6
Immunoaffinity Chromatography: Advantages and Limitations

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6.1
Introduction

Biochemistry is based on specificity of molecular interactions. Separation scientists have used this principle for the development of extremely specific techniques for the purification of biologically active molecules. The use of an immobilized "bait" molecule that binds noncovalently to a "target" molecule and pulls the target molecule out of a solution containing many other molecules is the basis of a purification approach referred to as "affinity purification."

A subset of this field, which uses the specificity of the antibody–antigen interaction, is termed immunoaffinity purification, and the most commonly used form is of immunoprecipitation (IP). As first described, IP gave a physical precipitant when a specific antibody is added at the right concentration to a solution containing its cognate antigen. The immunoprecipitate could then be removed by a physical method, such as centrifugation. However, in recent years, it is more common to link the antibody to some type of insoluble support (most commonly a bead or magnetic particle), and the antigen–antibody complex is recovered from the solution by simply recovering the bead. Thus, a scaled up version of the bead-bound antibody–antigen complex is termed immunoaffinity chromatography.

In most instances of immunoaffinity purification, the antigen is the object (target) of the purification, and the antibody is immobilized as the bait. However, there are situations where the roles are reversed, and the antigen is immobilized as the bait. This is especially useful when a preparation of highly homogeneous, monospecific antibody is desired.

This chapter will concentrate on immunoaffinity chromatography, where the goal is to purify a specific protein in a biologically active form, and the yield of the active protein is on the order of hundreds of micrograms to 1 milligram or more. Immunoaffinity chromatography is not inexpensive. However, because of the extreme specificity of the interaction between antibody and antigen, the fold purification can be as high as 1000- to 10 000-fold in a single step. Thus, one or two purification steps can result in a highly pure, active protein. We have over

Analytical Separation Science, First Edition. Edited by Jared L. Anderson, Alain Berthod, Verónica Pino Estévez, and Apryll M. Stalcup.
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30 years of experience with using immunoaffinity chromatography. Our experience is based on efforts to purify bacterial RNA polymerase, eukaryotic RNA polymerase II, and general transcription factors for RNA polymerase II. However, these approaches are generalizable to other proteins.

This chapter will cover the following general areas of immunoaffinity chromatography: (1) sources of antibodies, (2) properties of antibodies for immunoaffinity chromatography, (3) construction of an immunoaffinity matrix, (4) a generalized immunoaffinity chromatography method, (5) issues of nonspecific binding, (6) the use of epitope tags, and (7) recombinant antibodies.

6.2 Sources of Antibodies

The extraordinary specificity of antibodies, and the ability to biochemically manipulate them, makes them invaluable reagents for diagnostic, therapeutic, investigative, and purification purposes. An antibody is a large (150 kDa) protein containing four polypeptides (two identical heavy chains and two identical light chains). These proteins fall into several classes (IgA, IgG, IgM, and IgE) depending upon the polypeptide that comprises the constant region of the heavy chain. The basic structure is that of the IgG molecule, usually represented in the typical simplified "Y" structure (Figure 6.1). Heavy chains are 50 kDa and light chains are 25 kDa. Most of the antibody molecule is comprised of constant regions. However, due to genetic rearrangements, a unique N-terminal region (the variable region), which is antigen-specific, is found on each antibody species. Each of these unique N-terminal regions contains three loop-like features, the complementarity determining regions (CDRs), which form the actual binding motif. The antibody can be digested with the protease papain to yield two fragments, the Fc fragment and the Fab fragment.

Antibodies of the IgG class are most commonly used for immunoaffinity chromatography, although we have used IgM molecules [1]. Generally speaking, IgG molecules have more specificity, have higher affinity, and are more stable for use in a chromatography mode than are other antibody classes.

Antibodies are produced by lymphocytes of the B-cell lineage. Due to a large repertoire of possible antibody-producing genes, exposure of an animal to an antigen results in the selection of a specific antibody-producing B cell, which matures to a plasma cell, specialized for the production of this one antibody species. The specific biochemical grouping on the antigen that reacts with the antibody is called an "epitope." However, most proteins contain more than one epitope. Therefore, antibodies in the serum of an individual animal are generally a mixture of antibodies with various specificities. These specificities include both multiple antibody species that react with different epitopes on the injected protein and antibodies specific to epitopes on other antigens to which the animal has been exposed. This type of antibody preparation is generally referred to as a

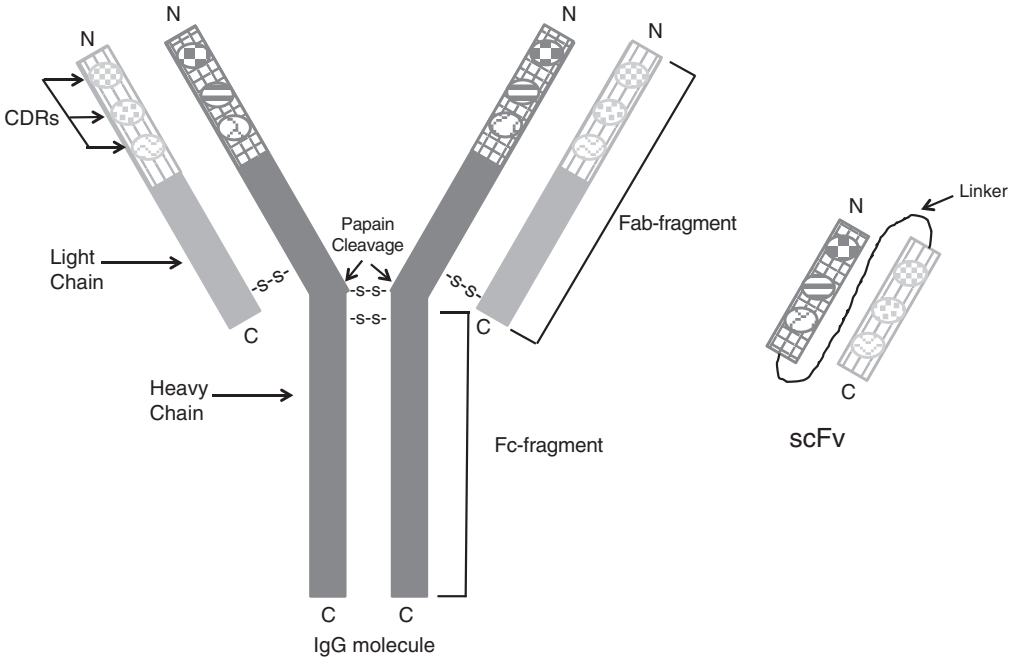

Figure 6.1 Schematic of an IgG molecule and an scFv engineered from it. Each molecule is made of two heavy chains and two light chains. The two heavy chains are identical and the two light chains are identical. The structure is stabilized by four disulfide bonds. The constant region of the heavy chain is solid dark gray, and the variable region of the heavy chain is patterned. The constant region of the light chain is light gray, and the variable region of the light chain is patterned. The epitope-binding region is comprised of the variable regions from the heavy and light chain. N and C refer to the N- and C-termini, respectively. The complementarity determining regions are depicted as differently patterned circles on the N-terminal domains of the heavy and light chains. The scFv consists of the variable regions from the heavy and light chains cojoined with a linker region of various sequences.

"polyclonal" antibody preparation. In order to isolate a homogeneous preparation of antibody from a polyclonal antibody source, it is necessary to immobilize the antigen (or hopefully the epitope) to a support, capture the antibody of interest, and elute it from the immobilized antigen. In fact, the first reported use of immunoaffinity chromatography [2] described the purification of bovine serum albumin-specific antibody from serum obtained from rabbits that had been injected with bovine serum albumin. In this case, bovine albumin was conjugated to diazotized *p*-aminobenzylcellulose and eluted with pH 3.2. Antibodies that are purified by affinity capture on an immobilized antigen can be used for immunoaffinity chromatography. However, this approach is rarely used because of the effort needed to purify these antibodies in a large quantity from polyclonal

serum. In addition, polyclonal antibodies have a limited quantity and reproducibility is not guaranteed.

Today, the most common way to produce antibodies for immunoaffinity chromatography is to produce them as monoclonal antibodies (mAbs). mAbs are usually made from an immunized mouse, but rat and rabbit mAbs are now quite common. A recent review has been published on the hybridoma method [3], but many general protocols are available in immunology manuals, such as Harlow and Lane [4]. Antibody-producing B cells or plasma cells are collected from the lymphoid tissue of the immunized animal and then fused to an established myeloma cell line. This results in a hybrid cell (hybridoma) that has the antibody-producing property of the plasma cell and the ability to grow indefinitely in culture from the myeloma cell. A metabolic selection is applied to ensure that only hybrid cells grow. Because each plasma cell produces only one species of antibody, the hybridoma produces that antibody species. The hybridomas are then screened for production of the antibody of interest, usually by an enzyme-linked immunosorbent assay (ELISA). The selected hybridomas are cloned several times to ensure monoclonality and stability of the cell line. Permanent stocks are frozen and stored submerged in liquid nitrogen. Thus, once the hybridoma is established, the mAb can be produced in large quantities for an indefinite period of time. However, it is wise to monitor mAb production because hybridomas do show instability.

Many mAbs are available from commercial sources; these are usually purchased in 100 μg quantities (at the time of this writing for about US $200–$300). While this amount of mAb is useful for analysis, such as IP followed by Western blotting, it is not enough to perform a reasonably sized immunoaffinity chromatography procedure, where recovery of active protein for further experiments is desirable.

If an mAb that has been demonstrated to work in immunoaffinity chromatography is available, it is wise to obtain either the hybridoma cell line or at least a few milligrams of the antibody to make an immunoaffinity resin. Sometimes, companies will sell unpurified antibody at a “bulk” rate. This usually requires a conversation with the company. However, if it is necessary to isolate a specific mAb for immunoaffinity chromatography, the “time line” in Figure 6.2 can be used as a general guide. We have divided the procedure into the parts, each of which takes several weeks. While it may be possible to shorten a few steps in the procedure, we have not found a way that is generally shorter than the one presented in Figure 6.2. If the hybridoma, or milligram quantities of the mAb, is available, it is possible to jump to the third phase, the purification phase. The entire process is admittedly lengthy, and, today, many researchers will choose to use an epitope-tagged protein purification system, which will be discussed in detail in the next section.

Finally, the ability to clone DNA sequences that express antibody-like molecules has opened a whole new biochemical field for antibody production. This will likely have a large impact on immunoaffinity chromatography in the future. Some aspects of this approach will be discussed next.

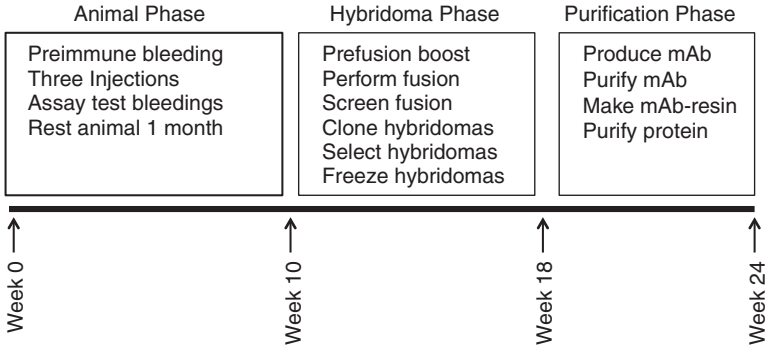

Figure 6.2 General scheme for the production of mAbs for use in immunoaffinity chromatography. The procedure is divided into three phases and the approximate time in weeks is given for each phase.

6.3 Properties of Antibodies for Immunoaffinity Chromatography

The ability to produce mAbs allows the production of a large amount of antibody with homogeneous properties. However, not all mAbs will be successful in immunoaffinity purification. Three of the most important parameters are ability to bind to an accessible epitope, high affinity, and elution conditions that will not inactivate the target protein.

6.3.1 Epitope Accessibility

When an animal is injected with an immunogen, generally several epitopes will be recognized as immunogenic, and antibody responses are mounted against these epitopes. Therefore, when hybridomas are prepared from the spleen cells of these animals, hybridomas can, theoretically, be isolated for each epitope recognized. Due to the processing of the immunogen by the animal for presentation of the epitope, some of the epitopes are likely to be buried in the folded protein. If the fusion is screened for mAbs by an ELISA, it is very common to identify mAbs that do not react with the native protein when it is solution. This is likely due to the fact that protein is distorted when it is adsorbed onto the plastic surface of the ELISA plate, exposing otherwise inaccessible epitopes. Thus, it is necessary for mAbs that are identified by an ELISA that immobilizes the antigen on a surface to be rescreened for the ability to react with the antigen in solution. This is best determined by perfoming a small-scale immunoprecipitation. The investigator should also be cautioned that a Western blot-negative mAb (supposedly an mAb that reacts only with "native" protein) will not necessarily work in immunoaffinity chromatography.

Another situation where the epitope is not accessible is if the protein forms a complex with another protein, and the second protein blocks the epitope from

the mAb. We found this to be the case with an mAb designated 8RB13 that reacts with the beta subunit of the bacterial RNA polymerase [5]. When the sigma subunit (promoter regulatory factor) bound to the core RNA polymerase, this epitope was blocked. It was later determined that the epitope constituted one of the two major binding sites on core polymerase for the sigma subunit [6].

Finally, epitope accessibility is also a concern if an mAb is to be used in a "immunodepletion" mode. In this case (which is also called negative immunaffinity chromatography), a specific protein is removed from an extract, rendering the mixture devoid of any activity. The purified protein, or a mutant protein, can then be added back to the reaction to restore the activity. This method has been especially useful to investigate transcription factors that do not have a measurable activity on their own but are part of a protein complex that has measurable activity [7,8].

6.3.2 High Affinity

The belief that an mAb to be used in immunoaffinity chromatography has to have low intrinsic affinity is false. In fact, if the target protein is present at a very low concentration in the starting material, the affinity of the mAb needs to be high in order to pull the target out of the solution. For example, if an antigen is present at the level of 1 ng/ml, the affinity (K_D) of the antibody needs to be around 10^{-9} M in order to pull it out of the solution. It is not necessary to know the exact K_D of the mAb to use it for immunoaffinity chromatography. Generally, the use of a large amount of antibody on the column can compensate for a lower affinity (within a reasonable range). Also, some antibody activity might be lost during the coupling of the mAb to the support matrix, thus resulting in a decrease in ligand efficiency. This is best decided by trial and error.

6.3.3 Elution Conditions

This has been the "Achilles' heel" of immunoaffinity chromatography. In a standard immunoprecipitation, the antigen is often eluted from the mAb with denaturing condition and then subjected to some analytical procedure, such as SDS-PAGE. However, in immunoaffinity chromatography, it is usually desired to recover the protein in an active form. Thus, harsh conditions, such as high or low pH values or denaturing agents, are usually not a good choice for eluting a protein, unless the protein of interest is resistant to these conditions. There are two basic ways to gently elute the protein from the immobilized mAb.

The first approach is to use a peptide containing the sequence of the epitope. The peptide competes with the protein for binding to the antibody. This usually requires high concentrations of the peptide, and excess peptide might need to be removed from the product. With today's technology, it is neither difficult nor too expensive to order a peptide with the required specificity from a commercial

source. However, this approach requires that the amino acid sequence of the epitope be known. Generally, the peptide contains two–three repeats of the known epitope, and is used at the level of several hundred micrograms per milliliter.

The second approach is to use a type of mAb that changes affinity under certain environmental conditions, where the new condition does not affect the activity of the protein. This can be as simple as increased salt concentration, or slight changes in pH values, that does not affect activity. It can also be due to changes in calcium concentration [9–11]. We have identified a specific type of antibody that we have termed "polyol responsive." The key advantage of these polyol-responsive mAbs (PR-mAbs) is that the conditions used to elute are very gentle, even stabilizing, and active enzyme or multisubunit complexes are easily isolated. We have been able to isolate a PR-mAb that reacts with almost every antigen that we have tested [5,12–19]. These PR-mAbs are listed in Table 6.1. By testing large numbers of antibodies, we have estimated that approximately 5–10% of mAbs are PR-mAbs.

Several reviews have been published about the properties, identification, and use of PR-mAbs in immunoaffinity chromatography [20–24]. Briefly, a PR-mAb is a naturally occurring antibody that was screened for the ability to lower its affinity for the antigen in the presence of a nonchaotropic salt and a small polyhydroxylated compound (polyol). The elution buffer is generally a Tris-HCl (50 mM) and EDTA (0.1 mM) buffer, pH 7.9 (TE buffer), which contains 50–750 mM salt and 20–40% polyol. The salt can be sodium chloride, ammonium sulfate, sodium acetate, or potassium glutamate, among others. The polyol is generally propylene glycol, but in some cases ethylene glycol or 2,3-butanediol. Generally, we use 500–750 mM sodium chloride or ammonium sulfate and 30–40% propylene glycol. However, one PR-mAb (8WG16) is responsive to just 50% glycerol [12]. This PR-mAb, which was isolated using wheat-germ RNA

Table 6.1 Polyol-responsive mAbs isolated by the Burgess Lab and used in immunoaffinity chromatography.

PR-mAb	Protein	IgG subclass	Reference
8WG16	Eukaryotic RNA polymerase II (CTD)	IgG2a	[12]
NT73	*E. coli* RNA polymerase (beta-prime subunit)	IgG1	[13]
8RB13	*E. coli* RNA polymerase (beta subunit)	IgG1	[5]
4RA2	*E. coli* RNA polymerase (alpha subunit)	IgG1	[14]
IIB8	Human RNAPII transcription factor B (TFIIB)	IgG2a	[15]
1TBP22	Human TATA-binding protein (TBP)	IgG1	[16]
1RAP1	Human RAP30 subunit of transcription factor IIF (TFIIF)	IgG2a	[17]
2ERR1	Human estrogen-related receptor alpha (ERRα)	IgG1	[18]
1EB1	Epstein Bar virus nuclear antigen (EBNA1)	IgG1	[19]

polymerase as the immunogen, reacts with the conserved heptapeptide repeat on the C-terminus of the largest subunit of almost all eukaryotic RNA polymerase II. Thus, this antibody was used to purify yeast RNA polymerase II on a large scale in sufficient purity to crystallize the enzyme [25].

It is possible to select mAbs that respond to salt/polyol elution by using a modified ELISA, which we have termed ELISA-elution assay [13,20,24]. This assay can be performed at the time the fusion is screened or after a number of antigen-specific mAbs have been collected. This assay can also be used to screen panels of mAbs for response to a variety of buffer conditions other than polyol/salt [26].

The gentle elution of target proteins has another benefit in that these conditions are also unlikely to inactivate the antibody on the immunoadsorbent. This prolongs the life of the immunoaffinity column, which is not an inexpensive commodity.

6.4
Construction of an Immunoaffinity Matrix

This section gives advice to investigators who are planning to create their own immunoaffinity matrix for use in immunoaffinity chromatography.

6.4.1
Ability to Produce Large Amounts of mAb

In order to prepare an immunoaffinity column of a reasonable size, it is necessary to prepare milligram quantities of mAb. In our laboratory, we generally conjugate our matrix at a ratio of 2.5 mg antibody per 1 ml resin.

mAbs are present in the cell culture fluid at a concentration of 1–10 μg/ml. While this is high enough for some purposes (such as Western blots, immunofluorescence, and some immunoprecipitations), regular cell culture supernatant is not useful for purifying the milligram levels of mAb needed for constructing an immunoaffinity chromatography column.

Most hybridomas will produce ascitic fluid when injected into the peritoneal cavity of mice. In years past, most researchers produced mAb for immunoaffinity chromatography in ascitic fluid, where it makes up over 90% of the antibody content and generally yields 1–10 mg of antibody per ml of ascitic fluid. However, in the university setting, it is getting exceedingly more difficult to produce mAbs in the form of ascitic fluids, due to the reluctance of the Institutional Animal Care and Use Committee (IACUC) to grant permission. Many antibody companies will produce an mAb as ascitic fluid on a contract basis if the investigator provides the hybridoma.

In our lab, we produce large quantities of mAbs in an *in vitro* quasi-continuous system. There are many of these systems available on the market. For our purposes, we use the CELLine Flask 350 (CL 350) manufactured by Integra Biosciences (Switzerland). In the United States, this product is distributed by Argos

Technologies, Inc. (Elgin, IL). The use of this method is described in detail in Thompson *et al.* [24]. Different hybridomas will produce differing levels of antibody per milliliter of culture fluid in the growth chamber; however, it is not uncommon to produce up to a milligram per milliliter, and thus recover 30–40 mg of antibody from the growth chamber over a 1 month period.

Large quantities of mAbs can also be produced by a variety of recombinant DNA methodologies. Some aspects of this approach will be discussed below. Once a construct has been developed that produces the antibody-like protein, milligram quantities can be prepared in a variety of protein expression systems.

6.4.2
Purification of mAbs for Conjugation

For most purposes, it is not necessary to have a highly purified preparation of mAb for conjugation to the resin. However, it is generally necessary to purify the mAb away from the majority of the contaminating proteins in the source material. These contaminating proteins will also couple to the resin (unless an immunoglobulin affinity ligand, such as protein A/G, is used), which reduces the ligand efficiency of the resin. The main contaminating protein in ascitic fluid and cell culture medium is albumin. Most of the albumin can be separated from the immunoglobulin fraction by a simple ammonium sulfate precipitation. At 45% saturation, the immunoglobulin fraction precipitates, leaving the albumin in solution. The precipitate can then be subject to an additional purification step, such as ion-exchange chromatography.

6.4.3
Indirect Conjugation of the mAb

Many manufacturers produce resins or magnetic beads that already have protein A or protein G conjugated to a bead. These two proteins were originally isolated from *Staphylococcus aureus* (protein A) and a *Streptococcus* sp. (protein G), and are thought to play a role in defense against the host's immune system. However, today most protein A and protein G are made by recombinant methods and produced in *Escherichia coli*. These two proteins have an affinity for the constant region of IgG molecules. However, some IgG molecules from certain species, or certain subclasses of IgG from certain species, do not bind well to one or both of these proteins. Tables are available that address the scope of binding. Thus, protein A/G resins have been valuable for the purification of antibodies and the production of immunoadsorbents. The antibody–protein A/G interaction is high affinity (depending upon the species and subclass of the antibody) but does not have the robust nature of a covalent bond. Thus, it is subject to dissociation. It is possible to cross-link the antibody to the protein A/G beads.

Magnetic beads have also been used to create the immunoadsorbent. Again, there are many different magnetic beads available on the market. These differ in bead composition, size, and IgG-binding capacity. The mAb is usually bound to

the bead by way of protein A or protein G. These magnetic beads are very useful for immunoprecipitations but quite expensive for large-scale purifications.

6.4.4 Direct Conjugation of Antibody to the Resin

Because the mAb must be immobilized on a support, the selection of a resin is an important consideration. There are a variety of supports available on the commercial market that contain a variety of coupling chemistries. Some coupling approaches orient antibodies so that the Fab region is extended away from the bead. This is the case if the bead is coupled to an antibody-binding protein such as protein A or protein G, which adsorbs the antibody through the constant region of the heavy chain. It is also true for chemistries that couple through the carbohydrate moiety of the heavy chain. We have shied away from the use of protein A or protein G resins because the cost of these products adds considerable expense to an already expensive procedure. We routinely use cyanogen bromide-activated Sepharose. When we began using immunoaffinity chromatography to purify RNA polymerase II, there were only a few choices of activated resins available on the market. Several years ago, we experimented with the coupling of mAb 8WG16 (one of our most successful mAbs for IAC) to different resins with different coupling chemistries. We then tested these resins for their ability to purify RNA polymerase II from wheat-germ extracts. The only correlation that we were able to make is that the higher the coupling efficiency, the better the resin worked. At that time, we were unable to identify a resin that worked better than cyanogen bromide (CNBr)- activated Sepharose. However, RNA polymerase II is a huge protein complex, and smaller target proteins might give different results.

CNBr-activated Sepharose, and several other coupling chemistries, react with amino groups on the mAb. It is fortunate that majority of the available lysine groups on most IgGs are located on the Fc protein. Thus, it is necessary to have the mAb in a buffer that does not have free amino groups. In most cases, this means that the antibody is in a carbonate buffer. In addition, it is important to consider the pH and ionic content of the buffer. This is also true for other coupling chemistries; therefore, buffer components and the pH of the coupling buffer should reflect the chemistry of the conjugation.

Finally, because different resins have different efficiencies of coupling, it is important to measure the protein concentration of the mAb before and after the conjugation. With CNBr-activated Sepharose, the coupling efficiency is generally greater than 90%.

6.4.5 Blocking and Washing

After conjugation of the mAb to the matrix, it is also important to block any unreacted coupling moieties. In the case of CNBr-activated Sepharose, the

blocking is usually accomplished with either ethanolamine or glycine, although high concentrations of Tris-HCl buffer also work. The uncoupled mAb and the other contaminants should be removed from the resin preparation by washing extensively. In the case of CNBr-activated Sepharose, this is usually done with alternatively washing with a buffer at pH 8 and 4, both of which contain high salt (0.5 M NaCl).

6.4.6
Storage of Immunoaffinity Resin

The mAb-coupled affinity resin does have a finite shelf life. Despite the fact that the mAb is covalently coupled to the resin, the antibody will eventually leach off. Different coupling chemistries will leach at different rates. The other problem is that the mAb is a protein, and although IgG molecules as a whole are generally fairly stable, they are susceptible to denaturation from chemicals and degradation from proteases. For general storage, we store our CNBr-conjugated mAb at 4 °C in the presence of 0.02% sodium azide. Under these conditions, we can store the resin for about 6 months. After this time, the resin still seems to work, except that leaching of the antibody becomes a problem. Recently, we have had the opportunity to store resin in a buffer containing 50% glycerol at −20 °C with encouraging results. However, a long-term study has not been performed as yet.

6.5
General Procedure for Immunoaffinity Chromatography

Step-by-step procedures for isolating and using PR-mAbs for immunoaffinity chromatography have been published [20,21,24]. This section addresses some common questions that have arisen over the years, mainly from students, about immunoaffinity chromatography. If the reader's particular system is commercial, these tips might not apply.

6.5.1
Starting Material

Theoretically, the crude starting material containing the target protein should be able to be applied directly to the mAb affinity resin. However, it is usually wise to perform some type of initial fractionation step to clean up the sample to prevent fouling the resin. In addition, if the target protein is expected to be at low concentrations, it might be wise to perform an ammonium sulfate precipitation to concentrate the material to improve binding to the antibody. Also, the viscosity of the starting material should be addressed at this point. In purifying a nucleic acid-binding protein, it is advisable to remove as much nucleic acid as possible to avoid copurifying DNA with the target protein.

6.5.2 Use of the mAb-Sepharose

The resin should be equilibrated in the buffer that the starting material is in. The resin can be used in either the batch or the column mode. One gram of dry CNBr-activated Sepharose will yield 3.5 ml of packed wet resin. For use in a column mode, we pack a disposable polypropylene column (BioRad) with about 2 ml of mAb-Sepharose. The starting material should be loaded slowly at a flow rate of about 0.5–1 ml/min.

For use in a batch mode, we generally use the 2 ml of resin in a volume of about 10–20 ml of target protein-containing solution and allow it to mix end-over-end for about 1 h (in a cold room, if necessary). The resin can then be collected on a fritted glass filter and washed. While the resin can be collected by centrifugation, some beads are susceptible to crushing or breaking, so this should be avoided. The advantage of the batch mode is that the unbound material, including any small particulate material, can be easily washed away; this is the type of thing that can accumulate on the top of the column and foul it easily. The disadvantage of the batch method is that every time the resin is transferred to a new container, some of the resin is lost on the sides of the container.

The unbound material should always be saved in order to assay for the binding of the target protein and to check to see if the resin was overloaded. It is important to know how efficiently the mAb resin binds the target protein. If the resin is overloaded, the unbound material can be reapplied to the mAb-conjugated resin after it is regenerated.

6.5.3 Washing the Resin

The initial wash is usually with the buffer that was used to apply the target protein. Generally, at least 10 resin volumes of buffer should be used. In some cases, it is advised to collect the first part of the wash for analysis. Remember that, in the column mode, some of the applied material is still in the column volume when you start the washing process.

A differential wash can now also be performed. This might be a higher concentration of salt to remove a contaminating protein or remaining nucleic acid. This can also be advantageous to recover proteins that interact with the target protein. For example, differential potassium chloride washes were used to recover yeast proteins that interact with yeast RNA polymerase II after the polymerase was bound to the resin by a polymerase-specific antibody [27].

6.5.4 Eluting the Target Protein

As mentioned above, recovering the target protein with high yield in an active form is the goal. Conditions of elution should be worked out before

the chromatography is performed. Otherwise, a precious protein sample might be wasted.

In a column mode, the buffer containing the eluting compound is slowly passed through the column, and the fractions are collected. In the batch mode, it is often advantageous to collect the washed resin on a fritted filter to remove the excess buffer in order to not dilute the eluting reagent.

For reasons that are not known, elution of the target protein with salt/polyol is more efficient at 23 °C than it is at 4 °C. Thus, if the immunoaffinity procedure has been performed at 4 °C, the resin should be moved to room temperature for the elution with the salt/polyol.

The SDS-PAGE shown in Figure 6.3 demonstrates the purification, essentially as described in Ref. [15], of the RNA polymerase transcription factor B (TFIIB) expressed in *E. coli*. The soluble fraction was treated with PEI to remove nucleic acids and applied to a IIB8-mAb Sepharose. The target protein was then eluted with buffer containing 0.7 M ammonium sulfate and 40% propylene glycol.

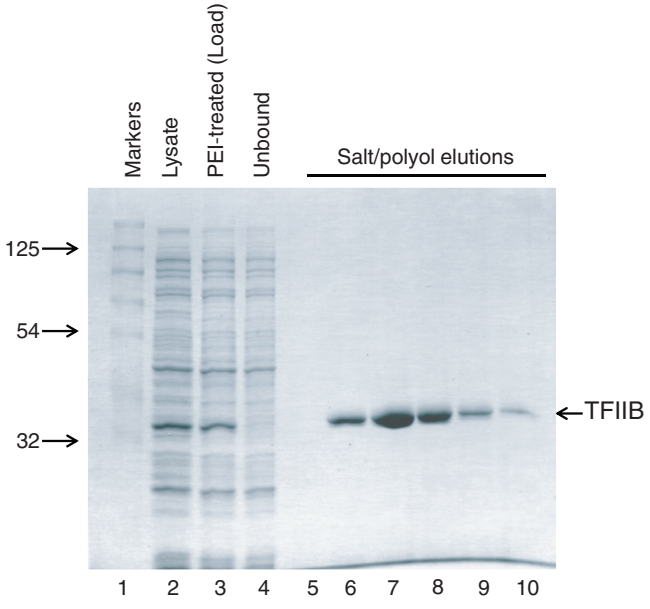

Figure 6.3 Analysis of the fractions from a purification using a polyol-responsive mAb. Fractions were analyzed by SDS-PAGE, stained with Coomassie blue. The target protein was human transcription factor B (TFIIB). TFIIB was expressed in *E. coli* as a soluble protein. The lysate (lane 2) was treated with polyethyleneimine (PEI) to remove the nucleic acid, which precipitated and was removed by centrifugation; the supernatant (lane 3) was loaded onto a 2 ml column of mAb IIB8. The unbound material was recovered (lane 4). After washing with starting buffer, the TFIIB was eluted with buffer containing 700 mM ammonium sulfate and 40% propylene glycol (lanes 5–10). Lane 1 contains molecular weight markers.

6.5.5
Removing the Eluting Reagent

The eluting reagent will usually interfere with activity assays. In most cases, the target protein is dialyzed into a suitable buffer. An alternative is to apply the target protein to a small buffer-exchange column, such as a spin column.

6.5.6
Regenerating the mAb Resin

Given the cost involved with immunoaffinity chromatography, it is wise to reuse the resin. After eluting the protein, the resin is washed with the starting buffer to remove any eluting agent and then treated with a chaotropic agent to remove any residual target protein and contaminating materials. As a general regenerating protocol, we treat the mAb- resin with TE buffer containing 2 M KSCN for about 5 min, followed immediately by an extensive TE buffer wash. The mAb-conjugated resin is then stored in a neutral buffer with 0.02% sodium azide.

6.6
Issues of Nonspecific Binding

Despite the specificity of immunoaffinity chromatography, contaminating proteins will often show up in the eluted target protein fraction. Depending upon the specific parameters of the protocol used, these can result from (1) proteins that cross-react with the mAb contained in the applied sample, (2) proteins that bind to the target protein that were not removed by a differential wash, (3) proteins contained in any particulate material that did not enter the column but were eluted from the particles by the eluting reagent, (4) proteins that adhered to the resin, the sides of the column, or other container that were not removed by washing but were removed by the eluting reagent, or (5) a breakdown product of the target protein or multimers of the target protein.

Detecting contaminating proteins can be frustrating; the best way to deal with them is to try to find out where they are being introduced. If the contaminant is coming from your resin, it may be that you will need to start with a new column. While immunoaffinity resin can be regenerated and used several times, eventually the resin will foul and will need to be replaced. In our experience, with CNBr-activated Sepharose, this usually occurs after about 8–10 uses.

6.7
The Use of Epitope Tags

Given the power of immunoaffinity chromatography, and the power of molecular genetics, it is not surprising that the two techniques have been used in

combination to generate epitope-tagged proteins. In this scenario, the genetic sequence of the target protein is fused with the nucleotide sequence that corresponds to the specific epitope. The expressed protein then contains the amino acid sequence of the epitope, usually on the N- or C-terminus of the expressed protein. Expression can be in a variety of systems such as bacterial, yeast, mammalian cells, or even intact animals.

Table 6.2 lists a variety of epitope tags, including routinely used commercial tags [10,28,29] and some polyol-responsive epitope tags that we have developed [6,30,31]. The two most common means of elution are by competition with a peptide or by changing buffer conditions to reduce the affinity of the antigen/antibody reaction (such as salt/polyol). It is sometimes preferable to incorporate two–three copies of the epitope onto the target protein. In most cases, the epitope tag does not seem to affect the activity of the protein. In some cases, after the successful immunoaffinity chromatography, the epitope tag is removed, usually by cleavage of the tag by a specific protease whose consensus sequence has been genetically engineered between the tag and the target protein.

Epitope tags were originally developed as a way either to distinguish a recombinant protein from an endogenous protein or to detect a protein for which there was not an existing antibody. The first epitope tag for use in immunoaffinity chromatography was the FLAG™ tag [29]. The sequence of the epitope (D Y K D D D D K) was selected in the hope that an mAb that reacts with this epitope would release the antigen in response to increased salt concentrations. However,

Table 6.2 Some epitope tags used in immunoaffinity chromatography.

Tag designation	Original antigen	Original mAb	Epitope	Elution method	Reference
HA-tag	Influenza virus hemagglutinin	12CA5	YPYDVPDYA	Peptide	[28]
Myc-tag	Human c-Myc oncoprotein	9E10	EQKLISEEDL	Peptide	[29]
Flag-tag	Peptide	4E11	DYKDDDDK	Low calcium or peptide	[10]
Softag1	*E. coli* RNA polymerase beta-prime subunit	NT73	SLAELLNAGLGGS	Salt/polyol	[30]
Softag3	Human TFIIB transcription factor	IIB8	TKDPSRVG	Salt/polyol	[31]
Softag4	*E. coli* RNA polymerase beta subunit	8RB13	PEEKLLRAIFGEKAS	Salt/polyol	[6]

it was fortuitously discovered that the mAb (4E11) released the antigen when calcium was removed from the buffer.

Some commercial enterprises offer kits that contain a specific mAb for use with a tagged protein and the eluting epitope-containing peptide. However, these kits are more on the scale of immunoprecipitations. Theoretically, these protocols can be run multiple times and the products from each run can be combined to yield a reasonable amount of target protein for biochemical experiments.

An elegant use of epitope-tagged proteins has been used to isolate transcription protein complexes from stably transfected cell lines [32].

6.8 Recombinant Antibodies

Antibodies, being proteins, have also been produced by recombinant protein technology. As described above, antibodies can be expensive and time-consuming to make. Therefore, efforts have been made to overcome these obstacles by producing antibodies, or antibody fragments, as recombinant proteins in various expression systems. Once the construct(s) is generated, the antibody can be expressed in large quantities, without the use of animals and sometimes even without the use of mammalian cell culture [33]. Recombinant antibodies are becoming widely used as pharmaceuticals. However, there is also great potential for their use in biotechnology, especially immunoaffinity purification.

Although the Fab can be generated from the whole antibody by cleavage with papain, it can also be produced by cloning the light chain and the N-terminal half of the heavy chain and coexpressing them in a protein expression system. This is most often a mammalian cell culture system, insect cells, or yeast cells. This type of system requires the expression of the two antibody chains and then the assembly of the chains with a disulfide bond, something that is not trivial in bacterial systems. The use of Fab has not been used extensively in immunoaffinity chromatography, probably because there is limited advantage over using the entire antibody.

However, further manipulation of the antibody genes yielded single-chain variable fragment (scFv), which are often expressed in bacterial systems. This fragment is constructed by genetically fusing the variable region of the heavy and light chains together with a linker region. A schematic is shown in Figure 6.1. There are two approaches to obtain a scFv. The first is to isolate the mRNA from the hybridoma, using a combination of polymerase chain amplifications and recombinant DNA techniques. We have prepared a scFv from the DNA sequence of the variable regions of the PR-mAb NT73 [34]. This construct was expressed in *E. coli* and refolded from the insoluble inclusion bodies. The scFv retained its polyol-responsiveness but lost considerable affinity for the antigen.

The other way to obtain a scFv is by screening a recombinant library, most commonly a phage-display library [35]. The phage-display library can be constructed either from the mRNA of an immunized animal or from a naïve source.

Usually, the scFv has low initial affinity but can be mutagenized to obtain a molecule with higher affinity, a process referred to as "affinity maturation." To our knowledge, this approach has not been documented for polyol-responsive mAbs.

In recent times, some researchers are using a heavy-chain antibody system derived from camels or llamas [36]. This group of mammals produces a species of antibody that lacks a light chain and also the constant domain of the heavy chain found on a canonical Fab. The molecule is often referred to as VHH (or HCAb or dsAB). The animal can be immunized with a specific immunogen, and then a library is generated by cloning the VHH genes from the peripheral blood lymphocytes. Selection of the specific antibodies is generally performed by phage display. In an excellent example of the power of this technology, Tillib *et al.* [37] describes the generation of a VHH library from a camel immunized with human lactoferrin, the screening of the VHH by phage display, and the subsequent use of the VHH to purify human lactoferrin from the milk of transgenic goats.

Antibody engineering has discovered nonimmunoglobulin alternatives for presenting CDR-like projections that can recognize epitopes. An alternative scaffold generally has not only a very stable structure, similar to the immunoglobulin fold, but also a "loopy region" that can be engineered with CDRs [38]. Finally, researchers are reporting the ability to manipulate the scaffold structure to augment the binding of a protein containing a post-translational modification [39].

6.9 Conclusions and Future Directions

Our increasing knowledge of protein–protein interactions and the ability to manipulate recombinant molecules are contributing to a new era in immunoaffinity purification. While it is still useful to employ some techniques that use whole antibody molecules, the future trend seems to be toward antibody-like molecules and their selective binding using combinatorial libraries and mutagenesis of these molecules for selected properties. These smaller antibody-like molecules will be easily expressed in bacterial systems and will probably have utility in a number of biochemical systems, including affinity purification.

Some have pointed to a futuristic view where every protein in the human proteome will have at least one cognate recombinant antibody, which has been isolated from a combinatorial library, such as phage display [40]. These combinatorial libraries can be automated, and in such a scenario, it would be possible to screen these recombinant antibodies for conditional release, such as polyol-responsiveness, thus allowing the purification of any target protein, possibly in a single step.

Acknowledgments

The authors thank the many individuals who have been in the Burgess Lab over the past 30 years and have contributed to the development of the PR-mAb work.

This work was supported at various times by grants from the National Institute of Health, Department of Defense, and the University of Wisconsin-Madison, USA.

Conflict of Interest

The authors disclose a financial interest in NeoClone, LLC (Madison, WI) that has marketed some of the PR-mAbs.

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