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 (NaN₃), 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
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 some 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

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.

To be coupled with another one to yield highly purified Ig fractions.
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 \(M\) 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; \(M\) 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-
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 IgG3 and mouse IgG1. 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

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-human 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. γ, IgA heavy chain; κ, λ, light chains.
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-
chain-Sepharose resin, anti-chain-Sepharose resin and anti-
chain-Sepharose resin and mixed as indicated before electrophoresis.
Resins were prepared according to the manufacturer’s instruc-
tions, from CNBr-activated Sepharose and commercially avail-
able goat anit-human , chain; , IgA heavy chain; , IgG heavy chain;
, light chains.

Affinity Chromatography using Immobilized
anti-Is

In this method, the immobilized binding molecules are Iggs (mouse, rabbit, goat, sheep) directed against Igg heavy and/or light chains. Using antibodies of various
specificities, it is possible to isolate either total Iggs (using anti- and - chains), particular Igg isotypes (using anti- , - or - chain) or Igg subclasses (using anti- , - or - 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- chain resin does not display any other heavy but 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 sub-
class. 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.

Affinity Chromatography using Immobilized
Antigens

A commonly used method for purifying and recovering antigen-specific, and antigen-free, Iggs from a polyclonal mixture of antibodies involves the use of matrix-bound antigens that bind specific, antibodies at physiological pH and salt concentration.
Affinity Chromatography Using Jacalin or Complement

Jacalin (a carbohydrate-binding molecule) allows the separation of both subclasses of pre-purified IgA (jacalin binds IgA1 but not IgA2). 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

<table>
<thead>
<tr>
<th>Starting material</th>
<th>Methods</th>
<th>Purposes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma, ascites</td>
<td>Affinity chromatography on protein G or A</td>
<td>Isolation of pure IgG</td>
</tr>
<tr>
<td>Plasma, ascitis, pre-purified immunoglobulin fractions</td>
<td>Affinity chromatography on purified antigens</td>
<td>Purification of monospecific antibodies</td>
</tr>
<tr>
<td>Plasma, ascitis, pre-purified immunoglobulin fractions</td>
<td>Affinity chromatography using monospecific antibodies ((\alpha)-, (\gamma)-, (\mu)-, (\kappa)- or (\lambda))</td>
<td>Isolation of immunoglobulins of a single isotype</td>
</tr>
<tr>
<td>Plasma, ascitis, pre-purified immunoglobulin fractions</td>
<td>(NH(_4))(_2)SO(_4)/DEAE Sepharose</td>
<td>Preparation of large amounts of relatively pure immunoglobulin fractions</td>
</tr>
<tr>
<td>Plasma, ascitis, pre-purified immunoglobulin fractions</td>
<td>(NH(_4))(_2)SO(_4)-Hydroxyapatite</td>
<td>Preparation of relatively pure IgM fractions</td>
</tr>
<tr>
<td>Plasma, ascitis, pre-purified immunoglobulin fractions</td>
<td>Gel filtration/DEAE Sepharose</td>
<td>Preparation of relatively pure IgM fractions</td>
</tr>
</tbody>
</table>

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.

Further Reading


