Chapter Four

Antibody production, purification and Characterization
Chapter-Four

4. ANTIBODY PRODUCTIONS, PURIFICATION AND CHARACTERIZATION

4.1 INTRODUCTION

4.1.a 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.,

4.1.a.i 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
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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.a.ii **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 cellularly 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.

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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.

![Diagram of primary and secondary immune response](image)

**Fig 4.1: Primary (a) and Secondary (b) Humoral Immune response to the foreign antigen.**

### 4.1.a.iii 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)
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e. 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. b Antibody Production

4.1. b.i 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 \( C_{II,2,3} \) regions while each L chain consists of one variable \( V_L \) and one constant \( C_L \) region. The Ag binding fragment \( F_{ab} \) 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 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. This 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.

Fig 4.2: Structure of Ab and various Ab-fragments.
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(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 (Francisco et al., 1993) followed by yeast cells (Sandhu, 1992).

4.1.b.ii 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). The alteration in the hapten chemical structure and spatial conformation should be minimum, 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
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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.b.iii 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 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, 1980). 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.c 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., Atrazine. Another target was to see if the labeled reagents work well with the antibodies produced. For this to achieve we aimed at following,

1. To Immunize animals (mice and rabbits) and characterization of antiserum collected using different haptens i.e, using BSA-MPAD, BSA-HAD and BSA-CAD conjugates as immunogens.
2. To determine the effect of hapten structure on the specificity and cross reactivity of antibodies obtained using class specific and compound specific haptens for immunization.
3. To determine the effect of Hapten density on the type and specificity of antibodies produced.
4. To purify antibodies in bulk using hapten affinity column chromatography.
5. Determination of specific activity and affinity constants for antibodies purified using ELISA.
7. To establish the usability of various labeled reagent, prepared earlier for development of immunoassay.
4.3 MATERIALS AND METHODS

Chemicals and reagents

Immunizing conjugates (BSA-HAD/CAD/MPAD) were prepared as described earlier, FCA, FIA, Ammonium sulfate, Goat anti-rabbit/mouse IgG-HRP conjugate, Protein-A Sepharose were purchased from Sigma, St. Louise, USA. Affigel-102 was purchased from Biorad, India. Elisa Plates were obtained from Griener, Germany. TMB/H$_2$O$_2$ substrate was purchased from Bangalore Genei, India. All other chemicals were Analytical Reagents Grade purchased Locally.

4.3.a Immunization and sera collection in mice and rabbits

Two groups of young female Balb/c mice and a total of 5 mice per group were immunized. Each mouse was injected peritoneally with 25 μg of immunizing antigen (BSA-hapten conjugate) prepared as 1:1 emulsion in Freund’s Complete Adjuvant (FCA). Further boosters were given at an interval of 21 days using 1:1 emulsion of antigens in Freund’s Incomplete Adjuvant (FIA). Blood was collected from the tail vein of the mice 4th 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. For bulk production of antibodies, two rabbits for each antigen were immunized sub-dermally with 250 μg of antigen (BSA-hapten conjugate) emulsified 1:1 with FCA. Boosters were given at the interval of 21 days and the blood was collected from the ear vein of the rabbit. Sera was prepared as mentioned earlier and pooled. The antibody titers were determined using ELISA assay.

4.3.b Purification of antibodies using affinity chromatography

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 form the serum.
4.3. b.i 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 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. Centrifuged the solution at 10000 g for 30 min and the precipitates were resuspended in minimum amount of PBS after removing the supernatant. Dialyzed the protein solution 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. b.ii 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) fall down to a minimum of 0.03 or lower. The elution was done using minimum amount of elution buffer i.e., Glycine-HCl (50 mM, pH=2.5). The buffer coming out of the column was monitored for pH change and the fractions were collected as soon as the pH started dropping. 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 elution buffer. The concentration of the purified antibodies was determined using OD$_{280}$.

Purification of Hapten specific IgG:

The total IgG fraction purified using Protein A affinity chromatography, contains different types of polyclonal antibodies and hapten specific antibodies were purified
using MPAD affinity column instead of protein-A for purification of Hapten specific antibodies.

4.3.b.iii Preparation of Hapten column: Hapten Affinity column was prepared using a high performance matrix (Affi-gel-102, Biorad) for the separation of specific antibodies from protein A purified whole immunoglobulin. Affi-gel-102 is an amino terminal cross linked agarose gel on which mercaptopropanoic acid derivative (MPAD) of atrazine was attached covalently via its activated carboxyl groups. The reaction mechanism involves first an activation of the carboxyl group on the MPAD by a carbodiimide (DCC), and then displacement by a nucleophile (R-NH₂), releasing the carbodiimide as soluble urea derivative. The activation was done by adding 50 μmoles (14.3 mg) MPAD, 75 μmoles (15.5 mg) DCC and 75 μmoles (8.6 mg) NHS in 1 ml DMF and the mixture was incubated for 4 hours at RT. Affigel-102 (1 ml) was taken and washed thrice with 5 ml acetate buffer (100 mM, pH 5.0) and finally suspended in a final volume of 2 ml. The activated MPAD solution was added drop wise over a period of 30 min and incubated overnight at RT. After the reaction was over the hapten bound Affi-gel was washed with 10 bed volumes of acetate buffer, followed by 10 bed volumes of DDW. Unbound hapten was further removed by successive washings with 10 bed volumes of 25%, 50% and 25% methanol in DDW. The overall reaction is shown in Fig (4.3)

4.3.b.iv Purification of Hapten specific antibodies: The Affigel-MPAD beads were washed with copious amounts of binding buffer and packed in the column with same buffer. The Protein-A purified immunoglobulin solution was then passed through hapten specific column at the ratio of 4 mg to 1 ml of matrix bed volume to obtain specific anti-atrazine antibodies. The actual amount of specific antibody bound to the matrix was calculated by subtracting the total unbound antibody from the initial amount. Subsequently, atrazine specific antibodies were eluted using different elution buffers, passing through the column at a flow rate of 1 ml/min. The eluted antibodies were dialyzed and its concentration was measured spectrophotometrically by taking OD280nm.

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Fig 4.3: Reaction mechanism for Preparation of Hapten Affinity column
4.3.c Characterization of Antibodies and labeled reagents

4.3.c.i Enzyme Linked Immunosorbent Assay (ELISA)

Coating antigen was coated 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 were 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 mouse-IgG (Biorad) was used as secondary antibody at a dilution of 1:10000 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/H$_2$O$_2$ as substrate (50 µl/well). The blue color development was stopped after 15 min using 50 µl/well 1N H$_2$SO$_4$ 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.

4.3.c.ii Competitive Inhibition ELISA

For competitive inhibition experiments the free antigen was added at different concentrations along with a fixed amount of antiserum (1:500) or antibodies (0.5 µg/ml) at the time of incubation of primary antiserum/antibodies. The colour was later developed as usual after incubation with secondary antibodies. The extent of antibody binding inhibition was measured from comparison the OD$_{450nm}$ of wells containing only antibodies and no pesticide with those containing known amount of pesticide.

4.3.c.iii Elisa Elution Assay (EEA):

For Elisa elution assay, after incubation with primary antibodies, another washing step comprised of Pre-elution washing buffer that depended on the choice of elution
buffer followed by a two-minute washing with the elution buffer was incorporated as an additional step. This followed a normal washing step i.e., three times with PBST and finally with PBS. The colour was developed using TMB/H$_2$O$_2$ after 1hr incubation with the HRP labeled secondary antibodies.

4.3.c.iv Determination of Affinity constant of antibodies

For determination of Affinity constant of different antibodies, dilution curves of the antibodies purified from the hapten affinity column were prepared using different concentrations of coated antigens in ELISA. The data was fed in the Ab Affi program for determination of antibody affinity developed by Raghava and Agrewala (1994). The software is freely available at [www.imtech.res.in/ragahva/progs/abaffi](http://www.imtech.res.in/ragahva/progs/abaffi).

4.3.c.v Biomolecular interactions using AFM

I. **AFM sensor description:** Atomic force microscope utilizes a microfabricated silicon cantilever to determine the surface topography or the interaction between biomolecules. The deflection of the cantilever produced is proportional to the force between the cantilever tip and the surface. The deflection of cantilever is recorded using a laser beam, which is aligned at the tip of the cantilever, and change in the position of the tip produces a corresponding deflection of the beam that is detected by a position sensitive photo detector (Fig 4.4).

![Fig 4.4: Schematic representation of working of Atomic Force Microscope](image)
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II. Sample preparation for AFM

a) Immobilization of antibody on the gold-coated slides: Optically polished gold-coated glass slides were kindly provided by Dr. H. Vogel [Laboratory of physical chemistry of polymers and membranes (LCPPM), Swiss Federal Institute of Technology, Lausanne, Switzerland]. Antibodies were immobilized on gold coated glass slides using protein-A immobilization method developed in our lab (Suri et al., 1995). In brief glass slides were dipped in 1.5N NaOH for 30min and washed with Distilled water followed by a 5 min. treatment with 1.2 N HCl. The slides were thoroughly washed with distilled water and dried at 70°C. The slides were then dipped in Protein-A solution (1 mg/ml) for two hours at room temperature. Non-specific binding sites were blocked using 0.1% BSA solution in PBS. The glass slides were then incubated in rabbit anti-MPAD antibodies (100µg/ml in PBS) solution for 3hrs at room temperature. Antibodies were then covalently coupled to the protein-A molecule by dipping in DMP solution (5 mg/ml in 0.2 M triethanolamine, pH=8.2) for one hour with gentle shaking and finally after washing with buffer the unused sites of the linker were blocked by treatment with 0.1 M ethanolamine solution for 10min. The glass slides were then washed with 0.5 M NaCl and finally with 0.1M Glycine-HCl, pH=2.5 to remove any non-covalently bound antibodies from the protein-A layer.

b) Immobilization of Hapten-protein conjugate on the cantilever tip: silicon nitride cantilevers (NT-MDT) were treated with 1.5 N NaOH for 30 min and rinsed extensively. Afterwards the cantilevers were treated with H2SO4/H2O2 solution (70:30 v/v) for 30 min and after extensive washings with deionized water dried the cantilevers at 100°C. The acid treated cantilevers were functionalized using silanization with 2% APTES solution in chloroform and evaporation of the solvent at 60°C for one hour and washed extensively with same solvent. Afterwards the silanized probes were treated with PBS containing 2.5% Glutaraldehyde for 2 hrs and washed extensively with PBS. Subsequently the cantilevers were put in drop of BSA-MPAD conjugate (0.1 mg/ml) and allowed to react overnight at 4°C.
III. Biomolecular Interaction Analysis using AFM

The force measurements were done between antigen coated cantilevers and antibody immobilized on the gold substrate in contact mode atomic force microscopy. Antibody coated glass slides were blocked using 2% Casein in PBST. The sample after washing was mounted on the substrate and placed in the liquid cell, which was then filled with 10 fold diluted blocking solution. A total of 90 force curves were obtained and each curve was obtained from the mean of three approach/retract cycles at 1 KHz at the point of measurement. To check the effect of presence of free analyte on the Ag-Ab interaction, the fluid cell was filled with diluted blocking solution containing 50 ppm atrazine. Deflection curves were again recorded as earlier after an incubation of one hour at room temperature.

4.3.c.vi Affinity binding studies using SPR sensor

I. Description of SPR sensors: Normal SPR sensors are based on the principle of interaction of evanescent wave, which is formed when at a critical angel of incidence light is totally reflected back while traveling from a denser to a rare medium, with plasmons at the surface of a thin metal layer placed in between the two media. The interaction of the plasmons with the evanescent field is monitored by change in intensity of the reflected light at a particular angel of incidence.

![Fig 4.5: Schematic representation of Resonant mirror SPR sensor and the coupling of incident light in the resonant layer.](image)
IASys (Thermolabsystems, Helsinki, Finland) is a commercial SPR sensor, which employs Resonant Mirror technique i.e., a combination of the enhanced sensitivity of waveguiding devices with simple construction and use of SPR phenomenon. Fig (4.5) shows the basic construction and working of resonant mirror SPR sensor. In brief, a resonant layer (100 nm) of high refractive index is placed below the sensing layer and is coupled to the prism through a thin (1 µm) coupling layer of low refractive index. Thus the resonant layer acts as a wave guide, having a layer of denser medium sandwiched between two layers of rare medium and multiple total internal reflections takes place in the resonant layer. The coupling layer is kept thin enough that the incident light through the prism is able to couple with the evanescent field at the sensing layer. This resonance coupling is dependent on the angel of incidence and at the resonance point the light enters the resonant layer and generates evanescent field at the sensing surface which interacts with the plasmons. The presence of biomolecules at the sensing surface brings about a change in the angle of resonance. The change in angle is measured to monitor the course of bimolecular interactions. The technique was described in detail by Cush and coworkers (1993).

II. Immobilization of Conjugate on the cuvette

The conjugate (OVA-MPAD) was immobilized on a carboxymethylated dextran (CMD) coated SPR cuvette provided by the manufacturer. The process consisted of activation of carboxyl groups of CMD by carbodiimide-active ester method and coupling of the conjugate through the amino groups. The activation of carboxyl groups was done using a water soluble carbodiimide EDC [1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide] and NHS [N-Hydroxy succinimide] using a 100 µl solution of both EDC and NHS (10 mM, 1:1) for five minutes followed by washing with PBS containing 0.05% tween-20 (PBST). After washing the surface was equilibrated with Acetate buffer (10 mM, pH=5.0) for 2-3 min. This was followed by addition of 100 µl of conjugate (100 µg/ml) prepared in acetate buffer. The coupling reaction was carried out for 10 min followed by a washing with PBST. The unused active sites were blocked using 100 µl of ethanolamine solution (1M) for five minutes. After another PBST washing four consecutive washings with guanidine hydrochloride were given to remove any adsorbed conjugate. The baseline shift was determined and the increase in the SPR signal was
correlated with the amount of conjugate immobilized on the surface. As per the manufacturers standards an increase of 600 arc-second in baseline response is equivalent to 1 ng protein/mm² of cuvette area and the area of the cuvette is 4 mm². The baseline shift was found to be between 400-900 arc seconds in different experiments and thus immobilization densities of 0.66-1.5 ng/mm² of cuvette were obtained. Fig (4.6) shows the sensor response during the various phases of the immobilization process.

Fig 4.6: Immobilization of OVA-MPAP conjugate on the SPR cuvette. The various phases are marked as (A) Addition of EDC/NHS (B) Addition of conjugate (C) Addition of Ethanolamine and (D) Washings with Guanidine-HCl. In between various phases the cuvette was washed with PBST.

III. Antibody binding and detection of Atrazine

The SPR response was obtained for binding of different amount of antibody on the surface of cuvette having immobilized conjugate on the surface. The control well was coated with OVA alone and during the analysis the binding to OVA was negated from binding to the conjugate immobilized on the cuvette. Only the binding region of the SPR curve was used for analysis. Typically the antibody solution with different concentration of Atrazine was added to the cuvette and the sensor response was recorded till the binding reached a stable level (approx. 1 to 5 min.). IN HCl was used for regeneration of the sensor surface in between different binding events.
**4.3.c.vii Dot blot Assay**

Either antigen (for checking antibody activity) or antibody (for checking the activity of labeled reagents) was coated on the Nitrocellulose membrane at different concentrations in carbonate buffer (50 mM, pH=9.4). Coating was done by placing a spot of 1 μl on the membrane and allowing it to air dry. The membrane was washed with PBS twice and nonspecific binding sites were blocked using PBS containing 5% skimmed milk. The membrane was washed with PBST thrice and was incubated with the primary antibodies or the labelled antigens for 3 hrs at 37°C. After incubation with primary antibodies membrane was again washed three times with PBST and finally with PBS.

1. For checking the activity of antibodies, the bound primary antibodies were detected by using HRP-labeled Goat anti mouse antibodies. Incubation with secondary antibodies was carried out for 1 hour at a predetermined dilution of 1:10000. Colour was developed using TMB/H₂O₂ substrate for dot blot.

2. For checking the activity of HRP labeled antigens. The binding of HRP-atrazine to the antibodies was confirmed by developing color using TMB/H₂O₂ substrate for dot blot.

3. For colloidal gold binding no color developing reagent was required and the typical red spots could be detected visibly wherever the antigen antibody interaction was taking place on the membrane.

4. The dot blot photographs were captured using a Pharmacia Gel doc system (Image Master VDS).
4.4 Results and discussion

4.4.a Immunological Characterization of Conjugates

4.4.a.i Immune response to various hapten structures in mice

Three different groups of mice, with 5 mice in each group, were immunized with conjugates prepared from three derivatives of Atrazine i.e., MPAD, HAD and CAD conjugated to Bovine serum albumin (BSA). The sera collected after giving booster doses was pooled and was checked for its activity against all the three haptens conjugated to a coating protein (OVA) that is known to have very little cross reactivity with BSA the carrier protein used for immunization. It was observed that all the antisera produced against each hapten was able to recognize the other two hapten structures. However, the anti BSA-MPAD antiserum showed less recognition for HAD and CAD hapten conjugate as compared to that shown by the anti HAD and anti CAD antiserum for MPAD conjugate (Fig 4.7).

![Graph showing OD at 450nm for different antisera](image)

**Fig 4.7:** The recognition of the different hapten structures by the antibodies produced against a single hapten. The results were obtained by using 1:500 dilution of pooled antisera produced against each hapten.
The greater recognition of haptens having related structure by anti HAD and anti CAD antisera as compared to that by anti MPAD antisera reflects the Class specific and compound specific property of the haptens used to produce anti-atrazine antibodies. So we expected anti MPAD anti bodies to be compound specific and anti HAD and anti CAD antibodies to be class specific. This was further confirmed by checking the cross reactivities of the anti-MPAD, HAD and CAD sera with different pesticides. It was observed that anti-MPAD sera showed minimum cross reactivities with other pesticides except with terbutryn while the cross reactivities were very high for all the pesticides tested in case of anti-HAD and anti-CAD anti sera. The results obtained for cross reactivities are summarized in Table 4.1

Table 4.1: Cross reactivity (CR) of antiserum generated against MPAD, HAD and CAD with various pesticides as compared to that of Atrazine

<table>
<thead>
<tr>
<th>S.No</th>
<th>Pesticide</th>
<th>CR* of Mouse anti BSA-MPAD sera</th>
<th>CR* of Mouse anti BSA-HAD sera</th>
<th>CR* of Mouse anti BSA-CAD sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Atrazine</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Simazine</td>
<td>19.8±13.2</td>
<td>93.03±9.25</td>
<td>51.7±17.4</td>
</tr>
<tr>
<td>3</td>
<td>Cyanazine</td>
<td>12.9±6.8</td>
<td>128.13±23.15</td>
<td>3.8±1.9</td>
</tr>
<tr>
<td>4</td>
<td>Prometon</td>
<td>51.9±9.5</td>
<td>36.63±1.13</td>
<td>155.6±32.7</td>
</tr>
<tr>
<td>5</td>
<td>Terbutryn</td>
<td>101.7±27.7</td>
<td>66.13±28.96</td>
<td>157.2±30.3</td>
</tr>
</tbody>
</table>

* The cross reactivities were determined by checking the percent inhibition of antibody binding at three different concentrations of free pesticide i.e, 0.1, 1.0 and 10 ppm. The inhibition by Atrazine was taken as 100% and the values denote the average cross reactivity (%) at all the concentrations for a particular pesticide and antisera. A fixed dilution of sera (1:500) was used for all the inhibition experiments.

4.4.a.ii Immune response to various hapten densities in mice

The low cross reactivity of anti BSA-MPAD antisera prompted us to carry out further investigations with respect to the immune response in mice against this particular hapten. The BSA-MPAD conjugates were prepared having different hapten densities. Different groups of mice having 5 mice in each group were immunized with each conjugate having different hapten density. The effect on titer and the type of antibodies produced were checked by ELISA. As shown in Fig (4.8) the antihapten antibody titer
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increased with increase in hapten density. As expected the titer in all the groups increased with successive booster doses.

Fig 4.8: The titer in the antiserum obtained from mice immunized with BSA-MPAD conjugates with various hapten densities. The graph shows the relative OD obtained at 1:500 sera dilution for each group.

It has been reported earlier that when conjugates having very high hapten density are used for immunization, they give rise to IgM type of antibody predominantly even in secondary responses which in general is characterized by higher IgG type of antibody. (Malaitsev and Azipha, 1993). We tried to see at what hapten density the IgM content starts increasing with booster dose. The sera of at least 4 individual mice from each group were tested to determine the IgM pattern (Fig 4.9). It was observed that the IgM content increased with increasing hapten density as observed on 4th day after booster. However it was observed to decrease for the intermediate hapten densities but increased in case of lowest and the highest hapten density conjugate used.
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Fig 4.9: The relative IgM content in antisera at 4th and 10th day after the booster dose was given to the mice. IgM content was found to increase with increasing hapten density.

Fig 4.10: Dilution curve for various sera collected from one of the rabbits immunized with BSA-MPAD. The Values are the mean OD values and SD values of three experiments done in triplicate.
4.4.a.iii Immune response in rabbits

The initial experiments on mice for production of Atrazine specific antibodies indicated that only MPAD hapten gave compound specific antibodies and other two haptnens, HAD and CAD, gave class specific antibodies which showed a good degree of cross reactivity with other related pesticides. So we concentrated our efforts on Anti-MPAD antibodies for development of immunoassay for Atrazine and production of bulk antibodies. Three rabbits were immunized with BSA-MPAD as antigen and sera was collected after subsequent booster doses. As shown in Fig (4.10) from the dilution curve for sera collected after booster doses a high titer (taken as 0.1 O.D.\(_{450nm}\)) was obtained even with second booster only, while the titer increased with successive boosters.

4.4.a.iv Anti Hapten and Anti Carrier response

It was observed that rabbits immunized with conjugates having different hapten density, anti-hapten antibody titer in the sera increased with increasing hapten density on immunizing conjugate, however the anti-carrier titer showed a decrease with increasing hapten density. The results are shown in Fig (4.11).

4.4.a.v Effect of Hapten density on the cross reactivity of antibody

Antibodies were purified from sera of rabbits immunized with BSA-MPAD conjugates having different hapten densities. These antibodies were checked for cross reactivity pattern with different pesticides having structures related to the target analyte Atrazine, these included Simazine, Cyanazine, Prometon, Hydroxy-atrazine and Terbutryn. It was observed that the antibodies produced from conjugates with low hapten densities showed a high degree of cross reactivity with most of the pesticides tested and in some case even more than the target analyte. The cross reactivities were calculated using the IC\(_{50}\) values determined for different pesticides by competitive inhibition assay. The IC\(_{50}\) value is the concentration of free antigen that decreases the maximal signal by 50%. The cross reactivities were determined using the following formula

\[
CR(\%) = \left[ \frac{C}{C^*} \right] \times 100
\]

C is the IC\(_{50}\) value for cross reactant and C* is the IC\(_{50}\) value for Atrazine as determined by the inhibition curve.
Fig 4.11: Anti Hapten and Anti Carrier immune response to immunizing conjugates having different hapten density. The hapten densities determined by chemical TNBS method are shown in parenthesis of Y-axis labels. The titer values are average of mean values from two independent experiments.
Table 4.2: The cross reactivity of the antibodies purified from rabbit sera of animals immunized using BSA-MPAD conjugates with increasing hapten densities.

<table>
<thead>
<tr>
<th>Cross-Reactant→ Conjugate used</th>
<th>Atrazine*</th>
<th>Hydroxy-Atrazine</th>
<th>Simazine</th>
<th>Cyanazine</th>
<th>Prometon</th>
<th>Terbutryn</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 (3)</td>
<td>100±6.98</td>
<td>8.4±3.57</td>
<td>42.6±8.81</td>
<td>196±3.61</td>
<td>9.8±6.31</td>
<td>98±7.33</td>
</tr>
<tr>
<td>M2 (5)</td>
<td>100±3.59</td>
<td>22.2±0.74</td>
<td>41.8±6.69</td>
<td>34.2±0.18</td>
<td>17.8±7.11</td>
<td>41±4.71</td>
</tr>
<tr>
<td>M3 (10)</td>
<td>100±5.59</td>
<td>------</td>
<td>1.3±0.72</td>
<td></td>
<td>0.96±2.20</td>
<td>54.2±2.44</td>
</tr>
<tr>
<td>M4 (14)</td>
<td>100±6.91</td>
<td>------</td>
<td>0.89±1.17</td>
<td></td>
<td>2.43±1.03</td>
<td>30±3.86</td>
</tr>
<tr>
<td>M5 (17)</td>
<td>100±6.24</td>
<td>2.45±1.8</td>
<td>29.06±3.48</td>
<td></td>
<td>26.30±2.96</td>
<td>150±3.88</td>
</tr>
</tbody>
</table>

*The cross reactivities were calculated using the IC$_{50}$ values determined from three different experiments with each experiment giving a mean value from samples run in triplicate.

The results for determination of cross reactivities are shown in Table 4.2. It was observed that the conjugates with intermediate densities i.e., 10-14 haptons per molecule of BSA showed minimum cross reactivity. Further increase in hapten density again showed an increase in the cross reactivity of the antibody with related pesticide molecules. So to produce good quality antibodies the hapten density may also be playing a crucial role, which is not generally taken into consideration since a higher titer of anti-hapten antibodies is obtained with increasing hapten density.
4.4. b Purification of Antibodies

4.4. b.i Total IgG purification

Total IgG antibodies were purified using Protein-A affinity chromatography matrix. We got an average yield of 9-11mg total IgG per ml of antiserum from batch to batch. It was observed that it was beneficial to use a precipitation step prior to Protein-A chromatography purification to separate out excess of unwanted proteins rather than directly using sera for purification of total IgG. This precipitation was carried out using either Saturated ammonium Sulfate or Caproic acid precipitation method according to the standard methods (Hudson and Hay, 1989). However except for removal of excess unwanted proteins that may clog the column, no additional advantage was observed in using an extra precipitation step.

SDS-PAGE

As shown in Fig (4.12) the purification of antibody from the serum proteins was confirmed using SDS page analysis. Under non-reducing conditions Ab molecules run intact and gave a single band at around 140-150 kD. Under reducing conditions it was observed that two bands were there at 50 kD and 25 kD corresponding to Heavy and light chains of antibody molecules which separate due to disruption of disulfide bridges under reducing conditions.

Fig 4.12: The SDS page of Pr-A purified antibodies. Lane (1 & 2) rabbit anti-MPAD antiserum dilution 1:40 & 1:50. Lane (3&4) 10 µg & 7.5 µg Ab under nonreducing condition. Lane (5) Standard Molecular weight marker. Lane (6&7) 10 µg & 7.5 µg Ab under reducing condition.
4.4. b. ii Purification of Hapten specific antibodies

Binding Analysis of different types of antibodies in total purified IgG

The total IgG purified using Pr-A affinity chromatography contains three different populations of antibodies which are a) anti-hapten (target) antibodies b) anti-carrier antibodies and c) normal IgG. We determined the content of different antibodies populations in the total IgG purified from the pooled sera and found that amount of different types of antibodies in the sera was a) 18-24%, b) 12-18% and c) 58-70% respectively. This was done by first passing a known amount of total IgG through a Sepharose-BSA (carrier-protein) column followed by passing it through an Affi-gel-Hapten (MPAD) column and determining the amount of antibody left and bound into the column at each step using OD$_{280}$ values.

4.4. b. iii Elisa Elution Assay

Development of a good immunoassay depends upon the homogeneity of the immunoreagents. The presence of different types of antibodies in the whole IgG fraction purified using Pr-A column made us to go for purification of Hapten specific IgG fragment from the total IgG and see if any better activity could be obtained using this fraction of antibodies. We tried to elute anti-hapten antibodies from the hapten column using normal elution buffer i.e., Glycine-HCl (50 mM, pH=2.5) but failed to get an elution of antibodies from the column. Some other elution buffers such as 2M Urea, upto 25% methanol etc. normally used for elution failed too.

Trying to find a suitable elution buffer using column consumed a lot of antibodies, time and reagents. The matrix was also rendered useless if a buffer failed to elute antibodies from the column matrix. So we tried to optimize ELISA assay for screening of elution buffers. Normal ELISA protocol was modified to incorporate an additional step after the binding of primary antibodies. Two minute of washing with excess (200 µl) elution buffer was performed after normal washing (three times with PBST and once with PBS) and a washing with pre elution buffer. This was followed by repetition of normal washing. After incubation with secondary antibodies and development of color, decrease in OD$_{450nm}$ in the wells washed with elution buffer indicated the dissociation of antibodies from the immobilized antigen as an effect of
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elution buffer washing. This decrease was taken as a measure of elution efficiency of buffer used and compared with the normal wells where no elution buffer was used. Table 4.3 shows the various elution buffers screened using this method. The elution efficiency of various buffers was determined using the equation

\[
\text{Relative elution efficiency (\%)} = \left(\frac{\text{OD}_{\text{REF}} - \text{OD}_{\text{EB}}}{\text{OD}_{\text{REF}}}\right) \times 100
\]

Where \( \text{OD}_{\text{REF}} \) is the optical density of antibodies eluted with reference buffer (PBS) and \( \text{OD}_{\text{EB}} \) is the optical density of antibodies eluted with various elution buffers. The relative elution efficiencies of various buffers screened are shown in Fig (4.13)

Table 4.3: Various Elution buffers with varying dissociation conditions

<table>
<thead>
<tr>
<th>Elution Conditions</th>
<th>ID</th>
<th>Elution buffers</th>
<th>Pre-elution wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. High &amp; low pH</td>
<td>A</td>
<td>0.1M Glycine-NaOH, pH 11.2</td>
<td>10 mM phosphate, pH 7.4</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.1M Triethylamine, pH 11.2</td>
<td>10 mM phosphate, pH 7.4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.1M Glycine-HCl, pH 2.5</td>
<td>10 mM phosphate, pH 7.4</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>0.1N HCl</td>
<td>10 mM phosphate, pH 7.4</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>0.1N NaOH</td>
<td>10 mM phosphate, pH 7.4</td>
</tr>
<tr>
<td>II. Ionic substances</td>
<td>F</td>
<td>2.5M Magnesium chloride</td>
<td>10 mM phosphate, pH 7.4</td>
</tr>
<tr>
<td>III. Chaotropic agents</td>
<td>G</td>
<td>2 M Guanidine chloride</td>
<td>Distilled water</td>
</tr>
<tr>
<td>IV. Organic solvents</td>
<td>H</td>
<td>50% ethylene glycol, pH 8.0</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>50% ethylene glycol, pH 11.2</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>5% methanol</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>15% methanol</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>25% methanol</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>5% methanol, pH 2.5</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>15% methanol, pH 2.5</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>25% methanol, pH 2.5</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>5 % methanol, pH 11.2</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>Q</td>
<td>15 % methanol, pH 11.2</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>25 % methanol, pH 11.2</td>
<td>Distilled water</td>
</tr>
<tr>
<td>V. Denaturants</td>
<td>S</td>
<td>2 M Urea</td>
<td>Distilled water</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>1 % SDS</td>
<td>Distilled water</td>
</tr>
<tr>
<td>VI. Antigen excess</td>
<td>U</td>
<td>Molar excess of the antigen (10ppm)</td>
<td>Distilled water</td>
</tr>
</tbody>
</table>

*ELISA plates and column were washed twice with pre-wash buffer solutions before giving treatment with elution buffers.
The effect of elution buffer on the immobilized antigen was also checked and it was observed that the binding of primary antibodies remains unaffected even if the antigen coated on the Elisa plate is subjected to a cycle of elution buffer washing before the primary antibody binds to the antigen surface. This confirmed that the loss of OD_{450nm} is due to the dissociation of Antibody from the coated antigen and not due to the leaching of entire Ab-Ag complex from the ELISA plate wells.
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To determine the suitability of results obtained from ELISA elution assay we compared the relative elution efficiency of different buffers, when used with immunoaffinity column with the relative elution efficiency data obtained using ELISA. The elution efficiency of the buffers was calculated by determining the total amount of antibody bound on the column and that eluted using a particular buffer, as under

\[ \text{REF} = \left( \frac{\text{Amount of antibody eluted}}{\text{Amount of total antibody bound}} \right) \times 100. \]

The buffers, which showed a minimum relative elution efficiency of 35-40% in the ELISA elution assay, were chosen for comparison of elution efficiency in affinity column. The antibodies eluted using different buffers were checked for the difference in binding activity of the various antibodies using normal ELISA assay. The results are summarized in Table 4.4.

**Table 4.4: Elution efficiency of different buffers and binding activity of antibodies eluted from hapten column using different buffers as determined by ELISA**

<table>
<thead>
<tr>
<th>S.No:</th>
<th>Elution Buffer Used for Eluting antibodies from the Immunoaffinity column</th>
<th>Efficiency of antibody elution from column (%)(^a)</th>
<th>Relative binding activity of eluted antibodies (%)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Protein-A purified Ab.</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>25% MeOH (pH=2.5)</td>
<td>80.9</td>
<td>39.7 ± 3.88</td>
</tr>
<tr>
<td>3.</td>
<td>15% MeOH (pH=2.5)</td>
<td>62.8</td>
<td>48.9 ± 2.96</td>
</tr>
<tr>
<td>4.</td>
<td>5% MeOH (pH=2.5)</td>
<td>45.8</td>
<td>84.4 ± 5.92</td>
</tr>
<tr>
<td>5.</td>
<td>25% MeOH (pH=11.2)</td>
<td>92.7</td>
<td>104 ± 8.06</td>
</tr>
<tr>
<td>6.</td>
<td>15% MeOH (pH=11.2)</td>
<td>60.7</td>
<td>92.9 ± 4.80</td>
</tr>
<tr>
<td>7.</td>
<td>5% MeOH (pH=11.2)</td>
<td>31.6</td>
<td>116.7 ± 6.79</td>
</tr>
<tr>
<td>8.</td>
<td>.1M Gly-HCl (pH=2.5)</td>
<td>32.5</td>
<td>10.9 ± 2.43</td>
</tr>
<tr>
<td>9.</td>
<td>.1M Gly-NaOH (pH=11.2)</td>
<td>61.9</td>
<td>7.70 ± 2.01</td>
</tr>
<tr>
<td>10.</td>
<td>.1M TEA (pH=11.2)</td>
<td>45.1</td>
<td>105 ± 7.88</td>
</tr>
</tbody>
</table>

\(^a\)The values are mean values of three independent elution experiments.
\(^b\)The values are mean values ±SD of three independent experiments, each done in triplicate.
This experiment proved that the antibodies produced against the BSA-MPAD antigen were high affinity polyclonal antibodies for two reasons

1) The Ag-Ab complex was not dissociated under normal dissociation conditions such as 2M Urea, Gly-HCl and high ionic strength 2.5M Magnesium Chloride, presence of organic solvent methanol (upto 25%) etc.

2) Second these antibodies were not suitable for displacement assay as they showed a higher affinity for the immobilized antigen as compare to the free antigen.

4.4.c Characterization of purified antibody.

4.4.c.i Effect of purification on the affinity constant of antibodies

The affinity constants for the polyclonal antibodies purified using different buffers from the affinity column, were determined using a solid phase ELISA based method developed by Raghava and Agrewala (1994). This method calculates the affinity constant from the dilution curve of antibodies binding with antigens immobilized on the ELISA plate at different concentrations (Fig 4.14). The software is freely available as Ab Affi from the website www.imtech.res.in/ragahva/progs/abaffi. The results showed that the antibodies varied in their affinity for the antigen when eluted using different buffers. It was observed that with increasing the amount of methanol in the elution buffer the

Fig 4.14: Dilution curve of antibodies eluted using different buffers from affinity column and their respective affinity constants are shown in the Inset box.
affinity constant showed a corresponding decrease. In general the affinity constants were in the range of 1.6-7.9X 10^8 M.

4.4.c.ii Determination of force of interaction between Ab-Ag using AFM

The antibody used for the development of immunosensor needs to be characterized first at the time of development of immunoassay under free conditions and second time when immobilized as bio-sensing element immobilized at the surface of the biosensor transducer i.e., Electrode, QCM or SPR transducer surface (kaur et al., 2004). We used Atomic force microscopy (AFM) to determine the binding of antibody molecule on the gold-coated surface usually used for immunosensors.

Determination of force distance curves

The biomolecular force interaction analysis was done using Atomic force microscopy. Rabbit anti-MPAD antibody was immobilized on gold-coated glass slides using Protein-A gold immobilization method for oriented immobilization of antibody molecules. BSA-MPAD antigen was immobilized on the silicon nitride cantilevers using Silanization method. The force interactions between the immobilized antibody and antigen (BSA-MPAD) functionalized cantilevers were studied. Interactions between protein-A layer and antigen (BSA-MPAD) functionalized cantilevers were also determined to check the specificity of antigen binding with the antibody only. As shown in Fig (4.15) the force curves were recorded in an approach/retract cycle at a rate of 1KHz both in absence and in presence of free antigen using contact mode. Pull off force was determined between antibody and antigen. The antibody-antigen interaction showed a wide range distribution of pull off forces ranging from 200 to 940 pN, with most values falling in 200-500 pN range averaging around 511.62±244.1 pN. The large SD in the pull off force values indicates the nature of the antibody to be polyclonal and presence of multiple antigen-antibody interactions. The force curve between antibodies and BSA-MPAD functionalized cantilevers in presence of saturating concentration of free antigen (Atrazine) gave a low mean pull off force of <50pN, which is significantly less compared to pull off force between antibody layer and BSA-MPAD functionalized cantilevers and confirms the specific interaction of the antibodies with the antigens.
Fig 4.15: Force curves between (A) Protein-A layer and BSA-MPAD functionalized immobilized cantilevers (B) Immobilized Anti-MPAD antibody and functionalized immobilized cantilevers and (C) Immobilized Anti-MPAD antibody and functionalized immobilized cantilevers in presence of excess free Atrazine.
The saw-tooth-like pattern of the force curves indicates the involvement of more than one binding, as the cantilever is being pulled apart. (Ros et al., 1998). The multiple binding sites on the antigen molecules and variation in the immobilization density of the antibodies are key players in determining the multiplicity and variations in the force interactions. We expected both because the BSA-MPAD used has a high hapten density, protein-A layer was formed at a high concentration and thus giving a high antibody immobilization density as well as the polyclonal nature of the antibody used lead to the great variation seen in the Ag-Ab interactions in this study. This study has already been published (Kaur et al., 2004).

4.4.c.iii Determination of antibody specificity using SPR studies

We used a surface plasmon resonance (SPR) based biosensor (IASys, Thermolabsystems, Helsinki, Finland) for detection of atrazine. IASys employs resonance mirror technique for generation of SPR signal i.e., change in angle of incidence with time and is expressed in arbitrary units of arc-seconds. This change in angle depends upon the refractive index at the sensing layer. The increase in binding of antibody molecule at the sensing layer causes a change in the refractive index of at the sensing layer (Voros, 2004) which in turn provides a corresponding shift in angle of incidence proportional to the amount of antibody bound at the surface. On an average binding of 1ng protein per mm² of the cuvette area gives a shift of 600 arc-seconds. Thus the sensor response gives a sensitive measurement of concentration/activity of biomolecules at the sensor surface.

Immobilization of conjugate on the sensor surface

It was observed that the immobilization density obtained was higher when we used a buffer with lower pH as compared to the normally used acetate buffer (50 mM, pH=5.0). We obtained a baseline shift of 400 arc seconds when using pH=5.0 buffer, which corresponds to an immobilization density of 0.66 ng/mm². The baseline shift was observed to be 970 arc-second when we used a low pH=3.0 buffer for immobilization of the conjugate on the dextran coated cuvette. Thus immobilization densities between 0.66-1.75 ng/mm² were obtained for different experiments.
Optimization of antibody binding

A typical Ab binding experiment allows for both association and dissociation of Ag-Ab immunocomplex formation to reach equilibrium interaction at the surface of the sensing layer. Fig (4.16) shows the course of Ab binding at the sensor surface. The association and dissociation curves are used for determination of the affinity constants of the Ag-Ab pair or protein-ligand interactions (Buckle et al., 1993). When relative activity has to be observed only the binding/association phase is used for comparative analysis, which saves time as the binding saturation reaches within 4-5 minutes at the sensor surface. Fig (4.17) shows the sensor response to binding of different amount Rabbit anti-MPAD antibodies at the sensor surface having an immobilized layer of OVA-HAD.

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**Ab-Ag Binding**

**Regeneration**

Fig 4.16: A typical SPR curve obtained for Ab-Ag interaction showing binding and dissociation of Ab on the Ag immobilized on SPR cuvette and regeneration of the immunosensor surface.
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Fig 4.17: SPR sensor response to binding of different amount of rabbit anti-MPAD antibodies to the immobilized HAD-conjugate. 100 μl of antibody solution was added to the test cuvette and the control cuvette. The signal from the control cuvette was subtracted from the test cuvette signal response at different concentrations of antibody. A) 6 μg/ml, B) 4 μg/ml, C) 2 μg/ml, D) 1 μg/ml and E) 0.5 μg/ml.

Antibody Sensitivity of the SPR sensor

The Ab binding response on the sensor surface in presence of atrazine is shown in Fig (4.18). The results show that the antibody used is highly sensitive to the presence of Atrazine in the sample and the antibody binding inhibition is pretty evident even at a concentration of 500 ppt atrazine.

Fig 4.18: Effect of presence of Atrazine on the binding of Rabbit anti-MPAD antibody with immobilized HAD-conjugate. The antibody binding was completely inhibited even at a concentration of 500 ng/ml concentration of atrazine.
4.4.d Biological Characterization of labeled immunoreagents.

4.4.d.i Antibody binding activity of HRP-hapten conjugates

Dot blot assay for checking the binding of HRP-hapten conjugates with various sera confirmed that antisera produced against various Hapten-protein conjugates recognized the haptons conjugated to the HRP enzyme. It was again observed that mouse anti-MPAD antisera showed lower reactivities towards the HAD and CAD-HRP conjugates while anti-HAD and anti-CAD antisera showed higher degree of cross reactivity. As shown in the Fig (4.19).

![Fig 4.19: Dot Blot assay for determination of labeling of HRP by various haptons. Strip I in for each type of sera was probed with HRP-MPAD strip II was probed with HRP-HAD and strip III was probed with HRP-CAD. Row (A) in each set had 1 µl spots of 1:100 dilutions of Normal Mouse Sera (NMS). Row (B) had 1 µl spots of 1:100, Row (C) had 1 µl spots of 1:50 and Row (D) had 1µl spots of 1:10 dilution of respective sera as indicated in each set.](image)

4.4.d.ii Recognition of colloidal gold hapten-protein conjugates by antibodies

The recognition of colloidal gold hapten protein conjugate by the antibodies was confirmed using dot blot assay.

![Fig 4.20: The binding of OVA-MPAD-nanogold conjugate with different antisera. Row A and B have 1 µl spots of 1:10 and 1:20 antisera dilution of 1) NMS 2) PBS 3) Mouse α BSA-CAD 4) Mouse α BSA-HAD 5) Mouse α BSA-MPAD antiserum.](image)
4.5 Bibliography

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