

Production of polyclonal antibody against tetracycline using KLH as a carrier protein

Siti Noraini, B.* and Nur Azura, M.S.

Biotechnology Research Centre, Malaysian Agricultural Research and Development Institute,
P.O. Box 12301, 50747 Kuala Lumpur, Malaysia

*Corresponding author: ainisnb@mardi.gov.my

Abstract

The production of polyclonal antibody against tetracycline was described using tetracycline - KLH conjugate (Keyhole Limpet Hemocyanin). Tetracycline was conjugated with KLH as a carrier protein because it was small molecule and unable to stimulate an immune response by itself. The UV absorbance reading of the tetracycline-KLH conjugate and KLH alone slightly shifted the reading of UV absorbance. Ammonium sulphate precipitation and Protein A affinity column were used in the antibody purification. Two peaks were obtained from affinity Protein A column. Peak 1 indicated the unbound material from serum sample and peak 2 was bound antibody with protein A which was eluted with elution buffer. Peak 2 was collected for titer antibody determination using ELISA method. Antibody level was higher at the fourth bleed which reached 1.2 absorbance (UV/nm) and equivalent with 1 mgmL⁻¹ concentration. The entire antibody level declined dramatically at dilutions greater than 0.0001 mgmL⁻¹ protein. The optimum and significant antibody concentration was at 0.00001 mgmL⁻¹ for use in ELISA or other assays

Key words: Antibody titer, ELISA, Keyhole Limpet Hemocyanin, polyclonal antibody, tetracycline

Introduction

Tetracycline is a group of broad-spectrum antibiotics used for medical purposes as well as animal husbandry. The tetracycline antibiotics have a broad range of activity against a variety of both Gram-positive and Gram-negative bacteria. Therefore, tetracycline and its derivatives (chlortetracycline & oxytetracycline) are widely used for prevention and control of diseases. Tetracycline antibiotic is also used as a growth promoting agent to improve growth performance in animals. Not only tetracycline is inexpensive, tetracycline antibiotic is also easy to administer and effective through oral dosing via water and feed (Kelly *et al.*, 2006). For these reasons, tetracycline antibiotic is extremely popular as a veterinary antibiotic. For example in chickens, tetracycline and its derivatives are being used up to 10 – 500 grams/ton of feed to obtain optimum rate of gain, improve feed

efficiency and egg hatchability and as a treatment for avian respiratory disease.

The use of these antibiotics has raised concerns as the presence of tetracycline residues in food may increase microbial resistance in humans which could lead to human health risks due to their carcinogenic potency. The emergence of resistance is related with the introduction of tetracycline for clinical, veterinary and agricultural uses (Aga *et al.*, 2003). Consequently, many countries have set the maximum residue levels (MRLs) to protect consumers. In Malaysia, the maximum residue levels for tetracycline as outlined in Food Act and Regulations 1983 are set at 100 ug/kg (in chicken's muscle), 200 ug/kg in eggs, 300 ug/kg in chicken's liver and 600 ug/kg for chicken's kidney. Excessive and abuse use of antibiotics in poultry have also drawn great attention among consumers and affects the acceptance of our livestock products when exported to other countries. For an example, upon the detection of excessive chemicals or

antibiotics in food commodity being exported, the food commodity will be rejected and returned. Besides paying the penalty fee and cost burden of product destruction, exporters may also be at risk in being blacklisted from entering the market.

There are several methods used to detect tetracycline residue. Microbiological assays are usually used for the measurement of tetracycline in food. However, these methods are complicated, time consuming and lack specificity (Kurtitu *et al.*, 2000). Instrumental analysis such as liquid chromatography-mass spectrometry (LC-MS) and high-performance liquid chromatography (HPLC) are sensitive and highly specific but require expensive equipment, derivatizing treatment and time consuming sample cleanup process. Therefore, they are not suitable to be used for routine screening of large quantity of samples and field detection. Enzyme-Linked Immunosorbent Assay (ELISA) is an immunological method known as a rapid, sensitive, specific and cost effective technique that needs the presence of specific antibody for residue detection (Zhang *et al.*, 2007). Another advanced and rapid immuno-based assay technique which also needs the presence of specific antibody in tetracycline detection is biosensor. But the challenge is to develop a high quality of the immunogen in the form of tetracycline conjugate. Tetracycline is small molecule that is unable to generate the immune response by itself. Therefore, conjugation to a carrier protein is required for immunization to stimulate and also producing a higher level of this antibody. The aim of this study was to produce a polyclonal antibody against tetracycline using KLH (Keyhole Limpet Hemocyanin) as a carrier protein to enhance *in vivo* immune response in rabbit.

Materials and Methods

Conjugate preparation

A 0.001g tetracycline (Sigma) and 0.02 g Keyhole Limpet Hemocyanin (mKLH Pierce, USA) was dissolved in 2ml and 1.5ml of water

separately, then were mixed in 10mL bottle. One mL of sodium acetate (pH 5.5, 3 molL⁻¹) and 37% (w/v) formaldehyde (0.4ml) were added to the reaction mixture and was stirred for 2 hours at room temperature under light-protected condition. The solution mixture was then dialyzed for 3 times with 0.01M phosphate buffer saline (PBS) pH 7.4. The final mixture was measured with the UV absorbance to determine the bonding of the tetracycline-KLH conjugate.

Immunogen preparation and Immunization schedule

A 200µg dried conjugate was dissolved in 0.5mL PBS. The conjugate suspension was mixed with 0.5ml of Freund's Complete Adjuvant and was injected subcutaneously in New Zealand white rabbits. The rabbits were rested for 3 weeks before injected with similar immunogen in 0.5ml of Freund's Incomplete Adjuvant. For antibody titer determination first bleed was taken one month after the injection. Blood was obtained by bleeding the central auricular artery of the rabbit's ear. A booster injection was given two weeks after the first bleed. The second booster injection was given 2 weeks after the first bleed. These booster injections and bleeds were repeated at two-week intervals until the fourth bleed.

Antibody purification

The collected blood was allowed to coagulate for three hours. The serum was then separated by centrifugation at 5000rpm for 15 minutes. Antiserum against tetracycline was diluted with distilled water (1:10) and then precipitated with saturated ammonium sulphate with continuous slow stirring to precipitate the serum protein. The serum mixture was then centrifuged at 5000rpm for 30 minutes at 4°C. The pellet was resuspended in PBS and dialyzed for 3 times in 0.01M PBS buffer to remove ammonium salt.

Partially purified antibody was then run through a protein A affinity column (nProtein

A Sepharose™) using AKTA prime Plus protein purifier instrument. Phosphate buffer (0.01M, pH 7) was used as the binding buffer and glycine buffer (0.1M, pH 3) was used as the elution buffer. The column was equilibrated with 5-column volumes before applying samples. Then, 3mL of partially purified antibody sample was injected into the column and run with a flow rate 2.0mLmin⁻¹ and 0.3Mpa pressure limit. Fraction tubes giving the highest absorbance reading at 280nm were collected. One molar of Tris-HCl pH 9 was used as neutralized buffer and dropped into the eluted sample. Purified antibody was run through dialysis again to remove salt. Antibody was then freeze-dried and kept at 20 °C for long term storage.

Titer determination

Titer determination was conducted using Enzyme-linked Immunosorbent Assay (ELISA). Microtiter plate was coated with antigen and incubated overnight at 4°C. The plate was emptied and washed 3 times with PBS-Tween (250µL per well). The well surface was then blocked by treating with 1:10 (v/v) solution of milk diluents in PBS for 30 minutes (250µL per well). Then, the plate was emptied and washed 3 times with PBS-Tween. Purified antibodies were added (100µL per well) and the plate was incubated for 2 hours at 37°C. The plate was emptied and washed 3 times with PBS-Tween. Anti-antibody-Alkaline phosphatase enzyme conjugate (1:1000) which was diluted in PBS was added in the well (100µL per well) and incubated for 30 minutes. Again, the plate was emptied and washed 3 times with PBS-Tween. Lastly, 100µL solution of P-Nitrophenyl Phosphatase diluents in diethanolamine (1:1000 w/v) was added and the plate was read at 405nm.

Results and Discussion

Tetracycline is a small molecule with a molecular size of 444.4 Dalton. Therefore, tetracycline itself is non-immunogenic and not able to elicit immune response in animals

for producing anti-tetracycline antibody. To make it immunogenic, it must be conjugated to a carrier protein before immunization (Zhang *et al.*, 2007). In this study, tetracycline was conjugated with Keyhole Limpet Hemocyanin (KLH) as a carrier protein to generate immune response and produce polyclonal antibody. KLH is a complex high molecular weight protein used as a carrier protein in antibody production because of its excellent immunogenicity and it confers to attach antigens (Nuria *et al.*, 2007). To obtain evidence of successful conjugation, UV absorbances recorded from 200 to 900 nm were measured for tetracycline, KLH and tetracycline-KLH conjugate as shown in Figure 1. It was observed that tetracycline had two peaks at 276 nm and 358 nm, KLH had one peak at 278 nm while tetracycline- KLH conjugate peaks were at 275 nm and 349 nm. Conjugation between tetracycline and KLH had slightly shifted the reading of UV absorbance. According to Lynn *et al.* (1998), UV absorbance for proteins changed during conjugation reaction conditions and the UV absorbance for hapten also changed when coupled to proteins.

The tetracycline-KLH conjugates were injected into New Zealand white rabbits with the addition of Freund's Adjuvant which was an inexpensive strategy for polyclonal production. Freund's adjuvant, which is paraffin oil based, had been used for stimulation of the immune system by Mycobacterium in Complete Freund's Adjuvant to generate high antibody titers (Trott *et al.*, 2008). Not only this adjuvant activated the immune system, it also retained the antigen to be released slowly into the injection site (Bollen *et al.*, 1996). Serum obtained from each bleed was purified using two main steps of antibody purification. First, serum was precipitated using saturated ammonium sulphate to precipitate antibody. Partially purified antibody was then run through Protein A affinity column to obtain pure IgG antibody against tetracycline. High yields of pure IgG antibody could be obtained using Protein A because it was very effective aseptically in purification strategies (Page and

Thorpe, 2008). Figure 2 shows the chromatogram of IgG fraction elution from Protein A affinity column with two peaks obtained from the graph. Peak 1 showed that the antibody had successfully bound with protein A while peak 2 showed that the bound antibody had successfully eluted. This finding

also suggested that the binding and eluted buffer used in this experiment were suitable to purify the antibody. The purified antibody fractions were collected via fraction tubes which showed the highest absorbance at 280nm.

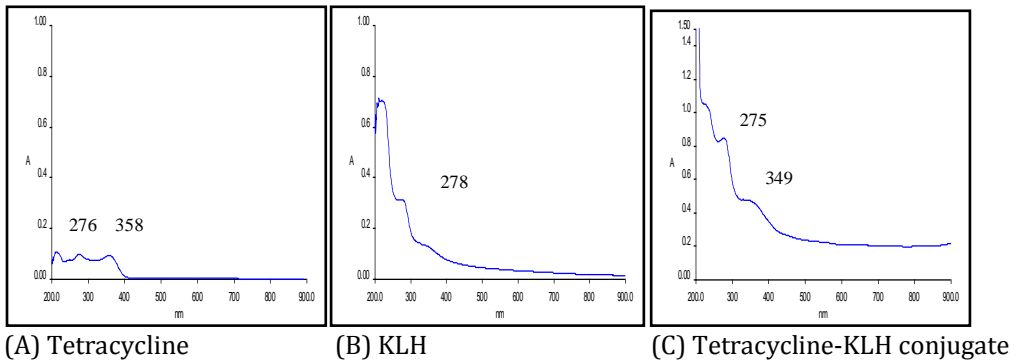


Figure 1. Synthesis of tetracycline-KLH conjugate when scanned through spectrophotometer wavelength. Two peaks of tetracycline was observed at 276 nm and 358 nm, (B) Peak of KLH at 278 nm and (C) Tetracycline- KLH conjugate peaks were at 275 nm and 349 nm.

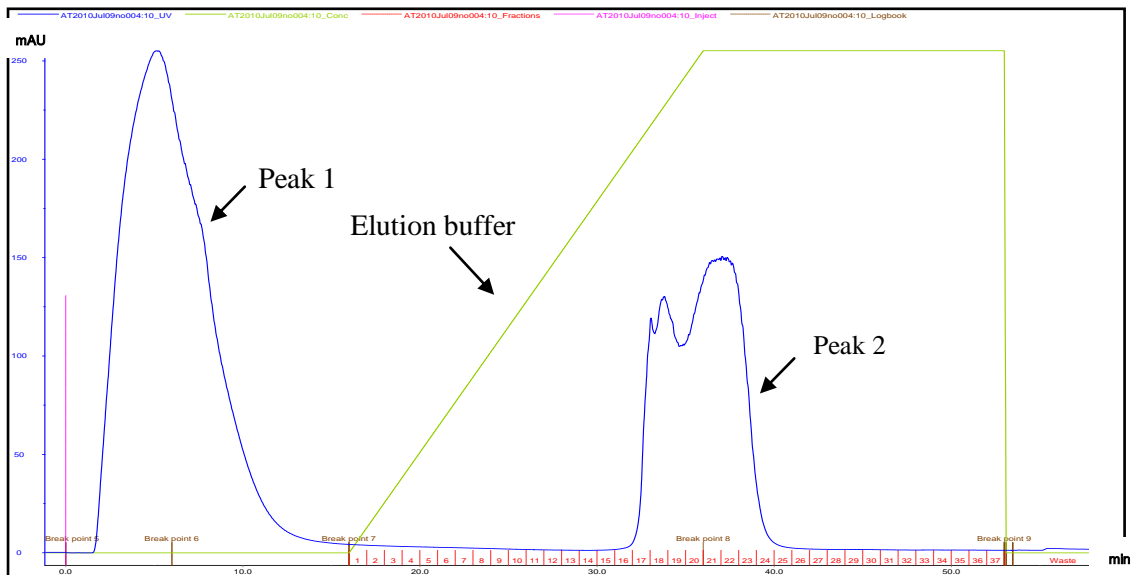


Figure 2: Chromatogram of IgG elution from Protein A affinity column using AKTAprime protein purifier

In the present study antibody titer determination was conducted through ELISA method. The higher antibody titer indicated the good quality of antibody produced. The results as shown in Figure 3 indicated that the antibody production against tetracycline was successful compared to the preimmune antibody. Preimmune was the serum taken before the rabbits were exposed to antigen. This suggested that the immunization and purification method used were successful. Figure 3 also shows the whole pattern of antibodies level in rabbits for all bleeds.

Absorbance reading and antibody titer increased from first to fourth bleed. The

booster injections given to the rabbits during immunization period had stimulated the body to produce more specific IgG against tetracycline, as previously reported by Faridah (2010). Antibody production was highest at the fourth bleed reaching an absorbance of 1.2 at 1 mgmL⁻¹ concentration. All antibody bleeds declined dramatically at dilutions greater than 0.0001 mgmL⁻¹ protein. The optimum and economic concentration of antibody was about 0.00001 mg/ml. Based on the antibody titer result, KLH can be considered as a good carrier protein for a small haptein molecule such as tetracycline.

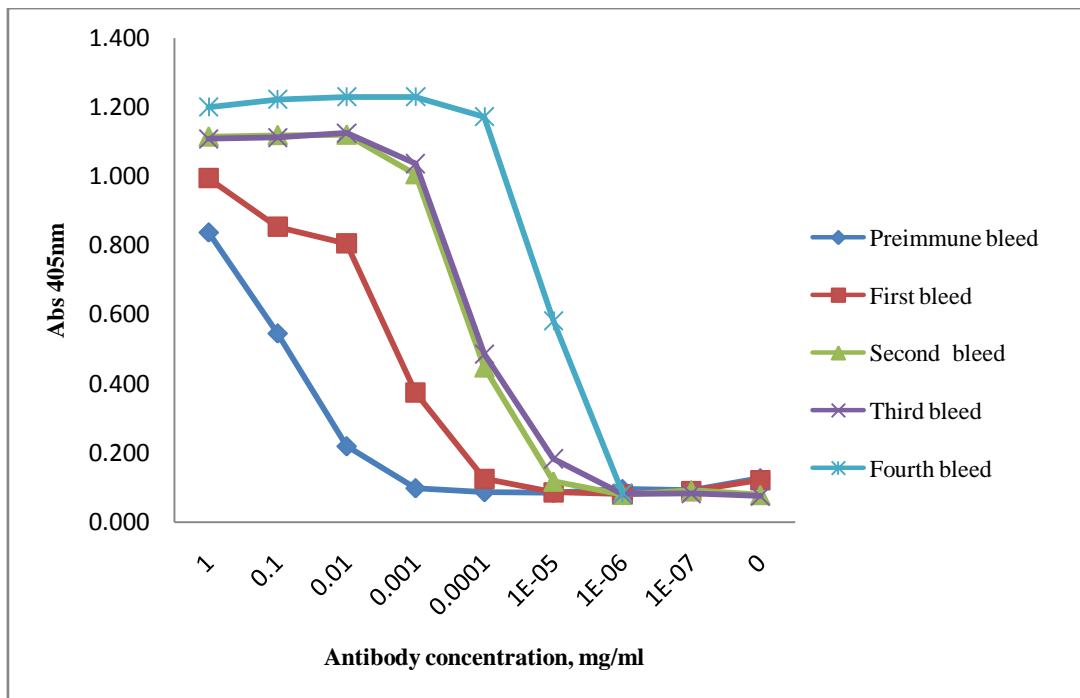


Figure 3: Pattern of antibody production against tetracycline in rabbit for all bleeds

Conclusion

The result of our study revealed that tetracycline was successfully conjugated with KLH as the carrier protein and was able to generate immune response when immunized in white rabbits with the addition of Freund’s adjuvant. Polyclonal antibody was

successfully purified using Protein A affinity column and the fourth bleed showed the best antibody titer sensitivity. Polyclonal antibody produced against tetracycline in this study has the potential to be used as biomaterial in the detection of tetracycline residue where the optimum and economic concentration of antibody is 0.00001mg/ml.

Acknowledgement

This work was supported by MARDI via WRM fund (JP-RB-0224).

References

- Aga, D.S., Goldfish, R. and Kulshrestha, P. 2003. Application of ELISA in determining the fate of tetracyclines in land-applied livestock waste. *Analyst*. 128: 658-662.
- Bollen, L.S., Crowley, A., Stodulski, G. and Hau, J. 1996. Antibody production in rabbits and chickens immunized with human IgG. A comparison of titre and avidity development in rabbit serum, chicken serum and egg yolk using three different adjuvants. *J. Immuno. Meth.* 191: 113- 120.
- Faridah, S. 2010. Development of immunosensor for *Salmonella typhimurium*. Ph.D. Thesis. Cranfield University.
- Kelly, M., Tarbin, J. A., Ashwin, H. and Sharman, M. 2006. Verification of compliance with organic meat production standards by detection of permitted and nonpermitted uses of veterinary medicines (tetracyclines antibiotics). *J. Agric. Food Chem.* 54: 1523-1529.
- Kurtittu, J., Lonnberg, S., Virta, M. and Karp, M. A. 2000. A group specific microbiological test for the detection of tetracyclines residues in raw milk. *J. Agric. Food Chem.* 48: 3372-3377.
- Lynn, L.J., Daniel, A. J. and Bruce, D.H. 1998. Development of an Enzyme-Linked Immunosorbent Assay for atrazine mercapturic acid in human urine. *Chem. Res. Toxic.* 11: 342-352.
- Nuria, P.N., Sergi, M., Angel, M. and Rosa, P. 2007. Synthesis of haptens and development of a sensitive immunoassay for tetracycline residues application to honey samples. *Analytica Chimica Acta.* 594: 211-218.
- Page, M. and Thorpe, R. 2008. IgG purification in immunochemical protocols, Second Edition, Ed. John D. Pound. New York: Humana Press. Pp: 95-111.
- Trott, D.L., Hellested, E.M., Yang, M. and Cook, M.E. 2008. Addition of killed whole cell bacteria preparations to Freund's Complete Adjuvant alter laying hen antibody response to soluble protein antigen. *Poult. Sci.* 87: 912-917.
- Zhang, Y., Shengxin, L., Wei, L., Zhao, C. and Rimo, X. 2007. Preparation of anti-tetracycline antibodies and development of an indirect heterologous competitive enzyme linked immunosorbent assay to detect residues of tetracycline in milk. *J. Agric. Food Chem.* 55: 211-218.