Immunoaffinity Chromatography with Affinity Supports

Immunoaffinity chromatography is a form of affinity chromatography in which antigens or antibodies are immobilized on a solid phase support. The bio-specific antigen-antibody interaction makes it possible to obtain a high degree of purification in a single step. However, this bio-specificity is often accompanied by extremely tight binding, making elution of active purified antigen or antibody very difficult. The following procedures are recommended to help to optimize conditions and to maximize yields.

PREPARATION FOR IMMUNOAFFINITY CHROMATOGRAPHY

Selection of Support for Immobilization

It is important to choose a support which meets the requirements listed below.

1. **Porous** — the matrix should be porous enough to allow both the antigen and the antibody to penetrate the pores. Immunoaffinity chromatography can be done on the surface of a support, but a porous matrix offers the advantage of providing a higher capacity support.

2. **Inert** — the matrix should be hydrophilic and inert, and should contain no chemically reactive groups or charged sites. This will minimize non-specific adsorption.

3. **Stable** — the matrix must be stable to all reagents used in the coupling and elution steps. Commonly used reagents include acids, bases, chaotropic salts, and organic solvents.

4. **Good Flow Properties** — Immunoaffinity chromatography is most often performed using column techniques. Flow properties are determined by the size, shape, and rigidity of the particles. Good flow properties are particularly important if relatively stringent conditions are used to elute labile molecules and exposure time must be minimized.

Bio-Rad offers a number of supports which are recommended for immunoaffinity chromatography. Affi-Gel® Hz and Affi-Prep® Hz hydrazide supports couple immunoglobulins via the carbohydrates located in the Fc portion of the antibody. This method of coupling yields highly active, oriented IgG. Affi-Gel 10, Affi-Gel 15, and Affi-Prep 10 activated supports spontaneously couple to primary amines. Use of these materials is described in detail in bulletins 1424 and 1085.

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Preparing Antiserum for Immobilization

Antiserum should be partially purified prior to immobilization. By removing the bulk of the non-antibody proteins, a higher capacity immunosorbent with minimal non-specific adsorption can be obtained. This can be optimized further by using an affinity purified antibody or a monoclonal antibody.

Bio-Rad has a variety of choices for immunoglobulin purification. DEAE Affi-Gel blue gel is recommended for single step purification of small volumes of serum, yielding a purified IgG fraction (see bulletin 1092). CM Affi-Gel blue gel is particularly useful for processing large volumes of serum or when maximum yields are desired (see bulletin 1092). A total globulin fraction is obtained by this method. Affi-Gel protein A and Affi-Prep protein A supports are ideal for the purification of all types of monoclonal antibodies or when the highest purity is desired.

Preparing Antiserum for Antibody Purification

If an antibody is to be affinity purified on an immobilized antigen support, it is also advantageous to partially purify the antiserum. DEAE Affi-Gel blue gel and CM Affi-Gel blue gel offer the advantage of removing complement factors which bind immune complexes. These methods also remove protease which can destroy valuable antibody during sample storage or decrease column life by destroying the immobilized protein. As a minimum purification, antiserum should be heat inactivated at 56°C for 30 minutes to destroy the complement factors.

PROCEDURES FOR IMMUNOAFFINITY CHROMATOGRAPHY

Adsorption of the Sample

It is advantageous to use only the required amount of affinity support. If excess matrix is used, sample elution becomes more difficult because the sample continues to bind and elute as it passes down the column. Stronger elution conditions become necessary, residence time is longer, the eluted peak is broader, and there is a greater risk of denaturation and poor recovery. If all potential binding sites are occupied, then once a molecule is eluted, it has no chance of further binding.

One method to insure that only the required amount of affinity gel is used is to apply the sample to the top of the column and elute using reverse flow (see Figure 1).

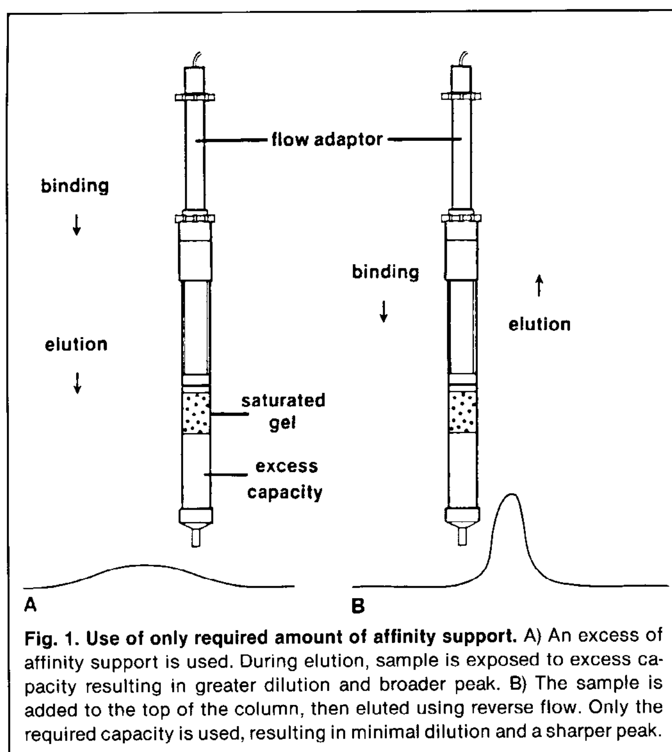

Fig. 1. Use of only required amount of affinity support. A) An excess of affinity support is used. During elution, sample is exposed to excess capacity resulting in greater dilution and broader peak. B) The sample is added to the top of the column, then eluted using reverse flow. Only the required capacity is used, resulting in minimal dilution and a sharper peak.

Another method is to titrate the gel with sample, checking the supernatant for unbound sample after each addition. This can be done either in a column or in a batch mode. Continue until the gel is saturated. This method can be used with a small amount of gel and sample to determine the capacity and, thus, the required amount of gel for the purification.

Removal of Unbound Solutes

Proteins or other solutes which are not bound or are weakly bound by non-specific interactions must be washed off prior to elution. This can be achieved by washing with mild chaotropic solutions (1M NaSCN, 1M guanidine hydrochloride, 1M urea), with salts (1M NaCl), or with detergents (0.5% Triton® X-100). In many cases, the elution buffer can be used, but at a lower concentration. This frequently neglected wash step eliminates proteins which may complicate final elution and helps yield a more highly purified product.

Elution Strategies

Elution is usually the most difficult step in immunoaffinity chromatography. Often, in an attempt to maximize yields, elution conditions are chosen which denature the proteins. The objectives of elution are to obtain high purity and high recovery of a stable and active product.

Antigens and antibodies are bound to each other by the forces of ionic bonding, hydrogen bonding, and hydrophobic interactions.[1] The strength of antigen-antibody complexes varies widely due to the different affinities and avidities of the antibodies. In addition, parameters such as coupling density, steric orientation, and non-specific interactions can become important. A wide variety of solvents have been reported as eluants in immunoaffinity chromatography and upon initial examination of the literature, the choice of an effective eluant appears empirical. There is, however, a logical strategy or sequence of eluants to consider when approaching a new immunoaffinity application. A logical sequence is:

1. **Specific Elution** with excess antigen or antibody should be considered first because, in theory, it will always work. It is, however, frequently impractical due to the cost and availability of the specific eluant. The other disadvantage is that an antigen-antibody complex will be eluted and the dissociation of the complex may be necessary and difficult to achieve.

2. **Acid Elution** is the most commonly employed desorption method and is frequently very effective. Eluants such as glycine-HCl, pH 2.5, 0.02M HCl, and sodium citrate, pH 2.5 can be used to disrupt the antigen-antibody interactions. In some cases, acid elution gives low recovery due to hydrophobic interactions between the antigen and the antibody. An eluant such as 1M propionic acid, or the addition of 10% dioxane[2] or ethylene glycol to the acid eluant, is more effective in dissociating such complexes.

3. **Base Elution** is less frequently used than acid elution, but, in some cases, is more effective. Elution with 1M NH_4OH or with 0.05M diethylamine, pH 11.5 has been shown to be effective with membrane glycoproteins and with certain antigens which precipitate in acid but are stable in base.[3] Organic solvents can also be added to basic eluants as described above with acid elution. An example in which an antibody to dinitrophenyl-bovine serum albumin (DNP-BSA) was coupled to an affinity matrix is described in Reference 4. DNP-BSA could not be eluted at all with acid or with acid plus organic solvents. Basic elution gave 69% yield and base plus dioxane gave 95% yield of purified antigen.

4. **Chaotropic Agents** disrupt the tertiary structure of proteins and, therefore, can be used to dissociate antigen-antibody complexes. Chaotropic salts are particularly useful because they disrupt ionic interactions, hydrogen bonding, and sometimes hydrophobic interactions. Chaotropic anions are effective in the order $SCN^- > ClO_4^- > I > Br^- > Cl^-$.[5,6] Chaotropic cations are effective in the order Gu>Mg>K>Na.[6] Eluants such as 8M urea, 6M guanidine hydrochloride (GuHCl), and 6M NaSCN are effective in disrupting most protein-protein interactions. The problem, however, is that in many cases, these strong chaotropes will destroy the activity of the antigen and/or antibody. For that reason, it is best to use the mildest conditions that will give acceptable recovery.

Once the eluant has been chosen, the elution conditions should be refined by optimizing concentration, time, temperature, and by combining the eluants described above.

No matter which eluant is selected, it is important to remove the eluted antigen or antibody from the eluant as quickly as possible to minimize the chance of denaturation. If acid or base is used, the samples should be neutralized immediately following elution. If a chaotrope is used for elution, it can be rapidly removed by desalting (Econo-Pac® 10DG desalting columns, catalog number 732-2010, Bio-Gel® P-6 DG desalting gel, catalog number 150-0738).

Special Considerations for Labile Antigens

Because there is a problem of stability with some antigens, whether immobilized or being purified, special considerations may be necessary. With labile antigens, the choice of the mildest possible elution conditions is desirable, with rapid elution and short exposure times being critical. One approach for labile *immobilized* antigen is to use relatively mild elution conditions, then do a more complete regeneration using a chaotropic salt after every fourth or fifth use

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of the column. This serves to increase the life of the column by minimizing exposure to stringent conditions and to periodically strip the column of bound proteins, therefore maintaining the capacity.

Alternative procedures have been published for eluting labile antigens from immobilized antibody columns. The use of deionized water has been reported,[7,8] but yields are generally low. Another method of increasing interest is electrophoretic elution.[9,10] An electrical field is applied and the adsorbed protein is electrophoresed away from the affinity matrix.

Renaturation of Eluted Proteins

Proteins which have been denatured during elution can often be renatured by the addition of a chaotropic agent such as GuHCl, followed by stepwise dialysis against decreasing concentrations of the chaotrope. The high concentration of GuHCl puts the protein into a random coil configuration. As the chaotrope is slowly removed, the protein will return to its native form.[11]

As an example of this renaturation procedure, goat anti-rabbit IgG was denatured by precipitation with TCA. It was then neutralized with base and GuHCl was added to make the solution 6M. It was then dialyzed against 4M GuHCl, then 1M GuHCl, and finally into phosphate buffered saline (PBS). Nearly all of the antibody activity was restored as is shown in Figure 2.

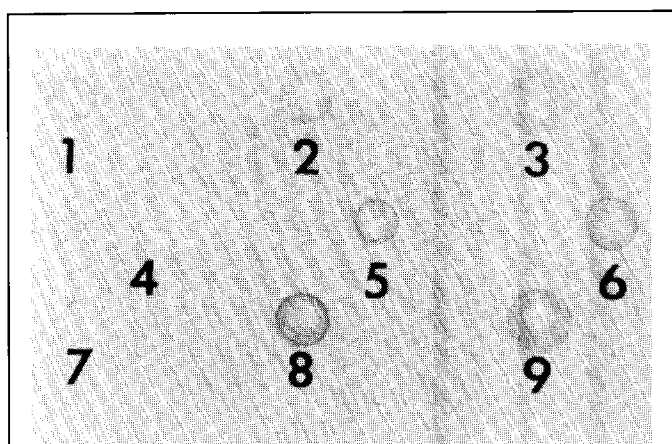

Fig. 2. Radial immunodiffusion of goat anti-rabbit IgG into agarose gel containing rabbit IgG. Wells 1, 2, and 3 represent 5, 10, and 20 λ, respectively, of antibody dialyzed against PBS. Wells 4 and 7 represent 10 and 20 λ antibody denatured by TCA precipitation. Wells 5 and 8 represent 10 and 20 λ antibody which was denatured by TCA precipitation and dialyzed stepwise into decreasing concentrations of GuHCl (see text). Wells 6 and 9 represent 10 and 20 λ native antibody dialyzed against decreasing concentrations of GuHCl.

Immunoaffinity Chromatography with Monoclonal Antibodies

Many of the problems encountered in immunoaffinity chromatography can be overcome by using monoclonal antibodies. Monoclonal antibodies provide a homogenous interaction, unlike normal antisera which contain multiple antibodies of varying specificities and avidities. Judicious selection of a monoclonal antibody with the right affinity and specificity can be used to make a high capacity immunosorbent that is free of the problems often associated with antisera derived antibodies. A monoclonal antibody with a relatively low binding strength can be empirically chosen so that milder elution conditions can be used and increased yields obtained. When monoclonal antibodies are used, nearly all the antigens dissociate under a narrow range of conditions.

Another major advantage of immunoaffinity chromatography with monoclonal antibodies is its reproducibility. Once a cell culture is established, there is an "unlimited" supply of homogenous antibodies. Since monoclonal antibodies bind with predictable affinity to a single antigenic determinant, problems of reproducibility between antisera-producing animals and from lab to lab are eliminated.

For example, a monoclonal antibody against interferon was immobilized on Affi-Gel 10 gel.[13] The immobilized antibody was then used to purify recombinant human leukocyte interferon. The result was 95% recovery and one thousand fold purification of the interferon.

Applications

Table 1. Immunoaffinity Chromatography

Ligand Bound to Affi-Gel 10	Applications	Reference
Monospecific IgG	Purification of chlamydia trachomatic-specific antigen	1
Serotype-specific vesicles	Purification of antibodies prior to ELISA	2
Antibody	Isolation of brush border aminooligopeptidase	3
Fibrinogen	Sorption of antibodies	4
Antibody	Isolation of microtubule protein	5
Anti-CEA Antibody	Solid phase radio immunoelectrophoretic assay of CEA	6
p-(arsonic) phenylazotyrosine hapten	Anti-hapten antibody purification	7
Hen egg white lysozyme (HEL)	Purification of HEL-reactive antibodies	8
Anti-interferon antibody	Purification of mouse interferon	9
Antibody to Con A, goat anti-rat light chain antibody, antibody to dinitrophenyl-ovalbumin	Purification of antigens	10
Antibody to mouse interferon	Purification of mouse interferon	11
Monoclonal anti-interferon antibody	Purification of recombinant human leukocyte interferon	12
Synaptic protein substrate for cyclic AMP	Purification of antibody	13

References to Table 1

1. Caldwell, H. D. and Kuo, C. C., *J. Immunol.*, **118**, 437 (1977).
2. Johnston, K. H., *Infection and Immunity*, **28**, 101 (1980).
3. Maze, M. and Gray, G. M., *Biochemistry*, **19**, 2351 (1980).
4. Chen, J. P. and Shurley, H. M., *Thrombosis Research*, **7**, 425 (1975).
5. Ikeda, Y. and Steiner, M., *J. Biol. Chem.*, **251**, 6135 (1976).
6. Saravis, C. A., Zamcheck, N., Kupchik, H. Z., McCabe, R. P. and Munjal, D., *J. Nat. Cancer Inst.*, **53**, 921 (1974).
7. Kanellpoulos, J., Rossi, G. and Metzger, H., *J. Biol. Chem.*, **254**, 7691 (1979).
8. Benjamin, C. D., Miller, A., Sercarz, E. E. and Harvey, M. A., *J. Immunol.*, **125**, 1017 (1980).
9. DeMaeyer-Guignard, J., Tovey, M. G., Gresser, I. and DeMaeyer, E., *Nature*, **271**, 622 (1978).
10. Brooks, K. H. and Feldbush, T. L., *J. Immunol.*, **127**, 963 (1981).

11. Gresser, I., et al., *Am. J. Pathology*, **102**, 396 (1981).
12. Staehelin, T., Hobbs, D. S., Kung, H-F., Lai, C-Y. and Pestka, S., *J. Biol. Chem.*, **256**, 9750 (1981).
13. Naito, S. and Ueda, T., *J. Biol. Chem.*, **256**, 10657 (1981).

References to Text

1. Van Oss, C. J., Absolom, D. R., Grossberg, A. L. and Neumann, A. W., *Immunol. Comm.*, **8**, 11 (1979).
2. Hill, R. J., *J. Immunol. Methods*, **1**, 231 (1972).
3. Parham, P., *J. Biol. Chem.*, **254**, 8709 (1979).
4. Andersson, K., Benyamin, Y., Douzou, P. and Balny, C., *J. of Immunol. Methods*, **23**, 17 (1978).
5. Avrameas, S. and Ternynck, T., *Biochem. J.*, **102**, 37c (1967).
6. Robinson, J. B., Strottman, J. M. and Stellwagen, E., *Proc. Natl. Acad. Sci. USA*, **78**, 2287 (1981).
7. Bureau, D. and Daussant, J., *J. Immunol. Methods*, **41**, 387 (1981).
8. Ternynck, T. and Avrameas, S., *Biochem. J.*, **125**, 297 (1971).
9. Morgan, M. R. A., Brown, P. J., Leyland, M. J. and Dean, P. D. G., *FEBS Letters*, **87**, 239 (1978).
10. Iobal, M. J., Ford, P. and Johnson, M. W., *FEBS Letters*, **87**, 235 (1978).
11. McCoy, L. F. and Wong, K. P., *Biochemistry*, **20**, 3062 (1981).
12. Kennett, R. H., *In Vitro*, **17**, 1036 (1981).
13. Staehelin, T., Hobbs, D. S., Kung, H-F., Lai, C-Y. and Pestka, S., *J. Biol. Chem.*, **256**, 9750 (1981).

Ordering Information

Catalog Number	Product Description
Products for Antibody Purification	
153-7307	**DEAE Affi-Gel Blue Gel,** 100 ml
153-7304	**CM Affi-Gel Blue Gel,** 100 ml
732-2027	**Econo-Pac Serum IgG Purification Kit**
732-2026	**Econo-Pac Serum IgG Purification Columns,** 5
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153-6160	**Affi-Gel Protein A MAPS II Buffers**
153-6153	**Affi-Gel Protein A Gel,** 5 ml
153-6154	**Affi-Gel Protein A Gel,** 50 ml
156-0006	**Affi-Prep Protein A Support,** 5 ml
156-0005	**Affi-Prep Protein A Support,** 25 ml
153-6164	**Affi-Prep Protein A MAPS II Buffers**
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153-6048	**Affi-Gel Hz Gel,** 4 x 25 ml
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156-0015	**Affi-Prep Hz Gel,** 5 ml
156-0016	**Affi-Prep Hz Gel,** 25 ml
156-0017	**Affi-Prep Hz Gel,** 500 ml
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153-6052	**Affi-Gel 15 Gel,** 4 x 25 ml
153-6098	**Affi-Gel 10/15 Combination,** 2 x 25 ml each gel
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