SECTION II

ISOLATION OF PROTEINS USING ANTIBODIES

Immunoaffinity purification is a powerful technique for isolating proteins. Purifications of 10,000-fold or more can often be achieved in one step. Immunoaffinity columns most often employ monoclonal antibodies covalently attached to a solid-phase matrix; polyclonal antibodies have been used successfully, but they usually lack the specificity required for a single-step protein purification. The amount of protein that can be purified is solely dependent on the amount and affinity of the antibody employed.

Immunoprecipitation (*UNIT 8.3*) is a scaled-down version of immunoaffinity chromatography. This procedure permits the identification of small amounts of a protein in a complex mixture by its interaction with antibody. The antigen is isolated, usually from a radiolabeled mixture (*UNITS 8.11 & 8.12*), by specific precipitation and analyzed by SDS-PAGE (*UNITS 8.4-8.6*) followed by autoradiography (*APPENDIX 3*). This permits detection of antigen, characterization of its molecular size, and identification of subunits and associated proteins. This procedure is usually applied to detergent lysates of cells (*UNITS 8.1 & 8.2*) and is frequently used to characterize the molecular species recognized by antibodies, in particular monoclonal antibodies (Chapter 2) and antipeptide antibodies (Chapter 9).

UNIT 8.2

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 8.3*)
Activated, quenched (control) Sepharose, prepared as for Ab-Sepharose (support protocol, *UNIT 8.3*) 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

In Current Protocols in Immunology. Greene Publishing Assoc. and Wiley Interscience, New York. 8.2.1 - 8.3.11, 1991.

Columns
Quick-seal centrifuge tubes (Beckman)

Additional reagents and equipment for preparation of antibody-Sepharose (*UNIT 8.3*), column chromatography (*APPENDIX 3*), SDS-PAGE (*UNIT 8.4*), silver staining (*UNIT 8.9*), and immunoprecipitation (*UNIT 8.3*)

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. 8.2.1).

   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.

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 post-nuclear 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 8.1 for alternative solubilization procedures.

Set up and wash the columns

5. Attach Sepharose precolumn to immunoaffinity column (Fig. 8.2.1).

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. 8.2.1). 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.

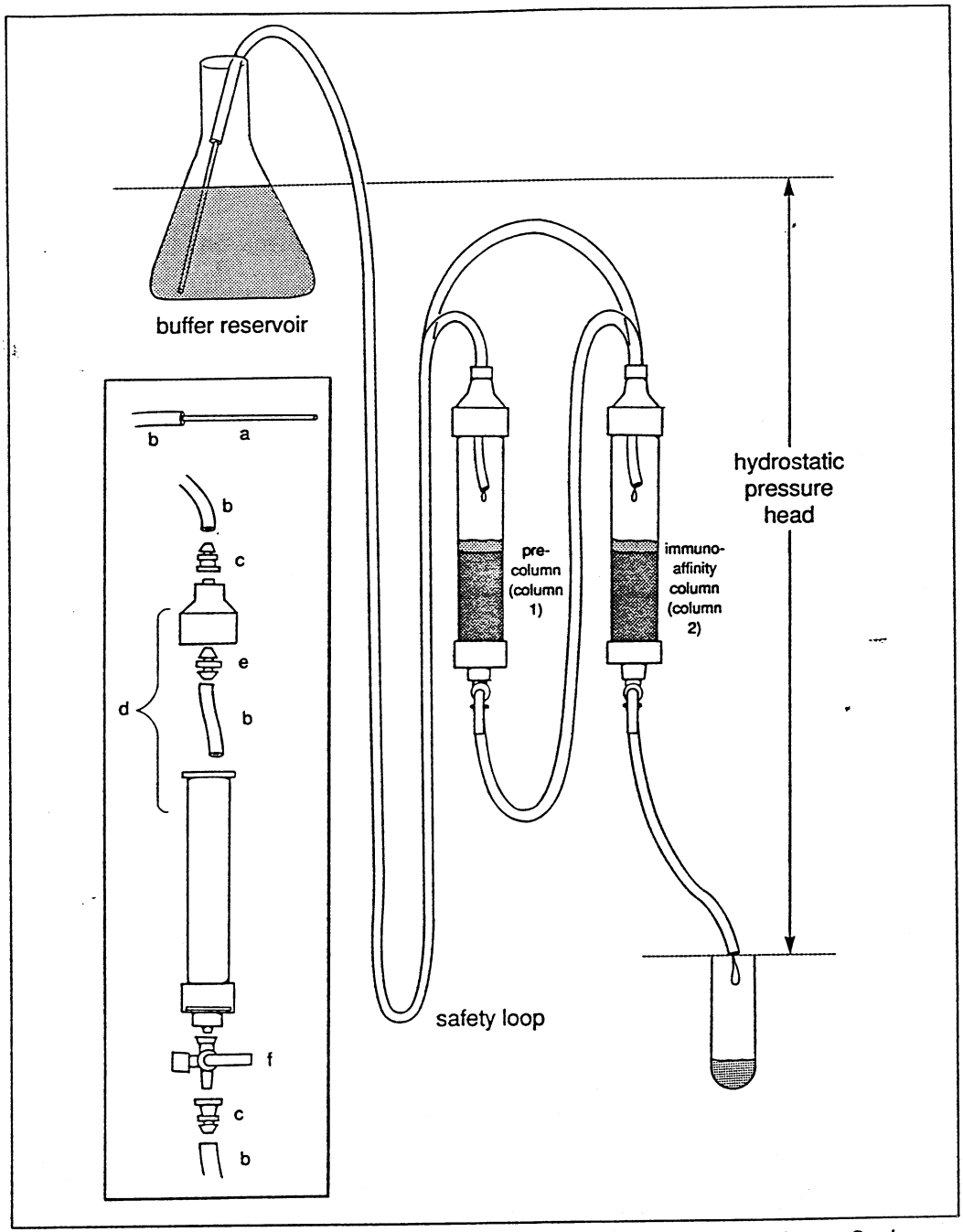

Figure 8.2.1 Immunoaffinity chromatography. During the application of the sample, two Sepharose columns, an immunoaffinity column (with covalently bound antibody) (2), and a Sepharose precolumn (without covalently bound specific antibody or with a covalently bound irrelevant antibody) (1), are attached 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.

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.

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 (about 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.).

ALTERNATE PROTOCOL

BATCH PURIFICATION OF ANTIGENS

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.

ALTERNATE PROTOCOL

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

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 pro-

tein 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 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. 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 8.4*) 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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Hjelmeland, J.M. and Chrambach, A. 1984. Solubilization of functional membrane proteins. *Methods Enzymol.* 104:305-318.

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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Immunoprecipitation

Immunoprecipitation consists of multiple ordered steps (Fig. 8.3.1): lysing the cell with detergent if the antigen (usually a protein) to be precipitated is membrane-bound; binding of a specific antigen to an antibody; precipitating the antibody-antigen complex; washing the precipitate; and dissociating the antigen from the immune complex. The dissociated antigen is then analyzed by electrophoretic methods (*UNITS 8.4-8.6*). The protocols for immunoprecipitation differ in the method of precipitation used and whether or not the antigen being precipitated is radiolabeled (*UNITS 8.11 & 8.12*).

The basic protocol details the immunoprecipitation of a radiolabeled antigen with a specific antibody (polyclonal or monoclonal) covalently linked to Sepharose. Preparation of Ab-Sepharose is described in the support protocol. The first two alternate protocols present methods for precipitating or isolating the soluble immune complexes formed between a specific antibody and a radiolabeled antigen. Immunoprecipitation is achieved with polyclonal anti-immunoglobulin (Ig) serum, anti-Ig-Sepharose, *Staphylococcus* protein A or *Streptococcus* protein G bound to Sepharose, or *Staphylococcus aureus* bacteria which contain protein A on the cell surface. The third alternate protocol should be used for immunoprecipitation of antigens that are nonspecifically associated with other proteins. The fourth alternate protocol describes immunoprecipitation of unlabeled protein antigens with Ab-Sepharose.

BASIC PROTOCOL

IMMUNOPRECIPITATION OF RADIOLABELED ANTIGEN WITH ANTIBODY-SEPHAROSE

This protocol follows the steps presented in Figure 8.3.1. It relies on the formation of an insoluble immune complex between a protein antigen and an antigen-specific monoclonal (or polyclonal) antibody bound to Sepharose.

Materials

Surface-labeled cells (with ^{125}I; *UNIT 8.11*) *or* biosynthetically ^{35}S-, ^{3}H-, or ^{14}C-labeled cells (*UNIT 8.12*)
Lysis buffer
Dilution buffer
Antibody (Ab)-Sepharose (see support protocol)
Activated, quenched (control) Sepharose, prepared as for Ab-Sepharose (support protocol) but eliminating Ab or substituting irrelevant Ab during coupling
Tris/saline/azide (TSA) solution
0.05 M Tris·Cl, pH 6.8
2× SDS/sample buffer (*UNIT 8.4*)

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

Lyse labeled cells and preclear the lysate

1. Incubate surface- or biosynthetically-labeled cells in lysis buffer (5×10^7 cells/ml) for 1 hr at 4°C.

2. Centrifuge the lysate 10 min at 3000 × *g* to remove nuclei and save the supernatant.

3. Centrifuge the supernatant 1 hr at 100,000 × *g* and save the supernatant.

Supernatants may also be prepared by microcentrifugation (10,000 × g) for 30 min.

The supernatant must be used within several days or stored at –70°C. The length of storage is limited by autoradiolysis and the half-life of the isotope. ^{3}H- and ^{14}C-labeled samples can often be stored frozen for years. Storage of ^{125}I-labeled samples

is usually limited to 1 to 2 months because of autoradiolysis. The usefulness of ^{35}S-labeled samples is usually limited to 6 months because of half-life. Repeated freezing and thawing may disrupt antigenic determinants and dissociate some protein complexes, especially those that are noncovalently associated.

4. Preclear supernatant to be used in one batch by adding 10 µl activated, quenched (control) Sepharose per 200 µl supernatant. Shake on an orbital shaker 2 hr at room temperature or overnight at 4°C. Centrifuge 1 min at 200 × *g* and save supernatant.

Preclearing removes nonspecifically absorbing material. Control Sepharose can be prepared without antibody or coupled with irrelevant (nonspecific) antibody. Irrele-

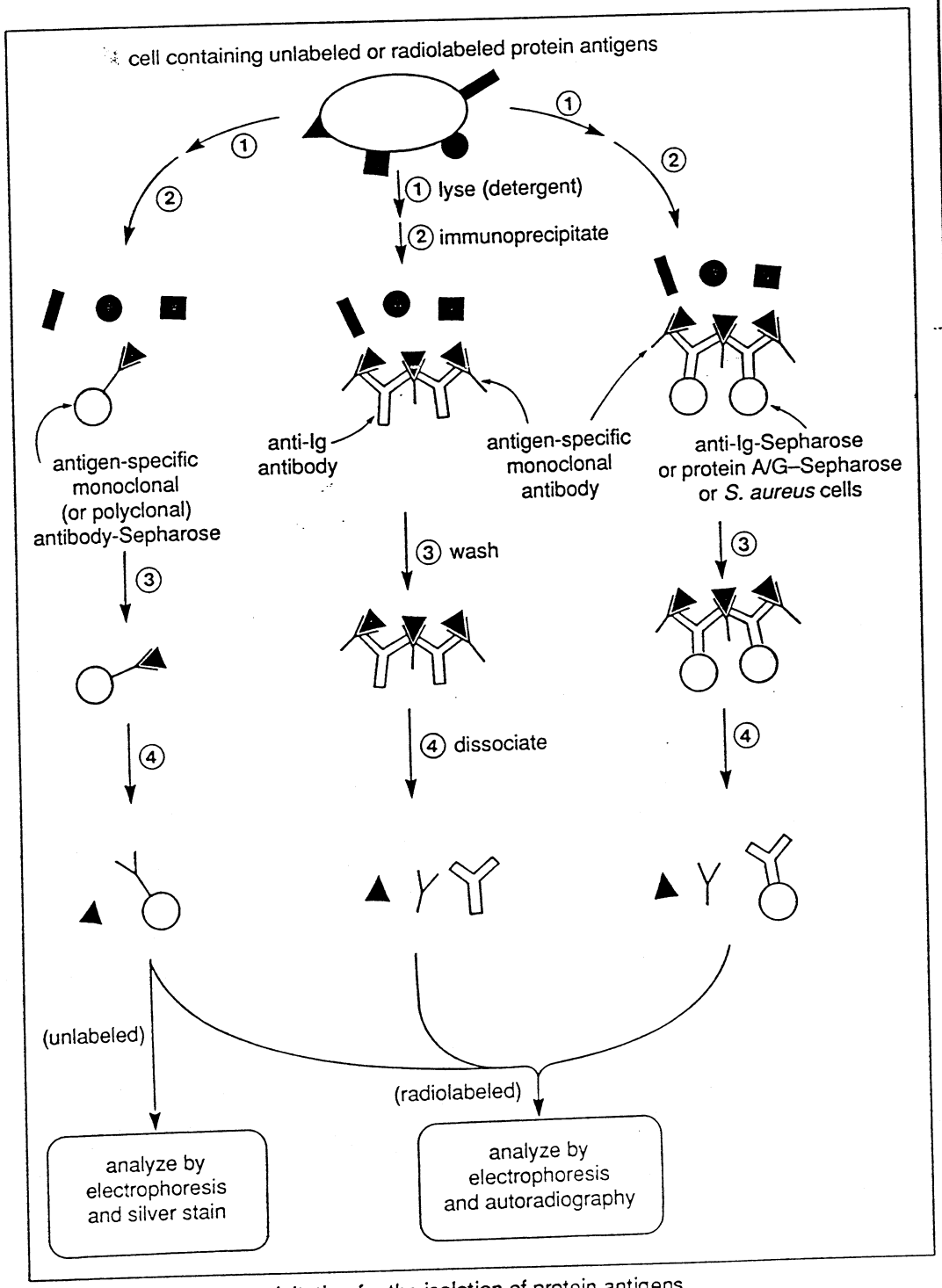

Figure 8.3.1 Immunoprecipitation for the isolation of protein antigens.

vant antibody is an antibody directed against an unrelated protein, and could also be whole IgG; it must not cross-react with the protein being immunoprecipitated.

Immunoprecipitate the antigen

5. Precoat 1.5-ml microcentrifuge tubes by filling with lysis buffer 10 min at room temperature. Remove the solution by aspiration.

   Precoating minimizes antigen absorption to the tube.

6. Add 10^5 to 10^6 cpm of radiolabeled (^{125}I or ^{35}S) supernatant containing antigen (from step 5) to a precoated microcentrifuge tube and bring the volume to 200 µl with dilution buffer.

   The recommended amount of radioactivity is appropriate for eukaryotic cells with >1000 molecules of antigen/cell. It is assumed that detection on slab gels of ^{125}I-labeled proteins will be carried out with enhancing screens and ^{35}S-labeled proteins with fluorography.

7. Add ~10 µl of a 1:1 slurry of Ab-Sepharose/dilution buffer and shake 1.5 hr at 4°C on an orbital shaker.

   The antibody coupled to Sepharose is antigen-specific. As described in the following support protocol, 5 mg/ml antibody per milliliter Sepharose is coupled, and the amount actually coupled can be estimated as described in step 10 of the support protocol. Shaking must be vigorous enough to suspend the Sepharose. Shaking may be extended to 3 hr; longer periods may increase background.

Wash, dissociate, and analyze the immunoprecipitate

8. Wash the Ab-Sepharose with 1 ml of the buffers listed below. After each wash, centrifuge 1 min at 200 × *g* or microcentrifuge 5 sec. Then, carefully aspirate the supernatant with a fine-tipped Pasteur pipet and leave 10 µl of fluid above the pellet. After the fourth wash, centrifuge again to bring down any residual drops on the side of the tube, aspirate, and leave 10 µl over the pellet.

   First wash: dilution buffer
   Second wash: dilution buffer
   Third wash: TSA solution
   Fourth wash: 0.05 M Tris·Cl, pH 6.8.

   Prepare a fine-tipped Pasteur pipet by pulling the pipet in a flame, scoring with a diamond pen, and breaking at the score.

9. Add 20 to 50 µl of SDS/sample buffer. Because the sample buffer has a higher density than the wash solution, it will sink into the Sepharose. Do not vortex because Sepharose may stick to side of tube above buffer level. Cap the tube securely and incubate 5 min at 100°C. Vortex and centrifuge 1 min at 200 × *g* or microcentrifuge 5 sec. Load the supernatant, carefully avoiding the Sepharose, into a gel lane and analyze by SDS-PAGE (*UNITS 8.4 & 8.6*). Detect labeled proteins by autoradiography (*APPENDIX 3*) with an enhancing screen (^{125}I) or by fluorography (^{35}S, ^{14}C, and ^{3}H).

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. It is necessary to first prepare the antibody and Sepharose separately. Next, the Sepharose is activated with cyanogen bromide (alternatively, CNBr-activated Sepharose can be purchased from Pharmacia LKB and used according to the manufacturer's instructions). Finally, the CNBr-activated Sepharose is coupled to the antibody.

Additional 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)
0.2 M Na_2CO_3
Cyanogen bromide (CNBr)/acetonitrile
1 mM and 0.1 mM HCl, ice-cold
0.05 M glycine (*or* ethanolamine), pH 8.0

Dialysis tubing (molecular weight cutoff >10,000)
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 (see APPENDIX 3).

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 be directly coupled 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 later use in step 10.

Prepare the Sepharose

4. Allow the Sepharose slurry to settle in a beaker and 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 cyanogen bromide

6. Transfer Sepharose to 50-ml beaker and add an equal volume of 0.2 M Na_2CO_3.

7. Activate Sepharose at room temperature 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 stirrer. 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. Wash with 2 vol of ice-cold 0.1 mM HCl. Hydrate the cake with enough ice-cold 0.1 mM HCl so the cake regains its sheen, but so 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, but the coupling capacity will be lower.

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 2). Stir gently with a magnetic stirrer 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, centrifuge to remove any residual Sepharose, and measure A_{280}. Compare absorbance to that of the A_{280} of the antibody solution from step 2 to determine the percentage coupling.

11. Store the Ab-Sepharose in TSA solution.

ALTERNATE PROTOCOL

IMMUNOPRECIPITATION OF RADIOLABELED ANTIGEN WITH ANTI-Ig SERUM

This protocol relies on the formation of soluble immune complexes between a protein and an antigen-specific antibody, followed by immunoprecipitation of the immune complexes by antibodies contained in anti-immunoglobulin (Ig) serum.

Additional Materials

Normal serum
Anti-Ig serum (Zymed Laboratories)
Antigen-specific antiserum *or* antigen-specific purified MAb *or* antigen-specific hybridoma culture supernatant

Follow the procedures in the basic protocol, with the following modifications at the indicated steps:

4a. Preclear by adding normal serum at a concentration of 2 µl/ml radiolabeled antigen. Add the proper amount of anti-Ig serum and let stand 12 to 18 hr at 4°C. Centrifuge 10 min at 1000 × *g* and reserve supernatant.

Normal serum is the source of carrier Ig. The proper amount of anti-Ig serum must be determined by titration with radiolabeled antigen or Ig. For high-titered anti-Ig serum, this amount would be 20× to 40× the volume of antigen-specific antiserum, 2 to 4 µl /µg purified MAb, or 1/3 the volume of hybridoma culture supernatant.

7a. Add 1 µl antigen-specific antiserum, 3 µg antigen-specific purified MAb, or antigen-specific hybridoma culture supernatant (30 µl cloned line or 100 µl uncloned line). Vortex and allow to stand 2 hr at 4°C. Then add the proper amount of anti-Ig serum, vortex, and allow to stand 12 to 18 hr at 4°C.

8a. Wash the immunoprecipitate as in the basic protocol, except centrifuge 7 min at 1000 × *g*.

9a. Add 20 to 50 µl of 2× SDS/sample buffer. Do not vortex, as immunoprecipitates may stick to side of tube above buffer level. Cap the tube securely. For immunoprecipitates, first incubate 1 hr at 56°C and then 5 min at 100°C. Load the mixture into a gel lane and analyze as in step 9 of the basic protocol.

The initial 56°C incubation enhances the dissolution of the immunoprecipitates by reducing irreversible aggregation which occurs when precipitated protein is rapidly heated to 100°C. Proteolytic degradation has never been noted, probably because of the high IgG protein concentration.

IMMUNOPRECIPITATION OF RADIOLABELED ANTIGEN WITH ANTI-Ig-SEPHAROSE, PROTEIN A– OR G–SEPHAROSE, OR *S. AUREUS* CELLS

ALTERNATE PROTOCOL

This protocol relies on the formation of soluble immune complexes between a protein and an antigen-specific antibody, followed by immunoprecipitation of the immune complexes by anti-Ig antibodies bound to Sepharose, by *Staphylococcus aureus* protein A or *Streptococcus* protein G bound to Sepharose, or protein A present on the surface of *S. aureus* cells. Monoclonal antibodies to rat and mouse κ chains are useful coupled to Sepharose or as adjuncts with *Staphylococcus* protein A or *Streptococcus* protein G, where antibodies do not couple successfully with protein A or G. In mouse and rat, 90% of immunoglobulins have κ light chains.

Additional Materials

1:1 (vol/vol) anti-Ig-Sepharose/dilution buffer (coupled at 10 mg/ml Sepharose as in support protocol), 1:1 (vol/vol) protein A– or G–Sepharose (Pharmacia LKB, Calbiochem, or Sigma)/dilution buffer, *or* 10% suspension *S. aureus* Cowan strain II bacteria

Follow the procedures in the basic protocol (see Fig. 8.3.1), with the following modifications at the indicated steps:

4b. Preclear as described below in one of the alternatives to step 7b (i, ii, or iii) as appropriate.

Preclearing removes nonspecific adsorbing materials.

7b. Add 1 µl antigen-specific antiserum, 3 µg antigen-specific MAb, or antigen-specific hybridoma culture supernatant (30 µl cloned line, 100 µl uncloned line). Then perform (i), (ii), or (iii) below.

(i) Add a 1:1 slurry of anti-Ig-Sepharose/dilution buffer.

(ii) Add a 1:1 slurry of protein A– or G–Sepharose/dilution buffer.

For (i) and (ii) above, use an amount 20× to 40× the volume of antiserum, 2 to 4 µl/µg MAb, or 1/3 the volume of hybridoma culture supernatants. Shake 1.5 hr at 4°C.

(iii) Wash a 10% suspension *S. aureus* Cowan II bacteria in lysis buffer in a low-speed centrifuge and resuspend at 10% in dilution buffer. Add 50 µl of the 10% suspension. Shake 10 min at 4°C.

8b. Wash the immunoprecipitates as in the basic protocol, except centrifuge slurries from (i) or (ii) (step 7b above) 1 min at 200 × *g* or slurry from (iii) 7 min at 1000 × *g*.

9b. Add 20 to 50 µl of SDS/sample buffer. Do not vortex since Sepharose and bacteria stick to side of tube above buffer level. Cap the tube securely and heat 5 min at 100°C. Vortex, microcentrifuge, and load the supernatant into a gel lane and analyze as in step 9 of basic protocol.

ALTERNATE PROTOCOL

IMMUNOPRECIPITATION USING MORE STRONGLY DISSOCIATING LYSIS AND WASH BUFFERS

This protocol should be used when protein antigens are suspected of being nonspecifically associated with other proteins after immunoprecipitation by the basic or alternate protocols.

Additional Materials

10% sodium deoxycholate (Na-DOC)
10% SDS
RIPA buffer

1. To the supernatant obtained after step 3 of the basic protocol, add 1/10 vol of 10% Na-DOC and 1/100 vol of 10% SDS to the lysate.
2. Repeat steps 3 to 7 of the basic protocol.
3. Repeat step 8 of basic protocol, except use RIPA buffer for first and second washes.
4. Repeat step 9 of the basic protocol.

ALTERNATE PROTOCOL

IMMUNOPRECIPITATION OF UNLABELED ANTIGEN WITH ANTIBODY-SEPHAROSE

Immunoprecipitation of unlabeled antigen followed by visualization with silver staining eliminates radiolabeling, one of the most tedious and expensive steps in immunoprecipitation protocols. Protein antigens present in greater than ~10^4 copies per eukaryotic cell may be detected by immunoprecipitation of unlabeled (i.e., not radiolabeled as in the basic protocol) cell lysates with Ab-Sepharose followed by SDS-PAGE (*UNIT 8.4*) and silver staining (*UNIT 8.9*). If antigen is eluted from beads in SDS lacking reducing agents, little antibody is coeluted.

Additional Materials

Modified lysis buffer
0.1% Triton X-100 in TSA solution
SDS/sample buffer without 2-mercaptoethanol (2-ME; *UNIT 8.4*)

1. Incubate 5×10^7 cells/ml in modified lysis buffer 1 hr at 4°C.
2. Centrifuge the lysate 15 min at 3000 × *g* to remove nuclei, and then centrifuge the supernatant 1 hr at 100,000 × *g*. Save the supernatant.

   Supernatants may also be prepared by microcentrifuging 30 min at 10,000 × g.

3. Preclear the lysate with 50 µl activated, quenched Sepharose (step 4, basic protocol) or irrelevant Ab–Sepharose/ml antigen by gently shaking 1 hr at 4°C. Centrifuge 5 min at 200 × *g* and save the supernatant.
4. Mix 25 µl of a 1:1 slurry of Ab-Sepharose/TSA solution per ml lysate and gently shake on an orbital shaker or mix by inversion 1 hr at 4°C.

   To control for antibody eluting from the beads, a control of Ab-Sepharose incubated with mock lysate should be run simultaneously. A control of lysate incubated with irrelevant Ab-Sepharose should also be run.

5. Wash as in step 9 of the basic protocol, except use the following buffers:

   First and second washes: 0.1% Triton X-100 in TSA solution
   Third wash: TSA solution
   Fourth wash: 0.05 M Tris·Cl, pH 6.8.

6. Add 20 to 50 µl of SDS/sample buffer without 2-ME. Do not vortex because Sepharose sticks to side of tube above buffer level. Cap tube securely and heat 5 min at 100°C. Microcentrifuge 5 sec to pellet Sepharose. Save the supernatant and apply to SDS-PAGE directly (nonreducing) or after incubation 1 hr at 37°C with 5% 2-ME (reducing). Load the mixture into a gel lane and analyze by SDS-PAGE (*UNIT 8.4*). Detect antigen by silver staining (*UNIT 8.9*).

Elution of antigen from Sepharose should be done under nonreducing conditions since antibody eluted from the beads under reducing conditions gives background staining. Theoretically, only 1 of the 4 chains in an IgG antibody molecule is covalently linked to the Sepharose, but interchain disulfide bonds keep all the chains linked under nonreducing conditions.

REAGENTS AND SOLUTIONS

CNBr/acetonitrile

To 25 g of cyanogen bromide *(CNBr should be white, not yellow, crystals)*, add 50 ml acetonitrile to make a 62.5% (wt/vol) solution. This may be stored indefinitely at –20°C in a desiccator over silica. Allow to warm before opening.

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

Dilution buffer

0.1% Triton X-100 (store at room temperature in dark)
0.1% bovine hemoglobin (store frozen)
Prepare in TSA solution (see below)

Lysis buffer

1% Triton X-100
1% bovine hemoglobin
1 mM iodoacetamide (freshly prepared)
Aprotinin (0.2 trypsin inhibitor U/ml)
1 mM phenylmethylsulfonyl fluoride (PMSF; add fresh from 100 mM stock in absolute ethanol)
Prepare in TSA solution (see below)

Modified lysis buffer

0.5% Triton X-100
1 mM PMSF
5 mM iodoacetamide
Aprotinin (0.2 trypsin inhibitor U/ml)
Prepare in TSA solution (see below)

RIPA buffer

1% sodium deoxycholate
0.1% SDS
Prepare in lysis buffer (see above)

Tris/saline/azide(TSA) solution

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

COMMENTARY

Background Information

Because of their high specificity, antibodies may be used to isolate specific antigens that are minor components of complex mixtures, such as cell lysates. Prior to isolation, membrane proteins must be solubilized into detergent micelles. Nonionic detergents are preferred over bile salts (see *APPENDIX 1*) as the latter precipitate at acid pH or in the presence of bivalent cations.

Both conventional and monoclonal antibodies may be employed for immunoprecipitation. Procedures used here are similar to those in Kürzinger and Springer (1982), Springer (1981), Ho and Springer (1983), Sanchez-Madrid et al. (1983), and Sastre et al. (1986). Monoclonal antibodies are usually nonprecipitating, as are most polyclonal antibodies at the low antigen concentrations employed. Therefore, a "sandwich" reagent is used to precipitate or isolate the antibody-antigen complex. The sandwich reagent may be anti-Ig serum, affinity-purified or monoclonal anti-Ig, *Staphylococcus* protein A or *Streptococcus* protein G coupled to Sepharose, or *S. aureus* Cowan strain II cells. Protein A methods are not generally applicable to either the rat (only its IgG1 and IgG2c subclasses bind) or the mouse (its IgG1 subclass binds too poorly for immunoprecipitation). In many cases protein G can be substituted for protein A as a conjugate (Table 8.3.1). However, protein G–Sepharose appears to have a lower binding capacity.

The identification of immunoprecipitated, radiolabeled protein antigen is achieved by SDS-PAGE followed by detection of radiolabeled proteins by autoradiography. Nonlabeled proteins are detected by silver staining (Dustin et al., 1986).

Critical Parameters

Anti-Ig serum gives very low backgrounds and is preferable to affinity-purified anti-Ig for immunoprecipitation. Anti-Ig precipitation gives lower backgrounds than *S. aureus* cells. Furthermore, there is no advantage to precoating *S. aureus* with antibody, since antibody-antigen complexes will bind preferentially over free antibody anyway (Kessler, 1975). Results comparable in excellence to anti-Ig serum are obtained with anti-Ig or protein A coupled to Sepharose. The use of monoclonal anti-Ig, such as anti-kappa monoclonal antibodies coupled to Sepharose, is also convenient.

The best results in terms of complete precipitation and lower backgrounds are obtained when an antigen-specific antibody is coupled directly to Sepharose. Sepharose CL-4B appears to give a lower background than CL-2B, perhaps because aggregates are better excluded.

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, by using an acid wash buffer (Gelb, 1973) as described in the support protocol,

Table 8.3.1 Relative Affinities of Proteins A and G for Various IgG Subclasses[a]

Antibody	Affinity for protein A	Affinity for protein G
Human IgG1	++++	++++
Human IgG2	++++	++++
Human IgG3	–	++
Human IgG4	++++	++++
Rat IgG1	+ or ++	+
Rat IgG2a	–	++++
Rat IgG2b	–	++
Rat IgG2c	++ or ++++	++
Mouse IgG1	+	++++
Mouse IgG2a	++++	++++
Mouse IgG2b	+++	+++
Mouse IgG3	++	+++

[a] Adapted from Harlow and Lane (1988).

activated groups remain stable for ≥30 min. 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 low amount of CNBr recommended is sufficient to obtain a coupling yield of 80% to >99%. Higher amounts of CNBr may result in multipoint attachment of IgG molecules to the matrix, thereby reducing accessibility to antigen. Most investigators purchase CNBr-activated Sepharose, while others, to achieve a higher coupling efficacy or to avoid the expense of the commercial product, prefer to prepare it themselves.

Quantities and ratios recommended in these protocols have been found to work well with several hundred monoclonal antibodies and more than 40 different antigens. However, titration of Ab-Sepharose or sandwich reagents versus the protein antigen may further optimize a given immunoprecipitation.

Reaction between antibody and antigen can be extended overnight, but this may increase the background when using Ab-bound Sepharose. Antigen-antibody complexes should not be left overnight in the midst of washing, as significant dissociation may occur.

In vitro translation products often appear aggregated and require pretreatment. Clear results have been obtained by incubation at 100°C in SDS followed by dilution in nonionic detergent to form mixed micelles prior to precipitation (Anderson and Blobel, 1983).

Troubleshooting

Distortion of SDS-PAGE separations can result from loading an excessive amount of unlabeled antibody used for immunoprecipitation, since wide zones of migrating heavy and light chains "push" background radioactivity ahead of them in the gel. This can give rise to artifact bands. In the presence of SDS and 2-mercaptoethanol, all IgH and IgL chains, except the 25% actually linked to Sepharose, will be eluted from Ab-Sepharose, which leads to contamination of the sample. Anti-Ig antibodies should always be affinity purified before coupling to Sepharose to reduce the amount of nonspecific antibodies coupled.

Anticipated Results

Provided that an monoclonal (or polyclonal) antibody directed against the protein of interest is available, it should be possible to immunoprecipitate the protein of interest from a complex mixture of proteins, using the basic or alternate protocols. Coupling yields for Ab-Sepharose are 80% to >99%.

Time Considerations

The basic, alternate, and support protocols each require ~1 day to complete. Most of the time involves waiting for cell lysis, centrifugation, nonspecific and specific binding reactions, or chemical reactions to be completed.

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The above references describe various detergents and solubilization conditions.

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