International Journal of Information Technology and Knowledge Management
July-December 2009, Volume 2, No. 2, pp. 461-464

PRODUCTION OF GOAT ANTI-RABBIT ANTIBODIES

Manish Kalra, Abhinav Goyal, Saaraj Gupta, Ketan & K Sweta*

Antibodies are a group of glycoproteins present in the serum as a part of our defence system. Specific antibodies are formed when an animal is immunized with an antigen. Antibodies can be produced experimentally by injecting any compound that contains at least one antigenic determinant and are termed as Primary Antibodies. Secondary antibody is an antibody which binds at *Fc* fragment of the primary antibody. The present work aims at purifying immunoglobulins from Rabbit serum and then using these as immunogens in Goats to develop Goat Anti-Mouse antibodies. These can be labelled as a probe that makes them useful for the detection, purification or cell sorting application. This being a highly specific reaction, antibodies has become an invaluable tool in the immunological research and clinical immunodiagnostics. Isolation of Goat Anti-Rabbit Antibodies has been successfully completed. Work is being done on production of Horse Radish Peroxidase enzyme. Further, conjugation of isolated Goat Anti-Rabbit Antibodies with Horse Radish Peroxidase is proposed to be done, so that these labeled antibodies can be used in indirect ELISA, indirect sandwich ELISA, indirect competitive ELISA, Immunostaining, and Immunocytometry.

Keywords: Antibody, Affinity Chromatography, Ion-Exchange Chromatography, Dialysis, Immunization, Double Immunodiffusion, Lyophilization.

1. INTRODUCTION

Antibodies are antigen binding glyco-proteins, secreted by plasma cells which are produced by *B* lymphocytes of the adaptive immune system. Antibodies attach themselves with one end to the antigen and other end to the phagocyte. The antigen-antibody complexes are then removed from circulation by macrophages through phagocytosis. Five distinct classes of Antibodies–*IgG*, *IgA*, *IgM*, *IgD* and *IgE* have been isolated which differ from each other physico-chemically in size, charge, amino acid and carbohydrate composition as well as immunologically. The basic structure [Frazer, J. K., and J. D. Capra. (1999)], [Schumaker V. N., Phillips ML *et al.* (1991)], [Amzel L. M., Poljak R. J. (1979)] is composed of four polypeptide chains, two identical heavy (*H*) chains, and two identical light (*L*) chains that are held together by disulfide bonds [Figure 1]. The regions of relatively constant sequence beyond the variable regions have been dubbed *C* regions, antigen-binding region are called Fragment antigen binding *(Fab region)* [Saul F. A., Amzel L. M. *et al.* (1978)]. The fragment crystallizable region (*Fc* region) is the tail region of an antibody (*Ab*) that interacts with cell surface receptors called *Fc* receptors and some proteins of the complement system. This property allows antibodies to activate the immune system. [Janeway CA, Jr. *et al.* (2001)]. Antibody response is generally observed when a foreign antigen used to immunize an animal species other than the origin of animal. Thus the antibodies can be produced by injecting an antigen into a mammal, such as a mouse, rat or rabbit for small quantities of antibody, or goat, sheep, or horse for large quantities of antibody. Blood isolated from these animals contains polyclonal antibodies—heterogeneous antibodies that bind to the same antigen—in the serum, which can now be called antiserum.

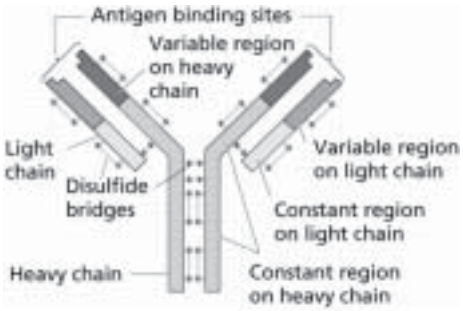


Figure 1: Structure of Antibody

An ideal antiserum should have high titre, high affinity and specificity. The affinity or Ka value should be between 10^9–10^{12} "per mole (1/mol)", then the detection range will be about 10^{-9} to 10^{-12} "mole per litre (mol/L)". Hybridoma technique is used in which tumour cells are fused with *B*-cells to produce polyclonal serum. By in-vitro culture techniques [Kohler, C. Milstein. (1975)], the cell clones which produce a specific and homogeneous antibody, can be selected to grow in-vitro and propagated to provide indefinite amount of required antibody. Antibody can be polyclonal or monoclonal. Monoclonal antibody [Schaffner G., Haase M. *et al.* (1995)] has uniform affinity, specificity and homogeneity.

Biotechnology, Kurukshetra University, Haryana, INDIA.
* Biotechnology, Uttar Pradesh Technical University, Uttar Pradesh, INDIA.
E-mail: manishkalra.20@gmail.com, gagan.bansal.15@gmail.com

2. METHODS AND MATERIAL

2.1 Colletion of Blood:

Serum is collected by inserting a vacutainer needle into the ear vein of rabbit. Swabbing the ear with cotton soaked with xylene & tapping the vein gently dilates the ear vein, making needle insertion easy. The blood is collected in a small beaker.

2.2 Preparation of Serum:

Collected blood in beaker was kept overnight in freezer. Post 24 hours, the collected serum from blood was centrifuged at slow speed and stored at –20 ºC. Serum samples from different rabbits were pooled together and processed further.

2.3 Purification of Immunogen (Rabbit *IgG*)

2.3.1 Precipitation of Proteins from Serum by Ammonium Sulphate:

Precipitation by salting out to remove non-specific protein is an effective method of crude purification of proteins. The procedure to be followed depends on a variety of experimental conditions, for example, degree of saturation, pH and temperature. Salts in solution in high ionic concentrations with strength much greater than that of tissue extract or serum will cause precipitation of the many proteins. As concentration of salt in protein solution increases after a certain level, salt begins to compete with protein for water molecules needed for their solvation. This leads to gradual precipitation of protein based upon their requirement of water molecules for remaining in solution form. For immunoglobulin purification 35%–40% ammonium sulfate is used. The concentration of ammonium sulphate is calculated by applying equation (1):

$$g = \frac{533(S_2 - S_1)}{100 - 0.3S_2} \qquad (1)$$

S_1 = Starting Concentration S_2 = Final Concentration.

A saturated solution of Ammonium sulphate (760 "Grams per litre (g/L)" at 25 "degree Celsius (ºC)" is made by dissolving in hot distilled water and cooling at room temperature. The *pH* is adjusted to 7.0 using ammonia solution and stored in presence of Ammonium sulphate crystals.

Precipitation occurs by neutralization of surface charges by the salt, by reducing the chemical activity of proteins and by diminishing the effective concentration of the water. This is known as "*salting out"* of proteins. Equal amount of saline was added to the serum followed by gradual addition of saturated ammonium sulphate solution under constant stirring at room temperature for 45 minutes. Centrifuged it at 2000 rotations per minute (rpm) in a refrigerated centrifuge for 20 minutes. The supernatant was discarded and the precipitates were re-suspended in 0.9 "Normal (*N*)" Sodium chloride. The procedure was repeated thrice.

2.3.2 Dialysis:

It is process of purification of solution containing electrolyte by keeping solution in bag made of cellophane and suspending the bag in water or buffer to remove ammonium sulphate or small molecules or ions. The solution is retained. [Figure 2]

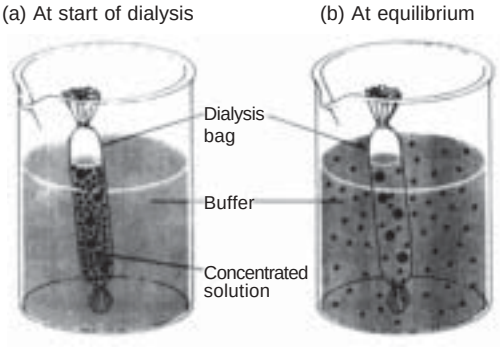


Figure 2: Purification by Dialysis

Washed the dialysis bag (10–12 "kilo Dalton (kDa)" in size) and checked for the leakage with PBS buffer. Ammonium sulphate precipitated crude rabbit *IgG* was then dialyzed in vertical position against Phosphate Buffer (*pH* 6.3) with constant stirring using magnetic stirrer for 48 hrs involving 3 buffer changes in between. The dialyzed sample was then put on ion exchange chromatography column for further purification.

2.3.3 Ion Exchange Chromatography:

Proteins can be separated on the basis of their net charge by ion-exchange chromatography [Martin, A. J. P., and Synge *et al.* (1945)], [Friesen, Bowman *et al.* (1983)]. If a protein has a net positive charge at *pH* 7, it will usually bind to a column of beads containing carboxylate groups, whereas a negatively charged protein will not. A positively charged protein bound to such a column can then be eluted (released) by increasing the concentration of sodium chloride or another salt in the eluting buffer because sodium ions compete with positively charged groups on the protein for binding to the column. Proteins that have a low density of net positive charge will tend to emerge first, followed by those having a higher charge density. Positively charged proteins (cationic proteins) can be separated on negatively charged Carboxy methyl (CM)-cellulose columns. Conversely, negatively charged proteins (anionic proteins) can be separated by chromatography on positively charged

Di-ethyl-aminoethyl(DEAE)-cellulose columns. The binding and release of compounds to ion exchange material is dependent on *pH* and salt concentration DEAE-Sephacel, an anion exchanger, was used because of its high flow rate for removing contaminating proteins, while *IgG* passes unadsorbed [Figure 2]. DEAE-Cellulose was filtered through a coarse filter using distilled water. The cake was transferred to beaker and 0.3 "Mole per litre (M/L)" KH_2PO_4 as added, and filtered through a funnel. It was again resuspended in 0.3 "Mole per litre (M/L)" Potassium dihydrogen phosphate KH_2PO_4 and aspiration was repeated. After washing with distilled water, the cellulose was suspended in 0.5 "Normal (*N*)" Sodium hyroxide and allowed to stand 3 hours, the alkali was aspirated through the funnel and the cellulose was washed with 0.5 "Normal (*N*)" sodium hyroxide. The resin was allowed to stand in a beaker with ethanol for 2 hours, washed once with ethanol, once with 0.5 "Normal *N*" sodium hyroxide and few times with water. The fraction was dialyzed for 2 hour against 100 "MilliMolar (*mM*)" phosphate buffer *pH* 8.

2.3.4 Protein-G Sepharose Affinity Chromatography :

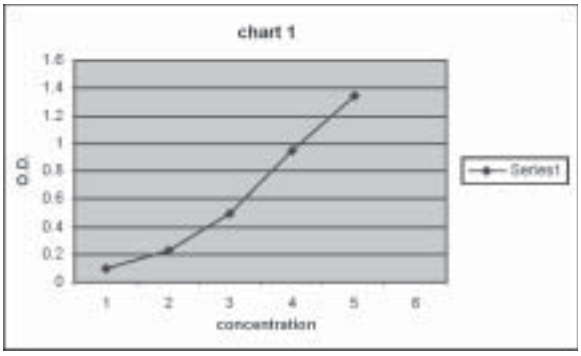
Affinity chromatography separates proteins by their binding specificities. The proteins retained on the column are those that bind specifically to a ligand cross-linked to the beads. After proteins that do not bind to the ligand are washed through the column, the bound protein of particular interest is eluted (washed out of the column) by a solution containing free ligand. Protein *G* can bind all subclasses of human *IgG* and *IgG* over a wide range of species. Protein *G* is a cell surface-associated protein from streptococcus [Sjobring U., Bjorck L., *et al.* (1991)] that binds to *IgG* with high affinity. It has three highly homologous *IgG*-binding domains. Protein *G* is an immunoglobulin-binding protein expressed in group *C* and *G* Streptococcal bacteria much like Protein *A* but with differing specificities.

2.3.4.1 Bradford Assay:

Estimation of the amount of the protein [Stoscheck, C. M. (1990)] to be loaded on the column in the fraction was done by Bradford Reagent [Bradford, M. M. (1976)]. A standard graph of Bovine serum albumin (BSA) reagent was prepared with three different concentrations of the sample [Table 1]. From the standard graph [graph 1], calculated the protein concentration in the sample.

Table 1
Protein Estimation by Bradford Reagent

S. No.	*1*	*2*	*3*	*4*	*5*
Conc. (ug/ml)	50	100	200	400	800
O.D. 595 nm	0.102	0.224	0.492	0.945	1.34



Graph 1 : From the Standard Graph [graph 1], Calculated the Protein Concentration in the Sample.

2.3.4.2 Purification:

Protein *G* Sepharose column [Janis U., Regmer F. E. (1989)] was washed with Glycine-HCl buffer and then equilibration of column was done with 100 "MilliMolar (mM)" phosphate buffer. The Immunoglobulin fraction sample obtained from DEAE-Sephacel column was loaded on the column, allowed to be adsorbed and unbound sample was taken in separate beakers. [Figure 3] Elution is done by Glycine-HCl buffer and 6 fractions were collected in different glass tubes containing Tris-HCl. The presence of proteins in a fraction was done by using Bradford reagent [Table 2].

Table 2
Protein Quantification by UV-VIS Spectrophotometry

Fraction	*O.D.(280nm)*	*Fraction*	*O.D.(280nm)*
B.F. 1.	0.1	E.F.1.	0.7
2.	0.29	2.	0.6
3.	0.161	3.	0.491
4.	0.05	4.	0.3
5.	0.0	5.	0.06
6.	0.0	6.	0.0

Different fractions were pooled and these were dialysed using 0.9% ammonium carbonate and dialysate was lyophilized [Theunissen, E. Stolz, *et al.* (1993)].

2.4 Immunization of Goats:

Prior to immunization of the Goats, control serum was collected by inserting a 22 gauge needle into the jugular vein of goat. Primary dose containing normal rabbit *IgG*, isotonic saline and Freund's Complete Adjuvant (FCA) was emulsified by repeatedly sucking in and out using syringe to form a stable emulsion. The preparation of the immunogen was checked in the cold saline and then injected intradermally. Booster of normal rabbit *IgG*, saline and Freund's Incomplete Adjuvant (FIA) was given after one month [Table 3]. Equal volumes of the emulsion were given on both sides of the neck via subcutaneous route. On seventh day after giving the booster, the test bleed was collected

from jugular vein. The serum, thus, separated was aliquoted into vials, stoppered and stored at –20 "Degree Celsius (°C)".

Table 3
Goat Immunization Record

Injection	*Antigen (Rabbit IgG)*	*Adjuvant 100ug/ml*	*Volume*	*Route of Injection*
Primary Dose	4 mg	FCA	3 ml	Intradermal
Booster 1	2 mg	FIA	3 ml	Intramuscular
Booster 2	2 mg	FIA	3 ml	Intramuscular

The test bleed was checked for the presence of antibodies by Double Immuno Diffusion method [Ouchterlony, O. and Nilsson, L. A. (1986)]. The second booster was given to the experimental animals after 15 days of the first booster. After 7 days of the second booster, sample bleed was collected for the purification of the antibodies. These antibodies were further purified by the above mentioned purification protocols and then lyophilized.

3. Observations and Conclusion

The titre of Goat Anti-Rabbit *IgG* antibody was calculated by Double Immuno Diffusion assay. Solution of Agarose, phosphate buffer containing Normal saline and Sodium azide (0.03%) was mixed and then boiled. Prepared the Petri dishes. Let it solidified as a gel. Using a glass borer or plastic tip, wells at the center and at sides were bored according to the pattern planned. Prepared Normal Rabbit Gamma Globulin (NRGG) and made different dilutions of antiserum with phosphate buffer and these were poured in different wells. Line of identity was observed between wells which shows the presence of the anti-rabbit gamma globulins in the sample.

4. Results and Discussion

Line of identity was observed between well 5 i.e. 1:10 Goat Anti Rabbit Serum and NRGG and in well 4 i.e. 1:20 Goat Anti Rabbit Serum and NRGG. Thus, it shows the presence of the anti-rabbit gamma globulins in the sample.

No line of identity was observed beyond 1:20 dilution of the antisera. Therefore, Ab Titre is 1:20.

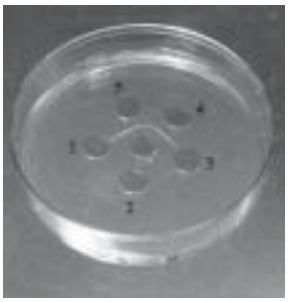


Figure 3: Result of Double Immuno Diffusion

Well 1-1:10 diluted normal Goat Serum.

Well 2- 1:50 diluted sample (Goat Anti Rabbit Serum)

Well 3- 1:30 diluted sample (Goat Anti Rabbit Serum)

Well 4- 1:20 diluted sample (Goat Anti Rabbit Serum)

Well 5- 1:10 diluted sample (Goat Anti Rabbit Serum)

Middle Well- NRGG (Normal Rabbit Gamma Globulin)

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