Immunoaffinity Chromatography

This unit describes the isolation of soluble or membrane-bound protein antigens from cells or homogenized tissue by immunoaffinity chromatography. This technique involves the elution of a single protein from an immunoaffinity column after prior elution of nonspecifically adsorbed proteins. Specifically, antibodies are coupled to Sepharose (an insoluble, large-pore-size chromatographic matrix). High-molecular-weight antigens pass freely into and out of the pores and bind to antibodies covalently bound to the matrix. 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 1) buffer. The use of batch purification of antigens shortens the column loading time (Alternate Protocol 2). The detergent octyl β-D-glucoside can be used instead of Triton X-100 for elution. Because octyl β-D-glucoside has a high critical micelle concentration (CMC) it can be readily removed by dialysis (Alternate Protocol 3). The procedure for covalently linking an antibody to Sepharose using the cyanogen bromide activation method is given in the Support Protocol.

ISOLATION OF SOLUBLE OR MEMBRANE-BOUND ANTIGENS

BASIC PROTOCOL

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)
Activated, quenched (control) Sepharose, prepared as for Ab-Sepharose (see Support Protocol) but eliminating Ab or substituting irrelevant Ab during coupling
Cells or homogenized tissue
Tris/saline/azide (TSA) solution (see recipe), ice cold
Lysis buffer (see recipe), ice cold
5% (w/v) sodium deoxycholate (Na-DOC; filter sterilize and store at room temperature)
Wash buffer (see recipe)
Tris/Triton/NaCl buffers, pH 8.0 and 9.0 (see recipe), ice-cold
Triethanolamine solution (see recipe), ice cold
1 M Tris·Cl, pH 6.7 (*APPENDIX 2E*), ice cold
Column storage solution (see recipe), ice cold

Chromatography columns
Ultracentrifuge
Quick-seal centrifuge tubes (Beckman)

Additional reagents and equipment for column chromatography (*UNITS 8.1*), SDS-PAGE (*UNIT 10.1*), and silver staining (*UNIT 10.5*)

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

Prepare the columns

1. Prepare an Ab-Sepharose immunoaffinity column (5 ml; 5 mg/ml antibody per milliliter packed Sepharose) and an activated, quenched (control) Sepharose precolumn (5 ml packed bed volume) linked in series (Fig. 9.5.1).

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

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

In Current Protocols in Protein Science. John Wiley and Sons, Inc. New York. 9.5- 9.11, 1995.

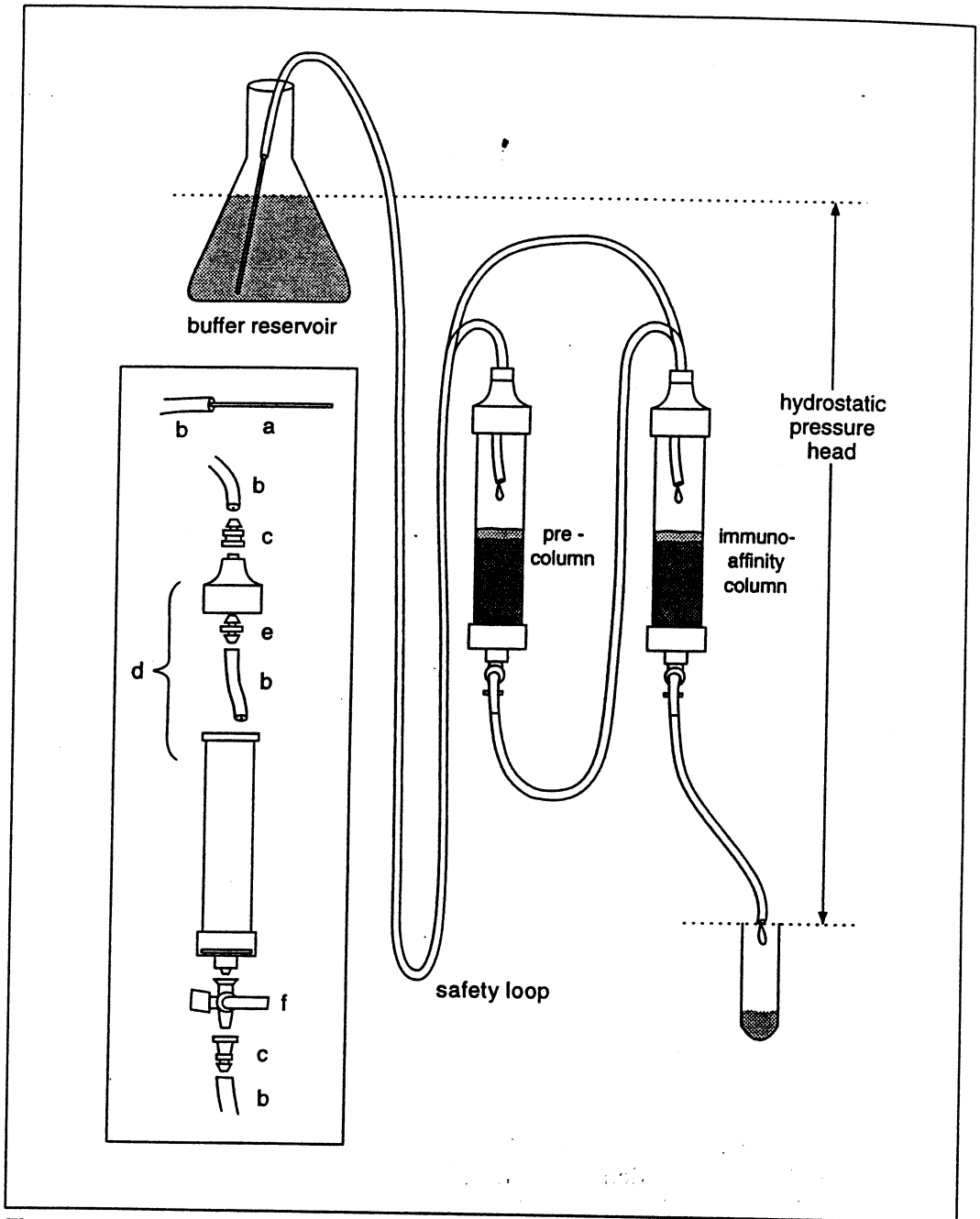

Figure 9.5.1 Immunoaffinity chromatography. During the application of the sample, two Sepharose columns, a Sepharose precolumn (without covalently bound specific antibody or with a covalently bound irrelevant antibody) and an immunoaffinity column (with covalently bound antibody), are attached in series 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.

Inset: 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), 1/16 in. (d) Kontes Flex-column (Kontes Glass). (e) Barbed nipple connector, polypropylene, 3/32-in. top, 1/16-in. bottom (Value Plastics, Series AD). (f) Luer-Lok two-way stopcock (Kontes Glass).

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 vol ice-cold TSA per vol packed cells or homogenized tissue. Add an equal volume of ice-cold lysis buffer and stir 1 hr at 4°C.

   For glycosylphosphatidylinositol (GPI)-anchored proteins (UNIT 12.5), incubate 10 min at 20°C for efficient solubilization.

3. Centrifuge 10 min at 4000 × *g*, 4°C, 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 needed only 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*, 4°C. Carefully remove supernatant and save.

Set up and wash the columns

5. Attach Sepharose precolumn to immunoaffinity column (Fig. 9.5.1).

6. Wash both columns using the following regimen:

   10 column volumes of wash buffer
   5 column volumes of Tris/Triton/NaCl buffer, pH 8.0
   5 column volumes of Tris/Triton/NaCl buffer, pH 9.0
   5 column volumes of triethanolamine solution
   5 column volumes of wash buffer.

Isolate the antigen

7. Apply the supernatant from steps 3 or 4 (reserving some for analysis as described in step 15 below) 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. 9.5.1). 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.

8. 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.

9. Wash the immunoaffinity column between each change of buffers (steps 10 to 14) 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 the 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.

10. Wash with 5 column volumes of wash buffer.

11. Wash with 5 column volumes of Tris/Triton/NaCl buffer, pH 8.0.

12. Wash with 5 column volumes of Tris/Triton/NaCl buffer, pH 9.0.

    Some nonspecifically bound proteins may be eluted at this step. In addition, some monoclonal antibodies may release some ligand at this pH. This should be checked.

13. 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).

14. 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.

15. 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 flowthrough 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 the 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.).

    Preliminary analysis of fractions can be done using A_{280} readings when octyl β-D-glucoside or sodium deoxycholate are used as detergents.

ALTERNATE PROTOCOL 1

LOW-pH ELUTION OF ANTIGENS

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 (also see Basic Protocol)

Sodium phosphate buffer, pH 6.3 (see recipe)
Glycine buffer (see recipe)
1 M Tris·Cl, pH 9.0 (*APPENDIX 2E*)

1. Prepare the columns and lysate, wash the columns, and isolate the antigen (see Basic Protocol, steps 1 to 11).

   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.

   TSA and triethanolamine solutions used here may also be modified by substituting 1% octyl β-D-glucoside for 0.1% Triton X-100 (see Alternate Protocol 3).

2. Wash with 5 column volumes of sodium phosphate buffer, pH 6.3.

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 (see Basic Protocol, step 15).

BATCH PURIFICATION OF ANTIGENS

ALTERNATE PROTOCOL 2

Batch purification of antigens shortens the column loading time. 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 (see 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. Suspend and centrifuge the cells and purify the membrane antigens (see Basic Protocol, steps 2 to 4) to obtain the postnuclear 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.
3. 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.
4. Wash the immunoaffinity column, elute the antigen, and analyze the fractions (see Basic Protocol, steps 9 to 15).

ELUTION IN OCTYL β-D-GLUCOSIDE

ALTERNATE PROTOCOL 3

The detergent octyl β-D-glucoside has a high critical micelle concentration (CMC) of 0.73% and can therefore be readily removed by dialysis. Also, adsorption of membrane proteins to surfaces for ELISA or adhesion assays is much more efficient when solutions containing membrane proteins are diluted so that the concentration of octyl β-D-glucoside is below the CMC. Because octyl β-D-glucoside is expensive, initial steps requiring large volumes may be done as described in the Basic Protocol; the initial detergent is then replaced by octyl β-D-glucoside in the wash step prior to elution.

Additional Materials (also see Basic Protocol)

TSA solution (see recipe) containing 1% octyl β-D-glucoside

1. Prepare the columns and lysate, wash the columns, and isolate the antigen (see Basic Protocol, steps 1 to 11).
2. Wash with 5 column volumes of TSA solution containing 1% octyl β-D-glucoside.
3. Elute with 5 column volumes of triethanolamine solution with 1% octyl β-D-glucoside substituted for 0.1% Triton X-100.
4. 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.
5. Wash the column and analyze the fractions (see Basic Protocol, steps 14 and 15).

SUPPORT PROTOCOL

PREPARATION OF ANTIBODY-SEPHAROSE

This protocol details the procedure for covalently linking an antibody to Sepharose (an insoluble, large-pore-size chromatographic matrix) using the cyanogen bromide activation method (also see *UNIT 9.3*). First, the antibody and Sepharose are prepared separately. The Sepharose is then activated with cyanogen bromide (alternatively, CNBr-activated Sepharose can be purchased from Pharmacia Biotech and used according to the manufacturer's instructions). Finally, the CNBr-activated Sepharose is coupled to the antibody.

CAUTION: CNBr is very toxic, releases toxic vapor, and is reported to create explosive side products upon storage; therefore, it should always be used in a hood and handled with appropriate CNBr- and solvent-resistant gloves. Only a fresh bottle of CNBr should be employed and it should be opened in the hood. Because of the problems involved in handling CNBr, it is best to dissolve a complete bottle in a defined amount of anhydrous acetonitrile. Short-term storage of this solution (several weeks) at 0°C is possible, but prolonged storage is not recommended.

Materials

1 to 30 mg/ml antigen-specific monoclonal or polyclonal antibody
0.1 M $NaHCO_3$/0.5 M NaCl
Sepharose CL-4B (or Sepharose CL-2B for high-molecular-weight antigens; Pharmacia Biotech)
2 M Na_2CO_3
Cyanogen bromide (CNBr)/acetonitrile (see recipe)
1 mM and 0.1 mM HCl, ice-cold
0.05 M glycine (*or* ethanolamine), pH 8.0
Tris/saline/azide (TSA) solution (see recipe)

Dialysis tubing (MWCO >10,000)
Ultracentrifuge
Whatman no. 1 filter paper
Buchner funnel
Erlenmeyer filtration flask
Water aspirator

Prepare the antibody

1. Dialyze 1 to 30 mg/ml antibody against 0.1 M $NaHCO_3$/0.5 M NaCl at 4°C with three buffer changes during 24 hr. Use a volume of dialysis solution that is 500 times the volume of antibody solution.

   Dialysis is performed to remove all small molecules containing free amino or sulfhydryl groups.

2. Centrifuge 1 hr at 100,000 × *g*, 4°C, to remove aggregates. Save the supernatant.

   Removal of aggregates is important. Because only some of the antibody molecules in an aggregate will directly couple to the Sepharose, the noncoupled antibody molecules may leach out during elution.

3. Measure the A_{280} of an aliquot of the solution and determine the concentration of the antibody (mg/ml IgG = A_{280}/1.44). Dilute with 0.1 M $NaHCO_3$/0.5 M NaCl to 5 mg/ml (or to the same concentration as desired for Ab-Sepharose) and keep at 4°C. Measure the A_{280} of this solution for comparison of absorbance in step 10.

Prepare the Sepharose

4. Allow the Sepharose slurry to settle in a beaker or the container in which it is sold; decant and discard the supernatant. Weigh out the desired quantity of Sepharose (assume density = 1.0).

5. Set up a filter apparatus using Whatman no. 1 filter paper in a Buchner funnel and an Erlenmeyer filtration flask attached to a water aspirator. Wash the Sepharose on the filter apparatus with 10 vol water.

   Sintered-glass funnels are traditionally recommended but rapidly become clogged unless coarse-porosity funnels are used.

Activate Sepharose with CNBr

6. Transfer Sepharose to 50-ml beaker and add an equal volume of ice-cold 2 M Na_2CO_3.

7. Activate Sepharose in an ice bath using 3.2 ml CNBr/acetonitrile per 100 ml Sepharose. Add CNBr/acetonitrile dropwise with a Pasteur pipet over 1 min, while slowly stirring the slurry with a magnetic stir bar. Continue stirring slowly for 5 min.

   Excessive and vigorous stirring may fracture the Sepharose beads; this may cause flow problems during column chromatography. The protocol uses 2 g CNBr/100 ml Sepharose. Two to four grams of CNBr/100 ml Sepharose can be used to couple 1 to 20 mg of antibody/ml Sepharose.

   CAUTION: *Activation should be carried out in a fume hood.*

8. Rapidly filter the CNBr-activated Sepharose as in step 5. Aspirate to semidryness (i.e., until the Sepharose cake cracks and loses its sheen). Wash with 10 vol ice-cold 1 mM HCl, then with 2 vol ice-cold 0.1 mM HCl. Hydrate with enough ice-cold 0.1 mM HCl so that the cake regains its sheen, but there is no excess liquid above the cake.

   Washing is most efficient if the wash solution is added evenly over the surface of the cake at about the same rate as the solution is removed by filtration. CNBr-activated Sepharose is very unstable at the alkaline pH necessary for activation; it is much more stable in dilute HCl. CNBr-activated Sepharose can be purchased from Pharmacia Biotech, but the coupling capacity will be lower.

   Destroy the contents of the filter flask, which contains excess CNBr, by slowly adding a 5-fold molar excess (relative to the total initial amount of CNBr used) of 1 M NaOH. Keep the reaction mixture cold by adding pieces of ice. Perform this decomposition in the hood, and keep the mixture there until all CNBr is destroyed.

Couple antibody to CNBr-activated Sepharose

9. Immediately transfer a weighed amount of Sepharose (assume density = 1.0) to a beaker. Add an equal volume of a solution of antibody dissolved in 0.1 M $NaHCO_3$/0.5 M NaCl (from step 3). Stir gently with a magnetic stir bar or rotate end-over-end 2 hr at room temperature or overnight at 4°C.

10. Add 0.05 M glycine (or ethanolamine), pH 8.0, to saturate the remaining reactive groups on the Sepharose and allow the slurry to settle. Remove an aliquot of the supernatant and centrifuge to remove any residual Sepharose. Measure the A_{280} and compare to the A_{280} of the antibody solution from step 3 to determine the percentage coupling.

11. Store the Ab-Sepharose in TSA solution.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

CNBr/acetonitrile

To 25 g of cyanogen bromide in the original bottle *(CNBr should be white, not yellow, crystals)* add 50 ml acetonitrile to make a 62.5% (w/v) solution. Store indefinitely at –20°C in a desiccator over silica. Allow to warm before opening.

CAUTION: *CNBr is a highly toxic lachrymator; handle in a fume hood.*

Column storage solution

Prepare in TSA solution (see recipe) either 1 mM EDTA/20 μg/ml gentamicin *or* 0.01% thimerosal (Aldrich).

Detergent stock solutions

Prepare 10% Triton X-100 *or* 5% sodium deoxycholate in water. Sterilize either solution by Millipore filtration. Both solutions remain stable for 5 years at room temperature; Triton X-100 solution should be stored in the dark to prevent photo-oxidation.

NP-40 can be used in place of Triton X-100.

Glycine buffer

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

Lysis buffer

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

IMPORTANT NOTE: *Iodoacetamide 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.*

NP-40 can be used in place of Triton X-100.

Sodium phosphate buffer, pH 6.3

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

Triethanolamine solution

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

Other organic solutions, such as diethylamine, may be used in place of triethanolamine. The pH of this solution should be determined for each antibody as elution conditions may vary (see Critical Parameters).

Tris/saline/azide (TSA) solution

0.01 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 and handle cautiously.*

Tris/Triton/NaCl buffer, pH 8.0 and 9.0

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

Wash buffer

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

CAUTION: *Sodium azide (NaN_3) is poisonous; wear gloves and handle cautiously.*

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 this 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 5 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 increased antigen size may constrain access to antibody in the pores of the affinity matrix. Because of its larger pore size, Sepharose CL-4B (a 4% cross-linked agarose) is far preferable to Sepharose 6B (a 6% agarose), which is the usual commercially available preactivated agarose. The cross-links in the CL series yield mechanically more robust beads and do not appreciably decrease activation with cyanogen bromide (CNBr).

The Support Protocol for coupling protein antigens to CNBr-activated Sepharose is a modification of the methods of Cuatrecasas (1970) and March et al. (1974). As originally described, the washing was done at alkaline pH. Because activated Sepharose is very unstable at this pH, it was originally recommended that washing, adding the protein ligand, and mixing be done in <90 sec (Cuatrecasas, 1970). However, with the use of an acid wash buffer (Gelb, 1973) as described in the Support Protocol, activated groups remain stable for ≥30 min. Wilchek et al. (1984) described an alternative activation procedure with CNBr triethanolamine in acetone/water that is highly efficient and minimizes side-reaction derivatization of the Sepharose. The 0.1 M $NaHCO_3$ buffer in which antibody is dissolved provides an optimal pH of ~8.4 after mixing with activated Sepharose. The small amount of CNBr recommended is sufficient to obtain a coupling yield of 80% to 95%. Higher amounts of CNBr may result in multipoint attachment of IgG molecules to the matrix, thereby inactivating

antigen binding sites and reducing accessibility to antigen. Coupling efficiencies on the order of 99% are usually associated with lower antigen binding capacity and are to be avoided; coupling efficiencies of 80% to 90% are ideal. Smaller amounts of CNBr per milliliter of Sepharose also result in lower derivatization of the Sepharose with charged groups. These charged groups can cause nonspecific binding.

The amount of CNBr used in commercially available activated Sepharose is not specified, but is probably high. Many investigators purchase CNBr-activated Sepharose; others activate their own. Commercially activated Sepharose is stabilized by drying. Not all Sepharose can withstand drying; e.g., Sepharose 2B (2% agarose beads) is less robust than Sepharose 4B (4% agarose) and is not available in a preactivated form. Activating your own Sepharose allows more control over coupling efficiency; enables use of CL (cross-linked) Sepharose, which is more robust and has faster flow rates; enables use of Sepharose CL-2B; and is more cost effective.

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 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, it 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) preserves membrane protein complexes (Helenius et al., 1979; Rivnay et al., 1982; Tsuchiya and Saito, 1984).

Glycosylphosphatidylinositol (GPI)-anchored proteins (*UNIT 12.5*) are resistant to detergent solubilization at 4°C (Schroeder et al., 1994). Preparation of the lysate (Basic Protocol, step 2) should be conducted for 10 min at 20°C for these antigens.

Octyl β-D-glucoside is an expensive detergent that is readily removable by dialysis. Furthermore, adsorption of proteins to substrates is enhanced by dilution below a CMC of 0.73%. In Alternate Protocol 3, the initial detergent is replaced with octyl β-D-glucoside prior to elution of antigen from the affinity column.

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), whereas 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; for others the reverse is true. For each antibody-antigen combination, the optimal 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 (see Alternate Protocol 1 and see Basic Protocol, respectively). The use of chaotropic agents (e.g., potassium thiocyanate and urea) for elution is a seldom-employed alternative (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 elution conditions used, the desired protein antigen may be contaminated with other proteins. It is very important to determine whether a contaminant is present if the protein is to be analyzed for amino acid composition or protein sequence. One-dimensional gel electrophoresis (*UNIT 10.1*) 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 ensure 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 (Kürzinger and Springer, 1982; Williams and Bar-

clay, 1986; Plunkett and Springer, 1986). Further purification can usually be achieved by a second cycle of immunoaffinity chromatography. Monoclonal antibodies are most convenient, 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 batch purification (Alternate Protocol 2) reduces the sample application time to 3 hr. Elution of an immunoaffinity column requires 5 to 6 hr.

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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.

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