Functional Purification of Proteins and Peptides by Affinity Chromatography

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A number of agarose bead derivatives useful in the purification of proteins by "affinity chromatography" are described. A variety of functional groups have been attached to these insoluble polymers: primary aliphatic and aromatic amines; carboxylic groups; bromoacetyl alkylating groups; reactive diazonium groups; tyrosyl peptides; and free sulfhydryl groups. These derivatives permit: the attachment of ligands to the gel through extended hydrocarbon chains which place the ligand at varying distances from the gel matrix backbone; the covalent attachment of ligands to agarose or polyacrylamide gels by a wide variety of techniques, including through amino, carboxyl, phenolic, or imidazole groups of the ligand; and the preparation of adsorbents containing ligands attached by bonds which are susceptible to specific chemical cleavage, thus providing means of removing the intact protein-ligand complex from the affinity adsorbent. Successful application of affinity chromatography in many cases will critically depend on placing the ligand at a considerable distance from the matrix backbone.

Insoluble adsorbents having selective biological specificity have recently been demonstrated to be powerful tools for the purification of a large variety of macromolecules (Cuatrecasas, 1971a; Cuatrecasas and Anfinsen, 1971; Cuatrecasas *et al.*, 1968). This method of purification, referred to as affinity chromatography, is based on the selective and reversible dissociation generally observed between macromolecules and specific small molecular weight ligands. Although the ideal case is that of an enzyme and a highly specific competitive inhibitor, the principles and methods are clearly applicable to virtually all interacting systems constituted of two or more components which are not restricted by size or complexity. For example, specific protein–protein, protein–membrane, or ligand–cell interactions can be exploited for the purification of one of the components.

Perhaps the most distinguishing feature of this general method of purification which contrasts it with the classical or conventional purification procedures is its fundamental dependence on the biological or functional rather than the physicochemical properties of the interacting species. Hence, one has the potential applicability of the method to a wide variety of biochemical systems and the ease with which dramatic results are possible in many cases. However, for the same reasons, the specific methodological details must be highly individualized and carefully tailored to reflect the

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specific biological properties of the particular interacting system under study.

It is therefore not the basic principle as much as the procedural execution which will determine the success of the functional purification method in any given case. The basic principles are indeed refreshingly simple and self-apparent. A selective adsorbent is prepared by properly attaching one of the components of the interacting system, *i.e.*, a specific ligand, to a carefully selected porous insoluble polymer or gel. The extract containing the component to be purified, *i.e.*, an enzyme, is passed through a column containing this adsorbent. The affinity of the enzyme for the ligand results in its binding to the column support while the remaining proteins in the mixture pass directly through with the eluate. Removal of the bound enzyme is accomplished with substrate containing buffers, by altering the pH or ionic strength of the buffer, or by adding protein denaturants to the buffer. This type of purification is related in principle to the use of immunoadsorbents, which have been used extensively in immunochemical studies (Cuatrecasas, 1971a; Silman and Katachalski, 1966). The application to enzyme–ligand systems and to other interacting systems of relatively low affinity poses many problems not encountered in immunochemical purifications; this probably in part explains the slow progress which, until recently, has been observed in the area of purification of enzymes (Cuatrecasas, 1971a).

A few examples of enzyme purification by selective adsorption procedures have appeared in the literature sporadically over the past 20 years (Lerman, 1953; Arsenis and McCor-

mick, 1964, 1966), but these have been generally considered merely as interesting unique cases. Attempts have been made in the last few years to generalize the approaches and to provide methodological procedures that can be readily adapted to a diverse group of protein–ligand systems (Cuatrecasas, 1970, 1971a,b; Cuatrecasas and Anfinsen, 1971). Techniques are not available to permit a systematic approach to the purification of virtually any enzyme by affinity chromatography. In addition, specific adsorbents may be prepared to purify antibodies, antigens, nucleic acids, vitamin binding proteins, transport proteins, repressor proteins, drug or hormone receptors, sulfhydryl group containing proteins, peptides formed by organic synthesis, and intact cell populations (Cuatrecasas, 1971a). Affinity chromatography may also be useful in concentrating dilute solutions of proteins, in removing denatured forms of a purified protein, and in separating and resolving protein components resulting from specific chemical modifications of purified proteins. Water insoluble adsorbents are also useful as primers for the synthesis of specific nucleic acids (Jovin and Kornberg, 1968; Sander et al., 1966; Gilham and Robinson, 1964; Erhan et al., 1965; Cozzareli et al., 1967).

Successful application of affinity chromatography depends basically on the proper: (a) selection of a water insoluble carrier and ligand; (b) attachment of the ligand to the matrix in such a way that it retains sufficiently strong, specific interaction with the protein; and (c) selection of experimental conditions to adsorb and elute the enzyme in a concentrated and undenatured form. Considerations of (c) are more difficult to generalize and systematize since these should reflect the specific peculiarities and properties of the particular system studied. Most of the comments in the following discussion will therefore focus on the selection of an insoluble support and on the manner in which ligands can be covalently attached to it.

An ideal insoluble carrier should display minimal nonspecific adsorption of proteins, exhibit good flow properties when packed in a column, be chemically and mechanically stable to conditions of ligand coupling and protein adsorption and elution, possess abundant chemically modifiable groups for linking of ligands, and form a loose porous network which permits uniform and unimpaired entry and exit of large macromolecules throughout the entire matrix (Cuatrecasas, 1971a; Cuatrecasas and Anfinsen, 1971; Cuatrecasas et al., 1968). A high degree of porosity is particularly important for protein–ligand systems of low affinity since it is necessary that all of the covalently attached ligands be freely accessible to the protein. Until recently virtually all water insoluble carriers have been derivatives of cellulose or polystyrene, and with few exceptions only enzymes or antigens have been the components covalently attached to these. Although useful in many cases, these derivatives lack many of the features enumerated above (Cuatrecasas, 1971a). Beaded derivatives of the polysaccharide polymer, agarose, have a number of desirable features (Cuatrecasas, 1971a; Cuatrecasas and Anfinsen, 1971), and in the past year they have been used extensively to prepare a number of effective adsorbents. It is not in the scope of the present discussion to enumerate all these examples, especially since recent adequate review articles are available (Cuatrecasas, 1971a; Cuatrecasas and Anfinsen, 1971). The problems related to the selection of an adequate ligand are described elsewhere (Cuatrecasas, 1971a; Cuatrecasas and Anfinsen, 1971).

A gentle general method is available for coupling compounds containing primary aliphatic or aromatic amines to
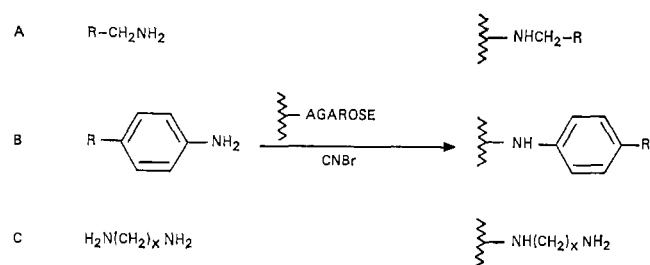

Figure 1. Coupling of ligands containing primary aliphatic amino groups (A), primary aromatic amino groups (B), and diamino aliphatic groups (C) to agarose by the cyanogen bromide procedure

insoluble carbohydrate derivatives (Cuatrecasas et al., 1968; Cuatrecasas, 1971a). Agarose is activated by reaction with cyanogen bromide at alkaline pH, washed, and coupled with protein or ligand at pH values between 6 to 10 (Figure 1). A large number of enzymes have now been purified by affinity chromatography with the use of adsorbents prepared in this way (Cuatrecasas, 1971a). The purification of chymotrypsin with D-tryptophan methyl ester derivatives of agarose is illustrated in Figure 2. The dissociation constant of this system is about 10^{-4} M. It is apparent that direct coupling of the inhibitor to the matrix backbone results in a relatively ineffective adsorbent (Figure 2B). When the inhibitor is placed at a longer distance, by virtue of an ϵ-aminocaproic acid extension, the enzyme binds to the column very tightly; the enzyme is eluted, in a very small volume, by passing through the column a buffer of low pH.

In contrast to this rather representative example (chymotrypsin), which is a relatively low affinity system, is the more unusual case of the biotin–avidin system which has a K_i of about 10^{-14} M (Figure 3). Avidin binds so strongly to biocytin–agarose columns that it is most difficult to recover the protein once it has adsorbed to the column. It is necessary to use extraordinary buffer conditions, 6 M guanidine.
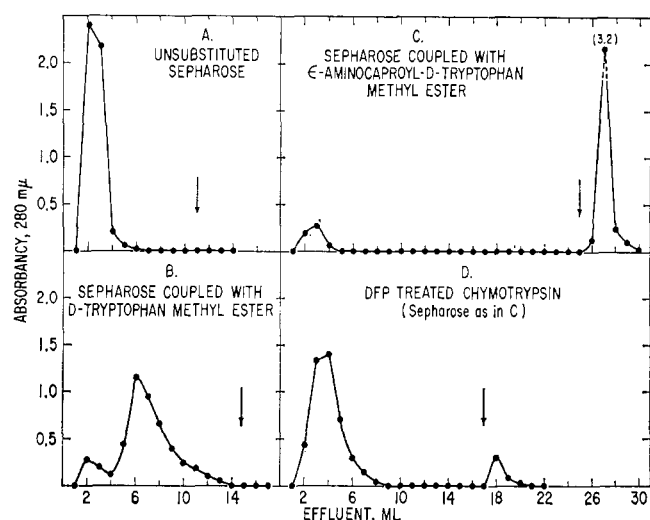

Figure 2. Affinity chromatography of α-chymotrypsin on inhibitor–Sepharose columns (2). The columns (0.5 × 5 cm) were equilibrated and run with 0.05 M Tris–Cl buffer, pH 8.0, and each sample (2.5 mg) was applied in 0.5 ml of the same buffer. One-milliliter fractions were collected in the flow rate was about 40 ml per hr, and the experiments were performed at room temperature. α-Chymotrypsin was eluted with 0.1 M acetic acid, pH 3.0 (arrows). Peaks preceding the arrows in B, C, and D were devoid of enzyme activity. [Reproduced with permission of: Proc. Nat. Acad. Sci. U.S.A. 61, 636 (1968).]
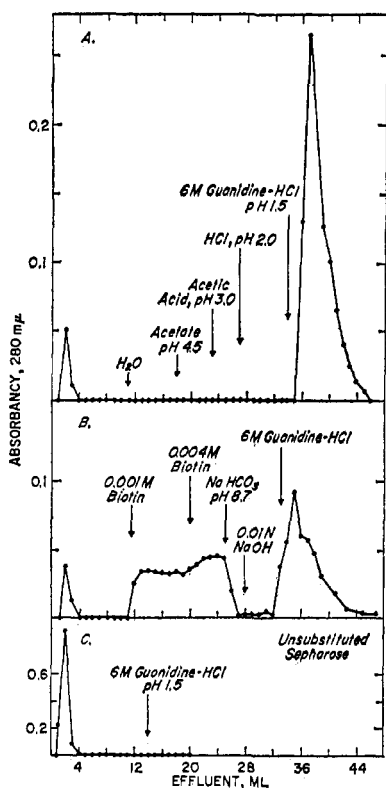
Figure 3. Affinity chromatography of purified avidin on biocytin–Sepharose (A,B) and unsubstituted Sepharose (C) columns (Cuatrecasas and Wilchek, 1968). The columns (0.5 × 5 cm) were equilibrated with 0.2 M NaHCO₃, pH 8.7, and 0.75 mg of avidin (in 0.5 ml of the same buffer) was applied to each column. One-milliliter fractions were collected, the flow rate was about 30 ml per hr, and the experiments were performed at room temperature. Elution was attempted by varying the conditions as indicated (arrows). The small protein peak that emerges early in A and B represents an impurity which does not bind biotin [14]C. [Reproduced with permission of: Biochem. Biophys. Res. Commun. 33, 235 (1968).]

Figure 4. Derivatizations of aminoalkyl agarose which can be used to attach ligands or proteins in the preparation of selective adsorbents for affinity chromatography (Cuatrecasas, 1970)
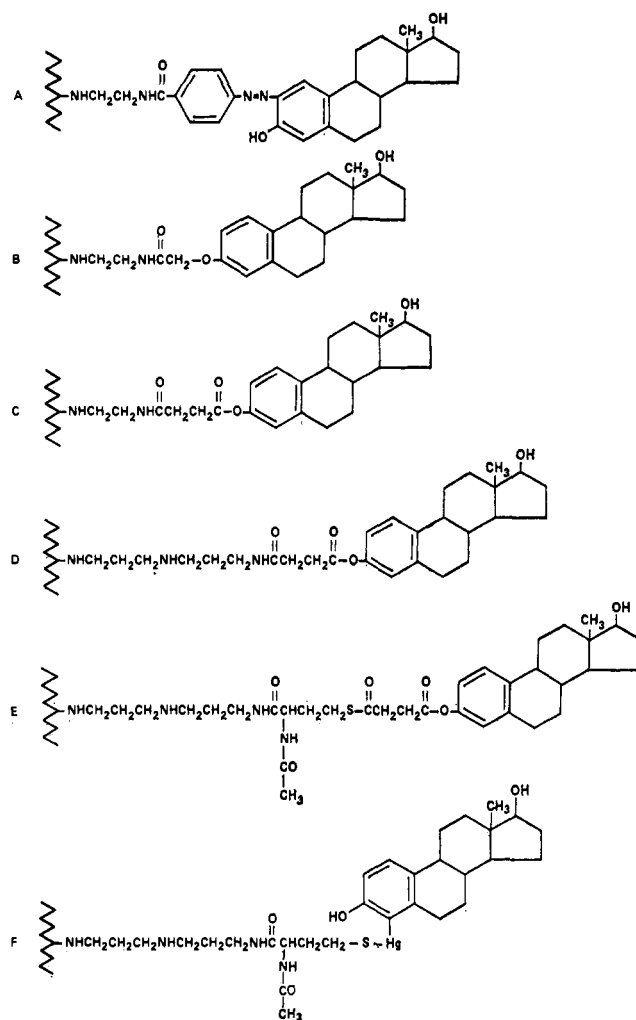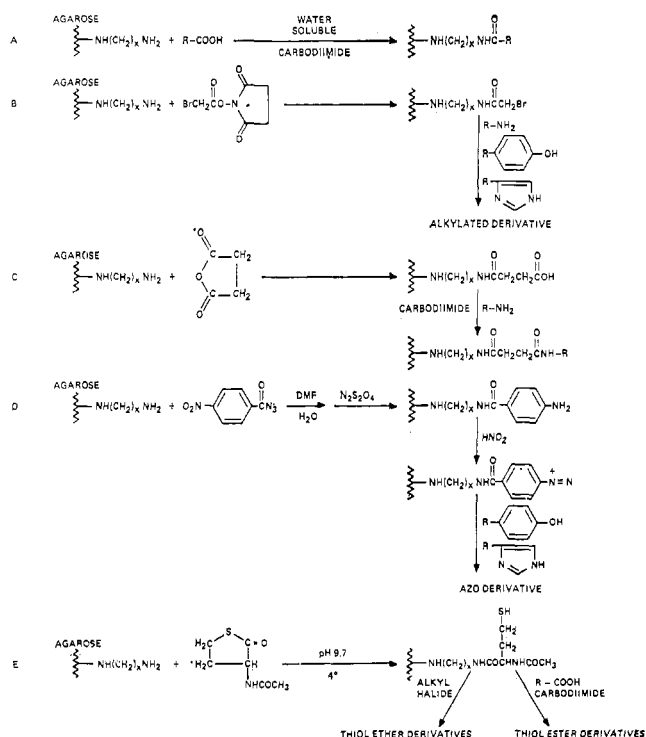

Figure 5. Various agarose derivatives of estradiol which are being used to purify the serum estradiol binding protein and uterine estradiol receptor proteins (Cuatrecasas and Puca, 1970)

HCl, pH 1.5. Luckily, the denaturation of this protein is reversible and it is possible to recover quantitatively the biotin-binding activity. In fact, pure avidin can be obtained from crude egg white in 70% yield in a single batchwise step using this adsorbent (Cuatrecasas and Wilchek, 1968). Many enzymes and biologically important proteins, however, cannot withstand such drastic conditions. It may therefore be important in certain cases to decrease the effective column affinity intentionally, for example, by attaching the ligand very close to the matrix backbone or through a functional group which contributes but is not essential to the binding interaction. Alternatively, the ligand can be attached to the support by chemical bonds which can later be selectively cleaved so as to remove the intact ligand–protein complex. Various means of achieving this will be described subsequently.

As suggested by the chymotrypsin example, it has become increasingly clear that successful purification by affinity chromatography will in many cases depend on placing the ligand sufficiently distant from the matrix backbone to minimize steric restrictions in the interaction between the ligand and the macromolecules to be purified (Cuatrecasas, 1970, 1971a). This is particularly important for protein–ligand complexes of relatively low affinity (K_i greater than 10^{-4} M), and for those involving multi-subunit or very large molecular weight proteins (Cuatrecasas, 1971a). As will be described,

this can be accomplished most easily by attaching the ligand to agarose derivatives to which long hydrocarbon extensions, or arms, have been linked.

Until recently (Cuatrecasas, 1970, 1971b) ligands and proteins have been attached to agarose only by the cyanogen bromide procedure described earlier (Figure 1A,B). A number of chemical derivatives of agarose which can be readily prepared under mild conditions extend the general usefulness and versatility of the methods (Cuatrecasas, 1970, 1971b). Specifically, these derivatives should permit the insolubilization of ligands lacking primary amino groups, the attachment of ligand through long arms, and the linking of ligands by chemical bonds susceptible to specific cleavage to permit selective removal of the intact ligand–protein complex. The preparation of these derivatives is principally based on the attachment of a diamino aliphatic compound of the general structure, $H_2N(CH_2)_xNH_2$, to agarose by the cyanogen bromide method (Figure 1C). An agarose derivative is thus formed which contains primary amino groups at some distance from the matrix backbone. The resulting derivative can then be subjected to a variety of chemical modifications designed to permit the linkage of ligands by any one of a number of functional groups (Figure 4).

Ligands containing carboxyl groups can be coupled directly to the aminoalkyl agarose derivatives with water soluble carbodiimide reagents (Figure 4A). Bromoacetyl derivatives of agarose, which can be prepared by reaction with the N–OH–succinimide ester of bromacetic acid, react with compounds containing amino, phenolic, or imidazole groups (Figure 4B). Various proteins can be attached to agarose in this way. Carboxylic acid derivatives of the aminoalkyl agarose, which are prepared on treatment of the latter with succinic anhydride, can be used to couple ligands containing primary amino groups (Figure 4C). Such ligands can therefore be attached to the agarose backbone through a long hydrocarbon extension. p-Aminophenyl agarose derivatives are prepared by reaction with p-nitrobenzoyl azide, followed by reduction with sodium dithionite (Figure 4D). The corresponding diazonium derivative, which results upon exposure to nitrous acid, reacts rapidly with imidazole or phenolic compounds. This method can therefore also be used to attach various proteins to agarose. The resulting protein– or ligand–agarose azo bond can be conveniently cleaved by treatment with sodium dithionite. Sulfhydryl agarose derivatives can be prepared readily by treating the amino agarose with N-acetyl homocysteine thiolactone (Figure 4C). In addition to the many potential uses of this material as such, it is possible to link various ligands to this material by thiol ester or ester bonds. The latter bonds can be selectively cleaved by exposure to alkaline pH or to neutral hydroxylamine. Thus, it is here possible to cleave the intact ligand–protein complex off the occupied selective adsorbent.

Ligands which contain primary aromatic amino groups can be diazotized with nitrous acid and coupled by azo linkage to agarose, to which the tripeptide, gly–gly–tyr, has been attached by the cyanogen bromide procedure (Cuatrecasas, 1970). These are also susceptible to selective reductive cleavage by sodium dithionite.

The versatility which these chemical derivatizations permit is illustrated by the various different derivatives of estradiol–agarose (Figure 5) which have been prepared in this laboratory (Cuatrecasas and Puca, 1970). These are being utilized to purify the serum estradiol binding protein as well as the uterine receptors for this hormone. The latter protein binds very tightly to some of these derivatives, and elution is
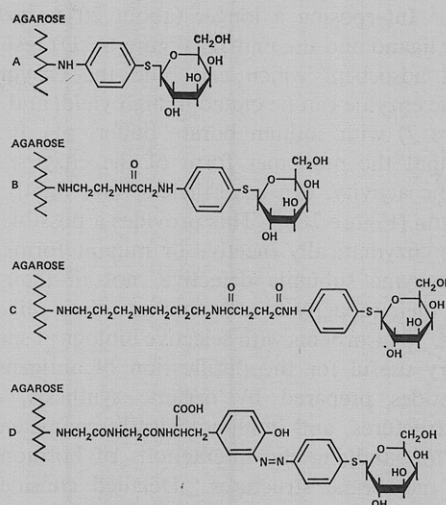

Figure 6. Agarose bead adsorbents prepared for studies of purification of β-galactosidases by affinity chromatography (Steers et al., 1971). p-Aminophenyl-β-D-thiogalactopyranoside is anchored to Sepharose 4B through extension of varying length and composition

difficult to achieve because the protein is particularly sensitive to denaturation by extremes of pH or by denaturants such as urea or guanidine. Some success, however, is being obtained with derivatives A, C, D, and E (Figure 5) because these can be selectively cleaved, as described earlier, under relatively mild conditions.

Studies of the purification of E. coli β-galactosidase illustrate well the importance of the distance which separates the ligand from the solid support, and the way in which this can be systematically resolved by the procedures described here (Steers et al., 1971). Figure 6 depicts four agarose derivatives which contain the weak (K_i about 5 mM) competitive inhibitor, p-aminophenyl β-D-thiogalactopyranoside, covalently attached at different distances from the support. The derivative containing the ligand coupled directly to agarose (Figure 6A) is totally ineffective as an adsorbent, and a short arm extension (Figure 6B) results only in a slight retardation in the downward migration of the enzyme through
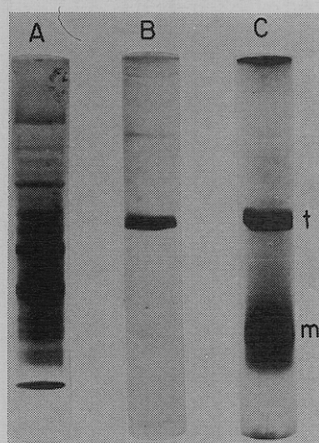

Figure 7. Polyacrylamide gel electrophoresis of crude E. coli extract (A) and of purified sample (B) eluted from a column containing the derivative shown in Figure 6C (Steers et al., 1971). Figure 7C is the material eluted from a column on which had been applied a sample containing a large proportion of catalytically inactive monomeric species of the enzyme; t designates the position of the tetramer, and m designates the position of the monomeric form. [Reproduced with permission of: J. Biol. Chem. 246, 196 (1971).]

the column. Interposing a longer (about 20 Å) extension between the ligand and the matrix (Figure 6C,D) results in a most useful adsorbent which can bind the enzyme with avidity. The enzyme can be eluted in high yield, and in pure form (Figure 7) with sodium borate buffer, pH 10. It is of interest that the monomer form of the enzyme, which lacks catalytic activity, can nevertheless bind tightly to the affinity column (Figure 7C). This provides a possible means of purifying enzymatically inactive or mutant forms of enzymes, or enzyme subunits defective, not in their active sites, but in their capacity to associate into their oligometric native forms. Adsorbents with selective biological specificity are also very useful for the purification of antigens, antibodies, peptides prepared by organic synthesis, specific ribosomal structures, and immunologically competent cells, as well as for studying the interactions of hormones and drugs with membrane structures. Detailed considerations of these uses can be found elsewhere (Cuatrecasas, 1971a).

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