Immunoaffinity Chromatography

This unit describes isolation of soluble or membrane-bound protein antigens from cells or homogenized tissue. Antibodies are coupled to Sepharose (i.e., a large-pore chromatography matrix). High-molecular-weight antigens pass freely into and out of the pores and bind to antibodies covalently bound to the matrix. In order to elute the bound antigen from the immunoaffinity matrix, the antibody-antigen interaction is destabilized by brief exposure to high-pH (basic protocol) or low-pH (alternate protocol) buffer. Another alternate protocol uses batch purification of antigens, which shortens the loading time of the column.

BASIC PROTOCOL

ISOLATION OF SOLUBLE OR MEMBRANE-BOUND ANTIGENS

Two different Sepharose columns in series—a precolumn to remove nonspecifically binding material and a specific column—are used to isolate antigens from a cell or tissue lysate. Column fractions are analyzed by SDS-PAGE and silver staining to detect the antigens.

Materials

Antibody (Ab)-Sepharose (see support protocol, *UNIT 10.16*)
Activated, quenched (control) Sepharose, prepared as for Ab-Sepharose (support protocol, *UNIT 10.16*) but eliminating Ab or substituting irrelevant Ab during coupling
Cells or homogenized tissue
Tris/saline/azide (TSA) solution, ice-cold
Lysis buffer, ice-cold
5% sodium deoxycholate (Na-DOC; filter sterilize and store at room temperature)
Wash buffer
Tris buffers, pH 8.0 and 9.0, ice-cold
Triethanolamine solution, ice-cold
1 M Tris·Cl, pH 6.7, ice-cold
Column storage solutions, ice-cold

Columns
Quick-seal centrifuge tubes (Beckman)

Additional reagents and equipment for preparation of antibody-Sepharose (*UNIT 10.16*), column chromatography (*UNITS 10.9-10.15*), SDS-PAGE (*UNIT 10.2*), silver staining (*UNIT 10.6*), and immunoprecipitation (*UNIT 10.16*)

NOTE: Carry out all procedures involving antigen in a 4°C cold room or on ice.

Prepare the columns

1. Prepare an activated, quenched (control) Sepharose precolumn (5 ml packed bed volume) and an Ab-Sepharose immunoaffinity column (5 ml; 5 mg/ml antibody per milliliter packed Sepharose) linked in series (Fig. 10.11.1).

   Irrelevant antibody can be coupled to the Sepharose in the precolumn.

   Column size can vary; adjust amounts of Sepharose and cells proportionally.

Prepare the lysate

2. Suspend 50 g of cells at 1-5 × 10^8 cells/ml in ice-cold TSA solution, or add 1 to 5 volume of ice-cold TSA per volume packed cells or homogenized tissue. Add an equal volume of ice-cold lysis buffer and stir 1 hr at 4°C.

In Current Protocols in Molecular Biology. Greene Publishing Association, New York. 10.11.1 - 10.11.7, 1987.

3. Centrifuge 10 min at 4000 × *g* to remove nuclei. Decant supernatant and save.

   For purification of cytoplasmic (soluble) antigens, it is not necessary to add detergents to the solutions and buffer used in subsequent steps. Detergent is only needed for cell lysis and solubilization of integral membrane proteins.

4. For purification of membrane antigens, add 0.2 vol of 5% Na-DOC to the postnuclear supernatant, and leave 10 min at 4°C or on ice. Transfer to quick-seal centrifuge tubes and centrifuge 1 hr at 100,000 × *g*. Carefully remove supernatant and save.

   See UNIT 10.3 for alternative solubilization procedures.

Set up and wash the columns

5. Attach Sepharose precolumn to immunoaffinity column (Fig. 10.11.1).
6. Wash both columns with 10 column volumes of wash buffer.
7. Wash both columns with 5 column volumes of Tris buffer, pH 8.0.
8. Wash both columns with 5 column volumes of Tris buffer, pH 9.0.
9. Wash both columns with 5 column volumes of triethanolamine solution.
10. Wash both columns with 5 column volumes of wash buffer.

Isolate the antigen

11. Apply the supernatant (reserving some for analysis as described in step 19 below) from steps 3 or 4 to the precolumn and allow it to flow through the precolumn and specific column linked in series at a flow rate of 5 column volumes/hr. Collect the flow-through fractions, each $^1/_{10}$ to $^1/_{100}$ the volume of the applied supernatant.

    "Fat" chromatography columns, filled with Sepharose to a height of ~2× column diameter, are used to maximize flow rates. A 10- to 20-ml syringe is used for 5 ml of Sepharose. The flow rate is adjusted with a hydrostatic head of up to 250 cm (Fig. 10.11.1). Sample loading can routinely take up to 2 days with no deleterious effect, but longer periods would suggest the column is clogged or the lysate is too viscous. The latter is usually due to the presence of DNA.

12. Wash with 5 column volumes of wash buffer, then close the stopcocks on both columns and disconnect the precolumn from the immunoaffinity column. Open the stopcock of the immunoaffinity column and allow fluid above the top of the column to drain out to bed level.

    The Sepharose has some elasticity and draining can continue until there is no buffer above the Sepharose bed. Draining until cracks appear in the Sepharose should be avoided.

    Fractions of this wash and washes obtained below should be saved.

13. Between each change of buffers (steps 14 to 18), wash the immunoaffinity column as follows. Close the stopcock and remove the end cap of the column. With a syringe connected to the outlet of the tubing from the buffer reservoir, aspirate all buffer from the tubing. Place tubing into the next buffer contained in another reservoir. Aspirating with a syringe, fill the tubing from the reservoir and remove the syringe. Crimp the tubing to regulate flow and rinse the inside wall of the column with the buffer. Open the column stopcock and drain the buffer to bed level. Put end cap loosely on column and allow buffer to drain into the column to a level several centimeters above the bed. Secure end cap and commence washes or elution.
14. Wash with 5 column volumes of wash buffer.
15. Wash with 5 column volumes of Tris buffer, pH 8.0.

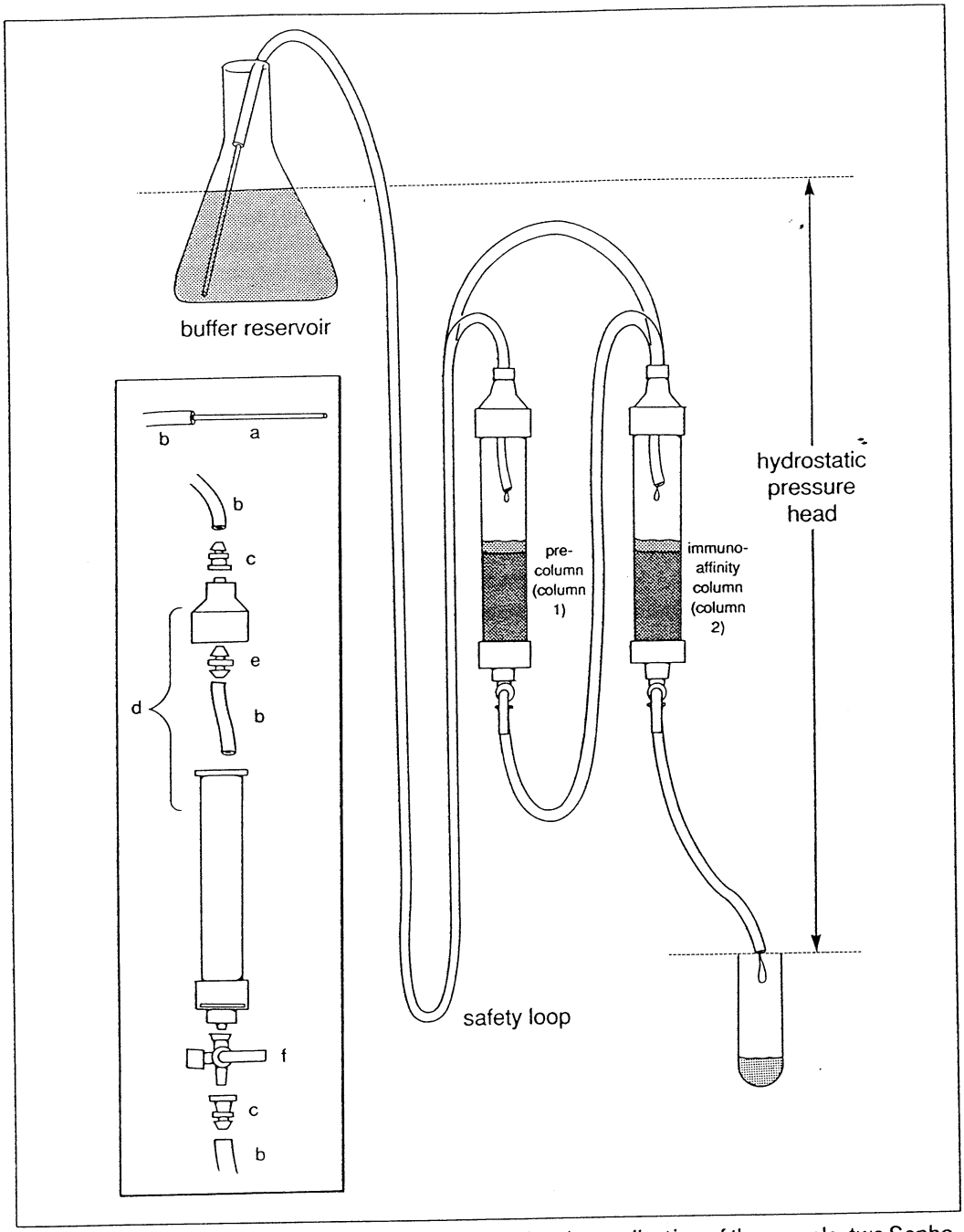

Figure 10.11.1 Immunoaffinity chromatography. During the application of the sample, two Sepharose columns, an immunoaffinity column (with covalently bound antibody) (2), and a Sepharose precolumn (without covalently bound specific antibody or with a covalently bound irrelevant antibody) (1), are attached in series to a buffer reservoir containing the sample. After the sample has been washed through, the precolumn is removed, and the tubing of the safety loop is connected to the immunoaffinity column. The hydrostatic pressure head is the distance between the top of the solution in the buffer reservoir and the tip of the tubing at the bottom of the immunoaffinity column. When the elution reservoir is emptied, the hydrostatic head becomes zero when the fluid level reaches the safety loop, preventing columns from running dry. Fluid remaining above the column beds can be removed by raising the safety loop. After rinsing the tubing, the next elution is begun by placing the end of the safety loop in another reservoir containing the next elution buffer.

Insert: Schematic diagram of an immunoaffinity column. (a) 50-µl disposable capillary micropipet. (b) Tubing: Tygon S-54-HL Microbore, 0.05-in. i.d., or Tygon R-3603 $^{1}/_{16}$-in. i.d. (softer tubing). (c) Female Luer fitting, white nylon, Value Plastics #FTL10, $^{1}/_{16}$ in. (d) Kontes Flex-column #K-420400. (e) Barbed nipple connector, polypropylene, $^{3}/_{32}$-in. top, $^{1}/_{16}$-in. bottom, Value Plastics Series AD. (f) Luer Lock two-way stopcock, Kontes #K420163-0000.

16. Wash with 5 column volumes of Tris buffer, pH 9.0.

    Some nonspecifically bound proteins may be eluted at this step.

17. Elute the antigen with 5 column volumes of triethanolamine solution. Collect fractions of 1 column volume into tubes containing 0.2 vol of 1 M Tris·Cl, pH 6.7, to neutralize the fractions collected.

    In some cases it may be desirable to lower the pH of the triethanolamine solution to preserve the functional activity of the ligand. The ideal pH gives complete release of the ligand, as verified by SDS-PAGE evaluation of a sample (~20 µl) of the eluted column bed (Ab-Sepharose) and eluate (50 µl).

18. Wash the column with 5 column volumes of TSA solution.

    A column may be reused many times and remain active for several years after storage at 4°C in TSA solution. It is important to prevent drying out of a column during storage. The use of column storage solutions inhibits the growth of microorganisms.

19. Analyze fractions for the presence of antigen—50-µl aliquots of each eluate fraction should be analyzed by SDS-PAGE and silver staining. Analyze 0.5- to 1-ml aliquots of the sample applied to the column and representative flow-through and wash fractions by immunoprecipitation with Ab-Sepharose and detect by silver staining to determine whether the column was saturated.

    If antibody leaches off the column during elution, it may be removed from eluate by passage through Protein A–Sepharose (Ey et al., 1978). Even the weakly binding mouse IgG1 subclass can be quantitatively removed at pH 8 (M. Dustin, pers. comm.).

BATCH PURIFICATION OF ANTIGENS

ALTERNATE PROTOCOL

The time required for loading a column is shortened by use of this protocol. This technique is valuable for viscous lysates that take too long to load on a column and for antigens especially susceptible to proteolysis, because less time is required to complete the steps. A precolumn is not utilized because the supernatant is mixed with Ab-Sepharose and poured into a column. The antigen is then eluted as in the basic protocol. The drawbacks of this protocol are that more "hands-on" time is required by the investigator and that nonspecifically binding material is not removed by a precolumn.

1. Follow steps 2 to 4 of the basic protocol to obtain the post-nuclear supernatant.
2. Suspend Ab-Sepharose in the supernatant in a flask. Shake gently on a rotary shaker for 3 hr. Stop shaking and allow the Sepharose to settle. Decant most of the supernatant. Pour the Ab-Sepharose and the remainder of the supernatant into a column and open the stopcock. Continue draining the column until all the Sepharose has been added. Allow the fluid to drain to bed level and close the stopcock.
3. Follow steps 13 to 19 of the basic protocol.

LOW-pH ELUTION OF ANTIGENS

ALTERNATE PROTOCOL

Some protein antigens may be eluted more completely with greater retention of native conformation and with fewer contaminants, when low-pH buffers are employed.

Additional Materials

Sodium phosphate buffer, pH 6.3
Glycine buffer
1 M Tris·Cl, pH 9.0

1. Follow steps 1 to 15 of the basic protocol.

   It is essential to remove sodium deoxycholate from the column before acid elution, because it precipitates or forms a gel at acid to neutral pH.

2. Wash with 5 column volumes of sodium phosphate buffer.

3. Elute with 5 column volumes of glycine buffer. Collect fractions into tubes containing 0.2 vol of 1 M Tris·Cl, pH 9.0.

   Mix each fraction immediately after collection.

4. Analyze fractions for antigen as in step 19 of the basic protocol.

REAGENTS AND SOLUTIONS

Column storage solutions

TSA solution (see below) containing:
1 mM EDTA + 20 µg/ml gentamycin
or
0.01% thimerosal (Aldrich)

Detergent stock solutions

10% Triton X-100 (store in the dark to prevent photooxidation)
or
5% sodium deoxycholate

Sterilize each solution separately by Millipore filtration. Both solutions are stable 5 years at room temperature.

Glycine buffer

50 mM glycine·HCl, pH 2.5
0.1% Triton X-100 (see detergent stock solutions above)
0.15 M NaCl

Lysis buffer

TSA solution (see below) containing:
2% Triton X-100 (see detergent stock solutions above)
5 mM iodoacetamide
Aprotinin (0.2 trypsin inhibitor U/ml)
1 mM phenylmethylsulfonyl fluoride (PMSF), added fresh from 100 mM stock solution prepared in absolute ethanol

NOTE: Iodoacetomide is a protease inhibitor and prevents oxidation of free cysteines to disulfide-bonded cysteines. It should be omitted for enzymes that require cysteines for activity.

Sodium phosphate buffer, pH 6.3

50 mM sodium phosphate, pH 6.3
0.1% Triton X-100 (see detergent stock solutions above)
0.5 M NaCl

Triethanolamine solution

50 mM triethanolamine, pH ~11.5
0.1% Triton X-100 (see detergent stock solutions above)
0.15 M NaCl

Tris buffer, pH 8.0 and 9.0

50 mM Tris·Cl, pH 8.0 or pH 9.0
0.1% Triton X-100 (see detergent stock solutions above)
0.5 M NaCl

Tris/saline/azide(TSA) solution

0.002 M Tris·Cl, pH 8.0 (at 4°C)
0.14 M NaCl
0.025% NaN_3
CAUTION: *Sodium azide (NaN_3) is poisonous; wear gloves.*

Wash buffer

0.01 M Tris·Cl, pH 8.0 (at 4°C)
0.14 M NaCl
0.025% NaN_3 (*handle cautiously!*)
0.5% Triton X-100 (see detergent stock solutions above)
0.5% sodium deoxycholate (see detergent stock solutions above)

COMMENTARY

Background Information

The review of affinity chromatography by Wilchek et al. (1984) discusses available methods for activation of solid supports, coupling of ligands, adsorption of proteins, and elution of protein from affinity columns. Table III of that review lists numerous examples of proteins that have been purified by immunoaffinity chromatography and the elution conditions for each purification.

Traditionally, purification of membrane proteins started with a membrane purification step and, in some cases, is still desirable. However, it is difficult to achieve more than a 5-fold purification of plasma membranes and yields are usually only 10% to 40%. Omission of membrane purification in this protocol (Williams and Barclay, 1986; Johnson et al., 1985) results in increased yield and decreased experiment time.

Purification to homogeneity or near-homogeneity can usually be achieved for protein antigens present in ≥10,000 molecules per eukaryotic cell. This protocol can be used for both membrane and intracellular antigens. However, for soluble antigens, immunoaffinity chromatography is completed without detergent.

Critical Parameters

Binding capacities of Ab-Sepharose columns (coupled at 10 mg monoclonal antibody/ml Sepharose) have been found to be 2% to 20% of the theoretical binding capacity. The lowest and highest binding capacity values were found for antigens of 150,000 and 18,000 M_r, respectively, suggesting that antigen size may constrain access to antibody in the pores of the affinity matrix. A binding capacity of 40% was reported for coupling at 2 to 3 mg antibody/ml Sepharose. Successful purification has been achieved using monoclonal antibodies with affinity constants ranging from 2×10^7 to 4×10^8 M^{-1}. The column should be saturated with antigen by allowing some of the antigen to flow through the column during loading. This will result in the highest antigen purity and a high-mass yield, and will diminish the relative level of antibody eluted along with the antigen when antibody is leaching off the column.

Sodium deoxycholate is used in the solubilization protocol (Johnson et al., 1985) because it dissociates proteins from the membrane more effectively than Triton X-100. However, because sodium deoxycholate releases DNA from nuclei, it must be added to the lysate after the nuclei are removed. Sodium deoxycholate forms a mixed micelle with Triton X-100. Although sodium deoxycholate can be substituted for Triton X-100 during high-pH elution, deoxycholate gels at low pH and in high salt. Both sodium deoxycholate and nonionic detergents may dissociate subunits of protein complexes, which interact within the membrane. Addition of phospholipid, low concentrations of Triton X-100, and mild detergents (e.g., digitonin, octylglucoside, and CHAPS) have all been used to preserve membrane protein complexes (Helenius et al., 1979; Rivnay et al., 1982; Tsuchiya and Saito, 1984).

Protein antigens eluted by acid or base can frequently be renatured by neutralization. However, some protein antigens are irreversibly denatured. The structure of certain antigens is preserved after acid, but not base, elution (Plunkett and Springer, 1986). The structure of other antigens is preserved after base, but not acid, elution (Johnson et al., 1985). Some antigens are eluted at low pH, but not at high pH, while for others the reverse is true. For each antibody-antigen combination, the opti-

mal pH for elution of specific antigens as well as contaminants, must be empirically defined. Antibody binding capacity is usually retained after repeated exposure to low and high pH elution buffers. The alternatives of elevated temperature and chaotropic agents (e.g., potassium thiocyanate, and urea) are seldom used as eluants (Johnson et al., 1985).

Troubleshooting

Immunoaffinity chromatography relies on the elution of a single protein from an immunoaffinity column after prior elution of all other nonspecifically adsorbed proteins. Thus, depending on the exact elution conditions used, a desired protein antigen may be contaminated with other proteins. It is especially important to determine if a contaminant is present if the protein is to be analyzed for amino acid composition or protein sequence. One-dimensional gel electrophoresis (*UNIT 10.2*) should be used to verify elution of contaminating proteins during washing of the immunoaffinity column, as well as the purity of the protein in the final eluate. If the protein is not pure, the wash steps must be optimized to assure that other contaminating proteins are removed.

Anticipated Results

Antigen yield is typically 40% to 70% of starting material (Kürzinger and Springer, 1982; Johnson et al., 1985) and purification factors of 1,000- to 10,000-fold may be achieved (Williams and Barclay, 1986; Kürzinger and Springer, 1982; Plunkett and Springer, 1986). Further purification can usually be achieved by a second cycle of immunoaffinity chromatography. Monoclonal antibodies are most convenient to use, but affinity-purified polyclonal IgG can also be used.

Time Considerations

Pouring the column takes a few minutes and lysate preparation requires ~6 hr. Purification proceeds over 1 to 2 days depending on the flow rate of the immunoaffinity column. The majority of this time involves loading the sample on the column, which may be done from a reservoir and requires little hands-on time. The use of the batch purification (alternate) protocol reduces the sample application time to 3 hr. The elution of an immunoaffinity column requires 5 to 6 hr.

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Key References

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Johnson et al., 1985. See above.

Describes the critical parameters involved in immunoaffinity chromatography.

Wilchek et al., 1984. See above.

Describes the mechanism of activation of Sepharose by CNBr and alternative activation procedures, and lists numerous examples of proteins purified by affinity chromatography.

Contributed by Timothy A. Springer
Center for Blood Research
Harvard Medical School
Boston, Massachusetts