ESSENTIAL GUIDES FOR ISOLATION/PURIFICATION OF IMMUNOGLOBULINS

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Introduction

The immunoglobulins (Igs), proteins produced by B lymphocytes, have been extensively studied both at molecular and genetic levels. They consist of two identical heavy chains and two identical light chains having therefore the same isotype and the same type, respectively. Igs are purified for three main purposes. (i) as therapeutic injections to patients; (ii) for use as a tool in research or clinical diagnosis; and (iii) for their biochemical analysis (specificity, isotype or clonal diversity). Most of these applications require that the binding activity of Igs be retained throughout all the purification procedures.

Purification of Igs can be performed according to their physicochemical properties, their biological activities or a combination of both. The technique used will depend on the desired degree of purity and the amount and nature of the starting material. The methods that have been described are generally directly applicable to crude materials such as serum, ascitic fluid or cell culture supernatant. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) affords an efficient way of evaluating the degree of purity reached in affinity purifications. Several aspects of 2D-PAGE analysis are described in detail in two other articles 'Electrophoresis/Two-dimensional PAGE' and 'Clinical Applications of Electrophoresis/Electrophoresis' in this Encyclopedia.

As a general rule, and independently of the technique used, the starting material should always be devoid of any insoluble substances and the purification be preceded by centrifugation or filtration. Viscous fluids, such as serum, may be diluted before use, especially for chromatographic procedures. The solutions should contain a bacteriostatic agent, such as 0.02% sodium azide (NaN_3), and be kept on ice. Ig solutions should be handled gently, avoiding bubbling or frothing, because such manipulations may be accompanied by denaturing effects, and may lead to protein precipitation.

Purification by Precipitation

Solubility of the proteins, particularly Igs, in water relies mainly on the ability to make hydrogen bonds between polar or ionic groups with water molecules (hydrophilic interactions), and on the capacity to maintain hydrophobic groups that cannot interact with water molecules buried inside the proteins. In addition, the solubility of Igs is temperature-dependent. Any external factor capable of modifying hydrogen bonds or decreasing the medium hydrophilicity will decrease the solubility of the proteins and may eventually lead to their precipitation. Each protein has its own physicochemical characteristic, including solubility. For this reason, several differential precipitation procedures can be developed to isolate Igs from various fluids. These procedures are presented below.

Differential Ethanol Precipitation

The first fractionation of plasma proteins for therapeutic use was described in 1949 by E.J. Cohn. The basic procedure, with few modifications, is still widely used in industrial fractionation centres. Basically, ethanol is added progressively to the medium to a final concentration varying from 8 to 40%. Subsequently, the temperature is decreased to $-3°C$ and then to $-5°C$. Finally, the pH is decreased from 7.3 to 4.8. These steps yield precipitation fractions, called Cohn's fractions I–V. Fraction II contains the γ-globulins or Igs. The treatment of this fraction with caprylic acid (see below) allows the preparation of Igs that are enriched in IgA and IgM. This approach is used when large amounts of Igs are needed, i.e. for therapeutic purposes (from up to 5000 L plasma) and will not be detailed further.

Ammonium Sulfate Precipitation

Small and highly charged ions, such as ammonium ions, replace bound water molecules when present at a sufficient concentration. This decreases protein

solubility and, when the ammonium sulfate concentration is increased stepwise, a sequential precipitation of proteins may be obtained.

Practically, a saturated solution of ammonium sulfate ($761\ g\ L^{-1}$; $4.1\ mol\ L^{-1}$) is slowly added to a stirred (in order to avoid local over-increase in concentration) solution of Igs, until the final desired concentration, usually expressed as a percentage of ammonium sulfate saturation, is reached. Although some interspecies variability is observed for Ig solubility, a 50% ammonium sulfate saturation is usually appropriate for most Ig precipitation procedures. Preprecipitation at 40% ammonium sulfate saturation may be useful to remove large protein aggregates and proteins that may precipitate at low ammonium ion concentration. The precipitation is allowed to occur at 4°C for 6–12 h. The precipitate is recovered by centrifugation at 2000–5000 **g** for 20–30 min. In general, the pellet is then gently solubilized in a minimal volume of a physiological buffer, and dialysed to remove the residual ammonium sulfate. Alternatively, Ig purity can be increased by washing the pellet with a 50% saturated solution and solubilization in PBS, followed by another round of ammonium sulfate Ig precipitation. When starting with serum or cell supernatant containing serum, this method allows the removal of most albumin and haemoglobin, but the precipitated fraction still contains several serum proteins in addition to Igs. In this case this approach must be coupled with another purification technique if pure material is needed. When starting with a serum-free cell culture supernatant, this method is convenient to isolate, and to concentrate, monoclonal Igs in one step. In addition, the method is easy, cheap and can be applied to large volumes.

Caprylic Acid Precipitation

The solubility of proteins is altered by the presence of some short chain fatty acids, such as octanoic acid (caprylic acid) at mildly acidic pH. Basically, caprylic acid increases medium hydrophobicity. In practice the pH of the starting solution must be adjusted to 4 by the addition of about 2 vol of a $60\ mmol\ L^{-1}$ sodium acetate buffer. Then, 0.04–0.07 vol of caprylic acid (depending on the starting material as well as on the animal species of the Igs) is added drop by drop while stirring, and the solution is incubated at room temperature for 30 min. Under these conditions, most of the serum proteins are precipitated, with the exception of IgG, which is recovered in the supernatant after centrifugation at 5000 **g** for 10 min. This method bears similarities with that of ammonium sulfate precipitation. In particular, it needs to be coupled with another one to yield highly purified Ig fractions.

Chromatographic Methods

In chromatographic procedures, compounds in solution are separated by allowing them to flow through a selective medium poured in a column. Differential interactions between molecules and matrix are responsible for them migrating at various speeds, or even completely immobilizing them. Separated molecules are recovered in the effluent of the column. Several commercially available preparations allow separation of proteins according to their various physicochemical properties. Detailed information about the use of such media is furnished by the manufacturers or can be found in the literature for particular applications.

Ion Exchange Chromatography

An ion exchanger consists of a positively or negatively charged group covalently bound to an insoluble matrix. Charged molecules with complementary polarity to that of the immobilized groups bind to the matrix through electrostatic interactions, whereas uncharged or similarly charged molecules pass freely through the matrix. Since the net charge of a protein depends on the pH, the starting experimental conditions must be carefully chosen. The bound proteins may be desorbed either by change in pH, or by change in the ionic strength. The former modifies the charge of the protein, whereas salts compete with the binding of the protein to the resin. Addition of NaCl is most frequently used for elution. As the strength of the protein–matrix interaction depends on the net protein charge, a sequential elution can be performed by gradually increasing salt concentration. Because Igs have a more basic isoelectric point than most other serum proteins, ion exchange chromatography can be used for their purification. Practically, the matrix should be extensively washed with $0.5\ mol\ L^{-1}$ HCl or $0.5\ mol\ L^{-1}$ NaOH before use, and then equilibrated with the binding buffer. In addition, the sample must be dialysed against the binding buffer before being loaded on the resin.

At pH 6.5 ($5\ mmol\ L^{-1}$ phosphate), Igs will not bear negative charges, and therefore will not bind to a positively charged matrix such as diethylaminoethyl (DEAE) matrix, which is not the case for other serum proteins. A bulk Ig fraction can therefore be recovered in the flow-through. In contrast, Igs will bear a negative net charge at pH 8.5 ($10\ mmol\ L^{-1}$ Tris), and thus will bind to a positively charged matrix (DEAE). Sequential elution of proteins bound to the matrix can be performed by increasing the concentration of NaCl from 0.05 to $1\ mol\ L^{-1}$. Igs are among the first serum proteins to be eluted, at salt concentrations usually below $0.5\ mol\ L^{-1}$. Ig isotypes can be

differentially purified using this method. Relatively pure IgG is usually recovered in the first eluted fraction; IgM, the last eluted Ig isotype, may also be recovered in quite a pure form, whereas IgD and IgA, with intermediate elution properties, are only poorly resolved with this method. Ion exchange chromatography can yield sufficiently pure antibodies if the starting material is a cell culture supernatant or an ascitic fluid, but it must be coupled to an additional purification step when samples such as serum are used. The method is also cheap and is convenient for large initial volumes.

Hydroxyapatite Chromatography

Immobilized hydroxyapatite (calcium phosphate hydroxide) is used for another kind of adsorption chromatography. At pH 6.8, Igs bind to the matrix, and are eluted when a linear gradient of phosphate buffer from 120 to 300 mmol L^{-1} is applied. When highly purified Ig fractions are needed, this technique must be coupled to another one, again depending on the starting material.

Gel Filtration Chromatography

A gel filtration matrix consists of beads containing pores of various sizes. As the sample flows through the matrix, the largest molecules are excluded from the beads. They stay only in the mobile phase, and move fast. The smaller molecules, depending principally on their sizes but also on their shapes, diffuse more or less inside the pores, and move more slowly within the column. Thus, this system allows the separation of the proteins according to their sizes and shapes. Various matrices with particular structures, pore sizes and excluding limits (the M_r at which the proteins are no more able to enter into the beads) are available commercially. Gel filtration can be used within broad pH ranges, with or without detergents such as 1% sodium dodecyl sulfate (SDS), or dissociating agents such as urea or guanidine. Igs purification is usually performed without sophisticated conditions, and allows the separation of IgM molecules that are considerably larger than IgG as well as many other serum proteins. Using an exclusion limit of 300–500 kDa, and a column volume of at least 20 times larger than that of the starting solution, IgM is easily recovered in the excluded peak. Due to the time needed to allow a complete passage, the use of a fraction collector is highly recommended. Fractions corresponding to 1–3 initial volumes should be collected. Gel filtration must usually be coupled with other methods to yield sufficient Ig purity, and is mainly limited to the purification of IgM.

Precipitation of Immune Complexes

The Precipitin Reaction

When antigens are added in adequate proportions to a mixture of antibodies, specific Igs and antigens will form a lattice which is susceptible to precipitate (the precipitin reaction). This macromolecular complex can then be easily recovered by centrifugation. The required amount of antigens to be added must be determined by establishing a precipitation curve. This implies that one should also have a procedure allowing the precipitate to be quantified. The use of radiolabelled antigens is particularly suitable for this when restricted amounts of antigens are available. Complement activation, which occurs in normal fresh serum, is able to inhibit the precipitation strongly. Therefore, it is necessary to make either a pre-purification of the Igs or to inhibit the complement cascade by the addition of ethylenediaminetetraacetic acid before performing a precipitin reaction. The precipitated lattice is subsequently re-solubilized either by incubation with serum or with an excess of free antigen, or by digestion with papain, which generates Fc fragments.

Polyethylene Glycol Precipitation

Soluble (nonlattice) immune complexes, either present naturally, or generated by adding a corresponding antigen, precipitate from serum in the presence of 3–4% polyethylene glycol (PEG; M_r 6000) after 2–12 h incubation at 4°C. This method has been widely used to isolate circulating immune complexes in various pathological situations. Other high molecular weight proteins, as well as aggregated Igs, can also precipitate using above-mentioned conditions. Therefore, additional steps of purification, using protein G or A (see below), are generally warranted before sufficiently pure material can be used. Solubilization of most PEG-precipitated immune complexes can easily be performed using most buffers that do not contain PEG. In contrast, dissociation of immune complexes requires quite harsh conditions, that are frequently not compatible with techniques allowing the further purification of free Igs devoid of antigens. Immunoprecipitation of specific antibodies is therefore mainly limited to analytical purposes that do not need biological activity. Ig light and heavy chains from immune complexes are easily solubilized in buffers containing SDS, and can be analysed by SDS-PAGE or 2D-PAGE.

Affinity Chromatography

In affinity chromatography, samples containing Igs are incubated in the presence of a matrix consist-

ing of an Ig-binding molecule covalently coupled to a bead support. Unbound molecules are removed by washing, and specifically bound Igs are then eluted using an appropriate buffer. The method is highly specific and high Ig purity is usually reached in a single step. Ig-binding molecules belong to three major groups: (i) bacterial protein A or protein G; (ii) specific antigens; and (iii) monospecific antibodies directed to epitopes on Igs (such as goat anti-human Ig antibody). Various bead supports and coupling procedures have been studied. The use of commercially available activated beads has now allowed preparation of affinity media within most laboratories. In particular cyanogen bromide (CNBr)-activated Sepharose beads can easily be used for most applications and detailed instructions are furnished by the manufacturers. After coupling, the resin should be extensively washed to remove all uncoupled Ig-binding molecule, and equilibrated in binding buffer. The binding capacity of each resin should be determined experimentally before use. If sample binding is indifferently performed in a container (batch procedure) fixed on a rotating wheel or through a column, washing and elution are best performed through a column. The use of a peristaltic pump is highly recommended to ensure a fixed flow.

Washing and elution steps are best followed online with a UV detector set to monitor at 280 nm. Alternatively, fractions may be collected and tested individually using either UV absorption or more specific procedures. When necessary (see elution conditions below), the eluate has to be collected in a neutralizing solution, and the resin immediately re-equilibrated in the binding buffer. Unused resins should be stored at 4°C in the presence of a bacteriostatic agent, such as 0.02% sodium azide or 20% ethanol.

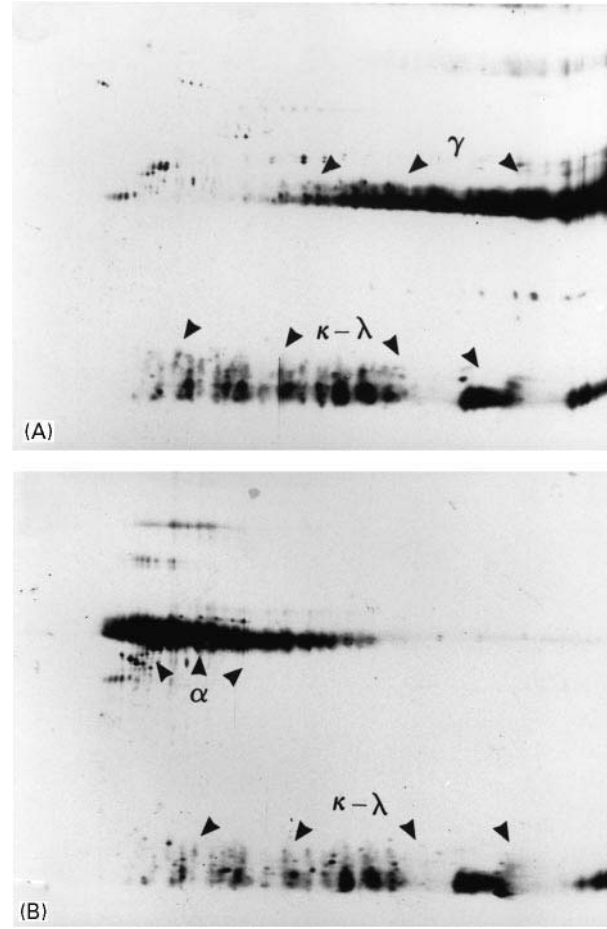

Figure 1 (A) 2D pattern of IgG purified over protein G-Sepharose. Human serum was incubated with commercially available protein G-Sepharose (Pharmacia), and IgG eluted as indicated in the text. γ heavy chains migrate within pIs ranging from 6 to more than 10, with a size of about 50 kDa. Light chains display pIs ranging from 5 to 10 and size between 21 and 26 kDa. γ, IgG heavy chain; κ, λ, light chains. (B) 2D pattern of IgA affinity purified over a homemade anti-α chain-Sepharose resin. The affinity resin was prepared from commercially available CNBr-activated Sepharose (Pharmacia), according to the manufacturer's recommendations, and commercially available goat anti-human α chain antibodies. α chains migrate with a pI ranging from 4.9 to 6.1 and a size of about 58 kDa. α, IgA heavy chain; κ, λ, light chains.

Affinity Chromatography using Immobilized Protein G and A

Protein A and protein G are present within the bacterial cell walls of *Staphylococcus aureus* and of group G *streptococci*, respectively. Both proteins have high affinity for the Fc region of IgG, but bind differentially IgG subclasses from various species. Whereas protein G bind all human and mouse IgG subclasses, protein A presents only low binding capacity for human IgG_3 and mouse IgG_1. Ready-to-use matrix-immobilized protein G or A is commercially available, and detailed information about the binding properties of these two proteins can be found in the literature or furnished by the manufacturers. Binding buffers usually contain 100 mmol L^{-1} Tris or 10 mmol L^{-1} phosphate with 0.15 mol L^{-1} NaCl, at pH 8 for protein A and pH 7 for protein G. After Ig binding to samples, the resin is washed with about 20 vol of the binding buffer until online UV absorption of the flow-through medium gives an optical density back to zero. Elution of IgG is best performed by addition of 100 mmol L^{-1} glycine pH 2–3 in tubes containing a suitable amount of a neutralizing buffer such as 1 mol L^{-1} Tris, pH 8 or 1 mol L^{-1} phosphate, pH 7. As illustrated in **Figure 1**, IgG is highly purified after a single-step procedure over protein G column, with no other heavy chain class detectable. The strength of the protein A(G)–Ig interaction, which is mainly based on hydrophobic

interactions, can be increased by raising salt concentrations of the binding and washing buffers to 3.3 and 3 mol L^{-1}, respectively. This high salt concentration method was initially described for the purification of mouse IgG_1 on protein A, but is now obsolete, due to the availability of protein G. The use of an excess of protein A allows IgG_1, IgG_2 and IgG_4 to be depleted from samples and IgG_3 to be purified using protein G in a second step. A sequential elution of all four IgG subclasses from protein A using a pH gradient has been described, but the resolution is quite low and subclass-specific purification of IgG_{1-4} is now best performed using immobilized monospecific anti-Ig raised against either γ_1, γ_2, γ_3 or γ_4 heavy chains of the IgG molecule.

The elution yield from protein G or A using standard procedures is never 100% (see below) and residual IgG can be eluted during a second run procedure and may contaminate the new IgG being purified with IgG from the previous run. In order to avoid any Ig contamination from a previous experiment, it is therefore highly advisable to use either the same batch of protein G or A for the same initial IgG preparation or to use a new batch of resin for each procedure.

Affinity Chromatography using Immobilized anti-Igs

In this method, the immobilized binding molecules are Igs (mouse, rabbit, goat, sheep) directed against Ig heavy and/or light chains. Using antibodies of various specificities, it is possible to isolate either total Igs (using anti-κ and -λ chains), particular Ig isotopes (using anti-μ, -γ, -δ, -α or -ε chain) or IgG subclasses (using anti-γ_1, -γ_2, -γ_3 or -γ_4 chains). The interaction between immobilized and targeted immunoglobulins is just a particular type of antibody–antigen interaction. Binding and elution are therefore basically performed using the same conditions as those used for immobilized antigen supports (see below). Figure 1B shows that IgA purified from a human serum sample over an anti-α chain resin does not display any other heavy but α chain isotope. The resolution obtained by 2D-PAGE in separating various Ig heavy chains is illustrated in **Figure 2**.

IgG subclasses can be purified using two different procedures named positive and negative isolations. In positive isolation, the desired subclass is immobilized on a resin, washed and recovered by elution, whereas in the negative isolation, all unwanted subclasses are bound on resin and the desired subclass is recovered in the flow-through. The advantage of this latter approach is that the final preparation is not exposed to strong nonphysiological conditions which may denature the purified IgG subclass. Disadvantages are that the methodology requires larger amounts of resin and several immobilized antibodies and that, when biological fluids are processed, the final preparation still contains proteins other than IgG.

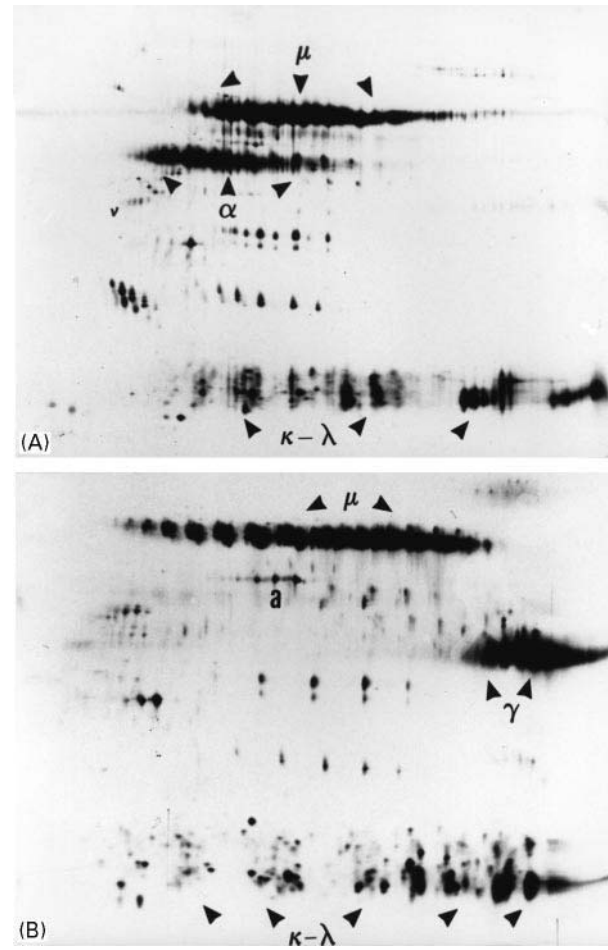

Figure 2 (A) 2D pattern of a mixture of affinity purified IgM and IgA; (B) 2D pattern of a mixture of affinity purified IgM and IgG. Immunoglobulins were purified over homemade anti-μ chain-Sepharose resin, anti α-chain-Sepharose resin and anti γ-chain-Sepharose resin and mixed as indicated before electrophoresis. Resins were prepared according to the manufacturer's instructions, from CNBr-activated Sepharose and commercially available goat anit-human μ, α and γ chains. Immunoglobulins were prepared from serum as indicated in the text. μ chains migrate with pIs between 5.6 and 6.4, and size of 72 kDa. μ, IgM heavy chain; α, IgA heavy chain; γ, IgG heavy chain; κ, λ, light chains.

Affinity Chromatography using Immobilized Antigens

A commonly used method for purifying and recovering antigen-specific, and antigen-free, Igs from a polyclonal mixture of antibodies involves the use of matrix-bound antigens that bind specific, antibodies at physiological pH and salt concentration

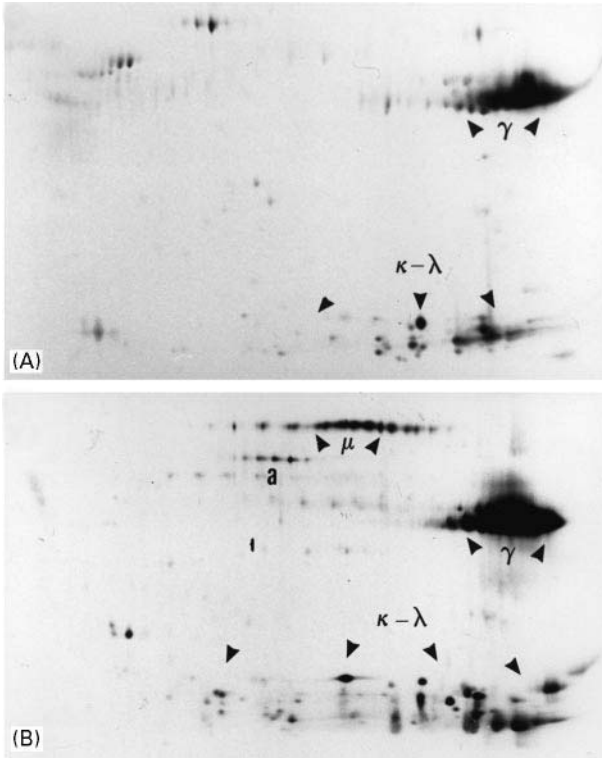

Figure 3 2D patterns of affinity purified anti-tetanus toxoid antibodies. Immunoglobulins were purified from two severe combined immunodeficient (SCID) mice (A and B) previously injected with human lymphocytes and boosted with tetenus toxoid. Homemade tetanus toxoid-Sepharose resin was prepared according to manufacturer's recommendations, from commercially available (Pharmacia) CNBr-activated Sepharose and a home preparation of tetanus toxoid. Igs were SDS-eluted, as indicated in the text. γ, IgG heavy chain; μ, IgM heavy chain; κ, λ, light chains, a, albumin.

(10 mmol L^{-1} Tris or phosphate buffer saline (PBS) 0.15 mol L^{-1} NaCl, pH 7.5). The unbound material is washed away with about 20 resin-volumes of binding buffer. Elution of bound antibodies is commonly performed by successive washes with 100 mmol L^{-1} glycine, pH 2–3 and 100 mmol L^{-1} triethylamine, pH 11–12. The eluted fractions are subsequently recovered into tubes containing neutralizing buffer. Elution solutions such as 5 mol L^{-1} LiCl/PBS, 3.5 mol L^{-1} $MgCl_2$/PBS, 1% SDS, 2–8 mol L^{-1} urea, 3 mol L^{-1} thiocyanate, 10% dioxane, or 50% ethylene glycol can also be used.

The purification of anti-tetanus toxoid Igs over a toxoid-coated resin is presented in **Figure 3**. Both 2D-PAGE light chain patterns shown depict a limited number of easily distinguishable spots, typical of oligoclonal Igs. These patterns are clearly different from that of total Igs (and purified total IgG, not shown) from the same serum, indicating that a subpopulation of Igs was purified. Whereas the anti-tetanus toxoid antibodies shown in Figure 3A consist only of IgG, some IgM anti-tetanus toxoid antibodies were also present in the case shown in Figure 3B.

Affinity Chromatography Using Jacalin or Complement

Jacalin (a carbohydrate-binding molecule) allows the separation of both subclasses of pre-purified IgA (jacalin binds IgA_1 but not IgA_2). Complement C1q will bind antigen-complexed Igs. Anti-complement Igs will bind immune complexes bound to components of the complement system.

Recovery from Affinity Resins

As already mentioned, elution from protein G or A-Sepharose may be incomplete. In our hands, purification of 6–12 mg batches of IgG from various sources resulted in a recovery of about 50%, using acidic elution. Similar recovery yields have also been reported by others, using similar elution. Purification of anti-tetanus toxoid antibodies on tetanus toxoid-Sepharose resulted likewise in a 50% loss of antibody activity. We further investigated antibody recovery yield using affinity-purified radiolabelled antibodies. When purification was scaled down to 10 μg Ig (an amount that allows enzyme-linked immunosorbent assay or electrophoresis techniques), recovery of bound material from protein G or tetanus toxoid-Sepharose was only about 10%. The percentage of lost Igs was roughly inversely proportional to the initial Ig amount. Some loss is acceptable when purifying large batches of monoclonal antibodies. On the other hand, when purifying antibodies for analytical purposes, one should keep in mind that antibody losses may skew the final results; the composition (isotype, subclass and diversity) of the eluted fraction may indeed no longer reflect the composition of Igs that were initially loaded on the resin. The problem of low recovery could be solved by heating Ig-loaded protein G- or tetanus toxoid-Sepharose in the presence of SDS and dithioerythritol; more than 97% of bound Igs could be recovered by this way. Of course, such treatment does limit further analysis to methods that do not require biological activity of Igs, such as electrophoresis, since Igs are denatured under such conditions.

Conclusion

Many different methods have been described over the years to purify Igs, and the most important have been briefly presented in this article. Of course, we have made a choice between the many methods available, and the list is not exhaustive. Numerous

Table 1 Summary of the major approaches for purifying immunoglobulins

Starting material	*Methods*	*Purposes*
Plasma, asciitis	Affinity chromatography on protein G or A	Isolation of pure IgG
Plasma, asciitis, pre-purified immunoglobulin fractions	Affinity chromatography on purified antigens	Purification of monospecific antibodies
Plasma, asciitis, pre-purified immunoglobulin fractions	Affinity chromatography using monospecific antibodies (anti-α, -γ, -μ, -κ or -λ)	Isolation of immunoglobulins of a single isotype
Plasma, asciitis, pre-purified immunoglobulin fractions	$(NH_4)_2SO_4$/DEAE Sepharose	Preparation of large amounts of relatively pure immunoglobulin fractions
Plasma, asciitis, pre-purified immunoglobulin fractions	$(NH_4)_2SO_4$-Hydroxyapatite	Preparation of large amounts of relatively pure immunoglobulin fractions
Plasma, asciitis, pre-purified immunoglobulin fractions	Gel filtration/DEAE Sepharose	Preparation of relatively pure IgM fractions

variations and/or combinations of methods may be used to satisfy a particular need, depending on the starting material, as well as for the purpose of the purification. However, for most current applications, affinity purification procedures appear to be the most elegant and selective methods. The binding capacities of affinity resins are usually high (up to 20 mg of immunoglobulins per mL resin), and their reusability allows the purification of quite large amounts of pure immunoglobulins in relatively short times.

Table 1 summarizes the most efficient methods of purifying Igs.

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