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Immunoaffinity Chomatography

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1. Introduction

Recent developments in recombinant DNA technology have enabled the synthesis of valuable therapeutic proteins in bacterial cells as well as in novel eucaryotic expression systems. However, the purification of proteins of interest from either the conventional sources, cell culture, or novel routes in a highly purified form necessitates the development of separation techniques capable of recovering proteins from these feed streams in a highly purified form *(1,2)*. Purification of therapeutic proteins from biological sources is usually complicated by the presence of endogenous proteins (2). Purification methodologies based on ion exchange or adsorption serve as excellent prepurification steps, but they fail to resolve complex protein mixtures to yield a homogeneous protein product **(1)** Purification techniques based on affinity interactions between molecules (i.e., immunoaffinity chromatography, IAC) have rapidly evolved using a variety of biological and synthetic ligands *(2)*.

1.1. Immunoaffinity Chromatography

Itnmunoaffinity chromatography is a process in which the binding affinity of an antigen (Ag) to a parent antibody (Ab) is utilized as a basis of separation. The antibody specific to the protein of interest is immobilized onto a rigid solid support to yield an active immunosorbent. A complex mixture of proteins is then passed over the immunosorbent whereby the antibody captures the protein of interest and the other nonproduct proteins are washed away in the column fall through *(1,5)*. The reversible interaction between the antigen and antibody can be disrupted to yield a highly purified product in the column eluate (2). This could be achieved by changes in pH or use of chaotropes such sodium or potassium thiocyanates/ureas as eluents. Due to the customized avid-

ity and specificity, monoclonal antibodies (mAbs) have become indispensable for both protein characterization and purification *(5)*.

Immunoglobulin (IgG) is an approximately 150,000 Dalton molecular weight glycoprotein. The association constant for the binding of antigens to antibodies (free to bound) is about 10^{-8}–10^{-10} M. The symmetry of the Fab fragments on the antibody molecule predetermines a theoretical antigen binding stoichiometry of 2:1 (molar basis) *(3)*. Primary amines (lysine residues) are found throughout the antibody molecule, whereas carbohydrate (CHO) moieties are mainly found in the Fc region. Most of the linker chemistries engineer covalent attachment of the antibody via the primary amines or the CHO residues.

1.2. Support Characteristics

The immunosorbent performance is dependent on the support matrix on which the antibody is immobilized. Efficient immunosorbents should possess mechanical/physical stability, good flow properties, acceptable pressure drop, minimal nonspecific binding, surface area for Ab–Ag interactions, and chemical stability *(10)*. Polymeric and/or agarose based supports are extensively used in affinity chromatography. Both of the aforementioned supports lend themselves to a variety of conjugation chemistries and offer reasonable physicall mechanical properties and are resistant to the various solvent systems used in affinity chromatography. The functioning of an immunosorbent column is dependent on the activation chemistry used to couple the antibody to the matrix.

2. Materials

2.1. Chromatographic Columns

Use jacketed columns from Pharmacia Biotech. These glass columns come in different sizes complete with flow adaptors, jackets, and fittings. Please read the instructions accompanying these columns for proper assembly.

2.2. General Laboratory Equipment

1. Bench-top laboratory centrifuge (operating speed 500–1000 rpm)
2. End-to-end rotator
3. Polypropylene bottles (Nalgene)
4. Masterflex peristaltic pumps

2.3. Reagents

All reagents are purchased at the best quality available. Use analytical-grade reagents and distilled water. All buffers must be filtered and degassed prior to use. Activated matrices supporting different activation chemistries are commercially available and can be purchased from appropriate vendors. Emphaze™ was purchased from Pierce Chemical Co. and Affiprep™ from Bio-Rad Laboratories.

2.4. Apparatus

Assemble a chromatographic station required for running the IAC that includes a peristaltic pump (Masterflex, Cole-Palmer), which supports flow rates between 0.5–5 mL/min, a flow-through UV-absorbance monitor (Bio-Rad or Rainin), chart recorder, columns with flow adaptors, and a fraction collector. Configure the system to enable sequential delivery of the feed and buffers through the pump, column, UV detector and fraction collector (see **Fig. 1).**

2.5. Antibody Sample Preparation

1. For a lyophilized antibody, dissolve the antibody in an antibody-coupling buffer. Make sure that the lyophilization buffer does not contain amines such as glycine or Tris.
2. For an antibody in solution (samples in buffers, IgG fractions, and so forth): Make sure that the antibody solution does not contain primary amines such as Tris or glycine. Remove any primary amines present by dialysis or gel filtration.

2.6. N-Hydroxysuccimide (NHS) Activation

1. NHS activated matrix (Note: Activated beads are supplied as a suspension in ethanol as 5070 (v/v) solution).
2. Antibody-coupling buffer (0.1 M 4-mortholinepropanesulfonic acid (MOPS),0.1 M NaCl, pH 7.2).
3. Blocking buffer (1 M ethanolamine, pH 8.0).
4. Ligand-loading buffer (10 mM Tris-HCl, 50 mM NaCl, pH 6.8).
5. Distilled water (Keep cold at 4°C.).

2.7. EMPHAZE™

1. Emphaze activated matrix.
2. Antibody-coupling buffer (0.05 M sodium phosphate, 0.75 M Na$_2$SO$_4$, pH 7.0).
3. Blocking buffer (1 M ethanolamine in 0.05 M sodium pyrophosphate, pH 9.3).
4. Ligand-loading buffer (10 mM Tris-HCl, 50 mM NaCl, pH 6.8).

3. Methods

3.1. Coupling Through Primary Amino (-NH$_2$) Groups

3.1.1. NHS Activation

1. Invert the bottles containing the activated matrix gently to obtain a well-mixed slurry. Pour 500 mL of well-mixed slurry on a sintered glass funnel and then suck the liquid through by gentle vacuum suction. This amount of slurry will yield a final sorbent volume of 250 mL. Stir the suspension with a glass rod to disperse the gel and take care to keep the gel moist at all times.
2. Wash the activated resin with 1.5 L of ice cold distilled water.. Repeat the wash step four to five times with distilled water to ensure the removal of ethanol. Drain water using gentle vacuum and transfer the moist gel to a 2-L Erlenmeyer flask
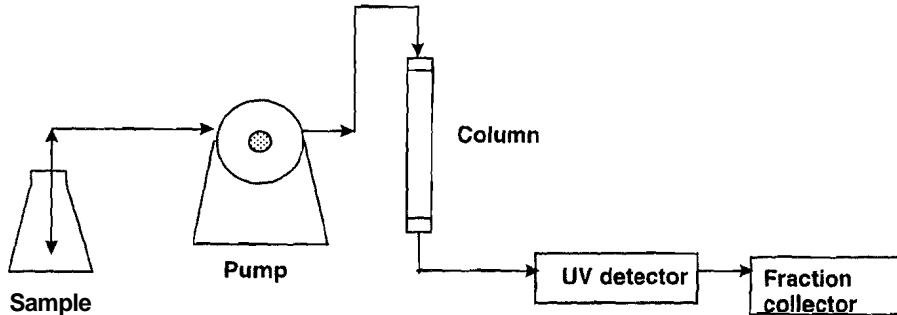
Fig. 1. Chromatographic apparatus and setup.

containing 1000 mL of a 3–5 mg/mL of antibody solution in coupling buffer. Carry out this step in the cold room. Place a medium-sized magnetic stir in the flask. Place the flask with the contents on a shaker and mix the contents on low speed for 24 h in the cold room or for 4 h at room temperature. Avoid vigorous shaking as that may lead to mechanical damage of the activated matrix (see **Fig. 2** for antibody coupling reaction).

3. Allow the gel to settle after completion of the coupling step (i.e., **step 2)** at room temperature (RT). Remove the supernatant by aspiration or decanting. Use care to retain beads in bottle. This supernatant should be saved for the determination of the amount of uncoupled antibody. The chemical compound released upon the reaction of the activated ester and the reactive amine to form a stable amide linkage interferes with A_{280} measurement and hence measuring A_{280} nm of the supernatant cannot be reliably used to estimate the amount of the uncoupled antibody.

4. Add 2.5 L of blocking solution to the immunosorbent and mix the contents of the flask on a shaker at low speed for 1 h at room temperature. Upon completion of the blocking step, allow the gel to settle at RT and remove the supernatant by aspiration or decanting. Use care to retain beads in bottle. To ensure complete blocking of unused activated sites, repeat the blocking step two times as before. The blocking-step supernatant should be saved for the determination of unbound antibody.

5. Add 1000 mL of ligand-coupling buffer to the antibody-coupled beads and mix the contents of the flask on a shaker at low speed for 1 h at room temperature. Repeat **step 5** four to five times to ensure that all of the blocking solution has been removed. Suspend the immunosorbent in 500 mL of ligand-coupling buffer after the final wash step and store at 4°C until further use.

6. Immunosorbent thus prepared is ready for use and can be packed in columns by gravity. In the event that the immunosorbent is being prepared for future use, store the immunosorbent in ligand-coupling buffer containing 0.02% sodium azide.
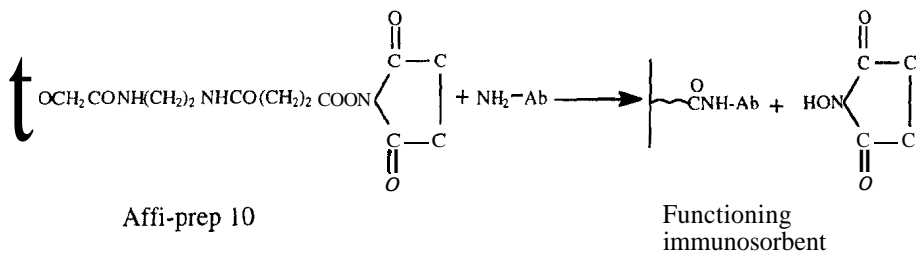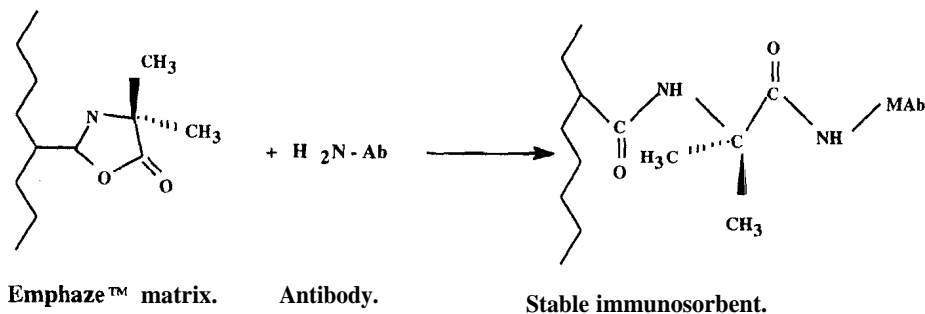
Fig. 2. Antibody coupling to NHS-activated matrix.

Emphaze™ matrix.   Antibody.   Stable immunosorbent.

Fig. 3. Antibody-coupling reaction to Emphaze.

3.1.2. EMPHAZE™

1. Refer to the individual lot swell volume value to determine the quantity of beads needed for the column. Typically 70–100 mg of dry beads give 1 mL of swelled gel. Weigh out 25 g of dry Emphaze to yield a final sorbent volume of 250 mL and transfer it to a 1-L polypropylene bottle (*see* **Fig. 3** for antibody coupling reaction). Take necessary precautions in handling dry sorbent.

2. Prepare 125 mL of an antibody solution at a concentration of 3–5 mg antibody per milliliter of coupling buffer and add it directly to the weighed sorbent in the bottle. Use antibody-coupling buffer to prepare the antibody solution. *There is no need to preswell the beads prior to use; the coupling buffer will swell the bends.* Gently rock or rotate the bottle to keep the beads suspended and carry out the coupling reaction for solution for 1 h at RT or for 24 h at 4°C. Avoid using magnetic stir bars because their use may damage the beads.

3. Centrifuge the contents of the bottle at 1200g for 5–10 min in a bench-top laboratory centrifuge until beads are pelleted. Centrifugation may be done at RT. Remove supernatant by aspiration or decanting. Use care to retain beads in bottle. This supernatant may be used to determine of the amount of ligand not coupled to the beads. Due to use of Triton X-100 surfactant in production of the beads, there may be interference with an $A_{280\ nm}$ measurement of the uncoupled protein.

4. Add 500 mL of blocking solution directly to the bottle to quench the unreacted azlactone sites. Gently rock or rotate the bottle for 2.5 h. Centrifuge the contents

of the bottle at $1200g$ for 5–10 min or until beads are sedimented. Repeat the blocking step three times as outlined in **step** 4 to ensure complete inactivation of unused azlactone sites. Remove and save supernatant after centrifugation to determine of unbound antibody. Decant the blocking solution at the end of the blocking steps to yield a moist immunosorbent.

5. Resuspend the immunosorbent in 500 mL of PBS. Rock or rotate sample to keep beads suspended in wash solution. Centrifuge samples as in **step 4.** Repeat **step** 5 three to four times and decant the supernatant to yield a moist immunosorbent.

6 Resuspend the immunosorbent in 500 mL of 1.0 M NaCl. Use of a high-salt wash solution, such as 1.0 M NaCl, will remove nonspecifically attached protein. Rock or rotate sample to keep beads suspended in 1.0 M NaCl for 30 min. Centrifuge samples as in **step** 4.

7. Resuspend immunosorbent in 500 mL of ligand-coupling buffer. Immunosorbent thus prepared is ready for use and can be packed in columns by gravity. In the event that the immunosorbent is being prepared for future use, store the immunosorbent in ligand-coupling buffer containing 0.02% sodium azide.

3. Typical Protocol for Immunoaffinity Chromatography

This is a typical protocol for the purification of antigen on an antibody column. Some antigens require more or less stringent conditions for dissociation from an immobilized antibody and conditions for elution may have to be determined experimentally.

3.2.1. Preparation of the Immunoaffinity Column for Use

1. Bring the prepared immunosorbent to room temperature.
2. Assemble a column with adapters. Pour the beads from the top with the help of a funnel and when packed place the top adapter and connect the tubing (see **Fig. 1).** A jacketed column from Pharmacia with an internal diameter of 5.0 cm is recommended
3 Equilibrate the column with 10 column volumes (cv) of antigen-loading buffer such as 10 mM Tris-HCl buffer, 50 mM NaCl, pH 7.0

3.2.2. Sample Application and Elution

1. Clarify and centrifuge the crude sample containing the antigen to be purified by low-speed centrifugation and filtering through a 0.45- to 0.2-µm filter. Failure to remove cell debris and particulate matter from the feed sample may lead to clogging and fouling of the immunosorbent. This may lead to shorter shelf life of the column.
2. Dilute the crude sample containing the antigen to be purified to total protein concentration 5 mg/mL with the antigen-loading buffer. Use OD_{280} nm to measure the total protein concentration of the crude sample.
3. Load the sample to the packed immunosorbent at a flow rate of 1 mL/min. Operate the column between linear flow rates (u) of 0.75 to 1.5 cm/min. For most

chromatographic operations u is defined as the volumetric flow rate in mL/min $\{Q\}$/cross-sectional area of the column in cm^2 {A} where $A = 3.14 \, (R^2)$; R is the radius of the column in centimeters.

4. Wash with the antigen-binding buffer until baseline absorbance at 280 nm is reached.

3.3. Selection of an Elution Strategy

   Antigens and antibodies are bound to each other by a web of forces, which include ionic bonding, hydrophobic interactions, hydrogen bonding, and van der Waals attractions. The strength of Ag:Ab complexes depends on the relative affinities and avidities of the antibodies. In addition, steric orientation, coupling density, and nonspecific interactions can also influence the binding. The objective of the elution step is to recover the specifically bound protein at a high yield, purity, and stability. Elution conditions, which might denature the protein product, have to be avoided. Examination of the current literature suggests a wide variety of elution conditions and the choice of an eluant seems empirical. However, a logical sequence of available elution strategies can be considered when selecting an appropriate elution protocol. The logical sequence as follows:

1. Specific elution: Certain antibodies bind to their respective antigens under high pH or in the presence of metals like calcium or magnesium or in the presence of chelating agents like EDTA. Antigens bound to such antibodies can be eluted under gentle conditions where lowering the pH or adding EDTA to the elution buffer or adding divalent metals to the elution buffer causes the Ag:Ab complex to disassociate.

2. Acid elution: This is the most widely used method of desorption and is normally very effective. The commonly used acid eluants are glycine-HCl, pH 2.5, 0.02 M HCI and sodium citrate, pH 2.5. Upon elution, quickly neutralize the pH of the eluant sample to 7.0 with 2 M Tris base, pH 8.5, to avoid acid-induced denaturation. In some cases increased hydrophobic interactions between antigen and antibody gives low recovery with acid elution. Incorporating of 1 M propionic acid, or adding 10% dioxane or addition of ethylene glycol to the acid eluant, is effective in disassociating such complexes.

3. Base elution: It is less frequently employed than acid elution. Typically, 1 M NH_4OH, or 0.05 M diethylamine, pH 11.5 have been employed to elute membrane proteins (i.e., hydrophobic character) and other antigens that precipitate in acid but are stable in basic conditions.

4. Chaotropic agents: These agents disrupt the tertiary structure of proteins and, therefore, can be used to disrupt the Ag:Ab complexes. Chaotropic salts are particularly useful as they disrupt ionic interactions, hydrogen bonding, and sometimes hydrophobic interactions. The relative order of the effectiveness of chaotropic anions is $SCN^->ClO4^->I^->Br^->Cl^-$. Chaotropic cations are effective in the order of Gu>Mg>K>Na. Eluants such as 8 M urea, 6 M guanidine-HCl, and

4 M NaSCN are effective in disrupting most Ag:Ab interactions. To avoid and minimize chaotropic salt-induced protein denaturation, rapid desalting or dialysis of the eluant is advised.

3.4. Regeneration of the Immunoaffinity Column

Wash with 3–4 cv of the 4 M NaCl or glycine-HCl buffer. Store at neutral pH in water or buffer containing a preservative, 0.02% sodium azide.

4. Notes

1. Before beginning antibody immobilization on commercially available matrices, a decision regarding the final immobilized antibody density has to be made. It is advisable to aim for immobilized antibody densities between 3–5 mg of antibody per milliliter of gel. Immobilizations at high densities > 8 mg/mL yield reduced antibody utilization due to steric hindrance and overcrowding effects.
2. It is recommended that a coupling chemistry based on the scale and the stringency of the purification is selected. We have routinely purchased commercially available activated matrices and provided the coupling instructions are followed. little or no problems were encountered.
3. It is recommended that antibody-coupling efficiency to the support matrix be evaluated. Antibody-coupling efficiencies in the range of 75–95% are acceptable. If coupling efficiencies less than 70% are attained, check the antibody-coupling buffers and the antibody samples for the presence of salts like Tris-HCl or glycine. Dialyze the antibody samples extensively to ensure the removal of salts like Tris and glycine. Alternatively, choosing another immobilization chemistry may give higher antibody-coupling efficiencies.
4. The amount of antibody immobilized on the support matrix is calculated as the difference between total antibody added to the gel and the uncoupled antibody recovered in the blocking step supernatants and wash pools, as measured by A_{280}. The antibody-coupling efficiency was calculated as the ratio of [coupled Ab:total Ab]. 100%. However certain activation chemistries (NHS or Emphaze) release substances as a result of the coupling reaction that may interfere with measurements at A_{280}. The following protocol may be used to estimate the coupling efficiency when immobilizing monoclonal antibodies (mAb). Coat Immulon II 96-well microtiter plates with 100 μL/well of 1:200 diluted anti-mouse whole molecule in 0.1 M $NaHCO_3$ (pH 9.3) for 24 h at 4°C. Wash and aspirate the wells with 0.05 M Tris/0.1 M NaCl/0.05% Tween (TBS-Tween) and block the residual reactive sites with TBS/0.1% BSA for 20 min at RT. Add various dilutions of standard and samples in TBS/0.1% BSA to the wells, 100 μL in each well and incubated for 20 min at 37°C. The concentration of the MAb standard in the assay ranges from 50 ng/mL to 0.78 ng/mL. Upon incubation, wash the wells four times. Add 1:1000 diluted horseradish peroxidase (HRP) conjugated goat antimouse IgG to the wells and incubate for 20 min at 37°C. Wash and aspirate the wells four times and add 100 μL of OPD substrate to each well. Stop the colorimetric reaction after 3 min by the addition of 100 mL of 3 N H_2SO_4 to each

well. Bound chromophore can be detected at 490 nm using an EL308 Bio-Tek Microplate reader.

5. The theoretical antigen binding efficiency (η_{Ag}) of immobilized monoclonal antibody (mAb) assuming a 2:1 antigen to antibody stoichiometry can be calculated as follows:

$$M = \text{Immobilized MAb density, mg antibody/mL of gel}$$

$$MW_{(mAb)} = \text{molecular weight of MAb, 150,000 Daltons}$$

$$MW_{(Ag)} = \text{molecular weight of antigen being purified, in Daltons}$$

$$V = \text{volume of gel, mL}$$

Theoretical maximum
$$\text{antigen binding} = [M] \times [V] \times \{MW_{(Ag)}/MW_{(MAb)}\} \times \{2/1\} \times 100 \quad (1)$$

Eq. 1 predicts the theoretical maximum antigen-binding capacity. The amount of antigen present in the eluate peaks can be measured by specific antigen assays and A_{280} measurement. Based on the amount of antigen eluted as determined by protein assays, the antigen-binding efficiency can be calculated as follows:

$$[\eta_{Ag}] = [\text{total amount of eluted antigen/theoretical maximum antigen binding}] \times 100$$

6. It is advisable to calculate the efficiency of the immobilized mAb for the antigen–antibody system being employed. If the antigen-binding efficiency is too low, selecting another coupling chemistry may prove beneficial. However, using current immobilization techniques, antigen-binding efficiencies in the range of 10–30% have been obtained. Selection of site-directed chemistries may offer better performance.

7. Also, check for antibody leakage in all column washes and eluants. Traditionally, CNBr-activated supports have shown to leach some percentage of anchored antibody due to the hydrolysis of the isourea linkage. Affi-prep and Emphaze show reduced or minimal antibody leaching.

8. Immobilization procedures outlined in this chapter are applicable to both polyclonal and monoclonal antibodies. Antigen-binding efficiencies in the range of 8–15% can be expected of polyclonal antibodies using procedures outlined in this chapter.

References

1. Chase, H. L. (1983) Affinity separations utilizing immobilized monoclonal antibodies. *Chemical Engineering Science* **39(7/8),** 1099–1125.
2. Velander, W. H. (1989) Process implications of metal-dependent immunoaffinity chromatography. *Bio/Technology* **5(3),** 119–124.
3. Stryer, L. (1981) *Biochemistry* 2nd ed., *W*. H. Freeman, San Francisco.
4. Milstein, C. (1980) Monoclonal antibodies. *Scientific American,* **234(4),** 66–70.
5. Pfeiffer, N. E., Wylier, D. E., and Schuster, S. M. (1987). Immunoaffinity chromatography utilizing monoclonal antibodies. Factors which influence antigen-binding capacity. *J. Immunol. Methods* **97,** 1–9.

6. Velander, W. H., Subramanian, A., Madurawe, R. D., and Orthner, C. L. (1992) The use of Fab-masking antigens to enhance the activity of immobilized antibodies. *Biotechnol. Bioeng.* **39,** 1013–1023.
7. Eveleigh, J. W. and Levy, D. E. (1977) Immunochemical characteristics and preparative application of agarose-based immunosorbents. *J. Solid-Phase Biochem.* **2,** 45–77.
8. Katoh, S. (1987) Scaling up affinity chromatography. *Tibtech* **5,** 328–331.
9. Hamman, J. P. and Calton, G. J. (1985) Immunosorbent chromatography for recovery of protein products. *ACS Symp. Ser.—Purification of Fermentation Products,* American Chemical Society, Washington, DC, pp. 105–113.
10. Narayanan, S. R. and Crane, L. J. (1990) Affinity chromatography supports: a look at performance requirements. *Tibtech* **8,** 12.
11. O'Shanessey, D. J. and Hoffman, W. L. (1987) Site directed immobilization of glycoproteins on hydrazide-containing activated supports. *Biotechnol. Appl. Biochem.* **9,** 488–496.
12. Schneider, C., Newman, R. A., Sutherland, R., Asser, U., and Greaves, M. F. (1982) A one-step purification of membrane proteins using a high efficeinecy immunomatrix *J. Biolog. Chem.* **257(18),** 10,766–10,769.