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Chapter 13
Purification and characterization of monoclonal antibodies

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The advent of the Köhler–Milstein method for the preparation of monoclonal antibodies [1] has produced a revolution in immunology and in diverse fields that utilize highly specific antibodies as assay reagents. Depending on the source, purification of such monoclonal antibodies can be anything from a simple task to an involved and difficult procedure. Certain advances in the past few years (such as serum-free growth medium) have considerably simplified previously described methods. Still, lack of (or incomplete) purification remains one of the stumbling blocks to obtaining clear results from such reagents.

A variety of methods exist for characterizing the purity and specificity of monoclonal reagents. The choice of which criteria is applicable depends to a large extent on the specific uses planned for the reagent. For example, a second-step labelled reagent used for radioimmunoassay must be free of any other binding specificity, but need not be free of other 'non-specific' IgG that co-purifies in an ion-exchange isolation. On the other hand, if a purified antibody is to serve as a target antigen in such an assay, then careful affinity methods will probably be necessary to eliminate contaminating antibody. In the final analysis, it is necessary to characterize the reagent in the intended application in order to determine which purification method is suitable.

Purification: from serum or ascites

Purification from serum or ascites offers the advantage of high yields of immunoglobulin, balanced by the disadvantage of numerous contaminating materials. In cases where hundreds of milligrams of antibody are required, such sources offer the only reasonable method (although growth in serum-free medium may become a reasonable alternative in the near future; see below).

Primary clarification

Serum or ascites is collected, allowed to clot and then stored frozen at either −20 °C or −70 °C [2]. On thawing just prior to purification, occasionally a fibrinogen clot forms (especially in ascites). This mass should be filtered out through fine-mesh nylon cloth (Nitex) or pelleted by centrifugation.

A major problem with some serum or ascites samples is the presence of lipids that clog columns and cloud samples. Often lipid can be eliminated in a centrifugation step (e.g. during the ammonium sulphate precipitation stage, as described below) or during a gel filtration step (eluting in the void volume). On the other hand, for very difficult samples, direct extraction with organic solvents is possible [3].

Purification method: delipidation technique

Equipment

Rotator ('Labquake', Labindustries, Berkeley, CA).
Centrifuge (RC-5, DuPont).

Materials

Extraction solution: 1 part butanol; 4 parts diethyl ether.

Procedure

1 Mix 1 vol. of serum/ascites with 1 vol. of a 1:4 mixture of butanol/diethyl ether and rotate gently for several hours at room temperature.
2 Transfer mixture to a well-capped polypropylene centrifuge tube and centrifuge to separate the two phases; remove organic phase (containing lipids).
3 Remove residual organics by applying water-aspirator vacuum for several hours or by blowing a stream of nitrogen across the liquid surface.

Notes and recommendations

Use good safety precautions when working with volatile organic solvents. Work in a well ventilated area away from sources of ignition. Ether is particularly hazardous due to a low flash point and a tendency to form explosive peroxides when stored for long periods.

Ion-exchange methods

Probably the most commonly employed method for the purification of immunoglobulin (monoclonal or heterogenous) from serum involves some application of ion exchange [4]. The most useful ion-exchange material for IgG antibody purification has been found to be some derivative of the weak basic ion-exchange group DEAE (diethylaminoethyl) [5]. A major disadvantage of these materials in the past was poor flow and clogging due to properties of the support matrix. Recently, DEAE on a bead cellulose (DEAE-Sephacel, Pharmacia), which has superior flow properties and stability, has become available. The method remains the same: IgG from serum binds to the ion-exchange support at low ionic strength; a salt gradient then selectively elutes immunoglobulins and, at higher ionic strength, albumin.

Unfortunately, certain immunoglobulins are not completely stable at low ionic strength so that they precipitate during the DEAE procedure. This is particularly a problem with certain isotypes, notably mouse IgG_3. Strongly basic ion-exchange groups such as QAE (quaternary amino ethane) remain charged at higher pH where IgG will remain bound at higher ionic strength and so offer a solution to this problem [6]. It is possible to design a batch absorption method whereby most contaminating proteins are absorbed out of an antibody serum, leaving IgG behind in the supernatant.

Purification method: ion-exchange gradient fractionation

Equipment

Fraction collector (FC-80K, Gilson).
Gradient former (GM-1, Pharmacia).
UV monitor (UV-1, Pharmacia).
UV-visible spectrophotometer (PM6, Zeiss).

Materials

DEAE-Sephacel (Pharmacia).

Procedure

1 Twelve millilitres of ascites containing IgG_{2a} is diluted 1:1 with 50 mM-Tris (pH 8.0).
2 Add slowly with stirring 24 ml of saturated ammonium sulphate (all at 4 °C); continue stirring for 2 h.
3 Centrifuge to collect precipitate: 10 000 rev./min. for 10 min.
4 Resuspended precipitate in 50 mM-Tris/Saline (to 12 ml).
5 Dialyse overnight versus 2 l of 50 mM-Tris at 4 °C; final volume 20 ml.
6 Pack and equilibrate DEAE-Sephacel column (1.5 × 20 cm) with 50 mM-Tris buffer at room temperature; wash column with 2 bed vols. of 50 mM-Tris.
7 Resuspend any precipitate formed on dialysing and apply to equilibrated column (if flow rate decreases, stir top of bed).
8 Wash through 2 bed vols. of 50 mM-Tris (collect fractions).
9 Run gradient: 250 ml of 50 mM-Tris in chamber A; 250 ml of 0.3 M-NaCl/50 mM-Tris in chamber B; IgG2a is eluted early in the gradient (after about 85 ml); the column profile for a representative monoclonal antibody is shown in Fig. 13.1.
10 Pool protein-containing fractions as determined by optical density (OD) at 280 nm and dialyse versus Tris/Saline (4 °C).
11 IgG_1 proteins are isolated as for IgG_{2a}; IgG_1 elutes later (about 110 ml).

Notes and recommendations

Purified immunoglobulin is often much more susceptible to denaturation than immunoglobulin in serum. Vigorous shaking of purified antibody solutions may lead to formation of precipitate. In particular, glass and polystyrene plastics should be avoided as much as possible. Storage in polypropylene tubes is probably least harmful.

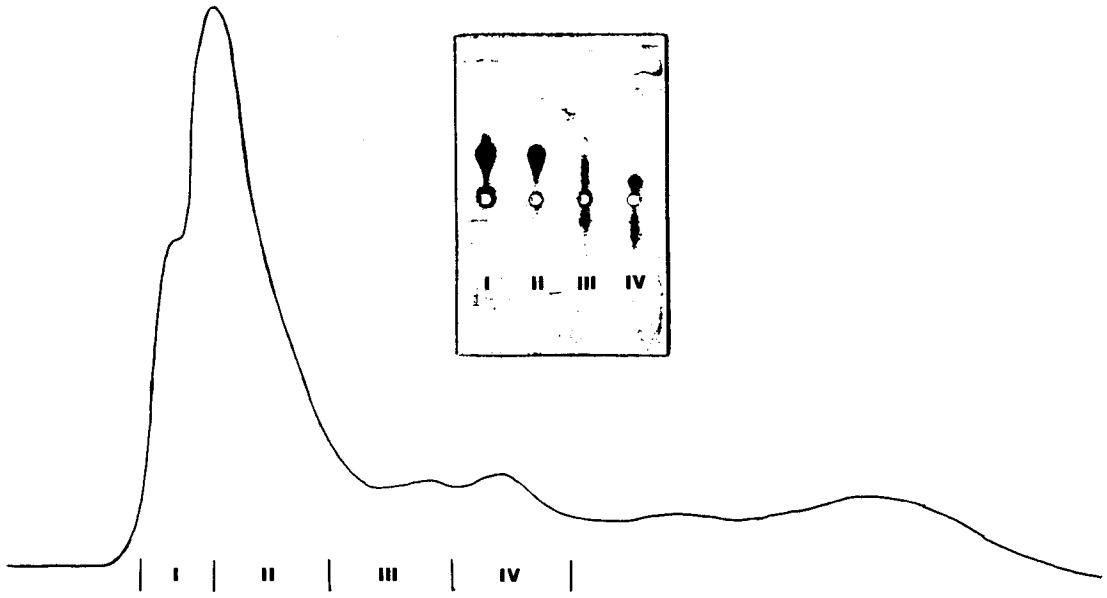

Fig. 13.1. DEAE-Sephacel gradient purification of monoclonal IgG2a. Inset shows agarose electrophoresis analysis of four column fractions. Fraction II is essentially pure immunoglobulin. Final broad peak (not numbered) is residual albumin.

Transferrin is often not well separated by the ion-exchange gradient method. During a 2 h ammonium sulphate precipitation relatively little transferrin should precipitate, but longer precipitation times will increase the amount of transferrin considerably. Gel filtration will then be necessary to eliminate the contaminating transferrin.

Purification method: QAE batch purification of monoclonal immunoglobulins

Equipment

Centrifuge.
UV-visible spectrophotometer (Zeiss).
Rotator ('Labquake', Labindustries, Berekley, CA).

Materials

QAE A-50 Sephadex (Pharmacia) Syringe, 5 cm^3 (Becton-Dickinson).
Polypropylene sample tube (17 × 100 mm, #2059, Falcon).
Desalting column (PD-10, Pharmacia).

Procedure

1 First, carry out 50% saturated ammonium sulphate precipitation at 4 °C by slow, dropwise addition of SAS to ascites or serum previously diluted 1 : 1 with buffer; continue stirring for 2 h.
2 Spin down (10 000 rev./min. in SS34 rotor/RC-5 centrifuge), precipitate and redissolve in Tris/saline buffer (volume about one-half original volume or less; see 'Notes and recommendations').
3a Dialyse versus buffer for at least 12 h to get > 1 : 100 dilution of ammonium sulphate.
3b Alternatively, for small-scale purifications, apply to desalting column (PD-10, Pharmacia) equilibrated with buffer and collect 0.5 ml fractions; pool protein fractions by optical density at 280 nm.
4 Pre-equilibrate QAE-Sephadex A-50 with buffer.
5 Mix 1 vol. gel with 2 vols. dialysed protein solution (this absorbs albumin). Continue mixing gently by rotating for 2 h.
6 Spin down gel and take supernatant; for maximum recovery spin liquid out of gel using syringe-centrifuge technique (see Fig. 13.2). Dialyse into appropriate buffer.
7 Characterize supernatant; it should contain mostly IgG (> 95%).

Notes and recommendations

Difficulties with transferrin, as described above, exist in QAE absorption also; limit precipitation to 2 h. In addition, this procedure seems to work best with

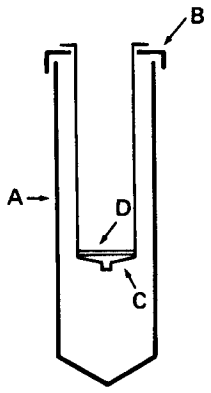

Fig. 13.2. Diagram of syringe centrifuge filtration apparatus. A: 50 ml disposable polypropylene centrifuge tube; B: cap with hole (made using cork borer); C: syringe barrel or used PD-10 column; D: plastic filter frit.

relatively concentrated antibody solutions (>2 mg/ml), so redissolving the ammonium sulphate precipitate in a minimum volume of buffer prior to dialysis or desalting is highly recommended.

Gel filtration (IgM antibodies)

Gel filtration is most commonly used in the purification of monoclonal IgM antibodies [6], since relatively few serum proteins have molecular weights of the order of IgM (900 000) while several have molecular weights of the order of IgG (150 000). In addition, the pI values for IgM vary widely, which makes ion-exchange isolation less predictable compared to IgG. A gel with a fractionation range extending beyond one million (AcA 22, LKB) is useful since protein aggregates and lipid micels often chromatograph in the void volume (effective molecular weights far greater than one million), while IgM will be in the included volume.

IgG can also be purified by gel filtration if a major objective is avoiding the ammonium sulphate precipitation steps. Small amounts (10–20 mg) of IgG can be highly purified by gel filtration (AcA 34, LKB) of delipidated ascites (or serum) followed by ion-exchange chromatography on DEAE-Sephacel (Pharmacia). One important use of gel filtration is in the removal of transferrin (occasionally a large contaminant), which often co-purifies with IgG by ion exchange [7].

Purification method: IgM by AcA 22 gel filtration

Equipment

Fraction collector (FC-80K, Gilson).
UV monitor (UV-1, Pharmacia).

Materials

AcA 22 gel (LKB).

Procedure

1 Pour an AcA 22 column approximately 70 cm long (2.5 cm diameter) and equilibrate in column running buffer (Tris/Saline).
2 Apply up to 5 ml of dialysed, delipidated ascites (or dialysed ammonium sulphate cut) and develop the column with running buffer.
3 Monitor the effluent at 280 nm and collect fractions.
4 IgM should elute just after the void volume.

Notes and recommendations

Some IgM antibodies are 'cryoglobulins' and will precipitate at 4 °C if stored in a relatively concentrated solution (>1 mg/ml). In general, low ionic strength buffers should be avoided.

Affinity chromatography

Affinity chromatography covers a broad range of purification methods, all of which involve specific interaction of immunoglobulin (or contaminating protein) with a binding molecule covalently attached to an insoluble support matrix [8]. When antibody-containing solutions are applied to such columns, this interaction will deplete from the pass-through fluid all proteins that bind to the matrix. Obvious applications include antigen (or hapten) columns [9], antibody columns (specific for certain classes of Ig) [10] and protein columns that interact specifically with Ig (e.g. lectins or protein A) [11].

Protein A is a membrane protein of the bacterium *Staphylococcus aureus* which specifically binds certain isotypes of immunoglobulins: in mouse, IgG_{2a}, IgG_{2b}, some IgG_1 and some IgM are bound; in human, IgG of subclasses 1, 2 and 4 are bound, as are certain IgM and IgA proteins [12]. There is differential binding (pH sensitive) for certain isotypes and this can be used in purification to separate these types. In this application, protein A is coupled to a solid support such as Sepharose 4B (Pharmacia).

Antigen (or hapten) affinity columns take advantage of the specificity of the immunoglobulin of interest to specifically purify it. In practice, antigen (or hapten) is coupled to a solid support such as Sepharose 4B (Pharmacia). This is possible by use of a number of coupling reactions, the choice of which is somewhat dependent on the antigen in question. For protein antigens, cyanogen bromide activation is commonly

used [13] and pre-activated gel is commercially available (CNBr-Sepharose 4B, Pharmacia). After application of Ig-containing serum or ascites to the column, the column is washed to remove non-adhering contaminating material, and then the antibody is eluted. Elution is often the most difficult step, especially when dealing with high-affinity antibodies. Elution may be accomplished with free hapten [14], by shifts in the pH of the eluting buffer [15] or by chaotropic agents (such as sodium thiocyanate) [16].

One obvious limitation of this technique is the requirement for relatively large amounts of purified antigen which may not be readily obtainable. In addition, high affinity antibodies may be very difficult to remove once bound. A possible solution to this problem is to use an analogue of the antigen which is bound at lower affinity by the antibody in question [17]. A final consideration is that conditions of elution may inactivate or in some way degrade the activity of the antibody. For example, antibody can be eluted from an antigen affinity column with hapten in order to obtain highly purified antibody preparations, but such preparations are then contaminated with hapten which may be difficult to dissociate from the antibody. Another problem occasionally encountered is irreversible denaturation of antibody under the conditions of elution.

A specific affinity absorbant occasionally employed in antibody purification is a gel with the organic dye Cibacron blue (F3G) which binds albumin and many proteases [18]. Passage of serum or ascites through such a column prior to ion-exchange purification eliminates much of the albumin which can contaminate certain monoclonal preparations. There is also a commercially available column that incorporates both ion exchange (DEAE) and Cibacron blue (Bio-Rad, Richmond, CA) which permits such absorption together with ion-exchange in a single step [19].

Purification method: protein A column

Equipment

Fraction collector (FC-80K, Gilson).
UV monitor (UV-1, Pharmacia).

Materials

Protein A–Sepharose CL-4B, Pharmacia.

Procedure

1 Pour protein A column, wash with low pH eluting buffer and cycle into running buffer (see 'Buffers and media').

2 Apply 1:2 diluted ascites or serum (diluted with running buffer) to the column and then wash column with running buffer.

3 Elute absorbed Ig with eluting buffer and collect into phosphate neutralizing buffer 1:1.

4 Dialyse protein-containing eluate against running buffer; determine purity of IgG; it should be greater than 95% immunoglobulin.

Notes and recommendations

Removal of antibody from low pH conditions is absolutely essential for maintenance of antibody activity. Certain antibodies may not tolerate the acid condition even for a short time and such immunoglobulins should be purified by thiocyanate elution or by ion exchange.

Purification method: DNP hapten column

Equipment

Fraction collector (FC-80K, Gilson).
UV monitor (UV-1 Pharmacia).

Materials

CNBr-activated Sepharose 4B (Pharmacia).
Basic ion-exchange resin (Dowex AG 1-X8, Bio-Rad, Richmond, CA).

Procedure

1 Prepare DNP hapten affinity column by mixing methyl ester of DNP-lysine with CNBr-activated Sepharose 4B (Pharmacia).

2 Apply ascites or serum containing DNP-specific antibody protein to the column and wash with running buffer until protein is no longer present in column effluent (OD 280 nm <0.05).

3 Elute antibody either with 0.05 M-DNP-glycine solution (pH 7.4) or with 1 M-sodium thiocyanate solution; remove DNP-glycine by passing eluted protein solution through basic ion-exchange resin (AG 1-X8); remove thiocyanate by dialysis or by passing down desalting gel filtration column; characterize protein.

Notes and recommendations

Removal of the final two equivalents of hapten from a very high-affinity antibody may not be possible by dialysis. If thiocyanate does not irreversibly denature the antibody, it is probably the method of choice.

Hybridoma supernatant

Monoclonal antibodies are commonly obtained from the supernatant medium in which the cells are grown [20]. Cells in tissue culture flasks grow at much lower density compared to the same cells growing in the peritoneal fluid (ascites) of a mouse, thus yielding a much lower concentration of antibody (typically 100-fold lower). However, yields of antibody in the 1–10 mg range are readily obtainable from reasonable volumes and, for some investigators, tissue culture is preferable to maintaining mice. In addition, certain hybridoma cell lines are derived from unusual strain combinations which make the choice of host animal complex [21]. Although rat–mouse hybrids will occasionally grow in nude mice [22], the author has generally found tissue culture supernatant to be a ready source.

The major problem to be overcome in purifying antibody from 'spent medium' is the low concentration of antibody, especially when compared to the other (serum-derived) proteins present in the medium. Affinity methods seem best suited to such isolation, and several are described in the following section. However, the recent development of serum-free medium [23], which permits the isolation of immunoglobulin by precipitation and ion exchange, may supplant affinity methods as high-density tissue culture methods become available.

Affinity chromatography of hybridoma supernatant

All the affinity methods described above for purification of antibody from serum can be applied to purification from supernatant. Protein A is perhaps the most widely used means of purifying immunoglobulins of the appropriate isotype from supernatant [24]. An important consideration with supernatant is that a large volume of liquid will be passed over the column, so that a short column with a large diameter is preferable to a long column with a small diameter. Another commonly used affinity column employs an immunoglobulin–anti-immunoglobulin matrix, such as a goat anti-rat IgG column [25]. Such columns are typically eluted with low pH glycine buffers, and an important consideration is the rapid neutralization of this buffer by direct collection into concentrated phosphate buffer.

Purification method: anti-rat Ig column

Equipment

Fraction collector (FC-80K, Gilson).
UV monitor (UV-1, Pharmacia).
UV-visible spectrophotometer (PM6, Zeiss).

Materials

CNBr-activated Sepharose 4B (Pharmacia).
Anti-rat Ig serum.

Procedure

1 The most convenient anti-rat IgG column has been prepared with selected goat anti-rat IgG. First, goat anti-rat IgG antiserum is absorbed on to a rat IgG column. Then the 'low-affinity' antibody is eluted by 0.1 M-glycine buffer (pH 3.2); this immunoglobulin is then coupled to column matrix by the standard CNBr coupling method.
2 Cycle the column into 0.1 M-glycine (pH 2.4) buffer, and then wash extensively with running buffer. Column should be used and stored at 4 °C. Always include a bacteriostatic agent in the buffer (e.g. 0.1% NaN_3).
3 Supernatant (typically 1 l or more) is applied to the goat anti-rat IgG column (typically 10 × 1.5 cm), and the column is washed afterwards with running buffer until protein is no longer detectable in the effluent (as monitored by optical density OD at 280 nm).
4 Elute the specifically bound rat IgG by applying eluting buffer; protein typically elutes in a broad band and may be relatively dilute.
5 Concentrate the rat IgG either by vacuum dialysis or by ammonium sulphate precipitation; characterize protein.
6 Cycle column with 0.1 M-glycine (pH 2.4) buffer, and then wash extensively with running buffer; column should be at low pH for the shortest time possible; such recycling is possible for at least 50 times, during which the capacity of the column will gradually decrease.

Serum-free medium

A major advance in the purification of monoclonal antibodies produced by cell lines that are difficult to grow in animals (e.g. rat–mouse hybrids) has been the introduction of defined serum-free media that can support the growth of most such lines. After a short period of adaption to the serum-free medium, hybridoma lines grow to high density and secrete as much antibody as when grown in serum-containing medium. Such media contain trace amounts of insulin and transferrin and considerable amounts of bovine serum albumin, but no other proteins [26]. Thus a simple ammonium sulphate precipitation yields a mixture of Ig and BSA, which can be further purified by ion-exchange absorption of the BSA.

Purification method: precipitation and batch absorption

Equipment

Centrifuge (RC-5, Dupont).
UV-visible spectrophotometer (PM6, Zeiss).

Materials

Serum-free medium (HB101 or HB102, HANA Biological).
Ammonium sulphate (Sigma).
QAE-Sephadex A-50 (Pharmacia).

Procedure

1 First, precipitate the protein from spent supernatant (typically 1 l) at 4 °C by addition of solid ammonium sulphate (350 gm/l is 50% saturation). Continue stirring for 2 h.
2 Spin down precipitate and redissolve in buffer (typically 0.1–0.5% of original supernatant volume).
3a Dialyse versus at least two changes of buffer.
3b Alternatively, apply to a small desalting column (PD-10, Pharmacia) equilibrated with buffer and collect 0.5 ml fractions; pool protein fractions.
4 Pre-equilibrate QAE-Sephadex A-50 with buffer.
5 Mix 1 vol. of gel with 2 vols. of dialysed protein solution (to absorb albumin). Continue mixing gently by rotating for 2 h at room temperature.
6 Spin down gel and take supernatant; for maximum recovery spin liquid out of gel using syringe-centrifuge technique (see Fig. 13.2). Dialyse into appropriate buffer.
7 Characterize supernatant; it should contain mostly IgG (>95%).

Notes and recommendations

Do not precipitate for more than 2 h since considerable amounts of transferrin will precipitate; transferrin is difficult to remove in subsequent ion-exchange steps.

Methods: characterization

Part of the purification technique for any monoclonal reagent should incorporate some method for characterizing the purity of the antibody and its binding specificity. This permits comparison with previous batches of the same reagent (if any) and serves as a method for comparison to different reagents. Methods routinely employed to assess purity and specificity should be simple enough to apply to all purification batches and sensitive enough to give reliable data. The methods described in the following sections include physical characterization of the purity and state of aggregation of the immunoglobulin, followed by binding assays to determine specificity of the antibody.

Electrophoretic methods

The most widely used methods for characterizing purified immunoglobulins are various forms of electrophoresis: the mobility of the protein is determined in a specific buffer–gel matrix. Probably the simplest method is agar electrophoresis in barbitol buffer [27]. This system takes advantage of the different charge on most immunoglobulins compared to most other serum proteins. One disadvantage of this method is lack of resolution of all immunoglobulin isotypes from serum proteins. In particular, IgM antibodies are often not well resolved. This method seems best as a quick, crude assay for the presence of concentrated immunoglobulin.

High-resolution methods for the characterization of antibodies include polyacrylamide gel electrophoresis in sodium dodecyl sulphate detergent (SDS-PAGE) [28], isoelectric focussing [29] and a combination of these two—two-dimensional (2-D) gel electrophoresis [30]. There are many good reviews of the methods involved in carrying out SDS-PAGE [31], isoelectric focussing [32] and 2-D gels [33]. A new method for visualizing proteins in such gels has been developed in the past few years which is considerably more sensitive than older dye methods. This is the silver stain technique [34] which yields sensitivities on the order of that previously obtainable with radio-labelled reagents. Kits are now available (Bio-Rad, Richmond, CA) which include all reagents necessary, together with protocols for this staining method.

Characterization method: agarose electrophoresis in barbitol buffer

Equipment

1000 V regulated power supply (Buchler Instruments).
Electrophoresis tank.
Paper wicks.

Materials

Agarose (Oxoid Ionagar No. 2).
Glass slides.

Procedure

1 See Fig. 13.3 for basic layout.
2 Prepare agarose slides: dip a rack of 2″ × 3″ glass

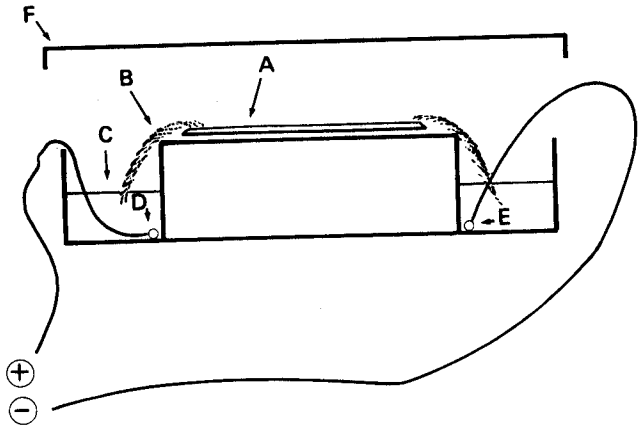

Fig. 13.3. Diagram of agar electrophoresis tank. A: agarose slide; B: paper wick; C: barbitol buffer; D: cathode; E: anode; F: lid.

slides in 0.1% Ionagar in distilled water; allow to dry; autoclave 2% Ionagar in distilled water and add 1 part this solution to 1 part prewarmed barbitol buffer; use 6 ml per slide; allow slides to set for at least 24 h; store in sealed box with moist filter paper.

3 Punch wells in agar with 15-gauge needle connected to water aspirator; use pre-drilled template to run many samples per slide; apply concentrated protein solution into well (typically 1 mg/ml or greater).

4 Each chamber of tank should be filled with 1 × barbitol buffer (the buffer is made up as a 2 × stock); it can be reused several times, but anode and cathode chamber solutions must be mixed before reuse.

5 Place slide in tank; place wet absorbent paper wicks to each end of slide and into the buffer solution; put lid on chamber.

6 Attach power leads and turn on power supply; run for 60–90 min at 20 mA per slide in current regulation mode (typically 50–70 V).

7 Turn power off; remove agar slide and stain in 'buffalo black' stain for 2–5 min; remove and wrap in moistened bibulous paper; dry in warm air dryer for 2 h; destain in 5% acetic acid for about 5–15 min; dry slide.

Liquid chromatography

Recently, methods of rapid gel filtration have been developed that permit analytical gel filtration to become a standard assay method [35]. These methods involve medium- to high-pressure pumps coupled with much stronger gel permeation matrices (which are not crushed by the high pressures developed in these systems). A number of manufacturers have designed modified versions of standard organic high-performance liquid chromatographs (HPLCs) specifically for aqueous systems. Columns are now available for standard HPLC analysis that permit aqueous phase fractionations over the 5–30, 20–250 and 100–2000 kDa ranges. Analyses typically require 15–30 min per run and up to 1 mg can be loaded for 'preparative scale' fractionation.

Size fractionation under standard buffer conditions adds important extra information about an antibody preparation. Specifically, the state of aggregation (or degradation) of the antibody can be readily assessed. Certain antibody preparations that look quite ordinary under reducing conditions on an SDS gel have been found to consist mainly of high molecular weight aggregates when analysed by HPLC. Such aggregates, possibly formed during low pH steps, often lead to unacceptably high non-specific background binding in assays. Thus HPLC may serve to localize problems with purification procedures that could not be easily visualized by other standard physical methods. A typical HPLC profile is shown in Fig. 13.4.

Binding assays: determining antibody activity

The other major question that must be answered for any purified antibody, after purity is determined, is that of specificity. To some extent, this is less of a problem with monoclonal reagents than with previously available heterogeneous antisera. With antisera, new lots had to be completely characterized with regard to specificity, and absorptions to ensure specificity had to be performed with each reagent. Monoclonals, while eliminating these problems, must still be checked with some degree of caution. This ensures that mix-ups will be detected early and that any changes in reagent activity (sometimes noted due to changes in levels of the non-specific NS-1 light chain; N. Warner, personal communication) will be quantified.

The most commonly employed assay for determining antibody reactivity with antigens available in

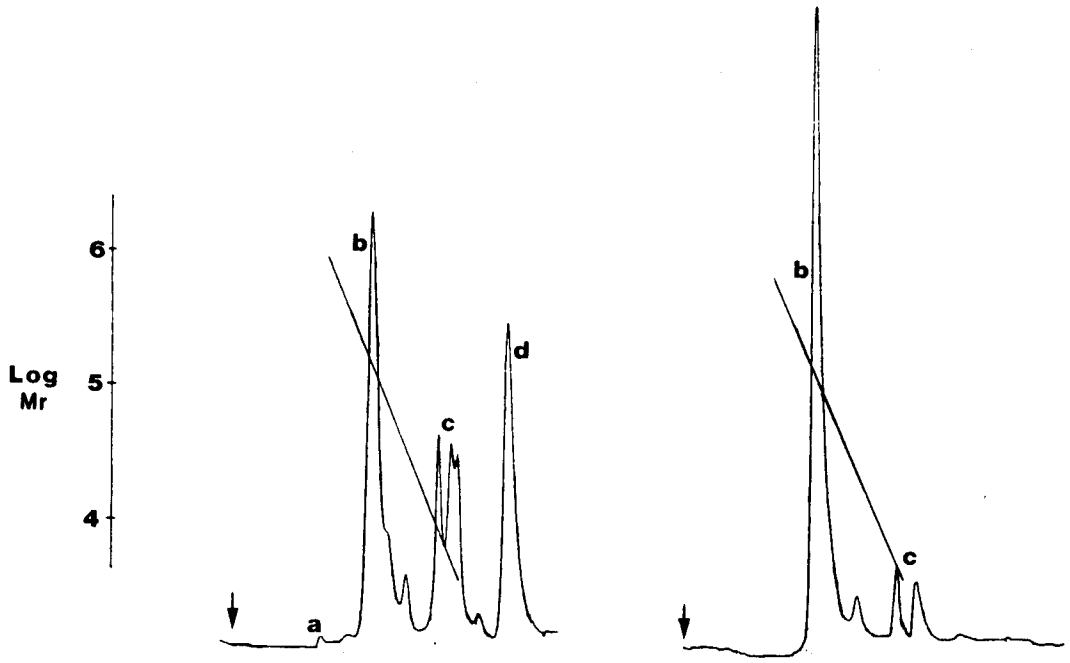

Fig. 13.4. Size-exclusion HPLC fractionation of purified monoclonal antibody using a TSK-250 column (molecular mass range approximately 250–20 kDa). The preparation analysed on the left is badly degraded. The preparation analysed on the right is typical of relatively pure antibody after several months storage and is mostly intact. A: aggregated protein; B: antibody peak; C: peptide fragments; D: sodium azide. Arrows mark the injection timepoint.

reasonably large amounts (greater than milligram lots) is a solid-phase plate binding assay [36]. In such a procedure, the wells of a microtitre plate are incubated with antigen (typically, 10–50 μg/ml in phosphate-buffered saline) for 1 h at room temperature. The unbound antigen is washed off and residual plastic sites are saturated with an excess of bovine serum albumin. Dilutions of antibody are next applied to the wells; dilutions of a standard antibody lot can serve as a control; after suitable incubation time, unbound antibody is washed away and an anti-immunoglobulin reagent is applied. This anti-immunoglobulin can be a second-step anti-immunoglobulin antibody (heterogenous or monoclonal) or it can be some other immunoglobulin specific reagent, like protein A. This reagent can be either radio-labelled [for RIA; 37] or enzyme linked [for ELISA; 38].

Methods for both RIA and ELISA assays are described in detail elsewhere [37,38]. Important controls for assaying purified immunoglobulins include previous lots of the same reagent (if available), wells coated with different target antigens, and wells with BSA coating alone. Depending on the choice of second-step reagent, further information can be gained about the reagent being characterized. For example, an isotype-specific assay (distinguishing IgG_1 from IgG_{2a}) is a very simple modification of the basic procedure [37].

Solid-phase binding assays are more difficult if the antigen in question is not readily available, as is often the case with many membrane proteins. A binding assay similar to the soluble antigen assay has been described using isolated solubilized cell membranes [39]. Another approach is to carry out a cell binding assay [40]. In this assay careful centrifugation keeps the cells in the microtitre wells throughout binding and washing steps. A variant of this technique employs a fluoresceinated final-step reagent (e.g. F1-protein A) and analysis by flow cytometry [41]. This is particularly useful in cases where the antigen is expressed on only a small subpopulation of the target cell population. A final procedure occasionally used couples morphological/anatomical criteria with staining: immunoperoxidase staining of frozen sections [42].

Characterization method: screening antibodies to cell-surface antigens

Equipment

Flow cytometer (Becton Dickinson, Coulter, Ortho). Centrifuge (CU-5000, IEC) with plate holders (Cooke

Microtitre System, Dynatech Labs, Inc., Alexandria, VA).

Materials

96-well PVC microtitre plates (Cooke Microtiter System, Dynatech Labs, Inc., Alexandria, VA).
Fluoresceinated second-step reagent (Vector Labs).

Procedure

1 Obtain a single cell suspension of target cells (i.e. mouse splenic lymphocytes, human peripheral blood lymphocytes, tumour cells, etc.).
2 Wash cells by centrifugation (200 ***g***, 4 °C) twice with staining medium and adjust cells to 2.5×10^7 per ml in this medium.
3 Prepare serial dilutions of the antibody solution in 25 μl aliquots in a 96-well microtitre plate; this is conveniently done directly in the plate.
4 Add 20 μl aliquots (5×10^5 cells) to each well; incubate for 15 min on ice; add 150 μl of medium; centrifuge at 1200 rev./min using microtitre centrifuge carriers to pellet cells; resuspend cells in 150 μl staining medium; centrifuge; repeat once more.
5 Add suitable amount of fluoresceinated second-step antibody in 25 μl to each well; include control of second step alone on cells; include control of cells alone.
6 After 15 min incubation on ice, wash as in step 4; resuspend finally in suitable volume and analyse on flow cytometer.

Specific applications in immunology

The applications of purified monoclonal reagents to work in immunology are clearly too numerous to list fully: these reagents serve in a vast array of immunoassays to quantify the levels of many different 'antigens' [43]. Another broad application is in immunostaining, either in section [44] or in solution [45], observed either by microscope [46] or on a flow cytometer [47]. An application that may come to the fore in the future is as tissue- or tumour-specific therapeutic agents [48]. Two specific applications, together with some of the special cautions that must be observed in these systems are described in the following sections.

Applications: RIA/ELISA reagents

Monoclonal antibodies can serve two functions in solid-phase binding assays: either as targets (antigens) or as revealing ('second-step') reagents. The standard of purity necessary is different for these two applications. In the case of antibody serving as an antigen (target) for anti-immunoglobulin assays [37], extremely high purity of the correct isotype (and/or allotype) is required. One means of achieving this level of purity is to take advantage of the antibody's binding specificity to 'plate purify' it: the plate is first coated with antigen, then the monoclonal target antibody, then the test sample [49]. Another approach is to purify immunoglobulin from serum-free medium in order to exclude 'background' serum immunoglobulin.

On the other hand, visualizing/revealing reagents need not be completely free of contaminating immunoglobulins, just free of any contaminating specificity. Thus most isolations of antibody from serum or ascites do not remove the variable amounts of 'background' antibody typically present. This makes the careful evaluation of the purified lot of antibody reagent very important, since different lots of antibody can contain considerably different amounts of 'background' antibody with different (unknown) specificities.

Applications: fluorescence staining reagents

A major application of antibodies directed against cell-surface determinants is in fluorescence staining, either for microscopy or by flow cytometric analysis or fluorescence activated cell sorting [50]. Reagents prepared for microscopy can have moderate background staining and still be perfectly usable, but applications in flow cytometry (where low levels of non-specific staining obscure weak positive staining) is more demanding. Several difficulties are occasionally encountered: one is that high label-to-protein ratios (> 3) can lead to inactivation and/or increased background staining; another is that even moderate degrees of labelling can lead to precipitation of the antibody [51]. High labelling ratios should be avoided as generally bad practice; usually collisional quenching limits the true fluorescence yield [52]. Thus determining F/P (fluorescein/protein) ratios and modifying labelling conditions to obtain low F/P conjugates is an important aspect of fluorescent labelling techniques.

Problems with inactivation and precipitation are often limited to certain antibodies and may be unavoidable: FITC may react preferentially with a lysine in the combining site. This seems to be more of a problem with rhodamine reagents (as compared with fluorescein reagents) [53]. These problems can be overcome by carrying out two-step staining using the biotin/avidin system: first biotinylate the antibody; reveal it with rhodamine–avidin [54]. Further applications of fluorescent reagents in cell sorting are described elsewhere in this handbook (Chapter 29).

Application: FITC labelling

Equipment

UV-visible spectrophotometer (PM6, Zeiss).

Materials

FITC (Molecular Probes, Inc.).
Dimethyl sulphoxide (Kodak, Spectro Grade).
Desalting column (PD-10, Pharmacia).

Procedure

1 Bring antibody to approximately 10 mg/ml in carbonate buffer by vacuum dialysis or apply protein to desalting column (PD-10, Pharmacia) equilibrated with carbonate buffer and collect 0.5 ml fractions; pool protein fractions (labelling works best at protein concentrations greater than 4 mg/ml; more dilute samples give variable F/P ratios).
2 Dissolve fluorescein isothiocyanate in dimethyl sulphoxide (10 mg/ml); add 20 μg FITC (2 μl) per milligram of antibody to antibody solution and rock: for 2–3 h at 25 °C; or 45 min at 37 °C; or 15 h at 4 °C. Protect from light by wrapping in foil for the duration of the reaction.
3 If protein concentration is lower, use more FITC: at 2 mg/ml use 60 μg per milligram of antibody; this is somewhat variable—try several ratios.
4 After alotted time, apply solution to desalting column (PD-10 or equivalent) previously equilibrated with Tris/Saline (apply no more than 2.5 ml per column); elute labelled antibody with Tris/Saline.
5 Pool yellow fractions, add 1/100 volume of 10% sodium azide solution, and pass through Millipore filter (0.45 μm).
6 Determine F/P ratio: measure OD at 280 nm and 495 nm of a suitable dilution.

Correction: fluorescein absorbs at 280 nm (31% of 495 OD)
Absorption constants:

IgG OD (0.1%) at 280 nm = 1.4
IgM OD (0.1%) at 280 nm = 1.2

Extinction coefficient: fluorescein (495 nm) = 68 000
Molecular weights: IgG, 150 000; IgM, 900 000

$$\frac{F}{P} = \frac{\text{OD 495nm/68 000 (for FITC)}}{\text{Ab (mg/ml)/150 000 (for IgG)}}$$

Notes and recommendations

For maximal specificity (minimal backgrounds) the antibody should not be over-conjugated with fluorescein. Typically F/P ratios in the 2–3 range are preferable, although occasionally higher ratios may be acceptable. Ion-exchange chromatography (exactly as in primary purification, described above with DEAE-Sephacel) may be useful in fractionating low and high F/P antibodies from a bulk preparation.

Buffers and media

Saturated ammonium sulphate (SAS)

Heat 1 kg ammonium sulphate in 1 l total volume distilled water in a 60 °C water bath for 2–4 h. Remove to refrigerator or cold room and store for at least 24 h at 4 °C before use.

DEAE ion-exchange buffer

50 mM-Tris
pH 8.0

General column running buffer (Tris/saline)

50 mM-Tris
0.15 M-NaCl
0.1% NaN_3
pH 8.0

Affinity column eluting buffer

0.1 M-glycine
pH 3.2

Affinity column cycling buffer

0.1 M-glycine
pH 2.4

Affinity column sample neutralizing buffer

0.5 M-potassium phosphate
pH 8.0

QAE batch absorption buffer

50 mM-Tris
0.15 M-NaCl
pH 8.1

Fluorescence staining medium

Deficient RPMI-1640 (-biotin, -riboflavin, -phenol red)
10 mM-HEPES
0.1% NaN_3

3% Fetal calf serum (filtered through 0.2 μm membrane)
pH adjusted to 7.40

Barbitol electrophoresis buffer (2 ×)

0.1 M-sodium barbitol
0.2% NaN_3
pH 8.2

Agarose electrophoresis staining solution

5 parts MeOH
5 parts H_2O
2 parts glacial acetic acid
1% Amido swartz (buffalo black)

Carbonate FITC coupling buffer

Sodium carbonate 1.7 g
Sodium bicarbonate 2.8 g
100 ml distilled water
pH should be about 9.5

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