

Purification of antibodies by the use of biochemical and immunochemical techniques

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For many biochemical applications including immunohistology, not only highly specific antibodies are of great importance, the degree of purity with respect to the bulk of contaminating proteins in sera is also a matter of concern. Even if sometimes hyperimmune sera may work quite well, it is preferable to get familiar with the most applied procedures and to select a dedicated way for the antibody purification. While the conjugation of antibodies with enzyme molecules will always need highly purified antibodies, in other cases such as in indirect immuno-stainings (in which the primary antibodies are not labeled) an immunoglobulin fraction of the hyperimmune serum containing the primary antibodies will be sufficient. In this connection it must be noted that partial purification (instead of a high degree of purification) is not under all circumstances an disadvantage because contaminating proteins (f.e. albumin) can act as protector colloids which will avoid aggregation of the antibodies.

The choice of an appropriate purification method is led by the necessary degree of purification, and the latter usually depends on the type of application. When labeled primary antibodies are to be used in direct immuno-staining techniques, then antibody preparations with a high degree of purity are essential in order to obtain reliable conjugates with which to achieve clear-cut staining results with lowest possible background. Some of the commonly used methods for antibody purification are listed in Table 1.

Table 1: Principles of common methods for antibody purification

Method	Application, quality	Advantage	Disadvantage
Ammonium sulfate	Bulk of serum γ -globulins Useful for concentration, not recommended as single step	Easy, cheap convenient for large volumes Low loss of antibodies	Poor purification degree, must couple with other purification step
DEAE ion exchange	Partial purification, not recommended as single step	Cheap and convenient for large volumes and concentration	Impure antibody fractions (but higher than with ammonium salt), must couple with other purification step
Caprylic acid	Moderately pure IgG, not recommended as single step	Cheap and convenient for large volumes	Impure antibody fractions, must couple with other purification step
Hydroxylapatite	Relatively pure antibodies, not recommended as single step	High yield of concentrated antibodies	Impure antibody fractions, must couple with other purification step
Gel filtration	Separation by molecular mass, not recommended	Relatively pure separation of IgM from other	Low capacity High dilution of antibody

	as single step	antibody molecules	fraction, not useful for IgG antibodies
Protein A affinity	Pure IgG with species and isotype selectivity Single step method	High purification degree High yield	Not suitable for all species and isotypes
Protein G affinity	Pure IgG with species and isotype selectivity Single step method	High purification degree High yield Broader application than with protein A	Not suitable for all species and isotypes
Antigen affinity	Pure antibodies by antigen selective method Single step method	High specificity and purification degree High yield	Pure antigen required Method associated with loss and inactivation of antibodies by elution procedure

Affinity adsorption is a method of separation by affinity chromatography which yields antibodies of highest purification degree. Affinity chromatography is a versatile technique by which either unwanted antibodies may be removed or the desired antibodies are selectively eluted. In the latter case, antigen is first coupled to a matrix. Then, antibodies from a hyperimmune serum are passed through, and the antibodies bound to the insolubilized antigen may be subsequently eluted by a solution that disrupts antigen-antibody binding. A popular way for the purification of antibodies is to make use of affinity binding by protein A or protein G coupled to a matrix. For practical use, several commercial antibody purification and clean up kits are available.

Salt precipitation (ammonium sulfate)

Ammonium sulfate precipitation is one of the earliest and most commonly used methods for removing proteins in solution. This method, however, yields antibody preparations of the lowest purification degree as compared with the other methods from Table 1.

Proteins in solution form hydrogen bonds with water through their exposed polar and ionic groups. When high concentrations of small, highly charged ions such as ammonium and sulfate are added, these groups compete with the proteins for binding water. This removes water molecules from the protein which results in reversible decrease of its solubility and precipitation. Factors affecting a particular protein to precipitate include the number of polar groups, the molecular weight, the pH of the solution and the temperature at which the precipitation is performed.

The concentration of ammonium sulfate at which antibodies precipitate varies from species to species. Rabbit antibodies for example can be precipitated with a 40% saturated solution, while mouse antibodies need 45-50% saturation; conveniently, a 50% saturation is used for most applications. Following ammonium sulfate precipitation, the resulting antibodies are not pure and are contaminated by high molecular weight proteins. Thus, ammonium sulfate precipitation is not a single-step purification and must be followed by other methods when pure antibody preparations are needed.

DEAE ion exchange (batch, chromatography)

Ion exchange chromatography is also a widely used method for antibody purification and often applied as second step after previous ammonium salt precipitation. The principle of ion exchange chromatography is based on differences in isoelectric points of antibodies and the majority of other serum proteins. When the pH of an anion exchange matrix such as DEAE-cellulose is kept below the isoelectric point of antibodies, then antibodies will not bind to the matrix. In another approach, the pH can be raised above the isoelectric point where the antibodies will bind to DEAE groups.

In DEAE ion exchange chromatography, the DEAE matrix is washed, equilibrated to the desired pH, for example above the pI of the antibodies, and filled into a column. After passing the antibody solution down the column, antibodies are sequentially eluted with increasing concentrations of NaCl in the original buffer. DEAE chromatography is not a single step purification method even if its purification degree is somewhat higher than with ammonium sulfate precipitation. Depending on the necessary antibody purity, DEAE chromatography must be coupled with a further purification step.

Caprylic acid

Caprylic acid is a short-chain fatty acid which under mildly acidic conditions will precipitate most of the serum proteins with the exception of IgG molecules. This type of precipitation is useful for large volumes, however, this purification step will yield impure antibody fractions. In order to enhance purification efficiency, the caprylic acid method must be coupled with other purification steps, for example DEAE ion exchange chromatography. If necessary, concentrate the obtained antibody fraction and purify further by ammonium sulfate precipitation.

Hydroxylapatite chromatography

A rapid procedure for large scale purification of antibodies is column chromatography on hydroxylapatite. Antibody yields are high and good purification degree is achieved with serum from hyperimmune animals or with ascites fluid (from monoclonal antibody production). Even if the purification degree with hydroxylapatite chromatography is quite high, this preparative step must couple with other purification steps in order to eliminate contaminating proteins. An advantage of the hydroxylapatite procedure is that antibodies are concentrated, and antibodies are eluted from the column in buffer systems which are compatible with most labelling reactions.

Gel filtration (Sephadex, Sepharose)

Gel filtration on Sephadex or Sepharose (medium-sized beads, exclusion limit of 300,000 to 500,000 daltons for globular proteins) is useful for the isolation of antibodies of the IgM isotype because these molecules are larger than IgG antibodies and other molecules in serum. Nevertheless, it is recommended to combine gel filtration with other techniques, for example ammonium sulfate precipitation, in order to concentrate the antibody preparation and to obtain pure IgM.

Protein A and protein G affinity

Apart from immunoaffinity purification of antibodies on an antigen column, chromatography of antibody solutions by use of protein A or protein G beads is one of the most effective purification methods for antibodies from many species. The properties of protein A and protein G are shown in Table 2.

Table 2: Protein A and G affinities for various antibodies and species

Species, antibody	Protein A affinity ^a	Protein G affinity ^b
Human IgG ₁	+++ ^c	+++
Human IgG ₂	+++	+++
Human IgG ₃	–	+++
Human IgG ₄	+++	+++

Mouse IgG ₁	+	+++
Mouse IgG _{2a}	+++	+++
Mouse IgG _{2b}	+++	+++
Mouse IgG ₃	++	+++

Rat IgG ₁	–	+
Rat IgG _{2a}	–	+++
Rat IgG _{2b}	–	++
Rat IgG _{2c}	+	++

Other species		
(alphabetical order)		
Chicken	–	+
Cow	++	+++
Goat	–	++
Guinea pig	+++	++
Horse	++	+++
Hamster	+	++
Pig	+++	+++
Rabbit	+++	+++
Sheep	+/-	++

- ^a from JJ LANGONE (*J. Immunol. Methods* **24**, 269, 1978); PL EY et al. (*Immunochemistry* **15**, 429, 1978)
- ^b from L BJÖRCK and G KRONVALL (*J. Immunol.* 133, 969, 1984); B ÅKERSTRÖM et al. (*J. Immunol.* 135, 2589, 1985); B ÅKERSTRÖM and L BJÖRCK (*J. Biol. Chem.* 261, 10240, 1986)
- ^c Affinity binding of protein A and protein G: – no; + moderate; ++ strong; +++ very strong affinity with the respective antibodies

Protein A is a 42,000 dalton polypeptide cell wall component produced by several strains of *Staphylococcus aureus*, and protein G is a bacterial cell wall component from group G streptococci. Protein A has four binding sites for antibodies, but only two of them can react at the same time. Both, protein A and protein G, have the ability to bind specifically with the Fc region of immunoglobulins (mainly IgG). Because the Fc domains of different classes and subclasses of antibodies are different, the affinity of protein A varies with the class and species of the antibody molecules. Hence, the interaction of protein A and protein G is not equivalent for all species (see Table 2). The binding characteristics of protein G can be used for the purification of monoclonal and polyclonal antibodies that do not bind sufficiently well to protein A.

Antibodies with high affinity binding sites for protein A are purified using the low-salt method, i.e. salt concentrations near physiological levels. Antibodies that do not have a high affinity for protein A are purified by the high-salt procedure by which the affinity of protein A for antibodies is raised by increasing the strength of the hydrophobic bonds. This achieved by raising the salt concentrations. In both methods (low-salt and high-salt methods), antibodies are eluted from the column beads by lowering the pH of the elution buffer.

Antigen affinity (immunoaffinity chromatography)

Immunoaffinity chromatography on an antigen column is the most common and most effective procedure to purify antigen-specific antibodies from sera, ascites fluid or culture media. In this procedure, water-insoluble immunoabsorbents are prepared by covalent coupling of pure antigen to a solid supports. One of the most popular methods is coupling of antigen to cyanogen bromide activated agarose beads which are subsequently filled into a column. The antibodies specific for this antigen are allowed to bind; unbound antibodies and contaminating proteins are removed by several washings. Finally, specific antibodies are eluted by low and high pH buffer cycles. Some high affinity antibodies may not elute under these conditions, then elution with chaotropic ions (e.g. KSCN) is advised. The major advantage of immunoaffinity chromatography is the unique ability to isolate antibodies from a mixed pool. Disadvantages of this procedure are the need of large quantities of pure antigen and that the elution conditions can lead to loss of antibody by inactivation.

Monitoring steps during antibody purification, storage of antibodies

In the course of antibody purification, several variables must be monitored which include the purity, the amount and the antigen binding capacity of the antibodies.

The easiest way to control the purity of an antibody is to run a sample of the isolated antibody in an SDS polyacrylamide gel electrophoresis; gels are either silver stained or stained by Coomassie blue. Also, controls by immunoelectrophoresis can be done. If the antibody is

pure, a convenient method to measure the total amount of protein is UV absorbance (absorbance measurement at 280 nm; 1 OD = approx. 0.75 mg/ml of purified antibody). The antigen binding activity can be measured by comparing the purified antibody with the starting material in a series of titrations (normalizing to the total amount of antibody in each preparation).

Isolated antibodies are found to be less stable than in mixtures with other proteins like in hyperimmune sera. The stability depends to a large extent on the method of isolation. For example, exposure to low pH or high and low salt concentrations (usually used during the purification process) has significant influence on solubility, stability and quality of long term storage of antibodies. Formation of insoluble precipitates and aggregation are probably signs of hydrophobic changes and denaturation which can be the reason for increased background stainings in immunohistological assays.

The storage temperature is a very important factor for long term storage. Stock solutions should be aliquotated and stored frozen at -20°C (or better at -80°C) in tightly capped tubes to avoid repeated freezing and thawing cycles. The addition of protecting agents such as glycerol can be helpful inasmuch as to avoid the formation of crystallization and the disturbance of the protein's water shells. Furthermore, antibody solutions with very low protein content (less than 1 mg/mL) can be stabilized by the addition of bovine serum albumin (0.5-1.0% w/v) or glycerol (10-20% v/v). Then, in order to inhibit microbial growth, the addition of sodium azide (e.g. 0.1% w/v) is recommended.

It has been found that the addition of certain biomolecules are an attractive alternative to chemicals as protective substances against a variety of stress factors. For example, *Ectoin* (a low molecular, zwitterionic and hygroscopic organic compound) and *Hydroxyectoin* isolated from the halophilic bacterium *Halomonas elongata* were shown to be useful as protecting and stabilizing agents for proteins (GALINSKI EA et al., 1985; KNAPP S et al., 1999).

Prediluted antibodies are usually stored at 4-8°C for short time (when antibodies, conjugates or entire staining kits are purchased, please, follow the recommendations by the manufacturer). In any case, avoid excessive light exposure. The material of storage containers should have negligible protein adsorptivity, for example polypropylene polycarbonate or borosilicate glass vials are to be preferred.

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Full version of citations in chapter *References*.