3

Protein Purification: Affinity Chromatography

INTRODUCTION

Many of the purification procedures discussed in Chap. 2 make use of one or more biophysical properties of proteins. The major drawback of these approaches is that proteins with similar properties can be separated only with difficulty by using methods based on minor differences. In general, affinity chromatography is based on the biological specificity a particular protein may have for a ligand. Proteins having very similar biophysical properties can have very divergent biological specificity, and if this can be used as a basis for separation, an effective purification procedure results. Although the term "affinity" chromatography has been popular for only about 20 years, the principle involved is much older, having first been used in 1951 in the form of "immunoadsorbents" for the separation of antibodies.

The general philosophy of affinity chromatography is that for a specific protein there are ligands that interact with that protein reversibly and which, if immobilized on a support material, specifically retard the chromatography of that protein and allow its purification. Depending on the strength of the immobilized ligand–protein interaction, the protein may effectively be immobilized onto the support material and measures to achieve its elution must be employed. There are many considerations that go into the selection and use of an immobilized ligand in affinity chromatography, and these are considered in this chapter. Although this method finds its widest application in the field of protein purification, it has also been useful in studying quantitative aspects of protein–ligand interaction, which is considered in detail in Chap. 18.

In its simplest conception affinity chromatography would appear to be the panacea for all problems in purifications of proteins. In a particular system, however, a variety of difficulties may be encountered. These include the inability to find a ligand with an appropriate affinity for the protein, the lack of a suitable derivative of the ligand for immobilization, and the existence of a variety of proteins with similar specificity for the immobilized ligand. The work that has been done in the past 10 years or so has provided answers to many of these problems and has led to the development of new affinity chromatography approaches.

At the start of this section the term "reversible" was used to describe the interaction of a ligand with a protein in affinity chromatography. In recent years, as discussed at the end of this chapter, a variety of covalent affinity chromatography procedures have been developed which depend on the "reversibility" of various chemical reactions with specific residues in proteins or peptides.

REQUIREMENTS OF A LIGAND FOR EFFECTIVE USE

The optimal situation for an affinity chromatography system is one where nonspecifically retarded proteins elute in the void volume of the matrix material, while specifically retarded proteins either remain associated with their specific ligand until elution is achieved by changing the chromatography conditions or are retarded sufficiently to achieve a complete resolution from void volume proteins or other proteins that may elute in the included volume of the column. Two parameters are important in determining the retardation of a protein on an affinity matrix: the dissociation constant, K_1, for the interaction of the protein and the ligand, L, given by

$$K_1 = \frac{[E][L]}{[EL]} \tag{3-1}$$

for the reaction $E + L \leftrightharpoons EL$, and second, the concentration of the immobilized ligand, L_0. If E_0 is the initial concentration of protein that can interact with the ligand, then

$$K_1 = \frac{[E_0 - EL][L_0 - EL]}{[EL]} \tag{3-2}$$

Where $L_0 \gg E_0$, this equation becomes

$$K_1 = \frac{[E_0 - EL]}{[EL]L_0} \tag{3-3}$$

We can now define the chromatographic distribution coefficient, K_d, as

$$K_d = \frac{[EL]}{[E_0 - EL]} = \frac{L_0}{K_1} \tag{3-4}$$

and the elution volume, V'_e, of the protein which interacts specifically with the ligand, is defined as

$$V'_e = V_0 + K_d V_0 \tag{3-5}$$

where V_0 is the void volume of the matrix. The retardation of the specifically interacting protein is therefore

$$\frac{V'_e}{V_0} = 1 + \frac{L_0}{K_1} \tag{3-6}$$

which is determined by the concentration of immobilized ligand and the dissociation constant of the protein–ligand complex. To achieve a retardation of 20 void volumes (i.e., $V'_e/V_0 = 20$), one would, of course, need a 19-fold excess of immobilized ligand concentration over the K_1 for the ligand–protein interaction. As discussed later, achievement of more than 1 to 2 mM ligand concentration on the matrix is usually impractical, giving useful upper limits for the dissociation constant K_1 of approximately 0.05 to 0.1 mM. If the immobilized ligand concentration can be increased, of course, larger values of K_1 can be accommodated.

If the matrix material has a fractionation range such that many of the proteins do not elute at the void volume, the parameter V'_e/V_e, where V_e is the elution volume of a protein of similar size but lacking specific interaction with the matrix, gives a better estimate of the efficacy of the immobilized ligand. From Eq. (2-4), V_e (the elution volume of the protein in the absence of specific interaction) is equal to $K_{av}(V_t - V_0) + V_0$, and hence the ratio V'_e/V_e is given by

$$\frac{V'_e}{V_e} = \frac{V_0 + K_d V_0}{V_0 + K_{av}(V_t - V_0)} \tag{3-7}$$

and knowledge of K_{av} is required to calculate the retardation. The retardation is still, of course, governed primarily by the ratio of L_0 to K_1.

As will be discussed shortly, these equations, and considerations of the affinity the desired protein has for the immobilized ligand, are often useful in designing effective conditions for the loading of protein onto, or the elution of protein from, an affinity column.

TYPES OF SUPPORT MATERIAL

Given that a suitable ligand can be found to be immobilized, a number of considerations go into the selection of the support material. The ideal support material for use in affinity chromatography has a variety of physical and chemical characteristics that give it optimal behavior. In terms of its physical properties it should have a high porosity, to allow maximum access of a wide range of macromolecules

to the immobilized ligand. It should be of uniform size and rigidity to allow for good flow characteristics, and it must be mechanically and chemically stable to conditions used to immobilize the appropriate specific ligand. In terms of its chemical properties, it should have available a large number of groups that can be derivatized with the specific ligand, and it should not interact with proteins in general so that nonspecific adsorption effects are minimized.

A diverse variety of insoluble support materials have been used, including cellulose, polystyrene gels, cross-linked dextrans, polyacrylamide gels, and porous silicas, but by far the most popular support materials in use are beaded derivatives of agarose. The physical and chemical characteristics of agarose were described in Chap. 2 and are not repeated here, but in many ways agarose derivatives fulfill the characteristics required of the ideal matrix support.

Although agarose derivatives approach the ideal, a common problem with the support material (one that is not restricted to agarose) is steric hindrance. Once the ligand has been immobilized on the support, simple steric hindrance may prevent the interaction of proteins having binding sites for the ligand with the immobilized ligand. To circumvent this problem, a "spacer arm" is usually included in the support material to allow the ligand to be extended away from the physical surface of the support and permit unhindered interaction of protein with the ligand (although we are considering the spacer arm as part of the support, for practical purposes it is usually incorporated into the ligand prior to immobilization onto the support). In principle, such a spacer arm readily overcomes problems of steric effects. In practice, its inclusion *can* lead to a major problem in affinity chromatography—that of nonspecific interaction of proteins with the support material. The most commonly used spacer arms consist of 6 to 10 carbon atoms. However, these have quite a hydrophobic nature that they impart to the immobilized ligand, which can lead to nonspecific binding. Two approaches have been used to overcome this problem. In one, spacer arms with a hydrophobic nature are employed, but the buffer systems contain organic solvents such as *N*,*N*-dimethylformamide in low concentrations to help prevent these nonspecific interactions with the spacer-arm ligand system. In the other approach, spacer arms with hydrophilic natures are used. In particular 1,3-diaminopropanol, which can be coupled to the matrix by standard methods, has proven useful. Not only does this derivative (see Fig. 3-1) have a hydrophilic nature, but it is also easily lengthened or derivatized, as required.

A rather more pragmatic approach to this problem of nonspecific adsorption to the affinity matrix is to worry about the consequences during the elution phase of the purification rather than try to block the nonspecific adsorption during loading. This is dealt with later in the section on elution procedures.

Commonly Used Methods for the Immobilization of Ligands

The majority of matrices used in affinity chromatography are either agarose based, which have attendant hydroxyl groups that can be used in derivatization, or polyacrylamide based, which have reactive amide nitrogens available.

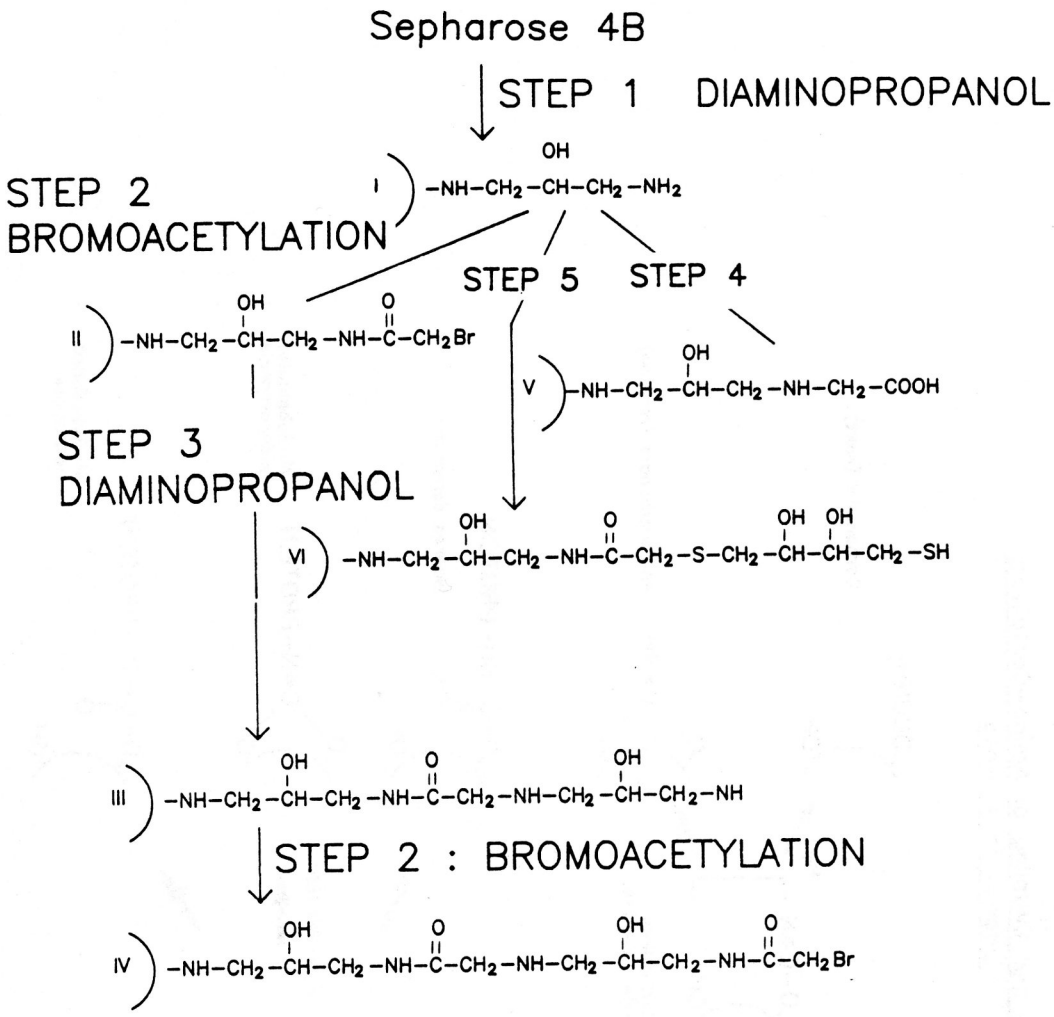
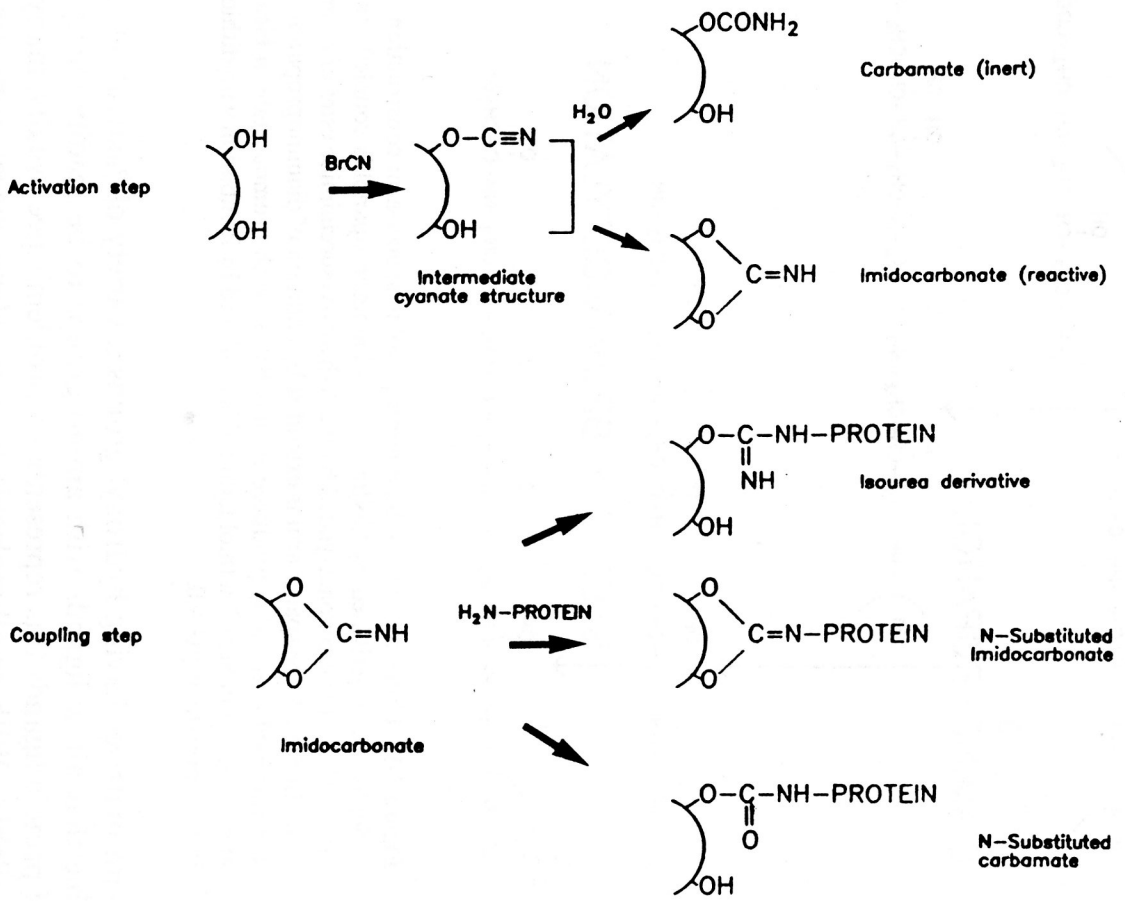


Figure 3-1 Utilization of 1,3-diaminopropanol in spacer-arm construction. In Step 1, Sepharose is activated by CNBr and 1,3-diaminopropanol is coupled. In Step 2, intermediate 1 is bromoacetylated by the *N*-hydroxysuccinimide ester of bromoacetic acid. In Step 3, the spacer arm is extended by addition of diaminopropanol. In Step 4, a carboxyl terminal is produced by incubation with bromoacetate in bicarbonate at pH 9.0, and in Step 5, a thiol terminal is produced by incubation with dithiothreitol in bicarbonate at pH 9.0.

With matrices having hydroxyl groups a variety of "activation" procedures are available that allow ligands with amino groups to be readily incorporated. In the case of protein ligands this represents no problem since usually, many amino groups are available. With small-molecule ligands, where spacer arms are usually incorporated into the ligand prior to immobilization, the spacer arm terminates in an amino group which is then used in the immobilization. The most commonly used activation procedures are (1) cyanogen bromide activation, (2) epoxide activation, (3) periodate oxidation, and (4) triazine activation. Each of these procedures is outlined briefly in Fig. 3-2.

Several methods are available for the activation and coupling of ligands to acrylamide, and these are outlined in Fig. 3-3. During coupling, especially with

Activation and Coupling of Amino-Containing Ligands to Agarose



a) by cyanogen bromide

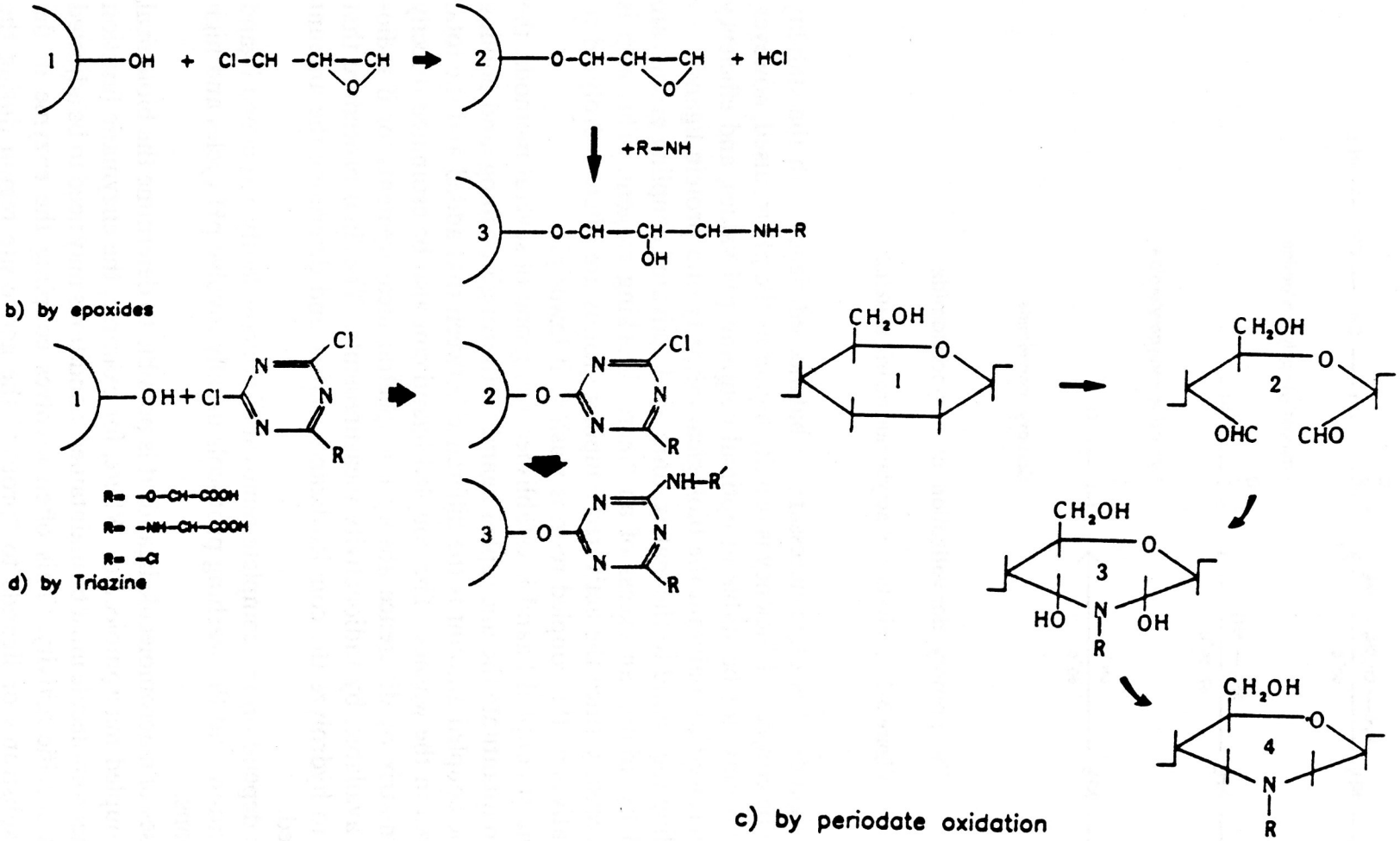


Figure 3-2 Activation and coupling of amino-containing ligands by (a) cyanogen bromide, (b) epoxides, (c) periodate oxidation, and (d) triazine.

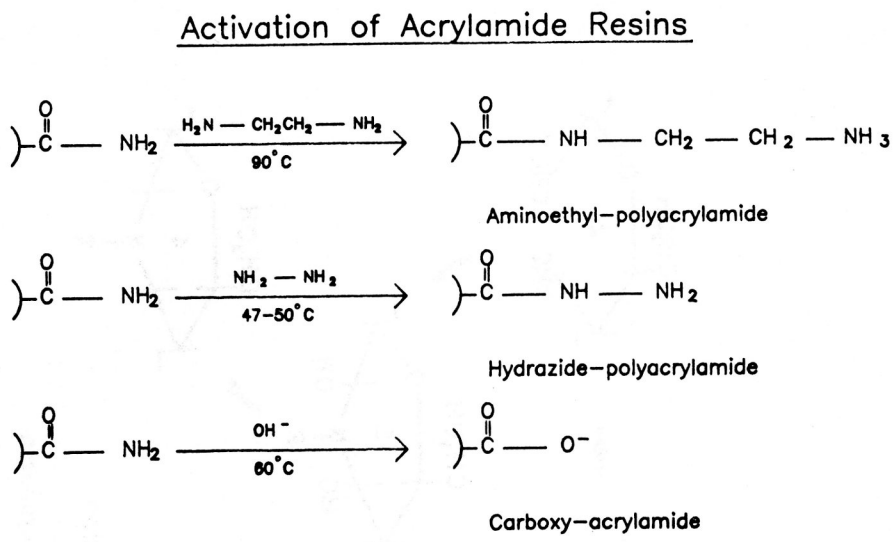


Figure 3-3 Activation of acrylamide matrix material.

macromolecule ligands, it is often necessary to balance efficiency with the stability of the protein to be coupled. Efficiency is usually higher as the pH is raised; however, the protein ligand may not be stable at optimal coupling pH values, and efficiency must then be sacrificed to maintain the biological integrity of a protein ligand.

After coupling the ligand to the matrix, unreacted activated coupling groups are usually blocked by adding an excess of an "inert" blocking reagent. This step is sometimes unnecessary since the activated coupling groups are often hydrolyzed to inactivate derivatives as the coupled resin is washed at lower pH.

The amount of coupled ligand is established using one of several methods, the simplest being to quantitate the uncoupled ligand by thorough washing (and pooling the washes). The coupled amount is the difference between that added and the total amount recovered in the washes. The coupled ligand can also be estimated directly on the washed matrix by difference absorption spectral measurements, or if radioactive ligand is available, by radioactivity measurements. The final procedure that may be used is to hydrolyze the coupled ligand–matrix and determine the amount of ligand released.

All of these depend on the complete removal of noncovalently associated ligand prior to quantitation, and the washing protocols usually involve pH cycles and high salt concentrations.

In some cases of macromolecule ligands it is possible to determine the biological activity of the coupled macromolecule. Where, for example, the enzymatic function of the coupled macromolecule must be maintained, conditions may need to be adjusted to maximize its specific activity. This often involves coupling the enzyme in the presence of its substrates or ligands to "protect" the active-site region during the coupling process. It is usually possible to use an immobilized enzyme in assays to determine its specific activity.

SELECTION OF APPROPRIATE LIGANDS AND CONDITIONS

There are three categories into which "ligands" used in affinity chromatography can be placed. The most obvious is where the ligand is a small molecule with affinity for a specific protein. In such a case it may be either a substrate (or substrate analog) or a regulatory ligand. The second category is where the ligand is a macromolecule that has a particular affinity for a specific protein. It may be a molecule with very specific affinity for the desired protein (such as an antibody) or may have specificity toward a class of molecules (e.g., concanavalin A) into which the desired molecule falls. The final category is covalent affinity chromatography, where the ligand contains a moiety that reacts covalently but reversibly with the wanted protein. This category will be dealt with later.

Small-Molecule Ligands

Many proteins have small-molecule ligands which may make suitable affinity chromatography ligands. Apart from the consideration of the dissociation constant the protein has for the ligand, several others go into the selection of the appropriate ligand. An important corollary to the dissociation constant is the requirement that the linkage process not adversely affect the affinity of the ligand for its target protein. The simplest way to check this is to use the spacer-arm ligand derivative (either before or after immobilization) as a competitive inhibitor of the normal ligand. If a simple kinetic or ligand binding assay is available, this gives an easy way to screen not only the affinity the immobilized ligand has for the protein, but also conditions of buffer, pH, temperature, and so on, that give optimal binding. Often, inhibition studies using underivatized analogs can give much useful information regarding the contribution certain parts of the potential ligand play in binding, thus allowing an informed choice to be made regarding which part of the ligand to modify by incorporation of the spacer arm.

A common problem with small-molecule ligands is that the ligand, although it may bind tightly to the wanted protein, has affinity for other proteins that recognize the same ligand. Consider, for example, the use of immobilized NAD in the purification of a dehydrogenase. Many dehydrogenases, and a number of other proteins, bind NAD with similar affinity and are retarded by such an affinity matrix. The solution to this dilemma has two aspects: (1) one must establish conditions such as pH, ionic strength, and temperature where binding to the wanted protein is optimal compared to binding to other proteins with affinity for NAD, and (2) if possible, a coligand that specifically enhances the affinity of the wanted protein for the matrix should be used in the buffer; frequently, a substrate analog accomplishes this through the formation of a ternary complex, which is of course far more specific for the wanted protein and leads to increased affinity relative to other proteins that may bind the ligand.

From the earlier equations for V'_e/V_e it should be apparent that anything that can be done to increase the affinity of the wanted protein relative to the affinities of potential contaminating proteins increases the retardation of the wanted protein on

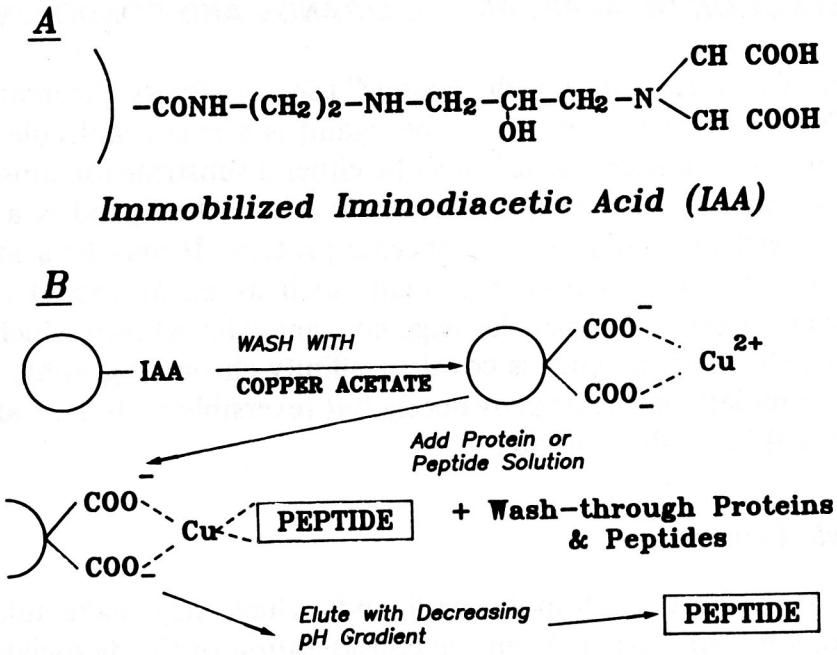


Figure 3-4 Use of metal affinity ligands in peptide and protein purification: (A) structure of iminodiacetic acid (IAA); (B) scheme for use in purification procedures.

the matrix. In situations where a variety of proteins bind to the matrix ligand but do so with quite different affinities, specificity of purification can be achieved during the elution phase, as described in the next section.

A unique approach to immobilizing a small-molecule ligand has been introduced where the small molecule is a metal ion. By immobilization of a chelating agent such as iminodiacetic acid, a variety of different metal ions can be chelated and used as affinity ligands for metal binding proteins. Such affinity resins have also found use in the purification of peptides that contain certain amino acids whose side chains show affinity for particular metal ions. This application is outlined in Fig. 3-4.

Macromolecule Ligands

Macromolecular ligands that may be of use in affinity chromatography can be divided into three categories, all of which have found significant use in protein purifications. In the first category are proteins that have specific affinity (and often very high affinity) for moieties which may be covalently attached to the wanted protein. Most frequently, this is useful in the purification of glycoproteins, where a wide range of plant lectins are available with differing specificities in regard to the sugars that they recognize. Most plant lectins that have been characterized with regard to their sugar specificity have also been immobilized and used to purify glycoprotein derivatives. A number of these are listed in Table 3-1 together with their specificities. Of course, the major problem with this approach is that any protein with the appropriate sugar covalently attached binds to the immobilized lectin, although this can partially be overcome during the elution phase. Lectin affinity

TABLE 3-1 Lectin affinity chromatography

Lectin	Specificity (competing sugar)
Concanavalin A	Complex (α-methyl mannoside)
Dolichos biflorus	*N*-Acetylgalactosamine
Soybean	*N*-Acetylgalactosamine
Helix pomatia	Blood group A (NAGal)
Limulus	Galactose
Garden pea	Glucose, mannose
Winged pea	Fucose
Wheat germ	*N*-Acetylglucosamine

chromatography is widely used in both general glycoprotein isolation and fractionation, and in purification schemes for specific enzymes that have a glycoprotein nature.

Although many lectins do not have well-characterized specificities for ligands of related structure, much work has been done on the specificity of concanavalin A, where carbohydrate ligands of related structure show quite a range of affinity for this lectin. As a result, concanavalin A–Sepharose can be used to give information on the type of carbohydrate chain recognized. Into this category also fall proteins such as avidin, which is found covalently linked to many biotin-requiring enzymes.

Although they do not have affinity for a small molecule conjugated to the macromolecule as in the previous instances, affinity ligands such as immobilized staphylococcal protein A must also be included in this category. Protein A binds the Fc portion of the IgG molecule with high affinity and has been used as an affinity ligand for the purification of IgG molecules and is also useful in "sandwich" affinity chromatography—the isolation of IgG–protein complexes.

The second category involves the use of immobilized antibodies as the ligand. Because of the precise specificity of many antibodies for their particular antigen, an immobilized antibody makes the perfect affinity ligand—it recognizes only the wanted protein. There are, of course, instances where polyclonal antibodies to a particular protein cross-react with other proteins having similar or related structural features (antibody to, for example, lactate dehydrogenase often reacts weakly with other dehydrogenases). The advent and ease of preparation of monoclonal antibodies, however, have made it easier to find a suitable antibody for affinity chromatography purposes.

The availability of monoclonal antibodies allows for some quite interesting applications of affinity chromatography. For example, anti-AMP antibodies have been used in the separation of partially adenylylated forms of glutamine synthase. Antibody is immobilized, and adhered glutamine synthase (which is adenylylated) is eluted using AMP gradients. Different monoclonals have been used, allowing the separation of glutamine synthase with fewer than three adenylated subunits per dodecamer from those with higher amounts of adenylylation.

The third category includes any protein that has a unique affinity for another protein and can thus be used as an affinity ligand, provided that the affinity is high

enough for that protein. A particularly interesting example of this type of macromolecule ligand is α-lactalbumin. This is a regulatory protein for galactosyl transferase, but interacts only weakly in the absence of coligands. However, when carbohydrate ligands such as *N*-acetylglucosamine are present, α-lactalbumin interacts strongly with the enzyme and has proved to be a most effective affinity ligand in its purification. Interestingly, galactosyl transferases from a wide variety of sources can be purified using immobilized α-lactalbumin, even though they do not physiologically interact with this protein. Similarly, calmodulin has been used to purify calmodulin-binding proteins.

Finally, we consider an application of affinity chromatography recently developed that illustrates some of the potential for affinity resins using macromolecule ligands. Anhydrotrypsin is a catalytically inert derivative of trypsin containing dehydroalanine in place of the active-site serine residue. This derivative has been shown to have an approximately 20-fold higher affinity than substrate peptides for product peptides produced by tryptic cleavage, and immobilized anhydrotrypsin has been used as an affinity ligand for the purification of tryptic peptides. COOH-terminal arginine peptides adhere more tightly than COOH-terminal lysine peptides. Further specificity may be obtained during elution, which usually involves decreasing pH gradients.

Dye Molecule Ligands

Several commercially available "dye–ligand" affinity chromatography media have been used in a wide variety of protein purifications. Some of these, such as Cibacron-blue or Procion-blue, have been shown to have affinity for enzymes requiring adenylyl-containing cofactors. In general, with this type of "nonspecific" affinity ligand, the specificity is provided by the adsorption and elution conditions employed in a particular purification, and these must often be worked out by trial and error.

ELUTION PROCEDURES

As has been indicated in a number of places, the elution phase of affinity chromatography can be used to give enhanced specificity in a purification. First, however, we must consider the physical basis for elution. Except in the case of covalent affinity chromatography, the interaction of the protein with the matrix ligand is reversible, and it is this interaction that retards the passage of the specifically adsorbed protein. If enough of the buffer used to load the column were washed through, the protein would eventually elute from the matrix and its position of elution could be estimated from the expression for V'_e/V_e given earlier. Except for cases where the protein does not interact strongly with the matrix and V'_e/V_e is a fairly low number (hence elution occurs in a reasonable volume), it is usually necessary, once contaminating proteins have been washed through, to assist elution of the wanted protein. This course involves taking measures that *increase* the *dissociation constant* for the matrix–

ligand–protein interaction. In general, there are two ways in which this is achieved. Any change in conditions such as buffer pH, ionic strength, or temperature that weakens the affinity of the matrix for the protein assists the elution process. In some instances where the protein has a very high affinity for the matrix–ligand and can be reversibly denatured, mild denaturing conditions can be used. For the elution of carbohydrate-containing molecules from immobilized lectins such as concanavalin A, quite harsh denaturing conditions are sometimes necessary. This represents a real problem for the use of those affinity ligands in the purification of proteins that may not withstand such harsh elution conditions. These nonspecific approaches cannot be used where the purification may depend on specificity during elution.

The addition of a competing ligand to the elution buffer represents a far more controllable approach. In cases where a small-molecule ligand is used on the matrix, it is possible to elute by inclusion of the free ligand in the elution buffer. If the dissociation constant K_1 for the immobilized ligand and the dissociation constant K_f for the free, competing ligand are known, the amount of free, competing ligand that must be included in the elution buffer to achieve rapid elution can be calculated. Using a concentration of free competing ligand to give $>90\%$ of the protein bound to the free ligand gives rapid elution of the desired protein in a small volume. Lower amounts of competing ligand still increase the elution rate but give more dilute protein. It is, of course, not necessary that the competing ligand be an analog of the matrix ligand, although this is often the case. It is sufficient simply that the added ligand "compete" with the matrix ligand. For example, leucine effectively elutes glutamate dehydrogenase from a GTP-Sepharose affinity column by binding to a separate regulatory site on the protein and blocking, via a conformational change, the binding of GTP.

The use of a competing ligand to achieve elution provides a way of increasing the specificity of purification in cases where more than one protein may interact with the column via affinity for the matrix ligand. Provided that the various proteins have a different affinity for the matrix (either naturally or through the inclusion of a coligand), they can be eluted separately from the column by using a concentration gradient of the competing ligand. The example of glutamate dehydrogenase elution from GTP-Sepharose by leucine provides the basis of a second method of increasing specificity during elution. Provided that a competing ligand which is *not* a structural analog of the matrix–ligand is available, specific elution is possible even when more than one type of protein has specifically interacted with the matrix.

We have discussed using a competing ligand for cases where the matrix–ligand is a small molecule. However, provided that a small molecule competitor of a protein–protein interaction is available, these considerations also apply to affinity chromatography using macromolecular ligands. Two examples illustrate this approach. Galactose is an excellent competitor of the interaction between immobilized peanut agglutinin (PNA) and glycoprotein ligands containing terminal galactose. This is to be expected since the interaction is mediated by a "small" ligand—the carbohydrate chain of the glycoprotein. A more interesting example is provided by protein A–IgG interaction, where it has been found from the crystal structure of the

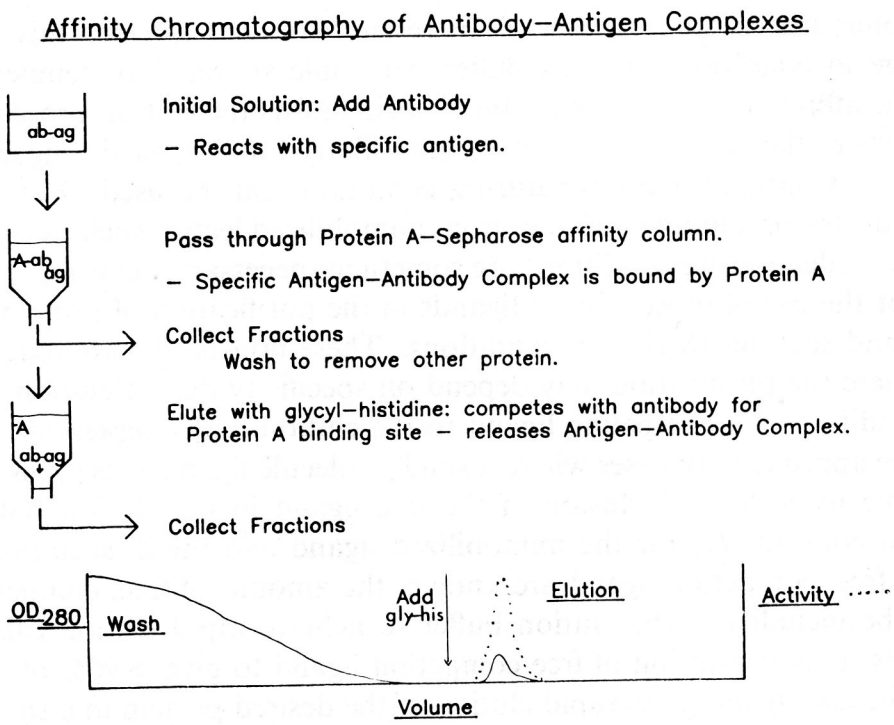


Figure 3-5 Use of proteinA–Sepharose in the isolation of antigen-antibody complexes.

complex that certain amino acid residues in each protein are involved in the interaction. As a result a variety of dipeptides, such as glycyltyrosine, glycylhistidine, and glycylphenylalanine, have been used as quite effective competing ligands for the protein–protein interaction that occurs with protein A–IgG. In this particular instance, and in other instances where such protein–protein interactions are mediated by hydrophobic bonds, reagents such as ethylene glycol, which disrupts hydrophobic interactions, have been used as nonspecific (but quite effective) elution procedures.

The use of protein A–Sepharose to isolate specific antigen–antibody complexes and the use of glycylhistidine to elute the complex is outlined in Fig. 3-5. In cases such as using α-lactalbumin–Sepharose to purify galactosyltransferase, where a coligand is required for affinity, elution can be achieved simply by omitting the coligand from the buffer.

SOME EXAMPLES OF AFFINITY CHROMATOGRAPHY PURIFICATIONS

Glutamate Dehydrogenase

Although glutamate dehydrogenase can be purified to homogeneity by conventional means, the use of affinity chromatography on a GTP-Sepharose matrix increases the yield, cuts down on the number of steps, and results in a more rapid and efficient purification. After initial ammonium sulfate fractionation and gel filtra-

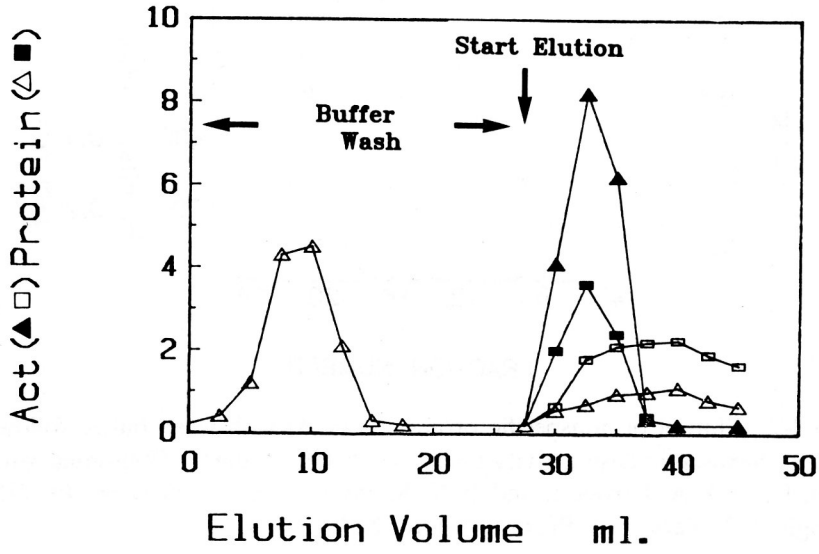


Figure 3-6 Typical elution profiles obtained during the purification of glutamate dehydrogenase with GTP-Sepharose: △, protein eluted during buffer wash or salt elution; ■, protein eluted by 5 m*M* leucine wash; □, activity eluted by salt wash; ▲, activity eluted by 5 mM leucine wash.

tion chromatography, active fractions are pooled, dialyzed against a 20 m*M* phosphate buffer at pH 7.0, and passed through a GTP-Sepharose column (5 ml bed volume). Nonspecifically retarded protein is washed through the column with starting buffer containing 50 m*M* sodium chloride (10 column volumes). Elution can be achieved in one of two ways: The pH of the buffer is raised to 8.0 and the NaCl concentration is raised to 100 m*M*, or leucine (5 m*M*) is included in the wash buffer. Figure 3-6 illustrates the different elution profiles obtained by these procedures and demonstrates the utility of the use of a competing ligand (in this case leucine) to achieve rapid elution.

Hexosaminidase

An affinity matrix suitable for the isolation of β-*N*-acetylhexosaminidase can be prepared by coupling asialofetuin to Sepharose 4B. This matrix has been used to enrich β-*N*-acetylglucosaminidase activity from rabbit sperm cytoplasmic droplets in a very simple procedure. The droplets, isolated by differential centrifugation, are freeze-dried, redissolved in buffer by sonication, dialyzed against 50 m*M* sodium acetate buffer (pH 4.5), and loaded onto the asialofetuin-Sepharose matrix. Figure 3-7 shows an elution profile of nonspecific protein washed through by the starting buffer, and a sharp elution of activity and its associated protein, using a gradient of NaCl from 0 to 500 m*M*.

Separation of Dehydrogenases

As discussed earlier, a problem often encountered in affinity chromatography is the resolution of a group of proteins all of which have affinity for the matrix ligand.

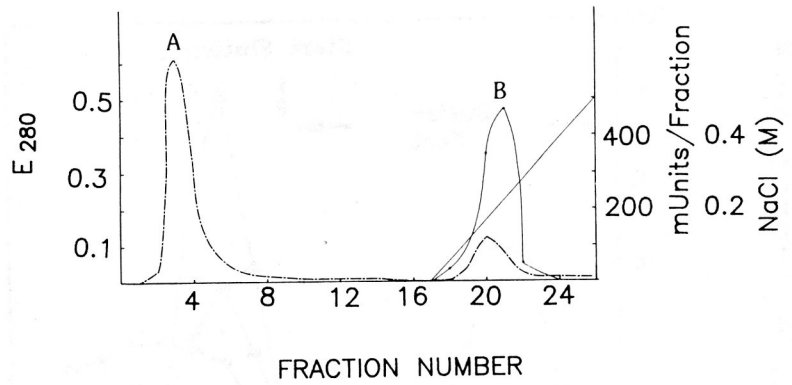


Figure 3-7 Elution of nonspecific protein (peak A) with wash buffer, followed by elution of hexosaminidase activity (peak B) by salt gradient. (Reprinted with permission from: A. A. Farooqui and P. N. Srivastava, *Int. J. Biochem.*, *10*, 745–748. Copyright 1979 Pergamon Press, Elmsford, N. Y.)

Figure 3-8 illustrates the separation of a mixture of BSA, malate dehydrogenase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase (H_4 isozyme), and yeast alcohol dehydrogenase using 5′-AMP–Sepharose (with an aminohexyl spacer arm). The mixture was applied in 10 m*M* phosphate, pH 6.0, and the column washed with this starting buffer. The BSA, which has no affinity for AMP, elutes immediately. The dehydrogenases are eluted from the matrix using a pH gradient as shown. Glucose-6-phosphate dehydrogenase elutes at low pH, yeast alcohol dehydrogenase at intermediate pH, and both malate dehydrogenase and lactate dehydrogenase at

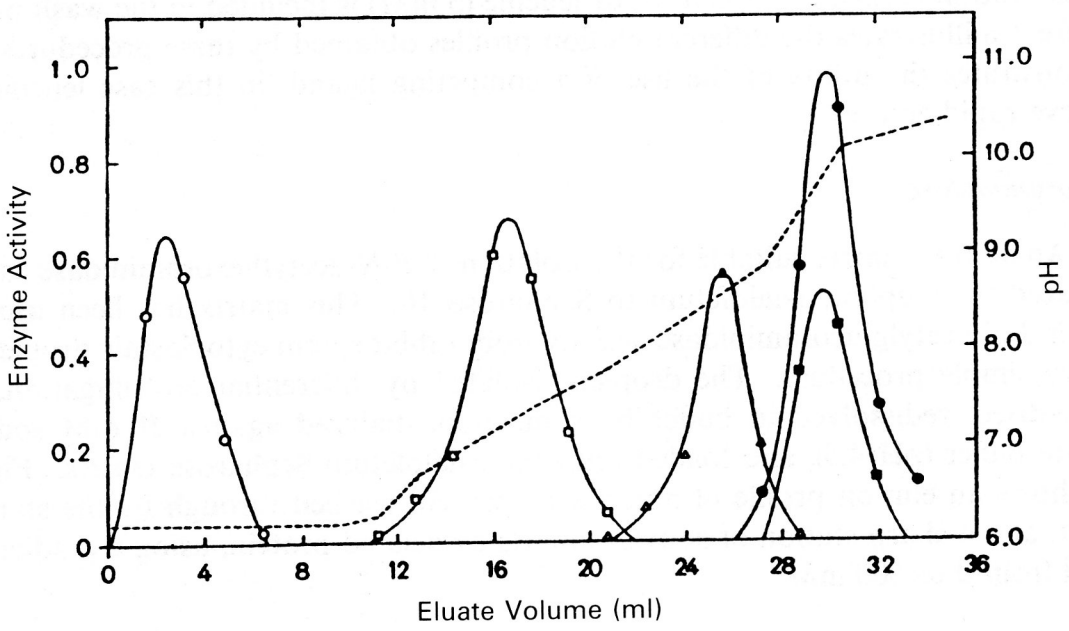


Figure 3-8 Differential elution of proteins adhering to AMP-Sepharose by use of a pH gradient: ○, BSA; ●, malate dehydrogenase; □, glucose-6-phosphate dehydrogenase; △, yeast alcohol dehydrogenase. [Reprinted with permission from: C. R. Lowe, M. J. Harvey, and P. D. G. Dean, *Eur. J. Biochem.*, *41*, 347–351 (1974).]

high pH. This example clearly shows the utility of gradient elution of affinity matrices to separate proteins with similar specificities but differing affinity characteristics.

COVALENT AFFINITY CHROMATOGRAPHY

Covalent affinity chromatography represents an aspect of affinity chromatography which has limited use in the purification of proteins but tremendous potential in the purification of peptides that might contain unique amino acids. The principle is simple: The matrix contains a reactive group that derivatizes reversibly a particular amino acid—any protein or peptide containing that particular amino acid reacts chemically with the matrix and is immobilized. Nonreactive peptides or proteins can be completely washed from the matrix without fear of eluting the immobilized material. Afterward, the immobilized material is eluted by inclusion in the buffer of a reagent that reverses the chemical modification. This idea was originally introduced using *p*-chloromercuribenzoate coupled to aminoethyl agarose, which reacts well with proteins having exposed, reactive sulfhydryl groups, and has been used in a variety of affinity separations, including the separation of calf thymus histone F_3 (which contains a cysteine) from other histones (lacking cysteine), and the fractionation of enzymatically active papain (intact sulfhydryl) from inactive papain (no intact sulfhydryl). In each case the reactive sulfhydryl containing material is eluted from the matrix by inclusion of β-mercaptoethanol or cysteine in the buffer.

Arylsulfenyl chlorides (specific chemical modification reagents for tryptophan) have been immobilized to cross-linked polyacrylamide and used to separate tryptophan containing peptides from peptides lacking tryptophan. The immobilized sulfenyl chloride reacts with the tryptophan side chain in the peptide as shown in Fig. 3-9,

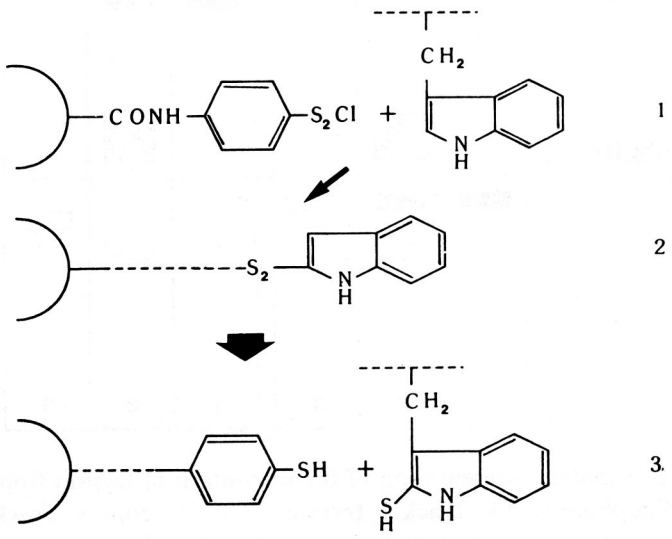


Figure 3-9 Reaction of tryptophan-containing peptides with immobilized arylsulfenyl chlorides and the release of peptide by thiol reagents.

and after elution of noncovalently immobilized peptides, the tryptophan-containing peptides are eluted by inclusion of a thiol in the buffer.

Although few such covalent affinity procedures have been detailed, the potential of immobilizing chemical modification reagents and using them for the purification of specific peptides containing unique amino acids remains.

AFFINOPHORESIS

The technique of affinophoresis is a recently introduced approach for the specific separation of macromolecules that makes use of some of the principles of affinity chromatography but uses as its separation method electrophoretic mobility. In this technique the specific affinity of a macromolecule is used to ligand to the macromolecule a polyelectrolyte containing an affinity ligand. The result of this specific interaction is that the target protein now carries increased charge and resultant increased electrophoretic mobility, allowing it to be readily identified and separated by electrophoretic methods. Cationic or anionic polyelectrolytes can be used and are linked to the affinity ligand in much the same way as a ligand would be immobilized on an insoluble matrix. Cationic polyelectrolytes are usually dextran derivatives with diethylaminoethyl groups as the cations, while polyacrylyl-β-alanyl carriers with sulfonate groups have been used as anions. As outlined in Fig. 3-10, affinophoresis has been successfully used to separate trypsin from "pronase" (which is a mixture of proteases, including trypsin) using a *p*-aminobenzamidine affinity ligand for trypsin on an anionic polyelectrolyte. In the absence of the affinophore (part A), trypsin, pronase (multicomponent), and the active-site-blocked TLCK-

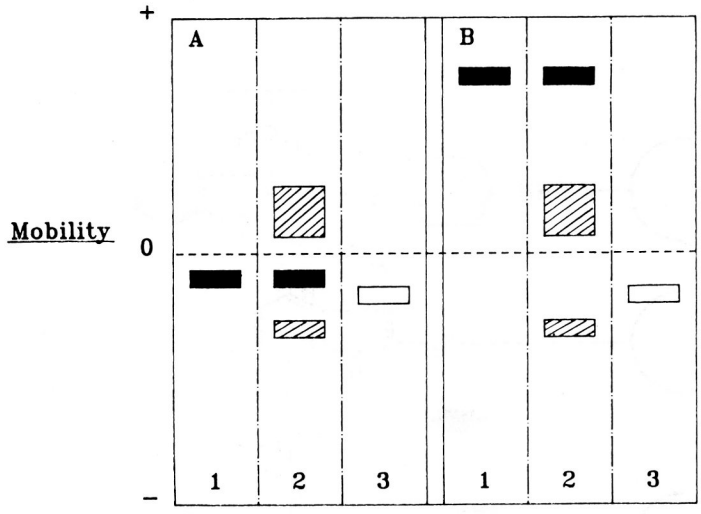


Figure 3-10 Schematic representation of the separation of trypsin from a pronase mixture by affinophoresis. (A) Track 1, trypsin; Track 2, pronase; Track 3, TLCK-trypsin. (B) Same as in part (A), but the electrophoresis is in the presence of an anionic affinophore. [Adapted from: K. Shimura and K.-I. Kasai, *Biochem. Biophys. Acta*, *802*, 135–140 (1984).]

trypsin show similar electrophoretic mobilities. In the presence of the affinophore (part B), trypsin and the trypsin component of pronase show greatly increased mobility, while the remaining pronase components and TLCK-trypsin (which cannot bind the affinophore) show unaltered mobility.

The various affinity techniques that we have discussed in this chapter are usually adapted to column chromatographic approaches. Where an extremely specific ligand is available, they are ideally suited to batch procedures. Affinity techniques are almost always used with some form of prior fractionation, usually because the sheer amount of protein obtained in initial homogenization or extraction is too large to employ the generally small bed volumes of affinity resins. This can lead to problems with nonspecific protein interactions or to premature elution of the retarded protein as a result of the large volume of material initially loaded onto the resin.

Affinity techniques are, however, of major use in many protein purifications and are now adapted to HPLC techniques for both analytical and preparative purposes. Because the elution phase can be closely controlled, affinity resins also provide a convenient means of concentrating dilute protein solutions.