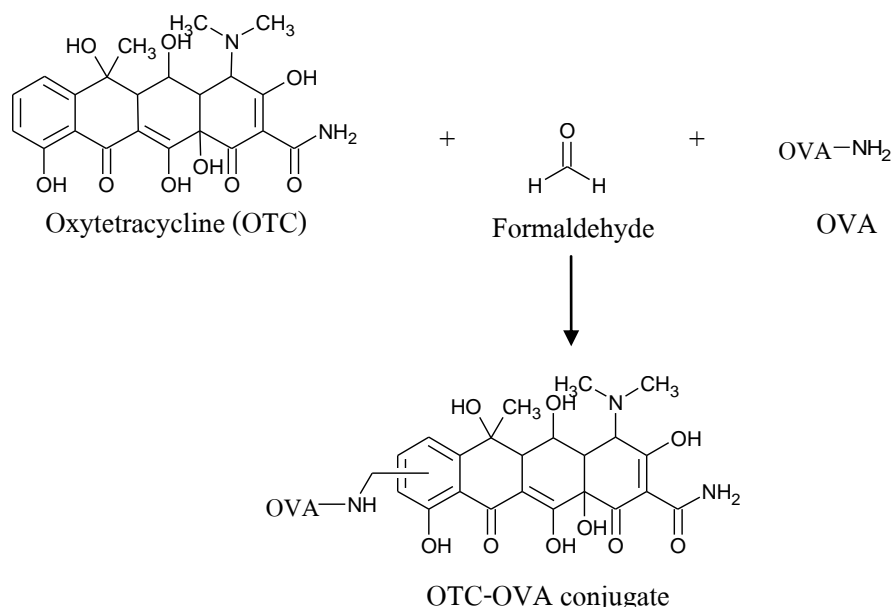
Figure 3.1 Conjugation of OTC to OVA using Mannich reaction (modified from Faraj and Ali, 1981)

3.3.1.2 Determination of Protein by BCA Protein Assay

The protein concentration of the OTC-OVA was quantified by BCA Protein Assay Kit. The assay procedure was employed according to the manufacturer's instructions. Briefly, standard BSA (0, 0.1 0.2, 0.4, 0.6, 0.8,1.0 and 1.2 mg/ml) and the OTC-OVA conjugate were diluted and then added 25 µl into the 96-well plate. Then 200 µl of working solution, prepared from the mixture of reagent A and B in the ratio of 50:1, was added into each well and incubated at 37ºC for 30 min. After that, the absorbance at the wavelength of 563 nm was measured with microplate reader.

3.3.2 Production of Monoclonal Antibody

3.3.2.1 Recovery Hybridoma Cells from Stock

The cryotube of hybridoma cell no. 2-4F producing MAb against OTC (Wirongrong Natakuathung, 2011) was taken from storage tank filled with liquid nitrogen and immediately immersed in water bath at 37ºC until liquidified. Cells were transferred carefully with pasture pipette into RPMI 1640 medium and centrifuged at 380 ×g for 5 min. The cell pellet was resuspended in RPMI 1640 medium supplemented with 20% FCS and cultured in the humidified 5% CO_2 incubator at 37ºC. Growing cells were subcultured every 2-3 days. (Nanthika Panchan, 2004)

3.3.2.2 Determination of MAb Production by Indirect ELISA

An Indirect ELISA (iELISA) was used to determine the antibody in the solution. The 96-well plates were coated with 100 µl of 2 µg/ml OTC-OVA and incubated at 4ºC overnight. The plates were washed 3 times with the washing buffer and then blocked with the blocking solution by incubation at 37ºC for 1 hr. After three washes, the culture media was added into the plate and incubated at 37ºC for 2 hr. Subsequently, the plates were washed again for three times and then incubated with 100 µl of goat anti-mouse IgG-HRP (GAM-HRP; 1/10,000 in PBS) at 37ºC for 1 hr. After another washing, 100 ml of the TMB substrate solution was added. The reaction was allowed to occur in the dark for 15 min at room temperature. The reaction was then stopped with 100 µl of the stop solution. Absorbance at 450 nm was measured using a microplate reader.
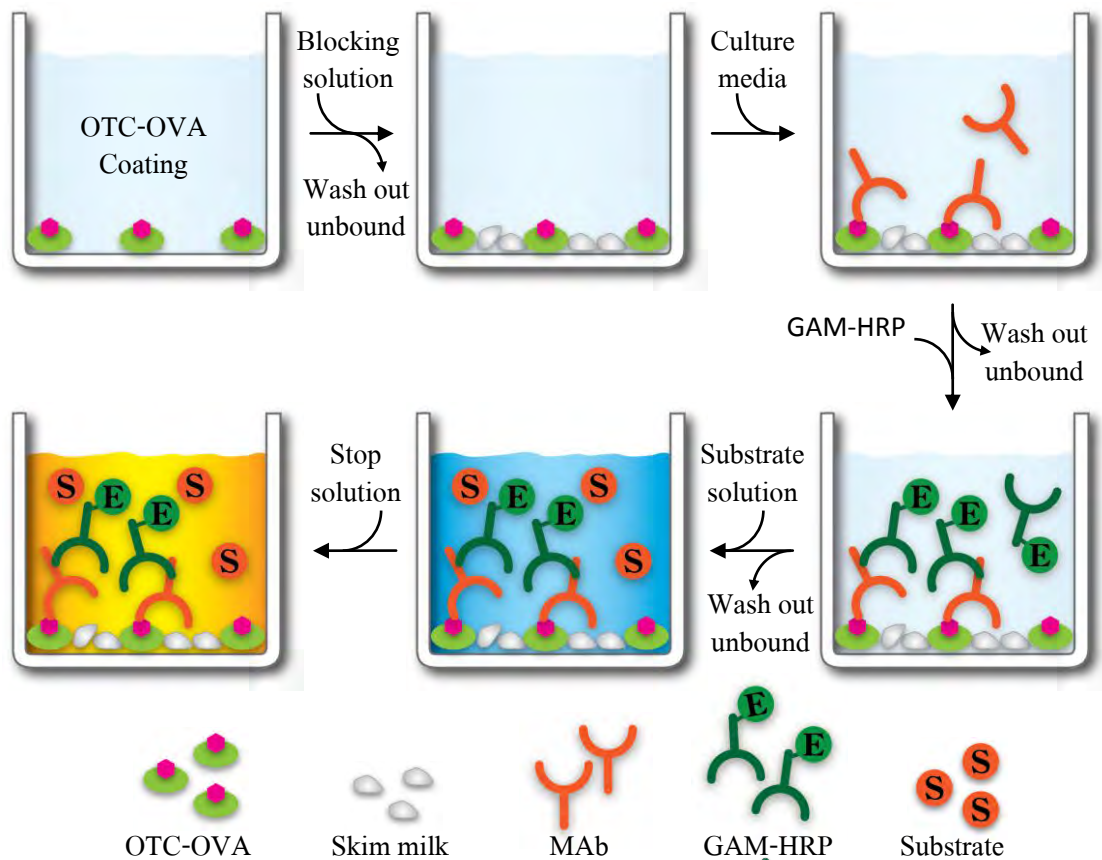
Figure 3.2 iELISA procedure

3.3.2.3 Production of Monoclonal Antibody

The growing hybridoma cells were cultured and expanded in 500 ml of hybridoma serum free medium using spinner flask and incubated in 5% CO_2 incubator at 37ºC until the color of medium was changed to yellow (approximately 5-7 days). The culture medium was collected by centrifugation at 380 ×g for 5 min. The culture supernatant was filtered through filter paper no.1 to remove cell debris and then the MAb in the culture supernatant was purified by affinity chromatography in the next step (Nanthika Panchan, 2004).

3.3.2.4 Purification of Monoclonal Antibody

The MAb in supernatant was purified by affinity chromatography using Protein G Sepharose 4 fast Flow. The protein G column was equilibrated with 2 mM phosphate buffer, pH 7.0, at a flow rate of 1.0 ml/min. The supernatant (500 ml) was loaded into the column, and then unbound protein was washed out using 30 ml equilibrated buffer. The bound MAb was

eluted with 0.1 M glycine-HCl buffer, pH 2.7, and collected fractionally for 30 factions (1 ml each) in 70 µl of 1 M Tris-HCl buffer, pH 9.0, to neutralize the pH. The protein concentration of each fraction was quantified by measuring the absorbance at 280 nm. MAb in each fraction was also confirmed by iELISA. Subsequently, the fractions with high protein concentrations were pooled and dialyzed against 0.01 M phosphate buffer saline, pH 7.4 (PBS) for five times. The MAb solution was then filtered through a 0.2 µm filter membrane and kept at -20ºC until use.

3.3.2.5 Determination of Protein by BCA Protein Assay

The protein concentration of the purified MAb was quantified by BCA Protein Assay Kit. The assay procedure was employed according to 3.3.1.2.

3.3.2.6 Determination of Molecular Weight of Antibody by SDS-PAGE

Molecular weight of the purified MAb was checked with slightly modification by SDS-PAGE using Laemmli's method (1970). The SDS-PAGE was set from a 1.5 mm thick slab gels with 5% stacking gel, 12% separating gel and 15-laned comb for applying samples. All reagents were prepared as described in the Appendix C. Total proteins of MAb (2, 3 and 5 µg) were denatured by mixing with equal volume of SDS staining dye, boiled for 5 min and then loaded into each well of the gels. Protein markers with a molecular weight ranging from 19 to 118 kDa were used as standard. Samples were electrophoresed at a constant voltage of 100 V for 90 min in running buffer. After electrophoresis, the separating bands of proteins in the gel were visualized by immersion in the staining solution for 30 min. The gel was then washed in destaining solution with 3-4 changes until the gel was cleared.

3.3.3 Development of ELISA

The ELISA formats that were evaluated for OTC detection ELISA could be divided into 3 formats:

- Antigen labeled direct competitive ELISA (dcELISA)
- Antibody labeled indirect competitive ELISA using GAM-HRP (icELISA-GAM)
- Antibody labeled indirect competitive ELISA using streptavidin-HRP (icELISA-SV)

3.3.3.1 Development of Antigen Labeled Direct Competitive ELISA (dcELISA)

3.3.3.1.1 Preparation of OTC-HRP Conjugate for Enzyme Tracer

OTC was conjugated to HRP with the same method as OTC-OVA (0). A solution mixture of HRP (5 mg in 1.0 ml DDI water) and OTC (60 µl of 100 mg/ml in DMF) was prepared. Then 3 M sodium acetate buffer, pH 5.5 (0.4 ml) and 7.5% (v/v) formaldehyde (0.4 ml) were added and stirred at room temperature for 2 hr. The mixture was dialyzed against PBS for five times. The protein concentration was determined by BCA protein assay kit (0). After that, the OTC-HRP solution was filtered through 0.2 µm cellulose acetate membrane and kept at -20ºC until use.

3.3.3.1.2 Optimization of Concentration Ratio of MAb to OTC-HRP

The ratio of coating MAb concentration to OTC-HRP concentration was optimized by checkerboard method. The concentrations of MAb were varied from 1.3 to 4.0 µg/ml by 1.25-fold dilution, and the concentrations of OTC-HRP were varied from 1.25 to 12.0 µg/ml by 1.5-fold dilution. The optimum ratio of MAb to OTC-HRP was defined as the ratio, which give the absorbance values ranging 0.8-1.2 unit (Pastor-Navarro et al., 2007). Four optimum ratios of MAb to OTC-HRP was selected and used in the dcELISA.

3.3.3.1.3 dcELISA Procedure

Each well was coated with 100 µl of the MAb at the optimal concentration and incubated at 4ºC overnight. Following coating, the plates were washed three times with washing buffer, blocked with blocking solution and incubated at 37ºC for 1 hr. After for three times washing, the plates were simultaneously added with 100 µl of the optimal concentration of OTC-HRP and 100 µl of the standard OTC (2-fold dilution from 1000 to 0.24 ng/ml) and incubated at 37ºC for 2 hr. After another washing, 100 µl of the TMB substrate solution was added. The reaction was allowed to occur in the dark for 15 min at room temperature. The reaction was then stopped with 100 µl of stop solution. The absorbance was measured at 450 nm using a microplate reader.
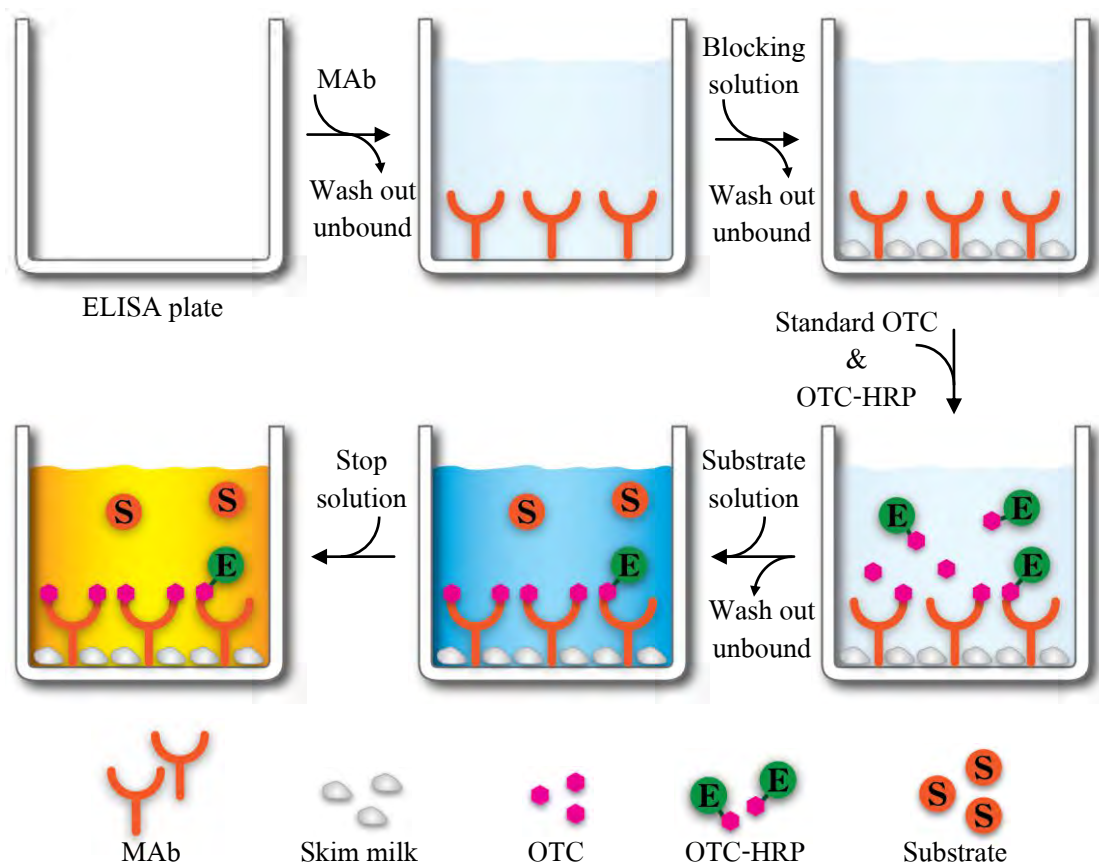
Figure 3.3 dcELISA procedure

3.3.3.1.4 Analysis of the Data

The data of the absorbance and the concentration of OTC were analyzed to generate the competitive calibration curve by program GraphPad Prism ver.5.0 software, which the log of OTC concentration was the X axis, and %B/B_0 value was the Y axis as show in Figure 3.4. The sensitivity (IC_{50} and LOD) was calculated and described below in 3.3.4.1. The obtained sensitivity of four optimum ratios of antibody and antigen were compared to select the best sensitivity of dcELISA format.
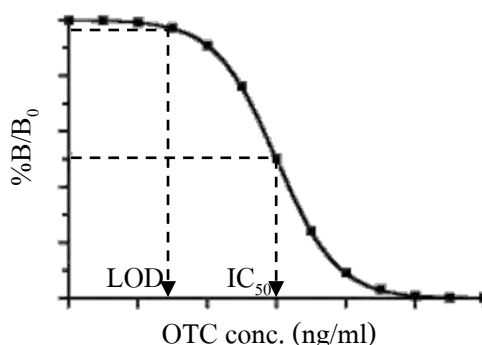
Figure 3.4 Example of competitive calibration curve

3.3.3.2 Development of Antibody Labeled Indirect Competitive ELISA Using GAM-HRP (icELISA-GAM)

3.3.3.2 .1 Optimization of Suitable Ratio of OTC-OVA to MAb

The concentration ratio of coating OTC-OVA to primary MAb was optimized by checkerboard method. The OTC-OVA at various concentrations from 0.25 to 8.0 µg/ml were coated, and the concentrations of MAb were varied from 0.003 to 0.4 µg/ml by 2-fold dilution. Four optimum ratios of the OTC-OVA to the primary MAb, which give the approximate absorbance values ranging 0.8-1.2 unit, were selected for icELISA-GAM.

3.3.3.2 .2 icELISA-GAM Procedure

Each well of 96-well plates was coated with 100 µl of the optimal concentration of OTC-OVA and incubated at 4ºC overnight. The plates were washed three times with the washing buffer, blocked with the blocking solution and incubated at 37ºC for 1 hr. After three times washing, the plates were simultaneously added with 100 µl of the optimal concentration of MAb and 100 µl of OTC standard (2-fold dilution from 0.24 to 1000 ng/ml) and incubated at 37ºC for 2 hr. Subsequently, the plates were washed again for three times and incubated with 100 µl of the GAM-HRP (1/10,000 in PBS) at 37ºC for 1 hr. After another washing, 100 ml of the TMB substrate solution was added. The reaction was allowed to occur in the dark for 15 min at room temperature. The reaction was then stopped with 100 µl of the stop solution. After measuring the absorbance at 450 nm, the data of the absorbance and the concentration of OTC were analyzed the sensitivity as same as in 0.

Figure 3.5 icELISA-GAM procedure

3.3.3.3 Development of Antibody Labeled Indirect Competitive ELISA Using Streptavidin-HRP (icELISA-SV)

3.3.3.3.1 Conjugation of MAb to Biotin (MAb-biotin)

Biotin was conjugated to the MAb according to the product information from Sigma-Aldrich. The MAb was dialyzed in 0.01 M phosphate buffer, pH 7.4 for three times. Biotin aminohexanoic acid N-hydrozysuccinimide ester (20 mg/ml in DMF) was added into the MAb solution at a ratio of 20 µl of biotin solution to 1 mg of the MAb (approximately 15-20 mole biotin per mole protein) and stirred at room temperature for 1 hr. The MAb-biotin conjugate was dialyzed against PBS for five times, and then determined the concentration by BCA protein assay. After that, the MAb-biot in solution was filtered through 0.2 µm cellulose acetate membrane and kept at -20ºC until use.

3.3.3.3.2 Optimization of Suitable Ratio of OTC-OVA to MAb-biotin

The concentrations ratio of the coating OTC-OVA to the MAb-biotin were optimized by checkerboard method. The OTC-OVA at concentration between 0.12 and 4.0 µg/ml by 2-fold dilution was coated into each well, and the MAb-biotin concentration was varied from 0.012 to 1.6 µg/ml by 2-fold dilution. The optimum ratio of the OTC-OVA to the MAb-biotin were defined from icELISA-GAM procedure, which give the approximate absorbance values ranging 0.8-1.2 unit where the OTC was absented.

3.3.3.3.3 icELISA-SV Procedure

Each well of 96-well plates was coated with 100 µl of the optimum concentration of OTC-OVA and incubated at 4ºC overnight. The plates were washed three times with the washing buffer, blocked with the blocking solution and incubated at 37ºC for 1 hr. After three washes, the plates were simultaneously added with 100 µl of the optimum concentration of MAb-biotin and 100 µl of OTC standard (2-fold dilution from 0.24 to 1000 ng/ml) and incubated at 37ºC for 2 hr. Subsequently, the plates were washed again and incubated with 100 µl of streptavidin-HRP (1/5,000 in PBS) at 37ºC for 30 min. After another washing, 100 ml of the TMB substrate solution was added. The reaction was allowed to occur in the dark for 15 min at room temperature. The reaction was then stopped with 100 µl of stop solution. After measuring the absorbance at 450 nm, the data of the absorbance and the concentration of OTC were analyzed the sensitivity as same as in 0.
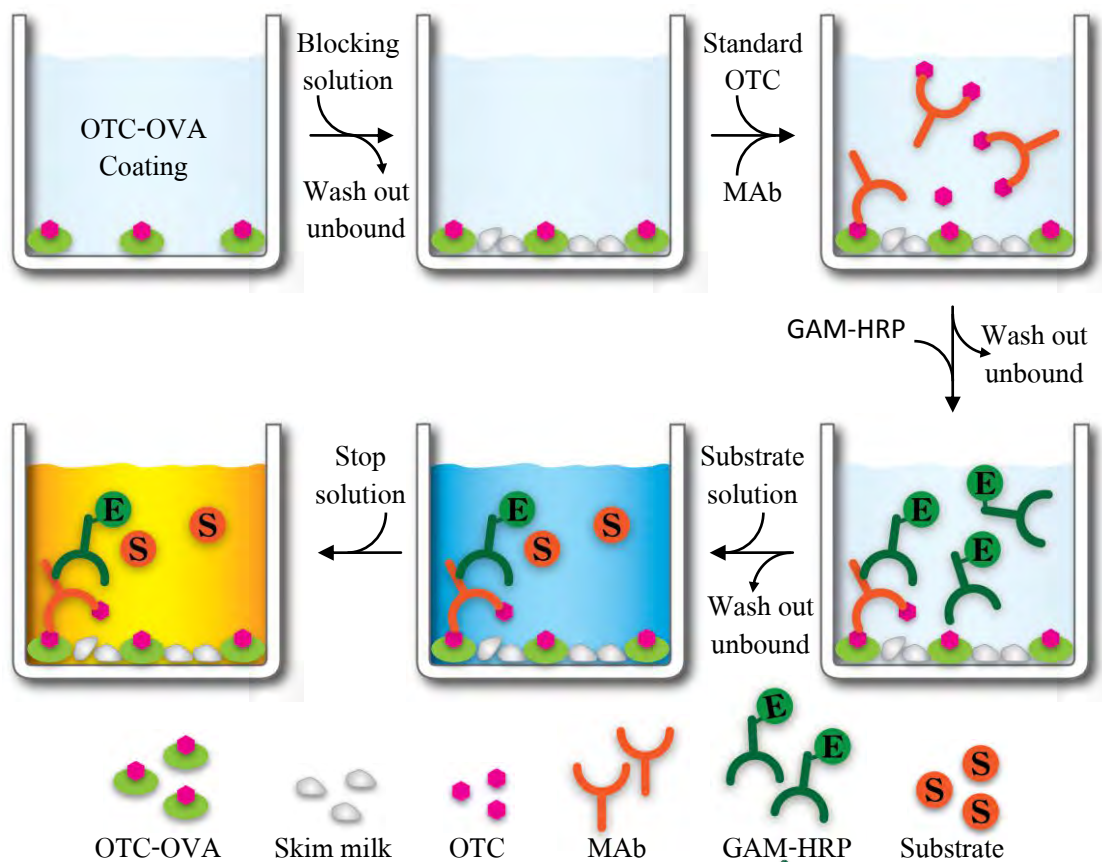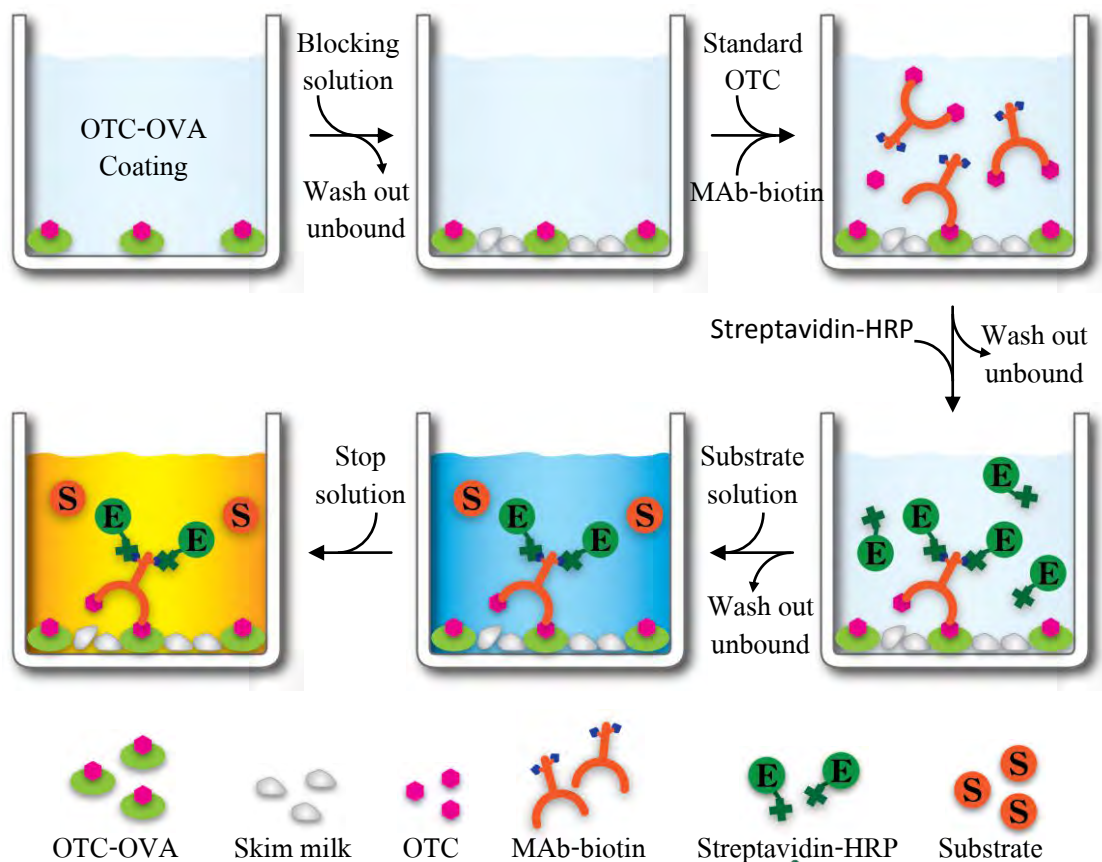
Figure 3.6 icELISA-SV procedure

3.3.3.4 Effect of Incubation Temperature and Competition Time

The selected ELISA format with the highest sensitivity was studied to find an optimized incubation temperature that practically suitable for the prototype ELISA format. The ELISA was performed with the incubation temperature of room temperature and 37ºC.

Using the selected incubation temperature, the practical competition time was also studied. The incubation time was varied from 30 to 120 min at 30 min interval and the sensitivity from all conditions more compared.

The ELISA condition that provides the highest sensitivity at optimal incubation temperature and optimal competition time was selected the prototype ELISA format.

3.3.4 Evaluation of Prototype ELISA

3.3.4.1 Sensitivity of ELISA

The inhibition curve or the competitive calibration curve was generated from the relation between the OTC concentration and the percent of inhibition ($\%B/B_0$), where B and B_0 were the average of the absorbances obtained from ELISA with and without different concentrations of OTC, respectively. The sensitivities of all ELISA formats were reported in terms of IC_{50} and limits of detection (LOD). The IC_{50} was defined from the OTC concentration that gave 50% of B/B_0. While the LOD was calculated from the OTC concentration at the mean of multiple blanks (n>20) determination signal (B_0) minus by three times its standard deviation (SD) (Cliquet et al, 2003 and Le et al, 2003).

IC_{50} (ng/ml)   : 50% B/B_0

LOD (ng/ml)   : B_0-3SD

3.3.4.2 Specificity of Prototype ELISA

The specificity of the prototype ELISA was evaluated in terms of cross-reactivity with other antibiotics. The IC_{50} of the compounds of interest were obtained in the same manner as previously described. The percentage of cross-reactivity was then calculated using the following formula:

$$\% \text{ cross-reactivity} = \frac{IC_{50} \text{ of OTC}}{IC_{50} \text{ of competitors}} \times 100$$

The competitors in this study were the antibiotic in the group of tetracyclines, including tetracycline, doxycycline, chlortetracycline and rolitetracycline, other antibiotics of interest such as chloramphenicol, norfloxacin and AMOZ and another compound as clenbuterol.

3.3.4.3 Analysis of Precision and Accuracy

The intra and inter variation of the assay were calculated from the OTC spiked samples at various concentration (0, 25, 50, 100, 200 and 400 ng/g). The accuracy was evaluated by the detection of OTC spiked samples at various concentrations (0, 25, 50, 100, 200 and 400 ng/g) by prototype ELISA. The %recovery was calculated using the following equation below.

$$\text{Percentage of recovery (\%recovery)} = \frac{\text{concentration of measured OTC}}{\text{concentration of fortified OTC}} \times 100$$

The acceptable accuracy was the %recovery of spike samples in range of 80 to 120% (Abbott et al., 2010).

The intra-assay variation was assessed as the average of OTC detection from the six replicates of a sample extraction and detection while the inter-assay variation was assessed as the average of OTC detection from the six replicates of different sample extractions and detections. The obtained percentage coefficient of variation (%CV) by replicate analyses (n=6) was calculated using the following formula:

$$\text{Percentage of coefficient of variation (\%CV)} = \frac{SD}{\mu} \times 100$$

Where SD was the standard deviation of the OTC detection and μ was the mean concentration of OTC in replicate analyses. If the %CV values are all below 20%, the level of precision is acceptable (Zhang et al., 2007).

3.3.5 Detection of OTC in Fortified Shrimp Samples Using Prototype ELISA

The sample extraction procedure was modified from the method of Zhao (2008). One-gram of homogenized shrimp tissue samples were fortified with OTC at the final concentrations of 25, 50, 100, 200 and 400 ng/g. Each sample was extracted with a mixture of 0.1 M EDTA (100 µl), methanol (1 ml) and acetonitrile (1 ml). Each homogenate was vortexed for 1 min and then centrifuged at 2,700 ×g for 10 min. After the supernatant was collected, the same extraction procedure was repeated twice, and the supernatants were combined. The combined supernatants were then evaporated under a nitrogen stream at 45ºC in water bath. The residue was resuspended in 10 ml PBS for ELISA analysis.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Conjugation of OTC-OVA

Oxytetracycline (OTC) has a molecular weight of 460.4 Da which is considered to be a small molecule and cannot or weakly binds to the surface of ELISA plate. Therefore, it must be conjugated to a carrier protein for this purpose. In this study, OTC was conjugated to ovalbumin (OVA) using the Mannich reaction. In this reaction, formaldehyde was used as a bridge between the amino groups of OVA and the phenolic moiety of the OTC molecule (Faraj and Ali, 1981). Protein concentration of the conjugated OTC was determined by BCA assay. The concentration of OTC-OVA based on protein concentration was 8.1 mg/ml as shown in Table 4.1. The level of conjugation was also assessed by calculating the molar ratio of OTC to OVA from UV‑VIS spectrum (Le et al., 2009) and MALDI‑TOF‑MS (Tanaka et al., 2007 and Kido et al., 2008).

Table 4.1 Protein concentration of OTC-OVA by BCA assay.

Dilution of OTC-OVA	Absorbance at 562 nm	Concentration of OTC-OVA (mg/ml)
1/8	1.13	6.9
1/16	0.73	8.2
1/32	0.47	9.2
Average		**8.1 ± 1.2**

Remark: The standard curve was shown in Appendix A.

OVA, OTC and OTC-OVA were prepared at 5.0 mg/ml (0.11 µM), 0.125 mg/ml (0.27 µM) and 2.5 mg/ml (0.056 µM), respectively. Their UV‑VIS spectra at the wavelength between 200 and 500 nm were scanned and shown in Figure 4.1. OVA and OTC showed the maximum wavelength (λ_{max}) at 280 and 337 nm, respectively, while OTC-OVA showed the λ_{max} at 230 nm. The shift in the λ_{max} indicated that OTC was conjugated to OVA (Le et al., 2009). Using the

absorbance intensity and the absorbance coefficient (ε), the molar ratio of OTC to OVA was calculated to be 1.95:1 (Appendix A).
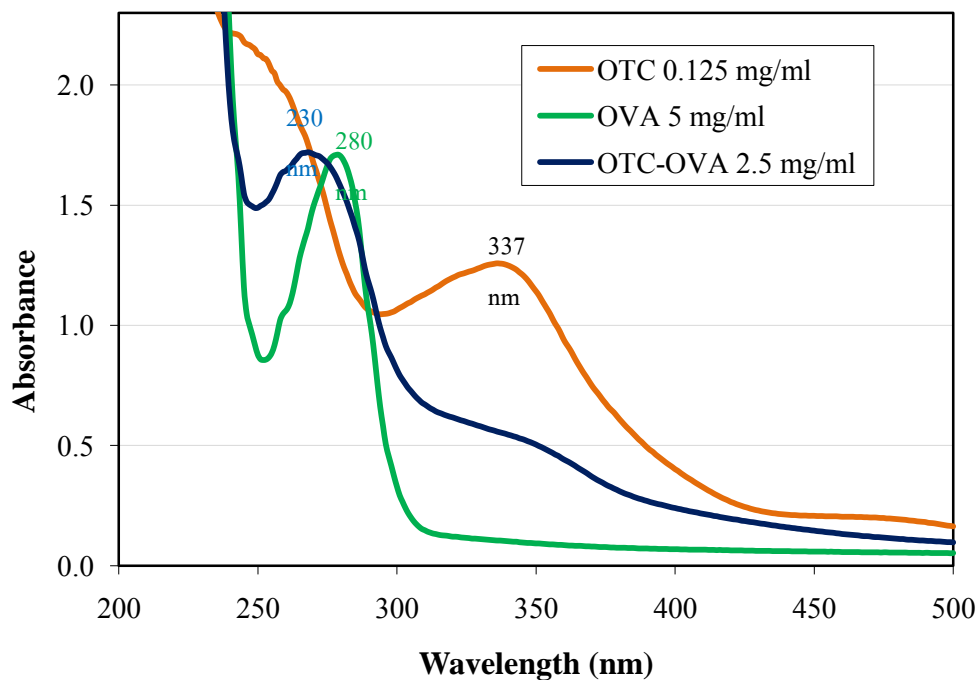
Figure 4.1 UV-Visible spectra of 0.125 mg/ml OTC (——), 5 mg/ml OVA (——) and 2.5 mg/ml OTC-OVA (——).

The conjugation of OTC to OVA was also confirmed by comparing the molecular mass of OVA before and after the conjugation reaction using MALDI-TOF-MS. The result showed the mass of OVA at 44,547 Dalton and that of OTC-OVA at 45,389 Dalton (Figure 4.2). This result indicated that the mass of the conjugate was higher than that of the OVA by 842 Daltons. Using the molecular weight of OTC at 460.4 Dalton, the molecular ratio of OTC to OVA in the conjugate was calculated to be 1.83:1. Based on the UV-VIS spectra and MALDI-TOF-MS analysis, it could be concluded that OTC was conjugated to the OVA molecule.
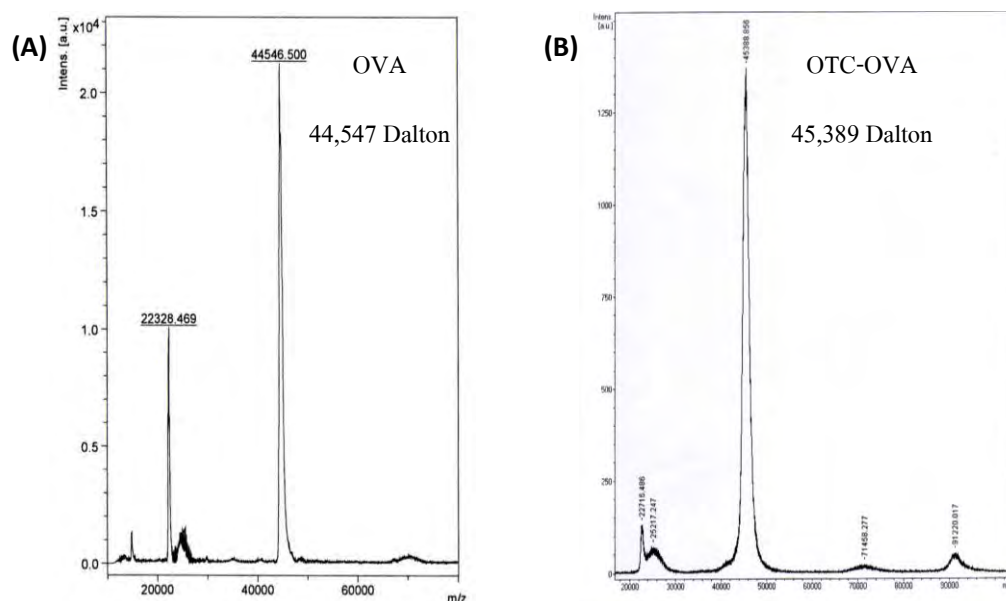
Figure 4.2 MALDI-TOF-MS analysis spectra of OVA (A) and OTC-OVA (B)

4.2 Production and Purification of MAb

After thawing from liquid nitrogen, the hybridoma clone no. 2-4F was cultured for 7 days. The hybridoma culture media was harvested and diluted to check for the monoclonal antibody (MAb) by iELISA. The result showed that the absorbance at 450 nm in the iELISA with respect to the decreased dilution of the MAb in the culture media (Table 4.2). This result indicated that the hybridoma clone 2-4F could still produce the MAb against OTC after storage in liquid nitrogen for 2 years. After the expansion, the MAb in the culture media was collected by centrifugation for further study.

Table 4.2 iELISA for MAb clone 2-4F

Source	Dilution	Absorbance at 450 nm
	1/100	1.367
	1/200	1.283
	1/400	1.060
Hybridoma culture media	1/800	0.821
	1/1600	0.512
	1/3200	0.310
	1/6400	0.189

RPMI 1640 media with 20% FCS	1/1	0.076

The isotype of MAb clone 2-4F was reported to be IgG1 (Wirongrong Natakuathung, 2011). Therefore, the MAb was purified by Protein G sepharose affinity chromatograpy. After the culture media was loaded into the column, the non-binding molecules were washed out by equilibrated buffer. The MAb was then eluted from the column by using the elution buffer at pH 2.7 and collected for 30 fractions (1 ml/fraction). The protein content of each fraction was determined by measuring the absorbance at 280 nm while the presence of MAb was determined by iELISA (Figure 4.3). The absorbance from both methods indicated that the MAb could be found in fraction no.5 to no.13. Therefore, these fractions were pooled together and dialyzed.

Figure 4.3 Chromatogram of MAb eluted from protein G sepharose affinity column: (——◆——) absorbance at 280 nm and (——●——) absorbance at 450 nm of iELISA

4.3 Determination of concentration and molecular weight of MAb

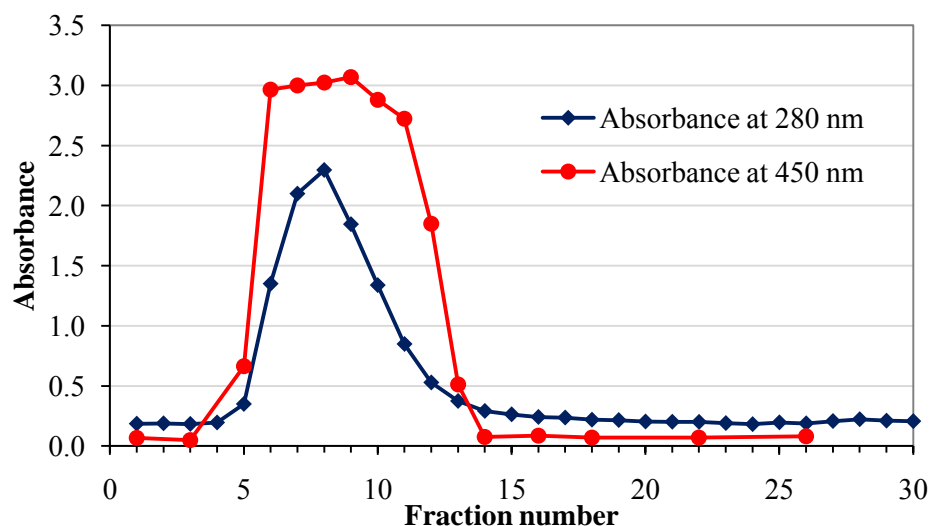Because only antibody can specifically bind to the Protein G column, the total protein content in the solution was assumed to be the MAb. Therefore, the MAb concentration was

quantified by the BCA assay. The protein concentration in the culture media was 2.3 mg/ml, while the concentration of the purified MAb was 1.6 mg/ml (Table 4.3).

Table 4.3 Protein concentrations in hybridoma culture media and purified MAb by BCA assay

Source of protein	Total Volume (ml)	Dilution of MAb	Absorbance at 562 nm	Concentration of Protein (mg/ml)
Hybridoma culture media	500	1/5	0.690	2.3
		1/10	0.410	2.3
		1/20	0.291	3.1
		Average		**2.5 ± 0.4**
Purified MAb	9	1/2	1.019	1.5
		1/4	0.617	1.7
		1/8	0.355	1.6
		Average		**1.6 ± 0.1**

Remark: The standard curve was shown in Appendix A.

After purification, the molecular weight and the purity of MAb were checked by SDS-PAGE as shown in Figure 4.4. The result showed that after the purification step, a large amount of proteins in the culture media (lane 5) was eliminated as compared to the purified antibody in lane 2, 3 and 4. However, four protein bands were found at 26, 29, 59 and 65 kDa. As compared to other report (Howard and Kaser, 2007), the two dark protein bands at 26 kDa and 59 kDa were expected to be the light and the heavy chain of the MAb. While the two faint extra bands may represent the post-translational products of heavy and light chains or the contaminated proteins existed in the medium. These results indicated that the MAb was partially purified; however, it could still be used for further study. Many reports have shown that the partially purified MAb still be used in the ELISA with high sensitivity (Umarphorn Chadseesuwan, 2011; Wang et al., 2007 and Kong et al., 2010).
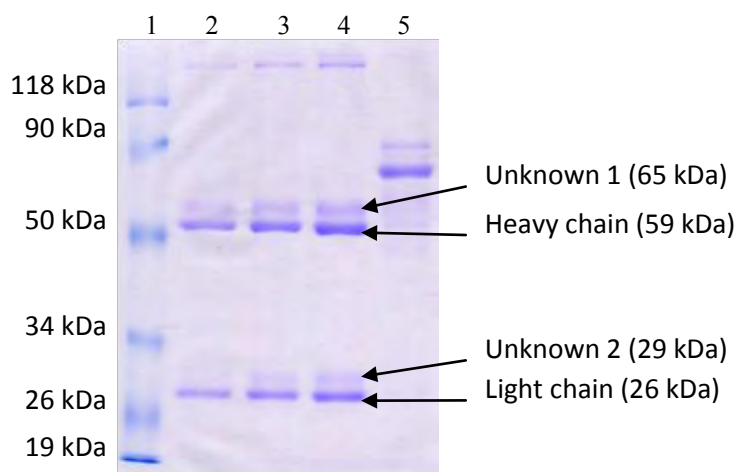
Figure 4.4 SDS-PAGE of purified antibody, lane 1: standard protein marker; lane 2, 3, 4: purified antibody at 2, 3, 5 µg, respectively and lane 5: serum free culture media at 5 µg.

4.4 Development of ELISA

4.4.1 Antigen Labeled Direct Competitive ELISA (dcELISA)

4.4.1.1 Conjugation of OTC-HRP

In order to prepare the OTC-HRP for enzyme tracer, OTC was coupled with enzyme HRP by Mannich reaction. After conjugation, the concentration of OTC-HRP was determined by BCA assay. The result showed in Table 4.4 that the concentration of OTC-HRP was 0.87 mg/ml. The OTC-HRP was used in the next study.

Table 4.4 Protein concentration of OTC-HRP by BCA assay

Dilution of OTC-HRP	Absorbance at 562 nm	Concentration of OTC-HRP (mg/ml)
1/2	0.658	0.91
1/4	0.384	0.86
1/8	0.261	0.85
Average		**0.87 ± 0.03**

Remark: The standard curve was shown in Appendix A.

4.4.1.2 Optimization of MAb and OTC-HRP Concentration

The appropriate concentration of the coating MAb and the OTC-HRP were determined by the checkerboard method. Four suitable ratios of the MAb concentration to the OTC-HRP concentration were selected for further study to develop an dcELISA. The least concentrations of the antibody-antigen pairs that gave the absorbance value closed to 1 (range of 0.8-1.2) were selected. According to the result shown in Table 4.5, the MAb concentrations at 1.3, 1.6, 2.0 and 2.5 µg/ml pairing with the OTC-HRP concentrations at 8.0, 2.0, 1.0 and 0.5 µg/ml, respectively, were selected.

Table 4.5 Optimization of the coating MAb and the OTC-HRP using dcELISA format

OTC-HRP	Coating MAb (µg/ml)					
(µg/ml)	1.3	1.6	2.0	2.5	3.2	4.0
0.13	0.227	0.182	0.210	0.247	0.297	0.342
0.25	0.378	0.288	0.363	0.438	0.519	0.611
0.5	0.567	0.498	0.646	**0.820**	0.939	1.111
1.0	0.766	0.783	**1.031**	1.320	1.548	1.792
2.0	0.895	**1.113**	1.527	1.942	2.416	2.550
4.0	0.925	1.290	1.865	2.377	2.927	3.229
8.0	**1.015**	1.295	1.691	2.223	2.696	3.211
12	1.178	1.423	1.844	2.399	2.933	3.125

Remark: the absorbance values with bold letters indicated the selected pairs of the MAb and the

OTC-HRP concentration.

4.4.1.3 Analysis of Sensitivity in dcELISA Format

In this format, the OTC-HRP conjugate competed with OTC in the sample for binding with the coating antibody. The enzymatic assay was used to quantify the bound OTC-HRP which was proportional to the amount of OTC in the sample. Thus, the higher the amount of OTC in sample, the lower the colorimetric intensity. In order to investigate the sensitivity, the selected concentrations of MAb and OTC-HRP were used in dcELISA with OTC standard as the

competitor. The competitive calibration curve obtained were compared and shown in Figure 4.5, the IC_{50} and LOD values obtained from the curve were averaged and summarized in Table 4.6. The result showed that using 1.6 µg/ml of MAb as coating agent and 2.0 µg/ml of OTC-HRP provided the highest sensitivity at the IC_{50} and LOD values of 25.5 ng/ml and 11.7 ng/ml, respectively.
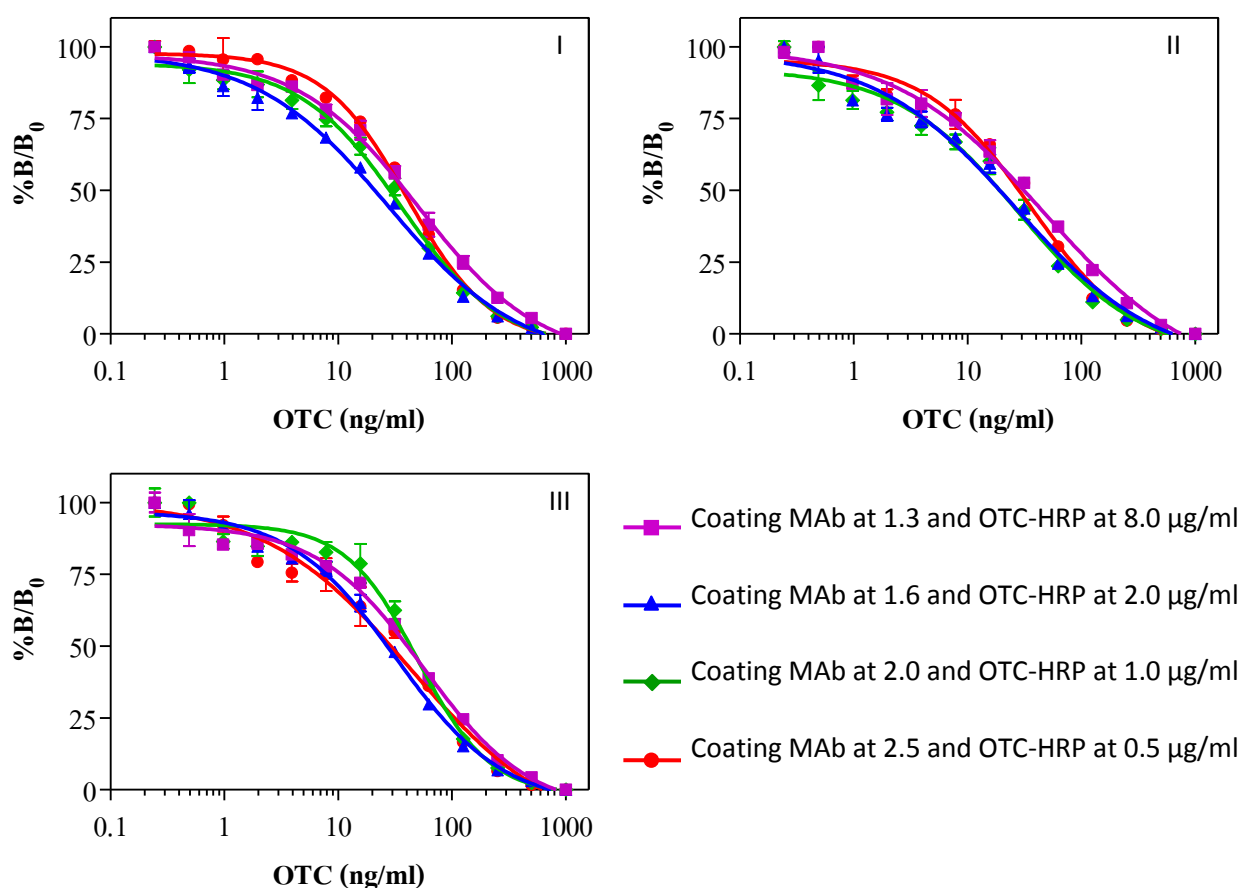

Figure 4.5 Competitive calibration curve of 3 replicated dcELISA with different ratios of MAb and OTC-HRP at 37ºC for 2 hr.

Table 4.6 IC_{50} and LOD of 4 different ratios of coating MAb and OTC-HRP in dcELISA

Coating MAb (µg/ml)	OTC-HRP (µg/ml)	$IC_{50} \pm SD$ (ng/ml)	$LOD \pm SD$ (ng/ml)
1.3	8.0	51.8 ± 4.1	23.1 ± 6.9
1.6	**2.0**	**25.5 ± 7.4**	**11.7 ± 7.3**
2.0	1.0	38.0 ± 11.1	16.1 ± 3.2
2.5	0.5	40.1 ± 3.9	15.2 ± 5.4

Remark: bold letters indicated the appropriate combination for the highest sensitivity

4.4.2 Antibody Labeled Indirect Competitive ELISA Using GAM-HRP (icELISA-GAM)

4.4.2.1 Optimization of OTC-OVA and MAb Concentration

The appropriate concentration of coating OTC-OVA and MAb were determined by the checkerboard method. Four suitable ratios of the OTC-OVA concentration to the MAb concentration were selected for further study of icELISA-GAM. The least concentrations of the antibody-antigen pairs that gave the absorbance value closed to 1 (range of 0.8-1.2) were selected. According to the result shown in Table 4.7, the OTC-OVA concentrations at 0.5, 1.0, 2.0 and 4.0 µg/ml pairing with the MAb concentrations at 0.32, 0.08, 0.04 and 0.02 µg/ml, respectively, were selected.

Table 4.7 Optimization of the coating OTC-OVA and the MAb using icELISA-GAM format

MAb (µg/ml)	Coating OTC-OVA (µg/ml)					
	0.25	0.5	1.0	2.0	4.0	8.0
0.003	0.095	0.101	0.146	0.182	0.223	0.256
0.005	0.111	0.132	0.171	0.264	0.331	0.362
0.01	0.138	0.172	0.236	0.408	0.544	0.583
0.02	0.172	0.252	0.390	0.658	**0.903**	0.970
0.04	0.212	0.355	0.630	**1.078**	1.350	1.423
0.08	0.303	0.522	**0.897**	1.395	1.563	1.581
0.16	0.420	0.713	1.238	1.586	1.615	1.612
0.32	0.571	**0.962**	1.446	1.624	1.636	1.635

Remark: the absorbance values with bold letters indicated the selected pairs of the OTC-OVA and the MAb concentration.

4.4.2.2 Analysis of Sensitivity in icELISA-GAM Format

In this format, OTC in the sample competed with the immobilized OTC-OVA for binding with the primary antibody. The OTC-OVA bound antibody was later quantified by the secondary antibody (GAM-HRP) which was proportional to the amount of OTC in the sample. Thus, the higher the amount of OTC in sample, the lower the colorimetric intensity. In order to investigate the sensitivity, the four selected concentrations of OTC-OVA and MAb were used in icELISA-GAM with OTC standard as the competitor. The competitive calibration curve obtained were compared and shown in Figure 4.6, the IC_{50} and the LOD values obtained from the curve were averaged and summarized in Table 4.8. The result showed that using 2.0 µg/ml of OTC-OVA as the coating agent and 0.04 µg/ml of MAb provided the highest sensitivity with the IC_{50} and LOD values of 6.1 ng/ml and 1.1 ng/ml, respectively.
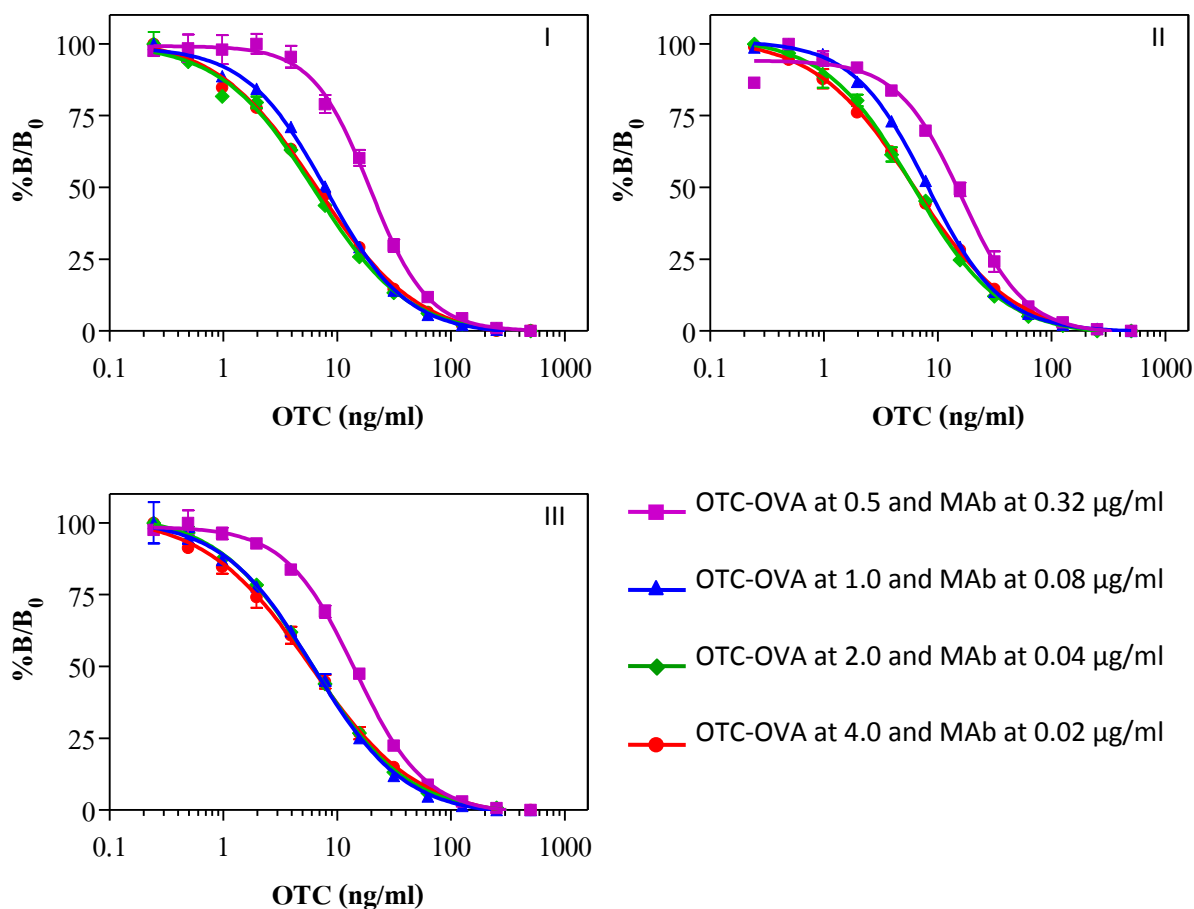
Figure 4.6 Competitive calibration curve of 3 replicated icELISA-GAM with different ratio of OTC-OVA and MAb at 37°C for 2 hr.

Table 4.8 IC$_{50}$ and LOD of 4 different ratios of coating OTC-OVA and MAb in icELISA-GAM

Coating OTC-OVA (μg/ml)	MAb (μg/ml)	IC$_{50}$ ± SD (ng/ml)	LOD ± SD (ng/ml)
0.5	0.32	16.6 ± 2.6	9.5 ± 1.8
1.0	0.08	7.5 ± 1.1	1.6 ± 0.9
2.0	**0.04**	**6.1 ± 0.2**	**1.1 ± 0.5**
4.0	0.02	6.2 ± 0.4	0.8 ± 0.3

Remark: bold letters indicated the appropriate combination for the highest sensitivity

4.4.3 Antibody Labeled Indirect Competitive ELISA Using Streptavidin-HRP (icELISA-SV)

4.4.3.1 Conjugation of MAb-biotin

Since four molecules of biotins can bind to one molecule of streptavidin, this binding could be applied to enhance the sensitivity of the ELISA (Lin et al., 2008). To do so, biotin was conjugated to the MAb. After biotinylation, the BCA assay was used to quantify the concentration of the MAb-biotin conjugate which was 1.08 mg/ml as shown in Table 4.9 (standard graph was shown in Appendix A)

Table 4.9 Protein concentration of MAb-biotin by BCA assay

Dilution of MAb-biotin	Absorbance at 562 nm	Concentration of MAb-biotin (mg/ml)
1/2	0.758	1.08
1/4	0.458	1.11
1/8	0.290	1.05
Average		**1.08 ± 0.03**

4.4.3.2 Optimization of OTC-OVA and MAb-biotin Concentration

The appropriate concentration of coating OTC-OVA and MAb-biotin were determined by the checkerboard method. Four suitable ratios of the OTC-OVA concentration to the MAb-biotin concentration were selected for further study of icELISA-SV. The least concentrations of the antibody-antigen pairs that gave the absorbance value closed to 1 (range of 0.8-1.2) were selected. According to the result shown in Table 4.10, the OTC-OVA concentrations at 0.5, 1.0, 2.0 and 4.0 µg/ml pairing with the MAb-biotin concentrations at 0.4, 0.1, 0.05 and 0.025 µg/ml, respectively, were selected.

Table 4.10 Optimization of coating OTC-OVA and MAb-biotin using icELISA-SV format

MAb-biotin	Coating OTC-OVA (µg/ml)					
(µg/ml)	0.25	0.5	1.0	2.0	4.0	8.0
0.013	0.104	0.136	0.181	0.270	0.437	0.708
0.025	0.125	0.194	0.334	0.610	**1.084**	1.236
0.05	0.165	0.297	0.585	**1.148**	2.059	2.174
0.1	0.244	0.457	**1.168**	2.351	3.403	2.954
0.2	0.529	0.736	2.009	3.362	3.574	2.739
0.4	0.647	**1.178**	2.778	3.673	3.398	2.559
0.8	1.153	2.437	3.419	3.393	3.101	2.420
1.6	2.445	3.201	3.067	3.040	3.093	3.134

Remark: the absorbance values with bold letters indicated the selected pairs of the OTC-OVA and

the MAb‑biotin concentration.

4.4.3.3 Analysis of Sensitivity in icELISA-SV Format

In this format, OTC in the sample competed with the immobilized OTC-OVA for binding with the MAb‑biotin. The OTC-OVA bound MAb‑biotin was quantified by the streptavidin‑HRP which was proportional to the amount of OTC in the sample. Thus, the higher the amount of OTC in sample, the lower the colorimetric intensity. In order to investigate the sensitivity, the four selected concentrations of OTC-OVA and MAb‑biotin were used in icELISA-SV with OTC standard as the competitor. The competitive calibration curve obtained were compared and shown in Figure 4.7, the IC_{50} and LOD values obtained from the curve were averaged and summarized in Table 4.11. The result showed that using 4.0 µg/ml of OTC-OVA as coating agent and 0.025 µg/ml of MAb‑biotin provided the highest sensitivity whit the IC_{50} and LOD of 7.2 ng/ml and 0.8 ng/ml, respectively.

Table 4.11 IC$_{50}$ and LOD of 4 different ratios of OTC-OVA and MAb-botin in icELISA-SV

Coating OTC-OVA (µg/ml)	MAb-biotin (µg/ml)	IC$_{50}$ ± SD (ng/ml)	LOD ± SD (ng/ml)
0.5	0.4	22.8 ± 1.8	10.8 ± 3.2
1.0	0.1	9.3 ± 1.2	3.3 ± 0.5
2.0	0.05	7.3 ± 0.1	1.4 ± 0.4
4.0	**0.025**	**7.2 ± 0.2**	**0.8 ± 0.5**

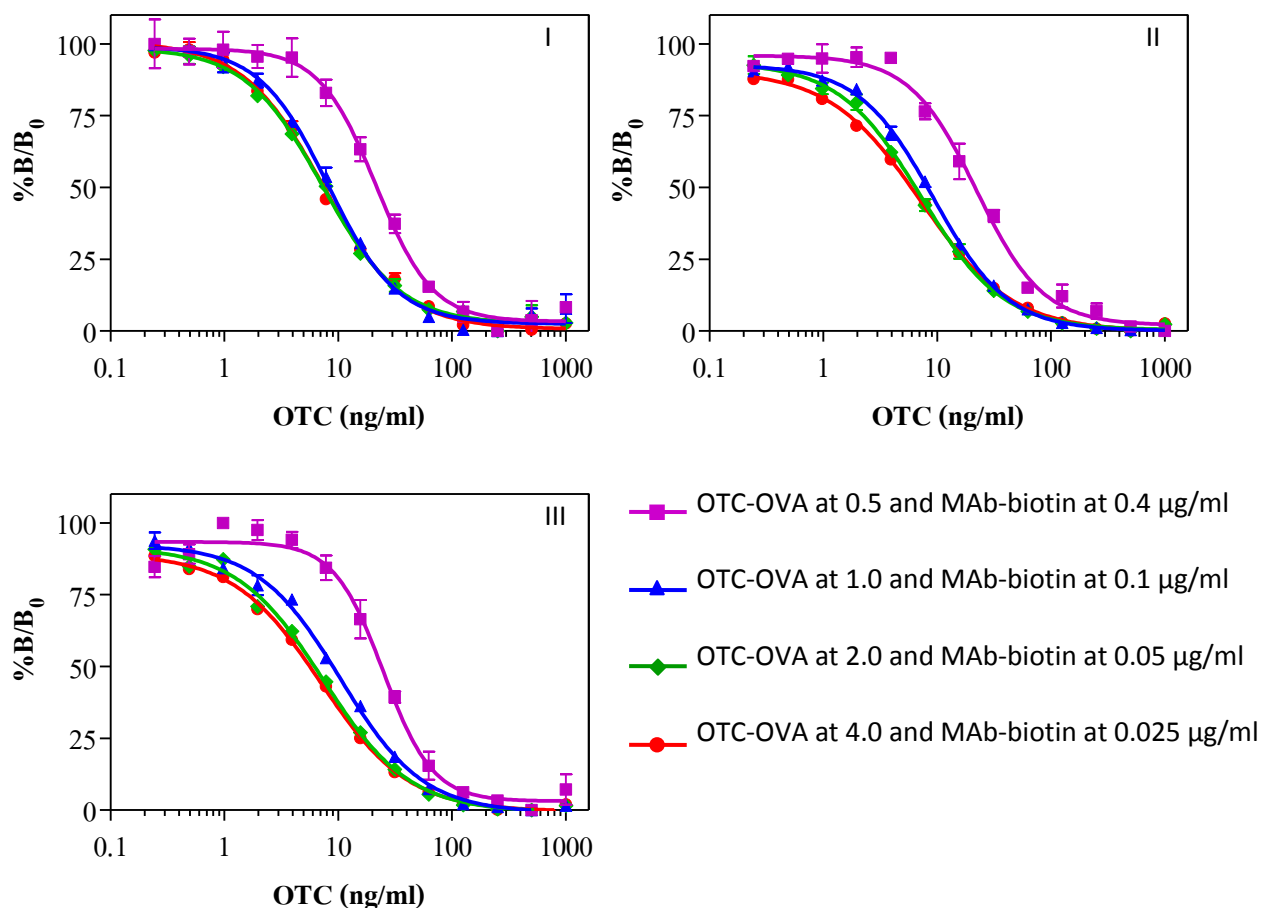Remark: bold letters indicated the appropriate combination for the highest sensitivity

Figure 4.7 Competitive calibration curve of 3 replicated icELISA-SV with different ratios of OTC-OVA and MAb‑biotin at 37ºC for 2 hr.

4.4.4 Comparison of Sensitivity among Different ELISA Formats

The sensitivities of all ELISA formats tested were summarized in Table 4.12. It could be obviously seen that the sensitivity of icELISA was better than that of dcELISA. This could be due to the fact that the concentration of the antibody to the antigen required to give the absorbance value closed to 1 is higher than those of icELISA. Therefore, a higher concentration of competition was required in the competitive binding or in order to decrease the absorbance. In spite of its lowest sensitivity, dcELISA gave advantages in the simplicity and short analysis time as compared to the icELISA.

In the case of the icELISAs, the icELISA-SV should give better sensitivity than the icELISA-GAM due to the signal amplification caused by the biotin-streptavidin system (Jeon et al., 2008). However, the result showed that both methods gave the same level of sensitivity. Based on the simplicity of the two ELISA formats preparation, the icELISA-GAM was selected as the prototype format for further study.

Table 4.12 Sensitivity of each ELISA format

ELISA Format	Antibody (µg/ml)	Antigen (µg/ml)	$IC_{50} \pm SD$ (ng/ml)	$LOD \pm SD$ (ng/ml)	Analysis time
dcELISA	1.6	2.0	25.5 ± 7.4	11.7 ± 7.3	2 hr 15 min
icELISA-GAM	0.04	2.0	6.1 ± 0.2	1.1 ± 0.5	3 hr 15 min
icELISA-SV	0.025	4.0	7.2 ± 0.2	0.8 ± 0.5	2 hr 45 min

4.4.5 Effect of Incubation Temperature and Competition Time

The main purpose of the ELISA development in this study was to use it as a screening detection method. Therefore, it should be suitable to handle a large number of samples at a reasonable time with minimum requirement for the instrument. Consequently, the incubation temperature at 37ºC and room temperature, and the times for binding competition from 30 min up to 120 min were studied.

The result showed that the sensitivity of the icELISA-GAM incubated at 37ºC and at room temperature were at the same level (as shown in Figure 4.8). As the result, the incubation at room temperature was selected for its simplicity.
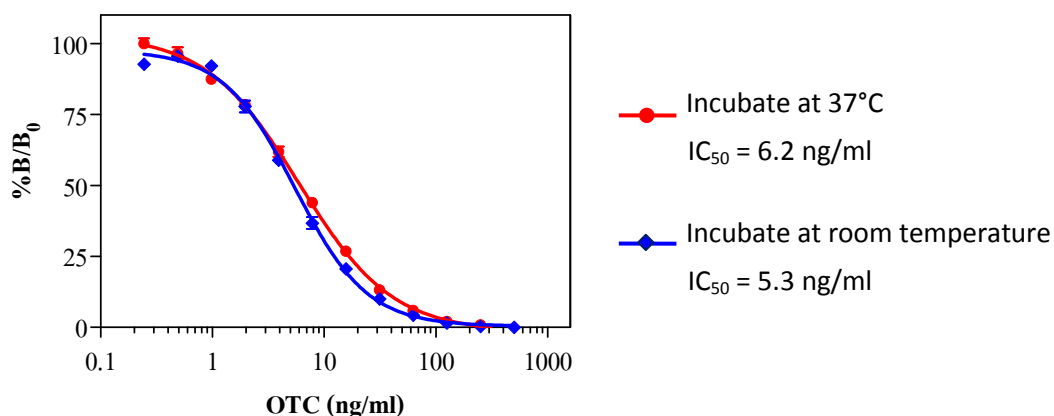
Figure 4.8 Competitive calibration curve of icELISA-GAM using 2.0 µg/ml of OTC-OVA as coating agent and 0.04 µg/ml of MAb with different incubation temperature at 37°C and room temperature for 2 hr.

In the case of the time for binding competition, the result showed that there was a decreasing trend of the sensitivity as the time decreased (Figure 4.9). However, all of the LOD values were still lower than the MRLs. The effect of the incubation time was further studied by using OTC-fortified shrimp samples.

Figure 4.9 Competitive calibration curve of icELISA-GAM at room temperature with different competition time.

The OTC concentration was varied in the range of 0-200 ng/ml while the incubation or competition time was between 30 and 120 min. The %recovery of OTC detected by icELISA-GAM at different competition times was shown in Figure 4.10. It was found that the incubation period of 30 min and 60 min yielded the %recovery in the range of 60-80 % which was lower than the accepta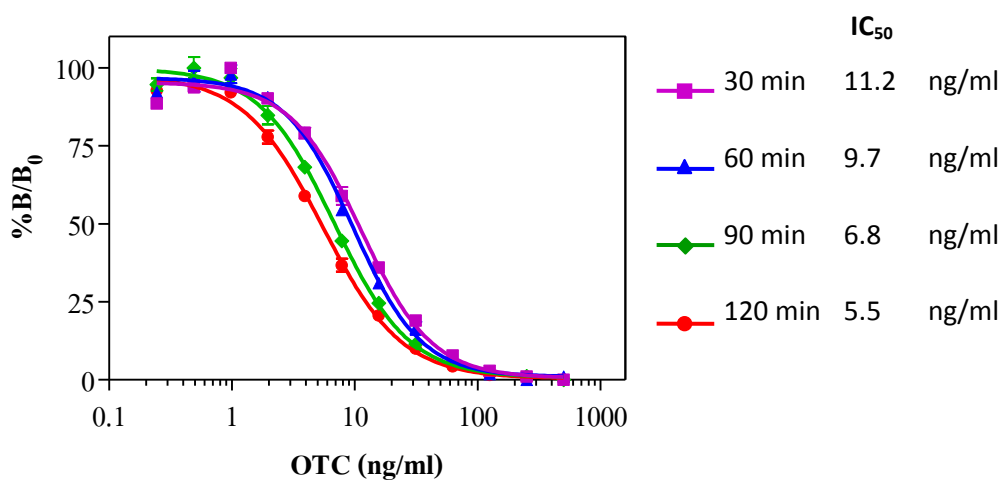ble level (80-120%). While the incubation period of 90 min gave higher %recovery between 117% and 129% but the standard variation was also too high (20-30%). The %coefficient of variation (%CV) was beyond the acceptable level of 20%. However, at the incubation period of 120 min, the % recovery and %CV was 96%-101% and 7.1%-12%, respectively. Therefore, the incubation period of 120 min. was selected for icELISA-GAM. It was possible that a certain period is required to obtain a complete competition of the binding between the coating Ag and the free Ag in the sample and the MAb.
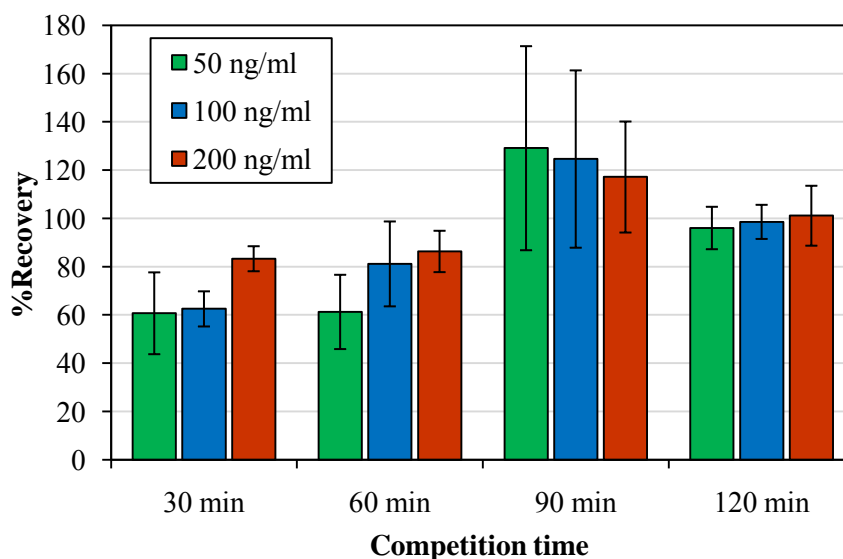

Figure 4.10 %Recovery of OTC detection with spiked OTC of 50, 100 and 200 ng/ml (*n*=6) in
sample extract at different competition time.

4.5 Evaluation of Prototype ELISA

4.5.1 Range of Working Concentration for Prototype ELISA

The condition of icELISA-GAM with the coating OTC-OVA of 2 µg/ml, the MAb of 0.04 µg/ml and 2 hr incubation period at room temperature was used to evaluate a suitable working range of the OTC concentration. The results shown in Figure 4.11, indicated that the working concentration of OTC was between 0.5 ng/ml and 32 ng/ml. Within this concentration range, the log of the OTC concentration varied linearly with the absorbance. The IC_{50} and the LOD were 5.5 ng/ml and 0.9 ng/ml, respectively.

(A)

(B)

$$y = -0.244\ln(x) + 0.9651$$
$$R^2 = 0.985$$
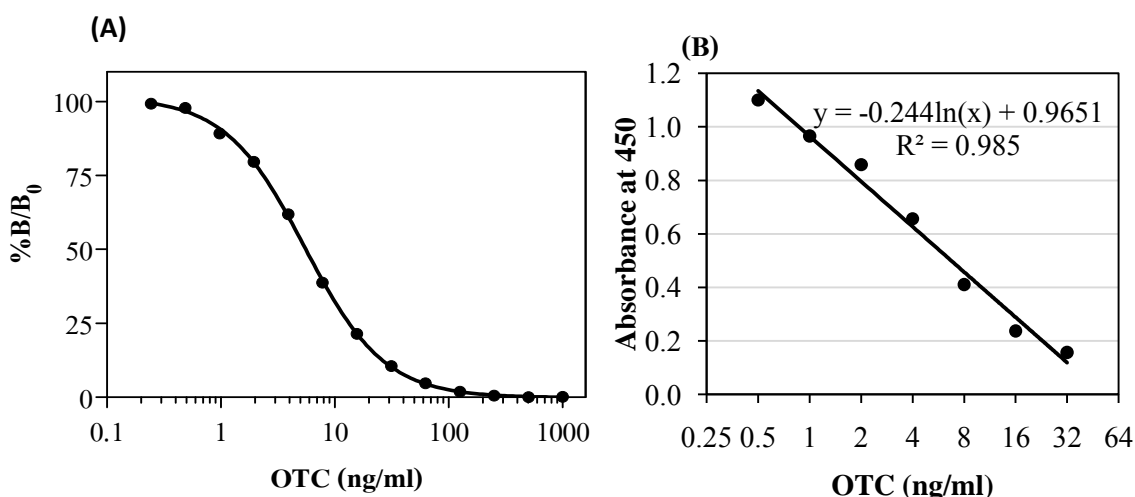
Figure 4.11 Competitive calibration curve (A) and standard curve (B) of prototype ELISA.

4.5.2 Specificity of Prototype ELISA

In this study, the specificity of prototype ELISA test kit was assessed by the cross-reactivity against several antibiotics in the group of tetracyclines and other unrelated groups. The percentage of cross-reactivity (%CR) was calculated from the percentage ratio of the IC_{50} of OTC to the IC_{50} of other interested compound. The %CR of all tested compounds was shown in Table 4.13. It was found that the MAb cross-reacted with RTC at 142% but only weakly cross-reacted with other TCs. Importantly, it did not cross-react with other compounds unrelated to the TCs group. It has been reported that the polyclonal antibody (PAb) raise against OTC showed strong

cross-reactivity with TC (1028%) and RTC (449%) (Cháfer-Pericás et al., 2010). This could be due to the fact that their structures are very similar to each other.

Table 4.13 Cross-reactivity of prototype ELISA with various compounds.

Compound	IC_{50} (ng/ml)	Cross-reactivity (%)
Oxytetracycline (OTC)	5.5	100
Rolitetracycline (RTC)	3.8	142
Doxycycline (DC)	67.1	8
Tetracycline (TC)	83.5	6
Chlortetracycline (CTC)	257.0	2
Chloramphenicol	> 2500	< 0.2
Norfloxacin	> 2500	< 0.2
AMOZ	> 2500	< 0.2
Clenbuterol	> 2500	< 0.2

4.6 Detection of OTC in Fortified Shrimp Samples Using Prototype ELISA

To evaluate the usability of the prototype ELISA, minced fresh shrimp samples were spiked with known amounts of OTC at the final concentrations varying from 25 ng/g to 400 ng/g. The samples were carefully extracted because OTC binds strongly to proteins and interacts with many ions to form stable complexes with tissue proteins (Barker and Walker, 1992) which cannot be detected by immunoassay. In almost confirmatory method, the McIlvain-EDTA buffer (0.1 M citric acid, 0.2 M Na_2HPO_4 and 0.1 M EDTA) and solid phase extraction (SPE) were used in the extraction and clean up step, respectively (Kijak et al., 1999 and Wu et al., 2011), that are time consuming, costly and require large sample volumes. In this study, the extraction procedure was simplified by solvent extraction with the metal chelating agent EDTA and does not require a complicated step to clean up sample.

The amount of OTC in the extracts was quantified using icELISA-GAM. The intra-assay variation was measured as the average of six replicated wells in one microplate of each concentration sample and the inter-assay variation was measured as the average of six replicated microplates of each concentration on different days. The analysis was summarized and shown in

Table 4.14. For the intra-variation assay, the %recovery was in the range of 82%-118%, while the %CV was in the range of 5.5-13.9%. In the case of the inter-variation assay, the %recovery ranged between 96% and 113% and the %CV ranged between 5.5% and 14.9%. These results indicated that MAb could be used in the prototype ELISA to detect OTC in shrimp samples within commonly acceptable ranges of accuracy (80%-120% recovery) and precision (CV of 0-20%).

Table 4.14 Analysis of OTC in fortified shrimp samples by prototype ELISA.

Spiked OTC (ng/g)	Intra assay (n=6)			Inter assay (n=6)		
	Measured OTC ± SD (ng/ml)	%R (%)	%CV (%)	Measured OTC ± SD (ng/ml)	%R (%)	%CV (%)
25	21.2 ± 1.6	85	7.4	24.1 ± 3.0	96	12.5
50	40.9 ± 5.7	82	13.9	56.5 ± 8.4	113	14.9
100	117.6 ± 8.5	118	7.2	107.9 ± 14	108	12.6
200	227.6 ± 14	114	6.2	215.1 ± 12	108	5.5
400	442.0 ± 24	110	5.5	418.1 ± 33	105	8.1

Note: Accepted accuracy at %R = 80-120% (Abbott et al., 2010)

Accepted precision at %CV < 20% (Zhang et al., 2007)

CHAPTER V

CONCLUSIONS

In this research, immunological-based methods, enzyme-linked immunoassorbent assay (ELISA) for the detection of OTC were developed. The hybridoma clone no. 2-4F was cultured in the spinner flask to produce the monoclonal antibody (MAb). The MAb in the culture media was partially purified by protein G sepharose affinity chromatography. The molecular weight of the MAb was analyzed by SDS-PAGE and found to be 26 kDa for the light chain and 59 kDa for the heavy chain.

In the case of ELISA, three different formats including antigen captured direct competitive ELISA (dcELISA), antibody captured indirect competitive ELISA using GAM-HRP (icELISA-GAM) and antibody captured indirect competitive ELISA using streptavidin-HRP (icELISA-SV), were evaluated for their sensitivity in terms of 50% inhibition concentration (IC_{50}) and limit of detection (LOD). Among the three format tested, the icELISA-GAM gave the highest sensitivity with the IC_{50} of 5.5 ng/ml and the LOD of 0.9 ng/ml. The working concentration of OTC that gave a linear standard curve between the absorbance value and the concentration was found to be 0.5-32 ng/ml.

Furthermore, the incubation temperature and binding-competition period were optimized. The incubation at room temperature was chosen since it gave the same level of sensitivity as the performed at 37ºC. The suitable binding-competition period was found at 2 hr. At shorter competition period, the sensitivity was reduced.

The accuracy and the precision of the icELISA-GAM was evaluated using shrimp samples fortified with OTC (0-400 ng/g). In the case of the intra-variation assay, the %recovery and the %coefficient of variation (%CV) were in the range of 82%-118% and 5.5%-13.9%, respectively. In case of inter-variation assay, the %recovery and the %CV was 96%-113% and 5.5%-14.9%, respectively. In both cases, the accuracy and the precision of the icELISA-GAM were in a generally acceptable range of 80%-120% and ±20%, respectively. These results indicated that the icELISA-GAM could be used to detect OTC in shrimp sample.

Summary of the OTC detection using ELISA

Detection format	Sensitivity	Detection range	%Recovery	%CV
icELISA-GAM	IC_{50}: 5.5 ng/ml	0.5-32 ng/ml	82%-118%	5.5%-14.9%
	LOD: 0.9 ng/ml			

Condition ; the coating OTC-OVA of 2 µg/ml, the MAb of 0.04 µg/ml and 2 hr incubation period at room temperature

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APPENDIX A

1. Calculation of molar ratio of OTC to OVA by UV‑Vis spectroscopy method

The hapten densities or molar ratio of OTC and OVA was calculated from molar absorbility or absorbance coefficient (ε) by this formula:

$$\text{Hapten density} = \frac{\varepsilon_{\text{conjugate}} - \varepsilon_{\text{protein}}}{\varepsilon_{\text{hapten}}}$$

From Beer‑Lambert law: $\varepsilon = \dfrac{A}{c \cdot l}$

When A is the absorbance, c is the molar concentration and l is the distance that light travels through the solution, generally that was 1 cm. Therefore, the molar absorbility was calculated and shown in Table A‑1.

Table A‑1 Table shows the molar absorbility of each compound.

Compound	Molecular weight (Da)	Concentration (mg/ml)	Concentration (μM)	Absorbance at 337 nm	Molar absorbility
OTC	460	0.125	0.272	1.257	4,626
OVA	45000	5	0.111	0.105	945
OTC-OVA	45000	2.5	0.056	0.555	9,990

$$\text{Hapten density} = \frac{9{,}990 - 945}{4{,}626} = 1.95$$

Therefore, the molar ratio of OTC and OVA was 1.95:1.

2. Determination of protein concentration by BCA protein assay kit

The protein concentration in solutions were calculate from the standard curve of BSA (Figure A-1) that was generated from the relation between BSA concentration and the absorbance at 562 nm in Table A-2.

Table A-2 Concentration of standard BSA and absorbance at 562 nm for determination OTC-OVA, OTC-HRP and MAb-biotin concentration.

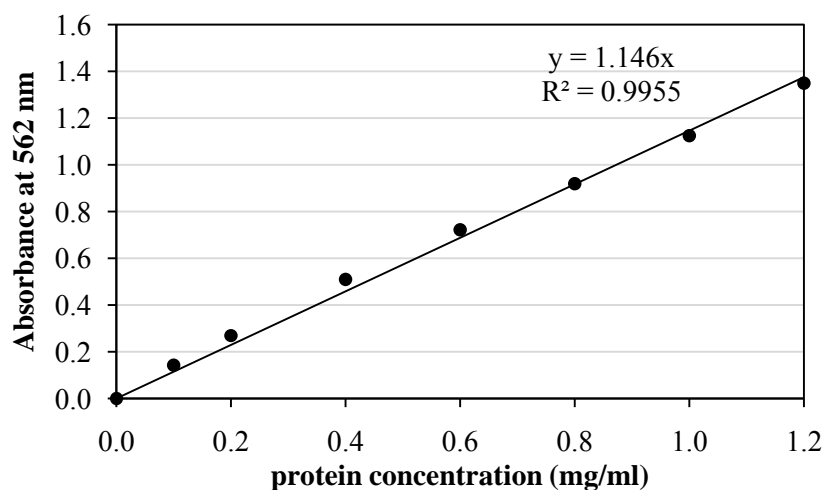| BSA concentration (mg/ml)	Absorbance at 562 nm
0	0.000
0.1	0.143
0.2	0.270
0.4	0.510
0.6	0.722
0.8	0.919
1.0	1.125
1.2	1.349

Figure A-1 Standard curve of BSA by BCA protein assay for determination OTC-OVA, OTC-HRP and MAb-biotin concentration.

3. Antibody purification using protein G column

Table A-3 Absorbance at 280 and 450 nm of MAb purification by protein G column

Fraction number	Absorbance at 280 nm	Absorbance at 450 nm
1	0.185	0.067
2	0.188	nd*
3	0.183	0.049
4	0.195	nd
5	0.349	0.663
6	1.350	2.965
7	2.099	3.001
8	2.296	3.023
9	1.845	3.068
10	1.338	2.881
11	0.848	2.723
12	0.528	1.849
13	0.374	0.512
14	0.291	0.077
15	0.263	nd
16	0.241	0.086
17	0.236	nd
18	0.218	0.069
19	0.215	nd
20	0.203	nd
21	0.201	nd
22	0.200	0.07
23	0.190	nd
24	0.181	nd
25	0.196	nd
26	0.188	0.082
27	0.207	nd
28	0.222	nd
29	0.210	nd
30	0.206	nd

*nd = non determine

4. Determination of MAb concentration by BCA protein assay kit

Table A-4 Concentration and absorbance at 562 nm of BSA standard for the determination of MAb
concentration.

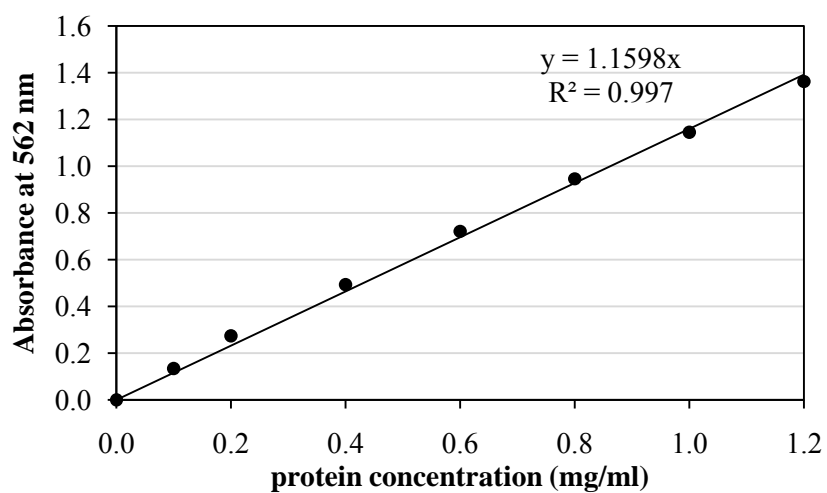| BSA concentration (mg/ml)	Absorbance at 562 nm
0	0.000
0.1	0.135
0.2	0.274
0.4	0.493
0.6	0.721
0.8	0.946
1.0	1.145
1.2	1.362

Figure A-2 Standard curve of BSA by BCA protein assay.

5. Determination of Molecular Weight

Table A-5 Molecular weight of protein markers and their relative mobility.

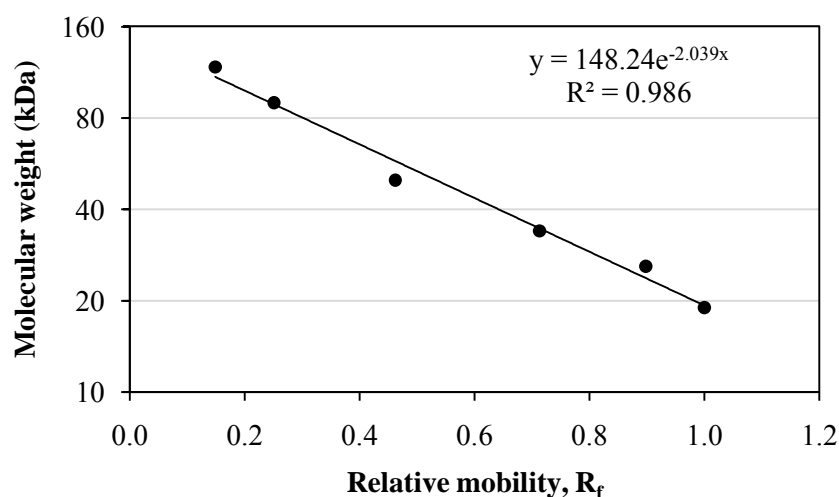| Protein marker (source)	Molecular weight (kDa)	Distance (cm)	Relative mobility, R_f
β-galactosidase (*E. coli*)	118	1.1	1.00
BSA (Bovine plasma)	90	1.8	0.90
OVA (Chicken egg white)	50	3.4	0.71
Carbonic anhydrase (Bovine erythrocytes)	34	5.2	0.46
β-lactoglobulin (Bovine milk)	26	6.5	0.25
Lysozyme (Chicken egg white)	19	7.3	0.15

$y = 148.24e^{-2.039x}$
$R^2 = 0.986$

Figure A-3 Standard curve of protein markers separated by SDS-PAGE.

Table A-6 Molecular weight of MAb calculated from standard curve of protein markers.

Band	Distance (cm)	Retention mobility, R_f	Molecular weight (kDa)
Unknown 1	3.0	0.41	65
Heavy chain of MAb	**3.3**	**0.45**	**59**
Unknown 2	5.8	0.80	29
Light chain of MAb	**6.2**	**0.85**	**26**