USING AFFINITY CHROMATOGRAPHY AND WESTERN BLOTTING – THE PURIFICATION OF POLYCLONAL ANTIBODY FROM RABBIT ANTISERA

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Antibodies are considered to be as antigen binding proteins that are present on the B-cell membrane and secreted by plasma cells. They are also called glycol-proteins. The main objectives of the work are: (1) Affinity purification of Anti E.Coli lysate antibody using Protein A Resin. (2) Affinity purification of partially purified Anti E.Coli lysate antibody using CNBr activated Sepharose 4B resin. (3) To check the purity of purified Anti E.Coli lysate antibody using SDS PAGE and Western Blot. (4) FITC labeling of purified antibody and removal of unbound FITC through GFC. Protein A is a wall component of Staphylococcus aureus that binds to Fc portion of IgG. The methods adopted are: (1) Pack the column using Protein A Resin; (2) Boric acid is used to equilibrate the column (10 column volume); (3) Then apply a small sample (1 ml) of Antisera; (4) Wash the column with 5 column volume of buffer A; (5) Elution was done using Buffer B. Collect the fractions into titrating diluent (e.g 1.0M Tris-Cl) and take the O.D. at 280 nm; (6) Pool the sample which shows the maximum O.D. and concentrated it in 10 k D centricon. In the present work, the author has successfully purified unlabelled as well as labelled protein (antibody) by Affinity Chromatography. The immune-activity of the purified antibody was confirmed by Western Blotting. Students of senior secondary level have been oriented using chromatographic techniques.

Key word: *Protein A, affinity chromatography, western blotting, SDS PAGE, boric acid.*

Introduction

Antibodies are hetero-dimers. It has a structure of four peptide chains having two identical light (L) chains of molecular weight 25,000Da and two identical heavy (H) chains of molecular weight 50,000Da. H and L chains are also regarded as immune-globulins. Light chain is linked to heavy chain by a disulphide (-S-S-) bond and by non-covalent structures such as salt linkages, hydrogen bonds and hydrophobic bonds to form a hetero-dimer (H-L). Similarly, two identical H and L chain linked by non-covalent interactions and disulphide bridges to form basic four chain (H-L)2 structure, i.e, a dimer of dimers. There

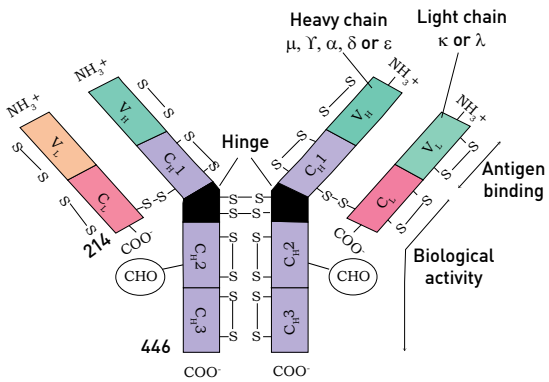

Fig. 1. Diagram of structure of immunoglobulin

are segments of highly variable sequence called V regions in both light (VL) and heavy chains (VH) respectively. Complimentary determining region (CDR) constitutes the antigen binding site of antibody molecule.

The above Fig. 1 is a diagram of structure of immunoglobulin derived from amino acid sequencing studies. Each heavy and light chain in an immunoglobulin molecule contains an amino-terminal variable (V) region (aqua and tan, respectively) that consists of 100–110 amino acids and differs from one antibody to the next. The remainder of each chain in the molecule—the constant (C) regions (purple and red)—exhibits limited variation that defines the two light-chain subtypes and the five heavy-chain subclasses. Some heavy chains (γ, δ, and α) also contain a proline-rich hinge region (black).

When the antibody IgG is treated with the enzyme papain it produces three fragments, two of which are regarded to be as identical fragments and the third is quite different. The fragments that are identical has the capability to bind to the antigens and are called Fab fragments (fragment antigen binding). The third fragment plays no role in antigen binding activity. It was found that the fragments crystallise during cold storage and regarded as Fc fragment, i.e., fragment crystallisable. When the IgG molecule is treated with mercaptoethanol, it breaks into heavy and light chains that are linked by a disulphide bond. The enzyme pepsin cleaves the IgG molecule below these bonds and the enzyme papain cleaves the bonds linking heavy chains.

The fragments produced by various treatments are also indicated. Light (L) chains are in gray and heavy (H) chains in blue.

Methods and Procedure

Affinity Purification of Anti E.Coli Lysate Antibody using Protein A Resin

1. Pack the column using Protein A Resin.
2. Boric acid is used to equilibrate the column (10 column volume).
3. Then apply a small sample (1 ml) of Antisera.

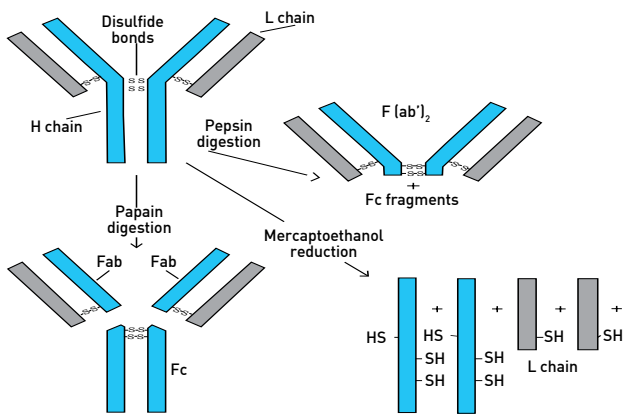

Fig. 2. Prototype structure of IgG, showing chain structure and inter-chain disulfide bonds

4. Wash the column with 5 column volume of buffer A.
5. Elution was done using Buffer B. Collect the fractions into titrating diluent (e.g., 1.0M Tris-Cl) and take the O.D. at 280 nm.
6. Pool the sample which shows the maximum O.D and concentrated it in 10 kDcentricon.

Affinity purification of partially purified anti E.Coli lysate antibody using CNBr activated Sepharose 4B resin

It is a medium used for the immobilisation (the covalent attachment of an enzyme on to the solid matrix) of ligands that contains primary amines. The resulting coupling reaction is spontaneous, rapid and easy to carry out.

To check the purity of purified Anti E.Coli lysate antibody using SDS PAGE and western blot

1. Take glass plate, notch plate and gasket and they should be properly wiped with methanol.
2. Put the notch plate down, cover the plate with the gasket on the walls of notch plate and properly put glass plate to it and fix it with clamps.
3. Perform leak test by pouring WFI between the glass plate and notch plate. Discard and soak it with Whatman filter paper.
4. Add resolving gel between the plates and a layer of saturated butanol so that the gel should not dry.
5. After 20 minutes, discard butanol, add stacking gel (4% acrylamide) and immediately place a comb over it so that the wells should form properly in which we are going to load the protein samples.
6. Take the running unit and in the lower and upper chamber add running buffer to it. Now load the protein samples into the wells and allow for electrophoretic run. The time taken by the gel for complete elution is 11/2 or 2 hrs. When the gel elutes, place it in the staining solution and perform silver staining.

FITC labelling of purified antibody and removal of unbound FITC through GFC.

Mix 500 mcl of Protein A purified antibody (Concentration 2mg/ml) and add 500 mcl of bicarbonate (HCO3) buffer, pH 9.0. So, this gives us the final pH 9.0 of the solution.

Making 20mg/ml solution of FITC in ethanol: Weigh about 2.0 mg of FITC and add 100 mcl of ethanol to it and cover the eppendorf with aluminium foil as FITC is light sensitive.

Labelling of Protein A purified antibody with FITC: Add 52.6 ml of above FITC solution (20 mg/ml) to 1.0 ml of Protein A purified antibody, pH 9.0 in a 2.0 ml eppendorf and cover it with aluminum foil as it is light sensitive and this will make 2.5 mM concentration of FITC in the final system. Incubate it for one hour at room temperature.

Results and Discussion

Affinity Purification of anti-lysate Antibody using Protein A Resin

Purification was based on affinity chromatography and Protein A was used

as a ligand which binds to Fc portion of immunoglobulin.

It was observed in Table 1 that out of the 19 fractions, fractions of the purified antibody that lies between 6-10 gives maximum absorbance at 280nm and those fractions were pooled and protein sample was concentrated using 10 kD centricon.

Table 1

Absorbance at 280 nm for Eluted Fraction of Protein A Purified Antibody

Fraction No	Absorbance at 280 nm	Fraction No	Absorbance at 280 nm
1	-0.011	11	0.027
2	-0.005	12	0.067
3	-0.002	13	0.093
4	-0.01	14	0.075
5	-0.006	15	0.04
6	0.08	16	0.024
7	1.229	17	0.012
8	1.385	18	0.004
9	0.223	19	-0.011
10	0.035		

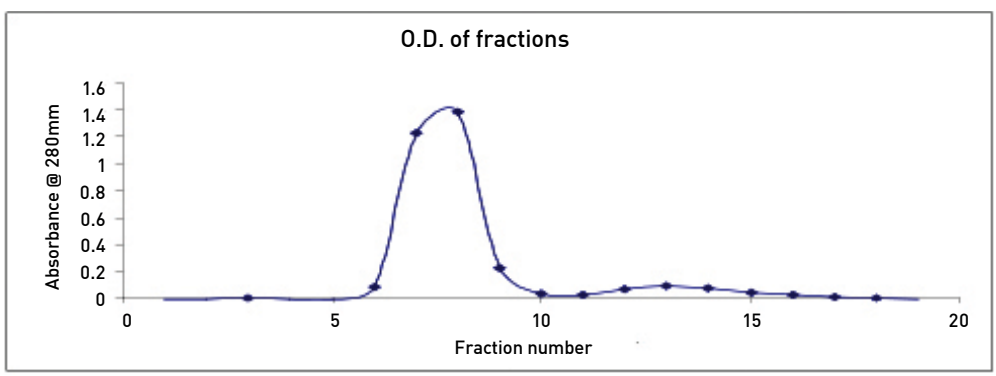

Fig. 3. Graphical representation of absorbance of protein A purified antibody

Affinity Purification of partially purified anti-lysate Antibody using CNBr activated Sepharose 4B.

Table 2

Absorbance at 280 nm of Eluted Fraction of CnBr Purified Antibody.

Fraction No	Absorbance at 280 nm	Fraction No	Absorbance at 280 nm
1	0	14	0.56
2	-0.003	15	0.54
3	-0.005	16	0.05
4	0.008	17	0.049
5	-0.002	18	0.047
6	0	19	0.043
7	0.046	20	0.042
8	0.096	21	0.046
9	0.125	22	0.045
10	0.113	23	0.04
11	0.092	24	0.039
12	0.072	25	0.04
13	0.57		

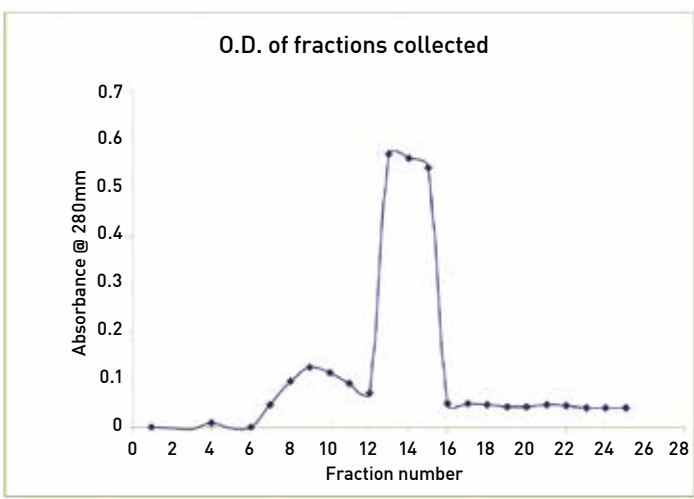

Fig. 4. Graphical representation of absorbance of CNBr purified antibody

To enhance the further purification of antibody that was obtained using Protein A, we followed the purification using CNBr Sepharose 4B as a resin. HCl was added to clean the resin. PBS was used to equilibrate the column. Stringency washes were done using 0.1M acetate, 0.1M Tris-Cl and 0.5M NaCl. Antiserum (1.0ml) was loaded and diluted it with 1X PBS. 0.1M Tris-Cl, 0.5M NaCl having pH 6.0 and 8.0, respectively, was used to remove non-specific binding of other proteins present in serum. Elution was done using 0.1M Glycine-HCl. Fractions 7-12 were pooled (mixed) as these fractions give maximum absorbance at 280nm and the protein sample was concentrated using 10 kDcentricon.

SDS PAGE and Western Blot to check

Purification achieved at each step: Purification was achieved in six steps. Antiserum loaded in the first well, then PBS wash-1 and PBS wash-2 in second and third. Fifth well consists of prestained marker. In sixth and eighth, CNBr purified antibody (NR) and CNBr purified antibody (R), respectively. When antibody is treated with non reducing agent, it gives the purified form of antibody because when it treated with reducing agents like DTT it dissociates into two heavy and two light chains which give the different bands on SDS-PAGE gel.

Purified antibody by western blot and silver staining: To check the effectiveness of the purified antibody, we use western blot.

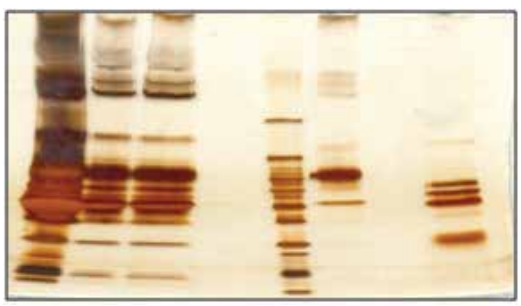

Fig. 5. SDS-PAGE Profile of Samples from Purification Process

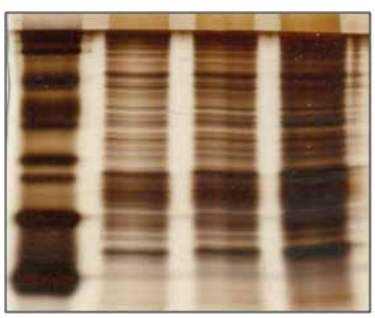

Fig. 6. SDS-PAGE Profile of E.coli lysate

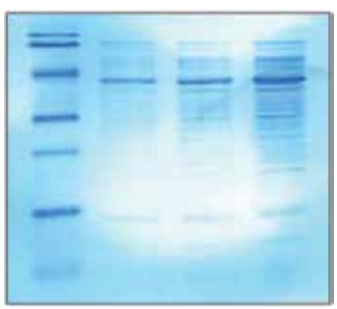

Fig. 7. Western profile of E.coli lysate

Table 3

Loading amount of SDS-PAGE and Western blotting

Lane	Sample ID	Sample buffer.	Loading Amt. (µg)
1	Prestained Marker	–	7µl
2	E.coli lysate	5X R	2.5µg
3	E.coli lysate	5X R	5µg
4	E.coli lysate	5X R	10µg

Primary antibody purified anti-lysate antibody and the secondary antibody was goat anti-rabbit HRP labelled antibody and was used to check the purity and the identity of the purified antibody.

As observed in Fig. 5, CNBr purified antibody was capturing the whole range of band which was detected in SDS-PAGE silver staining (Fig. 4), so this purified form of antibody is against this E.Coli lysate.

FITC labelling of purified antibody and removal of excess FITC using GFC

Labelling of purified antibody using FITC: Protein of interest is labelled using FITC. FITC has an absorbance at 495nm and protein has an absorbance at 280nm. Only those fractions were collected in which the protein bound to FITC, means that gives absorbance at 495 and 280nm, respectively, and the fractions were 3-7 and the protein sample was concentrated using 10 kDcentricon.

Removal of excess FITC using GFC: O.D. of FITC and protein gives maximum absorbance at fraction 5. When we overlap the fractions of proteins and FITC, the combination of it gives the maximum absorbance at fraction 5. As the number of fractions increases (Fraction no. 15–17), the O.D. at 280 nm was increasing slowly while O.D. of 495 was increasing very fast so this fraction contains only FITC molecule.

Table 4

Absorbance at 280 nm and 495 nm of Eluted fraction of GFC

Fraction No	O.D at 280 nm	O.D at 495 nm
1	-0.074	-0.044
2	-0.074	-0.04
3	-0.073	-0.043
4	0.819	0.88
5	1.988	2.106
6	0.305	0.348

7	0.016	-0.013
8	0.173	-0.03
9	0.242	-0.038
10	0.122	-0.041
11	0.005	-0.034
12	-0.058	-0.032
13	-0.076	-0.008
14	-0.056	0.074
15	0.105	0.555
16	0.184	0.873
17	0.291	1.246

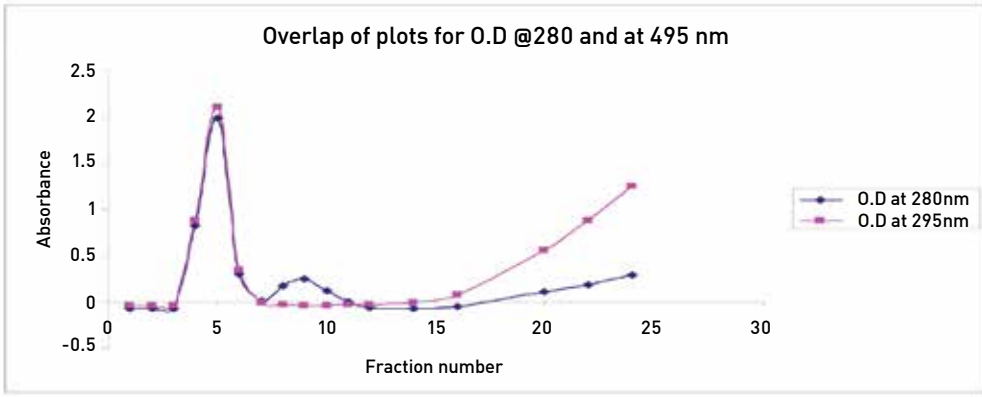

Fig. 8. Graphical Representation of Absorbance of O.D. 280 and 495 value of fraction of GFC

While fraction 8-11 has absorbance at 280 nm only so it may contain unbound protein or any other impurity.

Conclusion

In the present work we successfully purified unlabelled as well as labelled protein (antibody) by affinity chromatography. The immunoactivity of the purified antibody was confirmed by western blotting. The techniques used in the antibody purification can be correlated with the students of senior secondary level which can be studied.

References

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