Chapter-Four

Antibody Production, purification and Characterization
4.1. PREFACE

4.1.1. An Immune system

All organisms are continually under attack from other organisms both externally (by predators) and internally (by pathogens, bacteria, virus etc.). Vertebrates have developed **Immune system**, an elaborate protective mechanism against invading pathogens and foreign particles. The immune system has extensive ability to detect and respond to stimuli such as presence of foreign particles inside the body and to remember what it encountered over long period of time. Immunity in vertebrates is conferred by white blood cells collectively called as **lymphocytes**. These cells originate from stem cells in bone marrow like all other blood cells but unlike red blood cells they leave the blood vessels and patrol the intercellular space for presence of foreign intruders or macromolecules collectively called as ‘**Antigens**’. These cells get trained in the specialized lymphoid tissues such as Thymus, lymph nodes and spleen, where they learn to differentiate between self and non-self (Hudson and Hay, 1989). The presence of foreign material gives rise to two different types of immune responses i.e., cellular and humoral immunity.

4.1.1.1. Cellular Immunity

This type of immunity guards against the viral or bacterial infected cells, fungi, parasites and foreign tissue and is mediated by T-lymphocytes or T-cells so called because their development takes place in Thymus.

The cellular immune system, functions primarily to prevent the spread of viral infection by killing the virally infected cells. The cellular response begins by action of macrophages, which engulf and partially digest any foreign antigen. The resulting antigenic fragments are displayed on the surface of the macrophage in one of the two type of special surface proteins called as MHC (Major histocompatibility complex) proteins. MHC class I proteins are present on all vertebrate nucleated cells surface and when they display specific antigens, are recognized by receptors on the surface of cytotoxic T-cells. While class II MHC proteins are recognized by receptors on T-helper cells, T cell receptors can bind to the MHC proteins only when antigenic fragments are displayed on these proteins. The T-cells, which bind to MHC proteins displaying the
antigen, are induced to propagate selectively and the process is called as **clonal selection.** The clonal selection is brought about by the action of secretion of growth factors termed Interleukins (IL). Macrophages to whom T-cells are bound secrete IL-1 and IL-2 and these T-cells can also make IL-2 receptors as long as they remain attached to the macrophage leading to auto stimulation of macrophage bound T-cells. IL-1 and IL-2 thus control the proliferation of T-cells.

The matured T-cells are generated starting a few days after the first encounter with antigen. Cytotoxic T-cells, also called Killer T-cells, which bear receptors for MHC-I protein-antigen complex, specifically bind to the cells displaying this complex and release a 70 kD protein called as **perforin** that lyses the target cell by aggregating to form pores in their plasma membranes.

### 4.1.1.2. Humoral Immunity

This kind of immunity is conferred by the presence of diverse collection of related proteins in body fluids (*Humor: Fluid*), called as **Antibodies.** These antibodies or immunoglobulins are produced by B-lymphocytes or B-cells, which mature in bone marrow in mammals. This type of immunity is highly effective against foreign invading particles, bacterial infections and extra cellular phases of viral infections.

Humoral immune response is triggered whenever a B-cell, which display on its surface membrane bound antibodies and MHC-II proteins, encounters a foreign antigen. The membrane bound antibodies are engulfed after they bind to antigen, digested intra celluarly and the fragments are displayed along with MHC-II protein complex. This complex is recognized by receptors on helper T-cells. IL's secreted by bound helper T-cells stimulate the B-cells to proliferate and differentiate into plasma cells which produce large quantities of antibodies. This proliferation and differentiation of B-cells continues till the signal provided by helper T-cell last which in turn depend upon the continual presence of antigen.

Whenever an animal is exposed to a foreign antigen, B-cells are activated and after clonal expansion produce anti-antigenic IgM type of antibodies. These activated cells remain active for about two weeks and thereafter antibody titer decreases. If the same antigen is encountered again a fast secondary response is developed with high titer
of IgG type of antibodies, which arise due to faster expansion of memory B and T cells for this specific antigen. The cells activated by secondary exposure to the antigen build up very fast and remain active for quite a long time, more than 4 weeks, as compared to the primary immune response, which builds up slowly and remains for about 2 weeks.

4.1.1.3. Immune response to haptenated proteins

MHC proteins do not display haptens, being small molecules and hence they do not activate the T-cells. When haptens are conjugated to a carrier protein the peptide fragments covalently coupled to the hapten are displayed on the MHC proteins and thus activation of T-cells is conferred indirectly. This leads to maturation of immune response by proliferation and differentiation of B-cells thereby giving rise to the production of anti-hapten antibodies along with the anti-carrier antibodies (Weilteizen, 1996). However the occurrence of allergic reactions to small molecules such as drugs indicates that T cell activation is possible by small molecules associated with MHC proteins examples of such haptens are trinitrochlorobenzene, TNBS both of which are protein modifying agents, antibiotics like penicillin and metal ions (Koponen et al., 1986; Sinigaglia 1994) etc. The first proof of activation of T cells by synthetic hapten came in 1992, when Ortmen and coworker used MHC-specific-synthetic peptide hapten conjugates to activate the T cells. The current studies to the T-cell activation by haptens is restricted to a few types belonging to di/trinitrophenols, azido compounds, azobenzene arsonate compounds etc. the role of other small molecules capable of acting as haptens is still little understood and their role in affecting autoimmune disorders still needs extensive explorations (Weilteizen, 1996)

4.1.2. Antibody Production

4.1.2.1. Antibodies (Abs)

Antibodies are serum proteins and form a part of vertebrate defense system against invading pathogens or foreign particles i.e, antigens. Emile roux and Alexandre Yersin found that soluble fraction of microorganisms was responsible for generation of immunity and Emil Von Behring along with Shibasaburo kitasato (1890) proved that these toxins are responsible for production of special proteins in the immunized animals.
that were termed as 'antitoxins' now called as antibodies. It was later shown that not only toxins but also other molecules, termed antigens, which included proteins, nucleic acids and polysaccharides etc., produced antibodies. Karl Landsteiner showed that small molecules, which he called as haptens, attached to a macromolecule (protein) also gave rise to specific antibodies. This opened the field of Immuno-chemistry wide open because now antibodies could be produced practically against anything.

Antibodies are also termed as Immunoglobulins (Ig's) due to their globular protein nature and are classified into five major classes i.e., IgA, IgD, IgE, IgG and IgM. Of different types, IgG makes up for the 80% of the serum Ig's content and thus most immunoassays are developed using IgG type antibodies (Roitt and Peters, 1997). Studies by Rodney Porter and Gerald Edelman gave the chemical structure of the antibodies (Raju, 1999). As shown in Fig (4.2) the Ab molecule consists of two identical heavy (H) chains and two identical light (L) chains stabilized and linked by inter and intrachain disulfide bonds. Each H chain consists of one variable (V_H) and three constant (C_H1, 2, 3) regions while each L chain consists of one variable (V_L) and one constant (C_L) region.

The Ag binding fragment (Fab) of antibodies is formed by the association of parts if the variable regions of H and L chains, located at the amino terminal end. The variable region of both H and L chains contains three complementarity-determining regions (CDR's), which show maximum variability and associate to form the Ag binding site. These CDR's are separated by four framework regions, which show a high degree of conservation. The constant regions of the chains associate to give rise to crystallizable fragment (Fc) of the Ab molecule; is not necessary for antigen binding but are responsible for various effector functions of the antibodies such as complement activation.
Ab production, purification & Characterization

Fig 4.1: Structure of Ab and various Ab-fragments.

Ab production is conveniently carried out in warm blooded animals, e.g. rabbits, sheep, mice or chickens (Hock et al., 1995, Suri et al., 2002). Polyclonal antibodies (pAbs) are obtained from the serum and comprise a mixture of different Ab populations. Monoclonal antibodies (mAbs) consist of a single monospecific Ab population. These Abs are produced in cell culture by a single hybridoma cell derived from the fusion of B-lymphocytes with myeloma cells (Kohler and Milstein, 1975). The hybridoma cells can then be propagated almost indefinitely in culture and will continue to produce the Ab of the lymphocyte parent. Since an individual lymphocyte produces only a single Ab type, all of the Ab molecules produced by a hybridoma cell line derived from a single hybrid cell are identical and have the same binding properties. Therefore, the hybridoma technology guarantees the unlimited production of mAbs with constant characteristics (Alexander and Hughes, 1995). Owing to the great effort involved in mAb production many IAs still employ pAb's. A third-possibility for creating Abs using recombinant antibody (rAb) techniques has recently been emerged for immunoassay applications. Here, Ig genes can be cloned, introduced and expressed in inexpensive and relatively simple host systems (Hudson and Souriau, 2001;Yau et al., 2003). Although several non mammalian host systems (yeast, plant and insect cells) have been used to produce rAbs, the most common vehicle is Escherichia coli (Fransisco et al., 1993) followed by yeast cells (Sandhu, 1992).
4.1.2.2. Antigens (Ags):

A substance that after injection into the body of a vertebrate induces a specific Ab synthesis is called an Ag. Ags are principally macromolecules, for instance proteins, polysaccharides or nucleic acids. Synthetic polymers also belong to the antigens, i.e. they can be used as, or act as, Ags. Any ligand capable of binding to their specific antibodies is called as immunogen so that all antigens are immunogens but all immunogens are not antigens. Especially small molecules (haptens) such as pesticides, drugs, toxins etc with a molecular weight less than 1000D are not antigenic. These molecules need to be coupled to a macromolecular carrier to elicit an Ab response. (Hennion and Barcelo, 1998).

Hapten structure, Purity of hapten, site of attachment to carrier protein and hapten density on carrier protein is primary determinant of the type of antibody produced in terms of specificity and sensitivity. These two qualities are of paramount importance in development of immunoassays (Dankwardt, 2000). Their should be minimum alteration in the hapten chemical structure and spatial conformation, when a linker arm containing a functional group is attached at appropriate site, so that the unmodified target molecule is also recognized by the antibody in the assay system (Goodrow and Hammock, 1998). Purity of hapten used for conjugate synthesis decreases the chance of cross reactivity of antibodies. Change in specificity of antibodies due to change in site of attachment of linker arm has also been reported (Oubina et al., 1999). It has been observed that the specificity of the antibody is generally directed towards the part farthest away from the functional group attached to the carrier protein (Erlanger, 1980). An optimum number of hapten molecules on the carrier protein is an important factor in determining the type and quality of antibodies produced (Malaitsev and Azipha, 1993; Hock et al., 1995).

4.1.3. Antibody purification methods:

Polyclonal antisera raised against haptens, consist of antibodies with a varying degree of specificity and affinity, due to their origination from different B-cell clones. Total polyclonal IgG's have been used for development of immunoassays (Ahn et al., 2004; Shan et al., 1999) but affinity purification is shown to be necessary for immunodiagnostic applications, which provides antibodies of high specificity and
Ab production, purification & Characterization

sensitivity for the target molecule (Gonyea, 1977; Giraudi and Baggiani, 1996; Firer, 2001).

In the formation of antibody-antigen complex, multiple forces, such as hydrogen bonds, hydrophobic, van der Waals and Coulombic interactions, contribute to this interaction (Braden and Poljak, 1995; Hudson and Hay, 1989). Several chemicals/reagents are effective in disrupting the above interactive forces. Based on their physicochemical properties these disruptive reagents can be categorized broadly as the following elution conditions: extreme pH, chaotropic salts, ionic strength, denaturants, and organic solvents. Conditions of extreme pH (as low as pH 2 or as high as pH 11) weaken all forces except hydrophobic interactions. Chaotropic salts (potassium iodide etc.) effect elution by changing the structure of water in and around the site of the affinity interaction. These salts primarily effect hydrophobic interactions between ligand and target molecule. Ionic solutions (such as MgCl₂, LiCl, etc.) disrupt the charge-charge interactions. Denaturants like urea, guanidine chloride, SDS, etc., unfold the protein structures and thus alter the stability of interaction. On the other hand organic solvents such as ethylene glycol, methanol, etc., are polarity-reducing agents which are generally effective in breaking the immunocomplex formed with low molecular-weight organic molecules (Yarmush et al., 1992; Yang et al., 1999). However, some of these eluting reagents could cause dramatic changes in elution profiles when used to break an immunocomplex (Kummer and Li-Chan, 1998). It is therefore essential to select an optimal elution condition for the affinity purification of different biomolecules.

In most cases the choice of dissociation conditions are arbitrary, based on convenience, prior experience and end goal whether a functional product is required or not. Only few studies have been done to deliberately compare the effect of various dissociation conditions on the yield and specific activity of the affinity purified product (Tsang and Wilkins, 1991; Downham et al., 1992; Ben-David and Firer, 1996; Kummer and Li-Chan, 1998). In high affinity interactions such as Hapten-Ab the choice of elution buffer is very important because very stringent elution/dissociation conditions can change the structure and thus affect the activity of the antibodies (Narhi et al., 1997). Elution strategies should be based upon the type of interactions between antigen and antibody. Spinks and his colleagues (1999) used a reversed affinity chromatography
approach to refine the polyclonal antibody preparation for improving sensitivity and performance of enzyme assay for Paraquat.

4.1.4. Antibody characterization

Overall affinity and specificity of the antibodies are the primary determinant of the performance of the assay for a given target analyte. Antibodies need to be characterized thoroughly before using them for development of immunoassays / sensors. Functional affinity of polyclonal antibodies and the interaction forces between antigen and antibodies are prime determinant of the quality of the antibodies. (Dankwardt, 2000)

Various methods are used for determination of quality of antibodies which range from determination of functional affinity by equilibrium based ELISA method (Raghava and Agrewala, 1994), optical methods for real-time antigen-antibody interaction analysis such as Surface Plasmon Resonance (SPR) sensors (Malmborg et al., 1995), to direct measurements of actual force of interaction between antigen and antibody using atomic force microscopy (Kaur et al., 2004). These tools for determination of antibody properties have given a great leverage in fast and reliable characterization of antibodies available for development of sensitive and selective immunoassays and immunosensors

4.2. AIMS AND OBJECTIVES

The preparation and characterization of immunoreagents is a main aim of any immunoassay development, which in turn is advanced for development of immunosensors. The main aim of this part of the study was to prepare antibodies specifically recognizing the target molecule i.e., Parathion. For this to achieve we aimed at following,

1. To immunize animal (rabbit) and characterization of antiserum collected using BSA-AP and BSA-MPAD conjugate as immunogen.
2. Purification of amino parathion antibody and MPAD antibody using protein A-sepharose column.
3. Determination of relative affinity constant for antibodies purified using ELISA.
4. To determine the effect of hapten structure on the specificity and cross reactivity of antibodies.
4.3 MATERIALS AND METHODS

4.3.1. Chemicals and reagents

Hapten-Protein conjugates (BSA-AP and BSA-MPAD) were prepared as described earlier, Freund’s Complete Adjuvant, Freund’s Incomplete Adjuvant, Ammonium sulfate, Goat anti-rabbit IgG-HRP conjugate, Protein-A Sepharose were purchased from Sigma, St. Louise, USA. Elisa Plates were obtained from Griener, Germany. TMB/H₂O₂ substrate was purchased from Bangalore Genei, India. All other chemicals were Analytical Reagents Grade purchased locally.

4.3.2. Immunization and sera collection in rabbits

For production of antibodies, New Zealand white rabbits were immunized sub-dermally with 250 μg of antigen (BSA-hapten conjugate or BSA-MPAD) emulsified (1:1) with Freund’s Complete Adjuvant (FCA.). Further boosters were given at the interval of 21 days using 1:1 emulsion of antigens in Freund’s Incomplete Adjuvant (FIA). The blood was collected from the ear vein of the rabbit 5th day onwards at alternate days after the booster dose was given. The blood was allowed to clot for one hour at room temperature. The clot thus formed was punctured and allowed the serum to separate overnight at 4°C. Serum was collected after centrifugation for 30 min at 10000 rpm and pooled. The antibody titers in the pooled serum were determined using ELISA assay and found to be 1:128000.

4.3.3. Purification of antibodies using affinity chromatography

4.3.3.1. Purification of whole IgG using Protein-A:

The purification of IgG was done in two stages. The first stage included precipitation of IgG fraction and removal of unwanted serum proteins. In the second step Protein-A affinity purification was carried out to purify total IgG content from the serum.

4.3.3.2. Saturated Ammonium sulfate (SAS) precipitation:

The IgG fraction of serum proteins from the antisera was precipitated using Saturated ammonium sulfate (761g/L, pH adjusted to 7.0) precipitation method (Hudson and Hay, 1980). In brief the antiserum was centrifuged at 20000 rpm for 30 min to
Ab production, purification & Characterization

remove any cellular debris. To the serum supernatant added an equal volume of SAS slowly while stirring to achieve a final concentration of 50% ammonium sulfate and kept the mixture at 4°C overnight with constant stirring. Solution was centrifuged at 10000 g for 30 min and the precipitates were resuspended in minimum amount of PBS after removing the supernatant. The protein solution was dialyzed against PBS containing 0.01% sodium azide, overnight at 4°C with frequent changes of dialysis buffer. The dialyzed protein solution was centrifuged at 10000 g for 30 min and the protein concentration was determined by taking OD at 280 nm.

4.3.3.3. Protein-A affinity purification:

The SAS precipitated protein solution was diluted to a final concentration of 5 mg/ml and used for loading on the protein-A affinity column. The protein solution was passed through the Pr-A column twice, flow through was collected and the column was washed till the OD$_{280}$ of the washing buffer (PBS, pH=7.4) fell down to a baseline. The elution was done using minimum amount of elution buffer i.e., Glycine-HCl (50 mM, pH=2.5). One ml fractions were collected, till the OD$_{280}$ fall down to base line. The one ml fractions collected were neutralized immediately with 50 μl of 0.1 M Tris and kept at 4°C. The Fractions with maximum protein were pooled and dialyzed against PBS containing 0.01% sodium azide, overnight at 4°C with frequent changes of the buffer. The concentration of the purified antibodies was determined spectrometrically at OD$_{280}$.

4.3.3.4. Purification of Hapten specific antibodies:

The BSA column was equilibrated with binding buffer (PBS). The Protein-A purified immunoglobulin solution was then passed through BSA column at the ratio of 4 mg to 1 ml of matrix bed volume to obtain specific antibodies. Specific antibodies were eluted using different elution buffers, passing through the column at a flow rate of 1 ml/min. The eluted antibodies were pooled, dialyzed and its concentration was measured spectrophotometrically by taking OD$_{280nm}$ and 1.35 as extinction coefficient.
4.4. Characterization of Antibodies

4.3.1. Enzyme Linked Immunosorbent Assay (ELISA)

Coating antigen was immobilized on 96 well Elisa plate (Griener, Germany) at a concentration of 1µg/ml in carbonate buffer (50 mM, pH=9.6) by adding 50 µl per well and incubating overnight at 4°C. After washing twice with phosphate buffer saline (PBS) the non-specific binding sites was blocked with 5% defatted skimmed milk in PBS using 200µl/well and incubating for 2 hrs at 37°C. Plates were washed thrice with PBS containing 0.05% Tween-20 (PBST) and finally with PBS. 50 µl/well of primary antibodies (antiserum) solution prepared in PBS containing 0.1% defatted skim milk was added at various dilutions as required and incubated for 3 hrs at 37°C. The plates were thoroughly washed thrice with PBST and finally with PBS. HRP conjugated Goat anti rabbit-IgG (Biorad) was used as secondary antibody at a dilution of 1:5000 and taking 50 µl/well. Plates were incubated for one hr at 37°C followed by a thorough washing as in earlier step to remove any nonspecifically bound secondary antibody. Color was developed using TMB/H₂O₂ as substrate (100 µl/well). The blue color development was stopped after 15 min using 50 µl/well 1N H₂SO₄ as stop solution. The blue color is converted to bright yellow color which was read at 450 nm using a Molecular Devices micro plate reader.

4.4.2. Determination of Affinity constant of antibodies

For the determination of antibody affinity constant (Kₘₐₓ), microtiter plates were coated with different antigen concentrations (5, 2.5, 1.25 and 0.625µg/ml) prepared in carbonate buffer. After washing twice with phosphate buffer saline (PBS) the non-specific binding sites was blocked with 5% defatted skimmed milk in PBS using 200µl/well and incubating for 2 hrs at 37°C. Plates were washed thrice with PBS containing 0.05% Tween-20 (PBST) and finally with PBS. 100 µl/well of primary antibody solution (1000-0.05 ng/ml in PBS) was prepared in PBS containing 0.1% defatted skim milk and incubated for 2 h at 37°C. After washing with (1X) PBS, the plates were thoroughly washed thrice with PBST and finally with PBS. HRP conjugated Goat anti rabbit-IgG (Biorad) was used as secondary antibody at a dilution of 1:10000 and taking 100 µl/well. Plates were incubated for 1 h at 37°C followed by a thorough
washing as in earlier step to remove any nonspecifically bound secondary antibody. Color was developed using TMB/H₂O₂ as substrate (100 µl/well). The blue color development was stopped after 15 min using 50 µl/well 1N H₂SO₄ as stop solution. The blue color is converted to bright yellow colour which was read at 450 nm using a Molecular Devices micro plate reader. Kₐₛₚ values were determined based on the Law of Mass Action using the following relation:

\[ K_{eff} = \frac{(n-1)}{2} \left\{ n [Ab']_t - [Ab]_t \right\}, \quad n = \frac{[Ag]_t}{[Ag']_t} \]

Where, [Ag]₀ and [Ag']₀ are two different coated antigen concentrations and [Ab]₀, [Ab']₀ are the observed anti-amino parathion antibody concentrations at 50% of the maximum absorbance value.

4.4.3. Competitive Inhibition Immunoassay

The conjugate-coated ELISA plates were subjected to competitive inhibition experiments. The free antigen was added at different concentrations (0 ng/ml-500 ng/ml) along with a fixed amount of antibodies (10 µg/ml) at the time of incubation of primary antiserum/antibodies. The color was later developed as usual after incubation with secondary antibodies. The extent of antibody binding inhibition was measured from comparison the OD₄₅₀nm of wells containing only antibodies and no pesticide with those containing known amount of pesticide.

4.5 Results and Discussion

4.5.1. Immunological characterization of Conjugates

4.5.1.1. Immune response to hapten structure in rabbit

Rabbit was immunized with conjugate prepared from derivatives of Parathion i.e Amino Parathion and derivatives of atrazine conjugated to Bovine Serum Albumin (BSA). The sera collected after giving booster doses was pooled and was checked for its activity against hapten conjugated to BSA i.e. known to have very little cross reactivity with BSA, the carrier protein used for immunization (Fig 4.2, 4.3).
Fig 4.2. The titer in the antiserum obtained from rabbit immunized with BSA-AP conjugate. The graph shows relative OD obtained at 6.4x 10^4 dilutions.
Fig.4.3. Dilution curve for BSA column purified rabbit anti-MPAD antibody against BSA-MPAD, BSA, coated wells in ELISA.

4.4.1.2. Cross reactivity Studies

Indirect Competitive inhibition ELISA was used to determine the cross reactivity of immunoassay with different metabolites of Parathion and atrazine. Cross reactivity of related analogues were calculated on the basis of standard calibration curves in a range of ng mL\(^{-1}\) to sub ng mL\(^{-1}\) level. The data was normalized by %B/B\(_0\) transformation and the specific hapten concentration yielding 50% inhibition used to calculate the cross reactivity according to the formula:

\[
\% \text{ Cross Reactivity} = \frac{H}{C} \times 100
\]

Where, \(H\) is the concentration of standard hapten at 50% \(B/B_0\) and \(C\) is the concentration of cross reacting hapten/analog at 50% \(B/B_0\)
Table 4.1. %Cross Reactivity of antibodies generated against AP with various pesticide as compared to that of Parathion:

<table>
<thead>
<tr>
<th>Pesticide Name</th>
<th>% Reactivity</th>
<th>IC₅₀ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl parathion</td>
<td>100</td>
<td>31.25</td>
</tr>
<tr>
<td>Amino parathion</td>
<td>172</td>
<td>12.14</td>
</tr>
<tr>
<td>Maleimido amino parathion</td>
<td>138</td>
<td>23.41</td>
</tr>
<tr>
<td>Amino nitro phenol</td>
<td>200</td>
<td>15.62</td>
</tr>
<tr>
<td>Dithio phosphoryl chloride</td>
<td>235</td>
<td>13.86</td>
</tr>
</tbody>
</table>

Table-2: Cross reactivity of anti-MPAD antibody with the analogues of atrazine

<table>
<thead>
<tr>
<th>Atrazine and its analogues</th>
<th>IC₅₀ (ng mL⁻¹)</th>
<th>CR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPAD R₁ = S(CH)₂COOH, R₂ = ethyl and R₃ = isopropyl</td>
<td>0.10</td>
<td>100</td>
</tr>
<tr>
<td>Atrazine R₁ = Cl, R₂ = ethyl, R₃ = isopropyl</td>
<td>0.11</td>
<td>91</td>
</tr>
<tr>
<td>Prometon R₁ = methoxy, R₂ &amp; R₃ = isopropyl</td>
<td>0.15</td>
<td>67</td>
</tr>
<tr>
<td>Cyanazine R₁ = Cl, R₂ = ethyl, R₃ = cyanopropyl</td>
<td>2.00</td>
<td>5</td>
</tr>
<tr>
<td>Terbutryn R₁ = thiomethyl, R₂ = ethyl, R₃ = ter-butyl</td>
<td>1.05</td>
<td>10</td>
</tr>
<tr>
<td>Simazine R₁ = Cl, R₂ &amp; R₃ = ethyl</td>
<td>0.14</td>
<td>71</td>
</tr>
</tbody>
</table>
4.4.2. Characterization of Purified Antibody

4.4.2.1. Affinity constant of antibodies

The affinity constant for purified anti-amino parathion antibody using coating antigen at different concentration was determined by Law of Mass Action. The relative affinity constant of anti-amino parathion antibody was around $2.5 \times 10^6$ l/mol (Fig. 4.4).

Fig 4.4. Affinity constant for polyclonal antibodies purified using different concentrations.