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Review

Current trends in molecular recognition and bioseparation

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Abstract

Molecular recognition guides the selective interaction of macromolecules with each other in essentially all biological processes. Perhaps the most impactful use of biomolecular recognition in separation science has been in affinity chromatography. The results of the last 26 years, since Cuatrecases, Wilchek and Anfinsen first reported the purification of staphylococcal nuclease, have validated the power of biomolecular specificity for purification. This power has stimulated an explosion of solid-phase ligand designs and affinity chromatographic applications. An ongoing case in point is the purification of recombinant proteins, which has been aided by engineering the proteins to contain Affinity-Tag sequences, such as hexa-histidine for metal–chelate separation and epitope sequence for separation by an immobilized monoclonal antibody. Tag technology can be adapted for plate assays and other solid-phase techniques. The advance of affinity chromatography also has stimulated immobilized ligand-based methods to characterize macromolecular recognition, including both chromatographic and optical biosensor methods. And, new methods such as phage display and other diversity library approaches continue to emerge to identify new recognition molecules of potential use as affinity ligands. Overall, it is tantalizing to envision a continued evolution of new affinity technologies which use the selectivity built into biomolecular recognition as a vehicle for purification, analysis, screening and other applications in separation sciences.

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1. Introduction

Molecular recognition guides the selective interaction of macromolecules with each other in essentially all biological processes. The selectivity of biorecognition is embodied in binding domains of macromolecules and binding surfaces within these domains. Through current biological and biochemical research, the identification of macromolecules and their binding sites continues to expand. As this expansion occurs, the opportunity to understand the principles of molecular recognition is increasing.

For biotechnology, the discovery of recognition molecules and mechanistic understanding of their interactions provide starting points to develop therapeutics, diagnostics and separation methods. In separation science, perhaps the most impactful use of biomolecular recognition has been in affinity chromatography. This methodology has provided a means to purify a very large number of novel targets, for example receptors and enzymes, as well as known proteins produced by recombinant DNA technology. Starting with the pioneering work of Porath and his colleagues [1] on agarose and cyanogen bromide activation, chromatographic supports and ligand-immobilization chemistries have been developed which allow accessible binding of biological macromolecules with minimal nonspecific interactions (Fig. 1A) and their subsequent elution in a highly purified state. Anfinsen and his colleagues [2] demonstrated the first affinity chromatographic separation using porous gel technology for Staphylococcal nuclease (Fig. 1B).

Since this early preparative application of bioaffinity, increasingly sophisticated tools of affinity chromatography have evolved for macro-

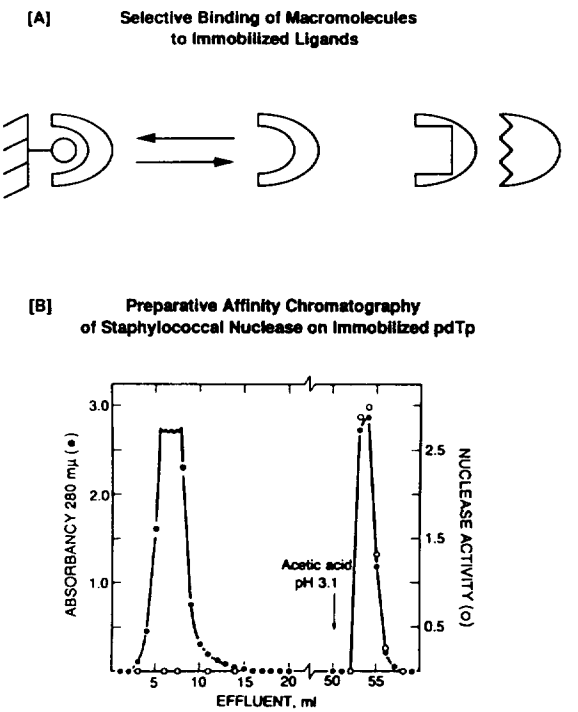


Fig. 1. Preparative affinity chromatography. (A) Schematic diagram depicting selective and reversible binding of eluting biomolecules to immobilized ligands in affinity chromatography. (B) Demonstration of preparative affinity chromatography by the purification of Staphylococcal nuclease on deoxythymidine 3′-phosphate, 5′-aminophenylphosphate–Sepharose [2]. Crude fraction from *S. aureus* broth was eluted, with most of the protein washed through the column without retardation. Nuclease, retained under the initial binding conditions, was subsequently chaotropically eluted with dilute acid.

molecular purification, including more rigid matrices such as cross-linked beaded agarose and cellulose, polymeric supports, silica, and controlled pore glass. At the same time, the success of affinity chromatography as a preparative tool has stimulated the development of analytical applications for biomolecular recognition, first analytical affinity chromatography and most recently molecular recognition biosensors (see Section 6). Affinity-based methods on other solid-phase surfaces such as blots and microtiter plates have further expanded affinity-based technology.

The present review is intended to be a representative, rather than exhaustive, summary of biomolecular recognition methods on porous matrices and how these are currently being used to devise novel separation methods for purification and analysis. This article focuses on types of immobilized ligands designed for biomolecular separation. First, some examples are discussed of protein-specific ligand supports of the type most traditional in the early development of affinity chromatography. Then, we discuss generic ligand supports capable of being used for an increasingly wide range of macromolecules and recombinant protein engineering approaches using Affinity-Tags which take advantage of generic affinity supports for their purification. We discuss the use of phage display methods to obtain affinity-matured ligands as separation tools as well as for other biotechnological applications. Finally, we discuss some analytical methods that have been spawned from the success of affinity chromatography and can be used among other things to help identify and design immobilized ligand systems for separation science.

2. Protein-specific immobilized ligands for receptors and enzymes

Design of the immobilized ligand for bioseparation is perhaps the most challenging aspect of engineering an affinity support. For isolating a cellular receptor, for example, the natural ligand might be an ideal choice for binding selectivity but may suffer from high intrinsic binding affinity. The latter could lead to inactivation of the receptor or ligand due to the harsh conditions needed for release from the affinity support. A more practical ligand may be a monoclonal antibody (MAb) to the receptor, which can be preselected for modest affinity and appropriate binding kinetics (*on* and *off* rates). Metal chelates, dyes and antisense peptides can be used as generic affinity ligands. For the purification of catalytic proteins, substrate derivatives, inhibitors and cofactors are potential choices.

Important parameters for a successful affinity sorbent for bioseparations include mechanical, chemical and biological stability as well as the potential for non-specific binding. Rigid matrices like polymeric supports, silica and controlled pore glass may suffer from non-specific binding and low recovery. Cross-linked beaded agarose or cellulose offers a good compromise between mechanical stability and non-specific binding. Product recovery is also excellent for these matrices. These matrices also have excellent chemical stability within the working range of biological separations. The interested reader is referred to other existing literature for discussion of core chromatographic matrices themselves [3,4].

Once a matrix is selected, the ligand should be immobilized via a stable covalent bond to avoid progressive leaching and consequent capacity loss. The most common procedure to activate agarose is cyanogen bromide (CNBr), which results in the coupling of an amine group of the ligand or spacer through an isourea bond. Matrices prepared by this procedure can suffer from the serious drawback of high ligand leaching. Alternatively, activation of the matrix by organic sulfonyl chlorides or epihalohydrins produces stable linkages [5]. Activation by epihalohydrin can be done in aqueous media.

Early development of affinity sorbents, such as pdTp–aminophenyl agarose for *S. nuclease* [2] and many others since then, were engineered with immobilized ligands chosen to be specific for a given protein and were able to separate that protein from complex mixtures such as cell extracts. As examples of protein-specific ligands, we will discuss two types, namely immunoaffinity and transition-state ligands. Both have been

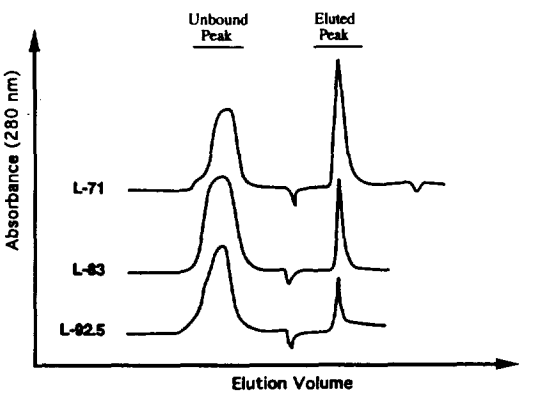
applied in our laboratories to the purification of a variety of protein molecules including enzymes and receptors.

2.1. Immunoaffinity separation and its application to soluble CD4

CD4 is a T cell receptor that is used by the human immunodeficiency virus (HIV) to recognize and subsequently infect T cells. Soluble CD4 (sCD4) has been considered as a possible therapeutic agent for AIDS by acting as a molecular decoy, that is binding to the gp120 coat protein of HIV and therein preventing cellular binding of HIV. In the search for CD4 variants with improved pharmacokinetic properties, a rapid generic purification scheme for sCD4 constructs was developed using immunoaffinity separation. In general, to prepare an immunoaffinity sorbent, MAb is preferred over polyclonal antibody, for two main reasons. First, the MAb can be obtained reproducibly once a hybridoma clone has been isolated. Secondly, the appropriate monoclonal can be selected with the desired binding properties to optimize biomolecular adsorption and elution. The desired elution conditions can be incorporated into the screening procedure to identify the most advantageous MAb. Since the binding constant varies from clone to clone, selection is necessary of a clone producing a MAb with a desirable binding constant. An antibody that gives a good response in either western blot or enzyme-linked immunosorbent assay (ELISA) is not necessarily the best ligand for the immunoaffinity sorbent. Interaction analysis using such current tools as optical biosensors can be used to screen MAbs for those with a good balance of sufficiently high affinity and finite *off* rate (see Section 6).

2.1.1. Selecting the monoclonal antibody

To prepare a robust immunoaffinity sorbent for the purification of sCD4 and a number of sCD4 mutants from either mammalian cell culture or microbial extracts, a series of MAbs were examined. Five different anti-CD4 monoclonal antibodies, from Becton and Dickinson (Mountain View, CA, USA) were immobilized through protein amino groups onto a Sepharose matrix containing an 11-atom spacer using active ester chemistry. All of the sorbents were evaluated individually on small test columns. In the cases of L-92.5 and L-83 clones, the binding was restrictively tight, while L-71, L-77 and L-104.5 showed moderate and more tractable binding affinity. Fig. 2 shows representative chromatograms to illustrate the point. Although L-92.5 sorbent bound the highest amount of CD4, the recovery was least of all the sorbents. On the other hand, the L-71 sorbent showed moderate binding but the recovery of sCD4 was quantitative. Thus, MAb L-71 was judged to be the most suitable candidate for immobilization to prepare a scale-up immunoaffinity sorbent to purify CD4 congeners. Recombinant proteins in *Streptomyces* broth could be loaded directly without



Ligand	L-71	L-83	L-92.5
Load (ug)	1400	1400	1400
Unbound	1062	1306	820
Eluted	336	55	61

Fig. 2. Absorbance (A_{280}) profiles of sCD4 binding to immunoaffinity column: Soluble CD4 (sCD4), at 0.1 mg/ml in N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer at pH 7.5, was loaded at 60 cm/h at 4°C onto a (1 × 6.4 cm) immunoaffinity column, pre-equilibrated with 0.05 *M* HEPES, 0.15 *M* NaCl, 0.01% PEG 3400, pH 7.5. To remove non-specifically bound proteins, the column was washed with two column volumes of HEPES buffer containing 0.5 *M* NaCl at the same flow-rate. Bound sCD4 was eluted with 0.1 *M* acetic acid, 0.15 *M* NaCl, 0.01% PEG 3400 at 60 cm/h. The inset shows quantities of sCD4 in various fractions determined by Bio-Rad protein determination.

prior clean-up, and the bound protein could be recovered with very high yield using 0.1 *M* acetic acid elution to obtain an electrophoretically homogeneous product. After neutralization, all congeners were highly active with respect to gp120 binding as judged by a radioligand-bead binding assay. The sorbent was also successfully used to purify full length CD4 in highly active form. The purified full length molecule displayed a K_a of 4.8 n*M* (± 0.4 n*M*), which was experimentally indistinguishable from the K_a of 5.2 n*M* (± 0.3 n*M*) found for purified soluble CD4 (unpublished data).

2.2. Using immobilized transition state analogues to purify proteolytic enzymes

The purification of proteolytic enzymes from either natural or recombinant sources can be demanding, especially when the concentration of the enzyme is low and multiple enzyme forms with homologous substrate specificity are present. A method to obtain highly purified and active protease is required if the enzyme is to be used for kinetic characterization and structural determination such as NMR and X-ray crystallography. The criteria of purity of a protease for clinical use as a drug are even more stringent. Immunoaffinity purification of proteases may not be appropriate because of ligand loss due to proteolytic degradation during chromatography. For the purification of enzymes, inhibitors and cofactors are more logical affinity ligand candidates. Usually, ligands used to prepare affinity sorbents for the purification of proteases are general inhibitors or substrate analogues [6–8]. A suitable ligand for purifying the protease should be highly specific and the complex formed should be stable enough to survive the washing procedure required to remove non-specifically bound contaminants and the rigorous cleaning and depyrogenating conditions needed to recycle the resin. Transition-state analogues offer both of these desired properties and hence are advantageous ligands for affinity sorbents. For serine proteases and aspartyl proteases, aldehydes and either statin or hydroxyethylene isostere, respectively, mimic the transition state [9–12]. A number of examples of potent transition state analogue inhibitors of proteases having inhibition constants in the nanomolar or sub-nanomolar range have been described. [13–18].

2.2.1. Serine proteases:

For serine and cysteine proteases, peptide aldehydes which mimic the transition-state have been used effectively. These proteases form a covalent bond between the protein and the ligand, resulting in a hemiacetal or thiohemiacetal. The recovery of the enzyme can be achieved either by incorporating a competitive inhibitor or an aldehyde modifying reagent. Use of a transition state analogue as a sorbent for the purification of serine proteases was reported first by Ishii and Kasai [19]. In this case the protease was recovered by reducing the aldehyde to an alcohol, or by adding a competitive inhibitor. These conditions are not very practical for protease purification where the demand for a large scale continuous supply of the enzyme is high. Subsequent purification of active proteases using transition-state affinity sorbents has been reported for trypsin [20], thrombin and urokinase [21], tissue plasminogen activator [22–25] and interleukin converting enzyme [26,27].

2.2.2. Aspartyl proteases and transition state separation matrices

In the case of aspartyl proteases, the carbonyl carbon of the substrate assumes a tetrahedral structure during catalysis. Umezawa and coworkers discovered the transition-state analogue, pepstatin A, during screening of cultures of *Actinomyces* in search of inhibitors of pepsin [28,29]. The general structure of pepstatin is RCO–Val–Val–AHMHA–Ala–AHMHA, where AHMHA is amino-3-hydroxy-6-methylheptanoic acid. Pepstatin is a transition-state analogue of aspartyl proteases and can potentially be used as a transition-state analogue affinity ligand to purify any aspartyl protease. In the case of acid proteases such as renin or HIV protease, the transition-state analogues are peptides containing the statin moiety or the hydroxyethylene isostere. In these cases, there is no

covalent bond formed between the protease and the ligand. The enhanced stability of the complex in the absence of the covalent bond derives from the added interactions between the protease and amino acid residues in the P′ sites. The use of pepstatin as an affinity sorbent ligand to purify hog renin was first described by Corvol et al. [30]. The use of a transition-state analogue, by design, for renin purification was reported by McIntire et al. [31]. Here, the ligand was a non-cleavable peptide inhibitor with the sequence D-His–Pro–Phe–His–Leu–^{R}–Leu–Val–Tyr, where R is a reduced isosteric bond (–CH_2–NH–).

HIV-1 protease, an aspartyl protease, also is inhibited by pepstatin, with an inhibition constant of 1.4 μM [13]. Taking advantage of this, Rittenhouse et al. [32] reported a method to purify HIV-1 and HIV-2 proteases using commercially available pepstatin–agarose as an affinity sorbent. Alternatively, reduced amide bond transition state analogues can be used effectively as ligands for the purification of HIV-1 protease. Heimbach et al. [33] described the use of such a peptide inhibitor containing a reduced amide moiety at the scissile bond (K_i = 1 μM). In both of these cases, additional chromatographic steps were needed to remove contaminants. For the purification of recombinant HIV protease produced in *E. coli*, a third kind of transition-state analogue has been used as an affinity ligand. The hydroxyethylene isostere shown in Fig. 3 was immobilized onto a Sepharose-based matrix containing a carboxylic acid functional group [34] using active ester chemistry. In this case, clarified *E. coli* homogenate was loaded straight onto the affinity resin at room temperature. After a series of washes (Fig. 4), the bound protease was eluted giving more than 1000-fold purification, with 73% yield. The protease was purified to homogeneity in a single step with an activity similar to protease purified by conventional multi-step chromatographic techniques. The affinity-purified protease also was suitable to prepare crystals of protease–inhibitor complex for structural determinations by X-ray diffraction.

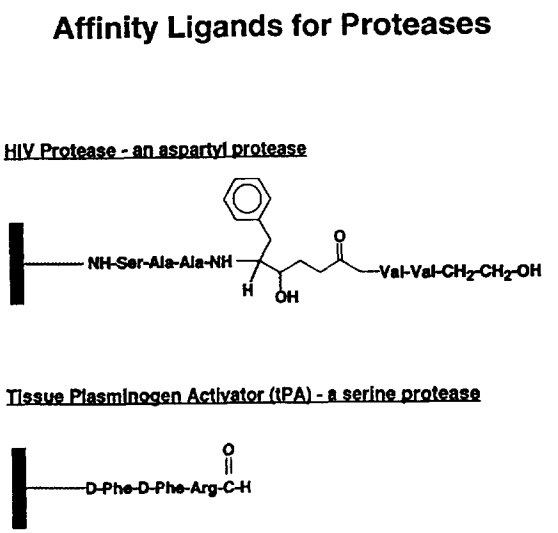


Fig. 3. Structures of immobilized transition-state analogues. Structures of transition-state analogue inhibitors immobilized onto an agarose-based matrix containing a carboxylic acid functional group using active ester chemistry.

3. Recombinant proteins and generic separation methods

3.1. Generic antibody separation methods

Affinity separation methods have found common application in the purification of natural and recombinant proteins with diverse functions from a variety of tissue and cellular sources. As illustrated in Table 1, affinity ligands have been successfully targeted to specific substrate or cofactor binding sites, unique protein to protein binding/recognition sites or, more generically, to post-translational structural modifications (e.g., carbohydrate or phosphorylation sites). When the macromolecular target for separation is known and is being produced by recombinant DNA cloning and expression, affinity chromatography can be useful, especially when expression is low and/or specific isolation of active components is required following protein refolding or when partial proteolysis has occurred.

As with isolation of natural proteins, generic binding ligands can replace protein-specific ligands for recombinant protein affinity systems.

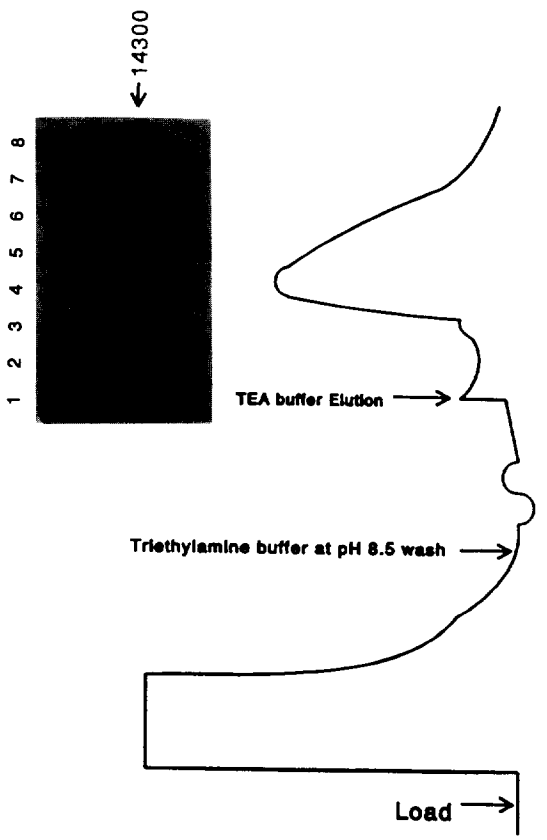


Fig. 4. Absorbance (A_{280}) profiles of HIV-1 protease purification using transition-state affinity chromatography. Filtered homogenate was loaded onto a 5 × 5 cm Pharmacia column pre-equilibrated with 50 m*M* 2-(N-morpholino)ethanesulphonic acid (MES), 0.5 *M* NaCl, 1 m*M* EDTA, 20% glycerol at pH 6.5. After indicated washes, the protease was recovered by 50 m*M* triethylamine (TEA) buffer at pH 9.3 containing 20% glycerol. The eluted protease was immediately neutralized to pH 6.0 with HCl. The inset shows silver stained SDS-PAGE of various fractions. Lane 1 = load (*E. coli* homogenate); 2 = unbound; 3 = MES wash; 4 = triethylamine pH 8.5 wash; 5 = zone before protease peak; 6 = start of protease peak; 7 = pooled protease peak; 8 = molecular mass standards. The eluted preparation showed a single band on silver stained SDS-PAGE (inset) The activity of the protease was consistently around 20 000 mU/mg of the protein with the turnover number of 50/s with the nonapeptide substrate Ac–Arg–Ala–Ser–Gln–Asn–Tyr–Pro–Val–Val–NH_2.

An example is the widespread use of the bacterial surface proteins Protein A and Protein G in the purification of a variety of natural and genetically engineered recombinant antibodies. Proteins A and G, isolated from *Staphylococcus aureus* and Group G *Streptococci* respectively, exhibit affinity for the Fc regions of many types of antibodies. Through their stability, specificity, and relatively low cost, Protein A and G make ideal affinity ligands for the isolation of polyclonal immunoglobulins from serum or plasma [46], as well as monoclonal antibodies from tissue culture or ascites [47]. An even broader application of the Protein A–Fc interaction has been made by the recent construction of fusion proteins containing either immunoglobulin Fc [48] domains or the binding domain of Protein A [49] to aid in purification or presentation of the protein of interest.

3.2. Isolation, selection and purification of MAbs targeting respiratory syncytial virus (RSV)–F protein

The principles and practical use of affinity chromatography with generic ligands are exemplified in approaches we have taken to isolate neutralizing human monoclonal antibodies to the important respiratory pathogen, respiratory syncytial virus (RSV). RSV is the leading cause of severe lower tract respiratory disease (pneumonia, bronchitis) in infants and young children in the USA. Currently there is no generally accepted antiviral treatment for this disease. Passive monoclonal antibody therapy aimed at treating or preventing disease shows promise in animal models of RSV infection. We are actively developing neutralizing human and humanized monoclonal antibodies targeted to the highly conserved viral fusion (F) protein for testing in humans. As one approach, we are utilizing the recently described combinatorial library technology where antibodies can be produced from large combinatorial repertoires of antibody fragments (FAbs) displayed on filamentous bacteriophage by selection with antigen (see Ref. [50] for review). In order to obtain the purified RSV target antigen, a MAb, directed to the viral F protein and pre-selected based on its binding properties as a suitable immunoaffinity reagent,

Table 1
Examples of affinity chromatography in protein purification

Affinity ligand	Target	Example	Ref.
Lectin (Ricin)	Glycoproteins	ECE	[35]
ADP	NADP/ADP dependent enzymes	NOS	[36]
Dye ligand (Blue-Seph)	NAD dependent enzymes	LDH	[37]
Avidin	Biotinylated proteins	Proprional CoA carboxylase	[38]
Protein A	Antibodies	Anti-TMS1 MAb	[39]
p-Amino-benzamidine (inhibitor)	Enzymes	TPA	[40]
Glutathione (substrate)	Enzyme or enzyme fusion proteins	GST	[41]
DNA	DNA binding proteins	Histones	[42]
M^{++} Chelate	Metal binding Proteins	α_2-Macroglobulin	[43]
Phosphotyrosine	Protein containing SH2 domain	Phosphotyrosine kinases	[44]
Calmodulin	Calmodulin binding proteins	Myosin light chain kinase	[45]

ECE = Endothelium converting enzyme; NOS = nitric oxide synthase; LDH = lactic dehydrogenase; TPA = tissue plasminogen activator; GST = glutatione-S-transferase; CAMBP68 = growth factor mediated calmodulin binding protein; Anti-CD4 MAb = monoclonal antibody against CD4.

was purified from myeloma-conditioned media to >90% purity (Fig. 5) utilizing the immunoglobulin binding selectivity of Protein A described above. A viral antigen-selective immunoaffinity resin was then prepared by immobilizing the isolated anti-RSV MAb to Sepharose using succinimide ester chemistry to minimize protein leaching. Viral F protein or a recombinant form of the protein expressed at very low levels (<1 mg/l of conditioned media) in a mammalian cell line could be purified to >80% purity in a single immunoaffinity purification step, demonstrating the selective power of this affinity method (Fig. 6). Subsequently, the purified F protein, bound to the surface of plastic microtiter plates, was successfully used to pan human antibody combinatorial libraries, selecting for phage displaying high affinity human antibody FAb fragments directed to the F protein [51]. To facilitate further biological characterization, the surface bound FAbs were finally expressed in *E. coli* as soluble proteins incorporating a C-terminal hexahistidine metal chelate peptide that allows for efficient affinity purification (see next section for discussion of hexa-histidine Tags).

4. Protein recognition tags based on affinity interactions

Purification of recombinant proteins has been greatly facilitated in recent years by affinity interactions with specific protein sequence recognition 'Affinity-Tags', genetically engineered into the protein of interest. This allows the expressed tagged protein to be purified by affinity chromatography techniques, often in a single step. The use of Affinity-Tags has allowed rapid purification of proteins expressed at both low and high levels in all common expression systems and has dramatically increased the rate at which new proteins can be brought from DNA sequence to purified reagents for screening or evaluation as therapeutic agents themselves and has developed into a cutting edge technology. There have been over 200 examples of purifications using Affinity-

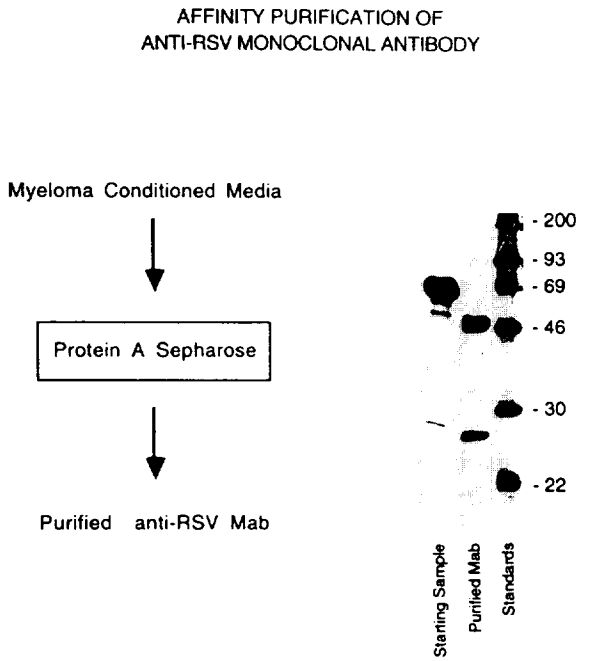


Fig. 5. Affinity purification of anti-RSV monoclonal antibody utilizing immobilized Protein A. Myeloma-conditioned cell growth media containing the anti-RSV monoclonal antibody is passed directly over a Protein A Sepharose column. Following a column wash to remove media contaminants, the bound antibody is eluted at low pH. The high purity (>90%) achieved in this single chromatography step is demonstrated by SDS-PAGE analysis comparing the crude starting sample to the Protein A elution fraction containing the highly purified MAb. SDS-PAGE was run under reducing conditions to separate the antibody into its heavy (approx. 50 kDa) and light chain (approx. 25 kDa) components, respectively.

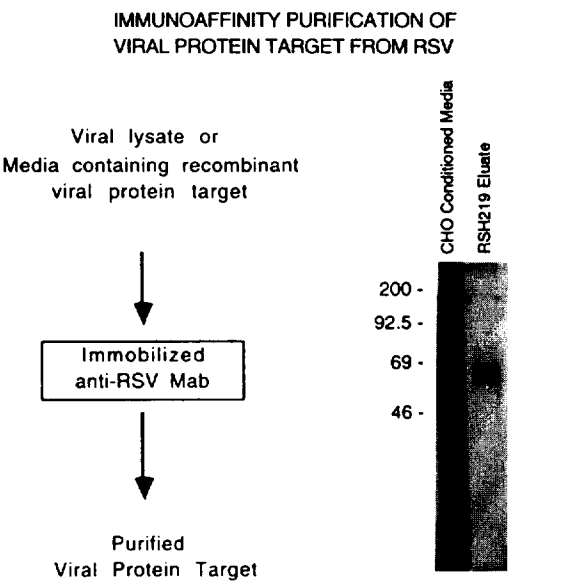


Fig. 6. Immunoaffinity purification of viral target protein. Viral lysates or conditioned cell growth media from Chinese hamster ovary (CHO) cells expressing the recombinant RSV fusion protein is passed directly over a column of the anti-RSV monoclonal antibody immobilized to Sepharose. After washing the column to remove cellular or media contaminants, the fusion protein is eluted at low pH. Again, high resolution one-step purification is demonstrated by SDS-PAGE analysis comparing crude starting material (in this case, CHO conditioned media containing the recombinant viral protein) to the elution fraction. The recombinant protein cannot be distinguished from background host protein in the starting material, however, highly purified (>80% purity) glycosylated viral fusion protein, running at an apparent molecular mass of approx. 69 kDa, is clearly observed in the column elution fraction.

Tag systems described in the literature since 1990. Selected examples of different types of Affinity-Tag systems are presented in Table 2. The Affinity-Tag approach has shown great utility and gained enough popularity for the development of commercially available kits which provide the DNA tools and affinity columns necessary to insert any DNA sequence of interest into a vector, express it as a tagged molecule and purify it on the provided affinity matrix. Companies such as IBI (New Haven, CT, USA), Qiagen (Chatsworth, CA, USA), Novagen (Madison, WI, USA), Invetrogen (San Diego, CA, USA) and Pharmacia (Piscataway, NJ, USA), to name a few, market such kits.

A subset of the general Affinity-Tags referred to above is the Immunoaffinity-Tag, where a short peptide sequence representing a linear epitope of an antibody is engineered into the protein of interest. Prerequisites to this approach should be that the epitope be linear rather than conformational and that the antibody have appropriate binding properties and be available in sufficient quantity for construction of immunoaffinity resins.

Once purified, it is often desirable to have native protein, without the Affinity-Tag. Consequently, protease-sensitive cleavage sites, such as the amino acid sequence IEGR for Factor Xa, DDDDK for enterokinase and others, have

Table 2
Literature examples of Affinity-Tag use for the purification of proteins. Examples of affinity-tags used for rapid protein purification

Tag	Affinity matrix	Ref.
Hexa-histidine	Ni-NTA	[52,53]
Choline binding domain	DEAE-Sepharose	[54]
Glutathione-S-transferase	Glutathione-Sepharose	[55]
IBI-flag	Anti Flag Ab	[56]
FC domain	Protein A-Sepharose	[57]
Gal carbohydrate recognition domain	Galactose-Sepharose	[58]
Biotin acceptor peptide	Avidin monomer	[59]
Protein A	IgG-Sepharose	[60]

routinely been engineered into fusion proteins between the Affinity-Tag and the N-terminus of the native protein. Specific limited proteolytic cleavage of the Affinity-Tag fusion protein with one of these proteases followed by re-chromatography on the immunoaffinity column will yield the protein of interest.

4.1. The advantages to the affinity-tag system approach are four-fold

The advantages of the Affinity-Tag system approach for rapid expression and purification can be divided into four main areas, namely expression, generic purification, assay development and removal of closely related translation errors. These areas, including examples, are discussed below.

4.1.1. Faster expression, evaluation and optimization

With an appropriate Affinity-Tag system, where an antibody that specifically recognizes the Tag part of the desired fusion protein is available, the evaluation and optimization of expression systems proceeds much more rapidly since expression can be monitored specifically by Western Blot techniques using existing reagents. Under normal circumstances the protein must first be expressed, small quantities of protein highly purified, then injected as antigen into rabbits to produce polyclonal antibody (PAb). It takes a few weeks time to obtain a high enough titer to use in Western Blots for subsequent expression evaluation. If the protein purified for this purpose has any impurities the PAb generated will also recognize the impurities, making it much less specific and less useful.

4.1.2. Rapid affinity purification

The expressed protein can be quickly purified using an affinity matrix which recognizes the Tag sequence on the engineered protein. Often purification can be accomplished in a single step. This affinity interaction can be especially important when the protein is expressed at low levels, where purification by a multi-step process using conventional methods may not be feasible. In some instances this also allows the purification of poorly expressed proteins without the need to increase expression levels, the latter of which takes time and effort. Another advantage of this approach is that it allows the set up of a generic purification procedure in a specific expression system, engineering the same Affinity-Tag on all proteins to be expressed in that system. This reduces the time needed to develop new purification schemes for new proteins and simplifies the purification process allowing individuals, not skilled in the art, to successfully purify their proteins without the need of extensive protein purification expertise.

Example: rapid generic purification of FAbs. With the use of the phage display technology, as mentioned earlier, many FAb fragments with the desired binding characteristics can be identified from each panning of the library. These FAbs need to be expressed and purified to allow

adequate analysis of their neutralization capabilities. Affinity-Tags are ideal for this application.

The simple generic purification scheme developed in our lab for hexa-histidine tagged FAbs (Fig. 7) exemplifies this. A hexa-histidine Tag was engineered into an expression vector, then we developed a generic purification scheme which was subsequently used by individuals, not skilled in the art, for the successful purification

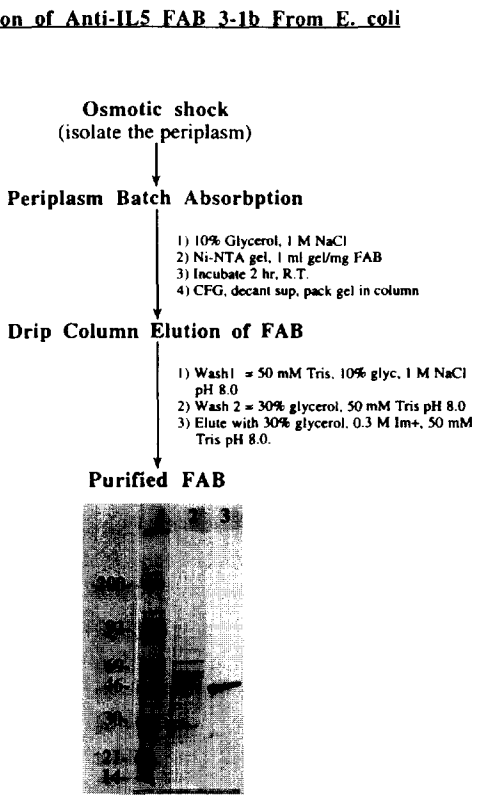


Fig. 7. Generic scheme for FAb purification. Anti-IL5 FAb 3-1b was expressed in *E. coli* via isopropyl-β-D-thiogalatopyranoside (IPTG) induction at 28°C as described previously [61]. *E. coli* cell paste was isolated, washed with phosphate buffered saline (PBS) solutions, then lysed by osmotic shock at 4°C in the presence of 20% sucrose and 1 mg/ml lysozyme. The periplasmic fraction (100 ml) was isolated by centrifugation, then treated with glycerol to 10% and NaCl to 1 *M*, final concentration. This solution was batch adsorbed on nickel nitrilotriacetic acid (Ni-NTA); at 1 ml of gel/mg of FAb, for 2 h at ambient temperature. The Ni-NTA gel had been previously equilibrated with 50 m*M* Tris, 1 *M* NaCl, 10% glycerol pH 8. The mixture was spun down at 1000 *g*, the supernatant decanted and the Ni-NTA gel slurried and poured into a 1 cm I.D. econocolumn. The resin was washed with 10 column volumes (10 CV) equilibration buffer, then 10 CV of 50 m*M* Tris, 30% glycerol pH 8.0. The purified FAb was eluted with 5 CV of 50 m*M* Tris, 0.3 *M* imidazole, pH 8. Reducing SDS-PAGE gel electrophoresis: SDS-PAGE electrophoresis was performed according to the method of Laemmli [62]. Samples (non-reduced) were electrophoresed on Bio-Rad Ready Gels (4-20% acrylamide gradient) under non-reducing conditions, then stained with Coomassie R-250. Lane 1 = Rainbow molecular mass markers: Lysozyme (14 kDa), Trypsin Inhibitor (21 kDa), Carbonic anhydrase (30 kDa), ovalbumen (46 kDa), bovine serum albumin (69 kDa), phosphorylase *b* (92 kDa) and myosin (200 kDa); lane 2 = periplasmic fraction; lane 3 = purified anti-IL5 FAb 3-1b eluted from Ni-NTA affinity column with buffer containing 0.3 *M* imidazole. Protein was purified to >95% homogeneity in a single step on Ni-NTA, with high recovery. The purified non-reduced IL5 FAb ran as a single band on SDS-PAGE at a molecular mass slightly higher than 46 kDa. This is consistent with the 48 kDa predicted by the protein sequence for this FAb. The FAb was active in binding assays without removal of the hexa-histidine Tag. This procedure was generic and could be used unchanged for the purification of many different FAbs.

of over 50 different FAb isolates. This allowed rapid identification of a neutralizing FAb in subsequent screening assays.

4.1.3. For direct incorporation into assays

If appropriately designed, Tags on the desired fusion proteins can be used to anchor the protein to surfaces allowing them to be directly adapted for quantitative in vitro assays, without need for additional modification. This allows rapid screening of biological activity for potential agonists or antagonists of a specific affinity interaction.

Example: purification of a hematopoietic receptor–Fc fusion and a hematopoietic receptor. Colony stimulating factors, such as erythropoietin (EPO), G-CSF, GM-CSF, IL-3, thrombopoietin (TPO) and IL-5 and their receptors are essential in hematopoiesis and drive the proliferation and maturation of multipotential cell progenitors to terminally differentiated cells, such as red blood cells, granulocytes, monocytes, megakaryocytes and eosinophils. Consequently, studying the interaction between these factors and their cognate receptors is of invaluable interest in understanding how these structurally related proteins are distinguished. To study this interaction requires large quantities of both receptor and ligands. In one example, a receptor was expressed as an Affinity-Tag fusion with the Fc domain of IgG. This fusion was purified in a single affinity interaction step on Protein A (Fig. 8) and subsequently used as a reagent in biological assays anchored through its Fc portion. As indicated in Section 4 the purified native molecule, without the Fc portion, can be prepared by enzymatic cleavage of the fusion protein. Here, the receptor molecule was prepared from a purified receptor–Fc fusion by Factor Xa cleavage of the protease-sensitive site, engineered between the receptor and the Fc portion of the fusion protein, then passed back over the Protein A column to bind the cleaved Fc Tag and any remaining fusion protein, providing a highly purified receptor (Fig. 8).

4.1.4. Removing fusion protein truncates due to protein synthesis errors

The use of Tags can often resolve very difficult purification problems arising during expression of recombinant proteins. One problem encountered in *E. coli* expression systems is the expression of truncated versions of the desired protein. These truncated proteins arise from errors such as internal initiation of translation or premature terminations during protein synthesis and result in truncated molecules that have physical characteristics very close to those of the full length molecules and can not be easily separated using standard purification techniques. Engineering of an Affinity-Tag on the N-terminus of a protein allows the selection of only molecules containing the correct N-terminus, but not truncated molecules arising from internal translation initiation errors or N-terminal proteolysis. Similarly engineering of an Affinity-Tag on the C-terminal allows the selection of only molecules containing the correct C-terminus, but not molecules which were prematurely terminated or proteolytically cleaved in the C-terminal region. A more rare occurrence in *E. coli* is observed when certain codons, poorly utilized by *E. coli* due to deficient levels of specific t-RNAs, cause protein synthesis to falter and frame shift at these codons, producing nonsense sequence from that point in the protein until the new C-terminus [63]. These molecules can also be very difficult to separate from the desired full length molecules. C-terminal Affinity-Tags, however, can allow rapid purification of full length molecules eliminating these side products.

Example: removal of premature terminations from an influenza vaccine candidate. Hemagglutinin on the surface of all influenza viruses is required for host infection. The extracellular domain of hemagglutinin is also the site of yearly genetic changes which the virus employs to evade the immune system. This requires that new vaccines be made each year, comprised of the strains which are considered likely to be prevalent in the world's population that year. A fusion protein comprised of a conserved region of the hemagglutinin-H1 subtype and a portion of the influenza non-structural protein (NS1) was an influenza vaccine candidate shown to elicit cross-strain protection in mice [64]. This fusion was expressed in *E. coli* but contained truncated forms of the fusion protein making purification difficult. With a C-terminal hexa-histidine Tag

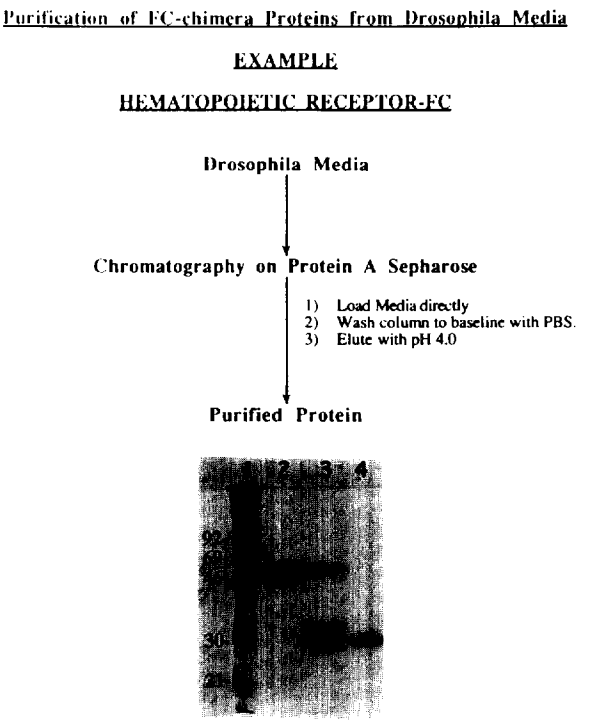


Fig. 8. Hematopoietic receptor–Fc fusion purification and Tag removal by Factor Xa cleavage: The hematopoietic receptor–Fc fusion was expressed in Drosophila via copper sulfate induction at 24°C. After 10 days, 10 liters of Drosophila conditioned media was harvested by centrifugation and sterile filtration. It was then pH adjusted to 8.0 and loaded onto a 2.5 × 5 cm Pharmacia Protein A column (25 ml) at 8.2 ml/min (100 cm/h) at 4°C. The Protein A column had been previously equilibrated with 0.1 *M* Tris pH 8.0. The column was then washed to baseline with equilibration buffer and purified receptor–Fc fusion eluted with 0.1 *M* glycine buffer pH 3.0. Collected fractions were immediately neutralized with 1 *M* Tris pH 9. The receptor–Fc fusion was cleaved by Factor Xa (Sigma) at a protein to Factor Xa ratio of 1/25 overnight at 4°C in 20 m*M* Tris, 0.1 *M* NaCl, 2 m*M* $CaCl_2$, pH 8.0. The digested product was subsequently purified over the Protein A column to remove the cleaved Fc domain. Reducing SDS-PAGE gel electrophoresis: SDS-PAGE electrophoresis was performed according to the method of Laemmli [62]. Samples were reduced and heated at 95°C for 1 min before being electrophoresed on freshly prepared 15% acrylamide gels, then stained with Coomassie R-250. Lane 1 = Rainbow molecular mass markers: lysozyme (14 kDa), trypsin inhibitor (21 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (69 kDa), phosphorylase *b* (92 kDa) and myosin (200 kDa); lane 2 = purified receptor–Fc fusion eluted in the glycine pH 3 buffer step; lane 3 = Factor Xa digest of purified receptor–Fc fusion; lane 4 = purified receptor in unbound portion of Protein A chromatography of Factor Xa digest, cleaved Fc remains bound to the column. Receptor protein was purified to >95% homogeneity in a single step on Protein A Sepharose, with complete recovery. The receptor–Fc fusion was active in the binding assays without removal of the Fc Tag. The Factor Xa cleavage worked well and provided pure receptor.

the purification by affinity chromatography was rapid and inclusion of a hexa-histidine C-terminal Tag eliminated the problems of contamination with C-terminal truncated forms (Fig 9).

4.2. Limitations of tags:

The use of Affinity-Tags does have some theoretical and practical limitations. These are discussed briefly in the following two sections. It should be pointed out, however, that these perceived limitations have not hindered the widespread use of Affinity-Tags, as demonstrated by the number of publications citing their use in purification.

4.2.1. Misfolding and/or loss of activity

There is some concern that placing unnatural amino acid sequence Tags on the protein of interest may cause misfolding or be responsible

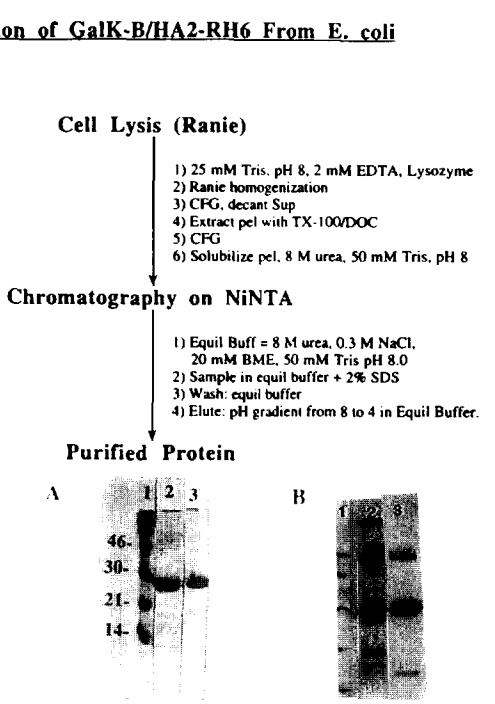


Fig. 9. Removal of C-terminal truncated versions of desired molecule: Part of an influenza hemagglutinin surface antigen was being purified as a potential flu vaccine candidate. During expression in *E. coli* it was found that there were alternate forms of the protein including internal starts with missing N-terminal sections and truncated molecules with a C-terminal portion missing. The hexa-histidine Tag on the C-terminus greatly facilitated purification by removing truncated C-terminal molecules (had no Tag). Flu D-RH6 was expressed in *E. coli* via temperature induction at 37°C. Fermentation broth was centrifuged and *E. coli* cell paste removed and stored frozen at −80°C until used. *E. coli* cell paste (16 g) was resuspended in 10 volumes of lysis buffer (25 m*M* Tris, 2 m*M* EDTA, pH 8). To this suspension was added DTT to 0.1 m*M* and lysozyme to 0.2 mg/ml of suspension. The suspension was stirred at ambient temperature for 1 h then homogenized at 10 000 p.s.i. (1 p.s.i. = 6894.76 Pa) on the Rannie. The resultant lysate was centrifuged at 25 000 *g* for 1 h, the supernatant discarded and the pellet extracted with 1% Triton X100 and 0.2% deoxycorticosterone (DOC). The resulting pellet was solubilized in 8 *M* urea, 50 m*M* Tris, pH 8.0 containing 2% SDS and 20 m*M* β-mercaptoethanol (BME). The solubilized pellet was loaded onto a 1.5 × 3 cm (5 ml) Qiagen Ni-NTA Agarose column at 0.5 ml/min (15 cm/h), which had been previously equilibrated with 8 *M* urea, 0.1 *M* phosphate, 20 m*M* Tris, 0.3 *M* NaCl, pH 8.0 buffer. The Ni-NTA column was washed with 3 CV of equilibration buffer, then purified Flu D-RH6 was eluted with a 6 CV pH gradient from pH 8.0 to 4.0 in equilibration buffer. Reducing SDS-PAGE gel electrophoresis: SDS-PAGE electrophoresis was performed according to the method of Laemmli [62]. Samples were reduced and heated at 95°C for 1 min before being electrophoresed on freshly prepared 15% acrylamide Gels, then stained with Coomassie R-250. Lane 1 = Rainbow molecular mass markers: lysozyme (14 kDa), trypsin inhibitor (21 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (69 kDa), phosphorylase *b* (92 kDa) and myosin (200 kDa); lane 2A,2B = urea solubilized *E. coli* lysate pellet; lane 3A,3B = purified Flu D-RH6 eluted from a Ni-NTA affinity column in the pH 8 to 4 gradient step; gel A is coomassie stained, gel B is a western blot of the same samples, probed with a primary rabbit pAb (anti-DPR8) directed against the flu viral hemagglutinin, and a secondary goat anti-rabbit-HRP Ab conjugate. The blot was developed with Sigma 4 chloro-1-naphthol reagent. Flu D-RH6 antigen was purified to >90% homogeneity in a single step on Ni-NTA agarose, with complete recovery. This particular fusion protein is expressed at very high levels (20% of total cell protein) and is localized in the insoluble *E. coli* pellet already >50% pure (lane 2A). The blot of the urea solubilized pellet (lane 2B) shows the presence of a Flu D-RH6 dimer at about 60 kDa and lower molecular mass species, which are truncated versions of Flu D-RH6, all recognized by the specific anti-Flu Ab. The blot of the purified Flu D-RH6 sample (lane 3B) shows that two of the lower molecular mass species are not present and thus do not bind to the Ni-NTA column, suggesting they are C-terminal truncates which do not contain the Tag, while a third truncate binds and is eluted from the Ni-NTA column suggesting it does have the Tag and therefore is a N-terminal truncate arising from an incorrect synthesis start in the middle of the protein. The Tag has allowed easy separation of these different species.

for loss of activity. There are numerous examples in the literature and in our own experience, where Tags have had absolutely no detrimental effect on activity of the native protein [65–68]. However, there will inevitably be some cases where activity is affected. From our experience we have identified two examples where a C-terminal Tag resulted in an expressed molecule with no activity, but the corresponding molecule with an N-terminal Tag was fully active. Both these observations were unexpected. Therefore with any protein to be expressed with a Tag, the potential loss of activity will have to be evaluated on a case by case basis. The more information about the protein one has, the better the prediction will be for which terminus to put the Tag on to preserve activity. However, if a Tag is on the N-terminus with a cleavage site between it and the native protein, specific limited proteolysis can yield native protein. A Tag on the C-terminus, however, is more of a problem, since a proteolytic cleavage site at this end of the molecule will leave 3 or 4 amino acids behind from the protease recognition sites. A Tag consisting of an arginine followed by histidines on the C-terminus can be removed by carboxypeptidase A, leaving only an extra Arg [69].

4.2.2. Physical characterization studies

When the protein of interest is targeted for X-ray crystallography, or other physical characterization studies, the native amino acid sequence and structure is preferred. Ideally under these circumstances, N-terminal Tags removable by specific limited proteolytic cleavage would be used.

5. Affinity ligand use in phage display technology

Phage display has been used increasingly to obtain antibodies against important anti-infective target antigens, for example infective agents such as human immunodeficiency virus (HIV) and hepatitis B virus [70]. As an application of this approach, a neutralizing human antibody to HIV exhibiting both enhanced affinity and broader strain cross-reactivity was developed by targeting complementarity-determining regions for random mutagenesis followed by selection for increased affinity using phage-display in a process that has been referred to as 'in vitro evolution' or 'affinity maturation' [71,72]. Further, competitive bio-panning procedures have been effectively incorporated to isolate FAbs directed to unique regions on a given target molecule, for instance, neoepitopes expressed on the activated complement component C5a but not found on native complement component C5 [61]. In another adaptation, the heavy and light chains of a rodent antibody were alternatively utilized as templates to guide the selection of human antibodies from phage display repertoires to a single epitope on human tumor necrosis factor [73]. The latter provided a potentially powerful alternative strategy to conventional methods of antibody humanization such as the grafting of antibody complementarity-determining regions.

In a further extension of phage display technology that is more analogous to the natural immune response, clonal selection and amplification of phage displayed antibodies has been achieved by linking antigen recognition to phage replication [74]. In this case, individual antigen-specific but non-infectious phage are made replication-competent by allowing a fusion protein of the antigen with the essential phage coat protein required for infection to bind to the displayed antibody fragment. By altering the time of contact with the fusion protein or altering incubation or wash conditions, such as pH or ionic strength, selection of tailor-made antibodies with desired binding properties is feasible. In addition to antibodies, broader application of phage display technology into the identification and optimization of other separation ligands is also evident. Peptide libraries presented on phage potentially represent a general source of affinity ligands for protein purification [75] and in one recent report, the immunoglobulin-binding domain of Protein A, mentioned earlier, has been displayed on the surface of phage, raising the potential for screening mutant forms of Protein A with improved specificity or milder elution properties [76]. Similarly, altered binding

properties of other proteins including some with direct therapeutic potential, such as human growth hormone [77], have also been achieved using this technology.

The emergence of phage display technologies, on the backdrop of now-classical affinity chromatography methods, emphasizes the central role of biorecognition and its continuing innovative application in modern pharmaceutical research.

6. Affinity analysis: analytical affinity chromatography to biosensors

The success of preparative affinity chromatography has spurred development of analytical methods for characterizing biomolecular recognition. This effort was started with analytical affinity chromatography [78–80] and has been reviewed in the literature [81–84]. In this methodology, quantitative elution properties of macromolecules on immobilized ligand supports, with and without competing soluble ligands, can be used to determine equilibrium affinity constants for interaction of macromolecules with both the immobilized and soluble ligands. The methodology can be used to help design biospecific affinity matrices as well as to learn about macromolecular interaction properties.

Analytical affinity chromatography arose in large part from the observation that affinity chromatography provided a powerful means to purify proteins and other biomolecules with a basic two-step retention/chaotropic elution procedure. Successes of preparative affinity chromatography suggested several key features of immobilized ligand interactions with eluting macromolecules, namely *accessibility* of immobilized ligand, *selectivity* of ligand interaction with soluble macromolecule and *reversibility* of macromolecule binding which allows its elution without denaturation. Based on this, it was demonstrated experimentally by Dunn and Chaiken [78,85] that isocratic elution of a macromolecule on an immobilized ligand support (that is elution with a non-chaotropic buffer at conditions allowing a dynamic equilibrium between association and dissociation) was directly dependent on the equilibrium constant for the immobilized ligand–macromolecule interaction. Hence, by measuring elution volume of a macromolecule on a column with immobilized ligand, affinity was determined (Fig. 10A). Furthermore, competitive elution of the macromolecule was shown to be a function of both the matrix and solution interactions of macromolecule and ligand. The analytical use of affinity chromatography for Staphylococcal nuclease (Fig. 10B) was achieved on the same kind of affinity support as used preparatively but under conditions (in particular decreased concentration of immobilized ligand) which allowed isocratic elution. Similar findings have been reported by now in many other systems [81–84]. Interaction analysis on affinity columns can be accomplished over a wide range of affinity and size of both immobilized and mobile interactors and can be achieved on a microscale, dependent only on the limits of detectability of the interactor eluting from the affinity column.

The analytical use of immobilized ligands has been adapted to methodological configurations which allow for automation. An early innovation of analytical affinity chromatography was its adaptation to high-performance liquid chromatographs. High-performance analytical affinity chromatography [86] provides a more rapid macromolecular recognition analysis, at a more microscale level, and potentially using multiple post-column monitoring devices to increase the information learned about eluted molecules. Simultaneous multi-molecular analysis also is feasible, for example by weak affinity chromatography [87].

The principles of analytical affinity chromatography recently have been adapted for a non-chromatographic affinity technology, namely real-time optical biosensors for interaction analysis. As a real-time method, biosensors offer the opportunity to measure not only the equilibrium affinity constant but also the *on* and *off* rate constants for interactions of biological macromolecules.

A key technological breakthrough for interaction biosensors was the surface plasmon resonance (SPR) biosensor developed by Pharmacia, called BIAcore™. This instrument contains the

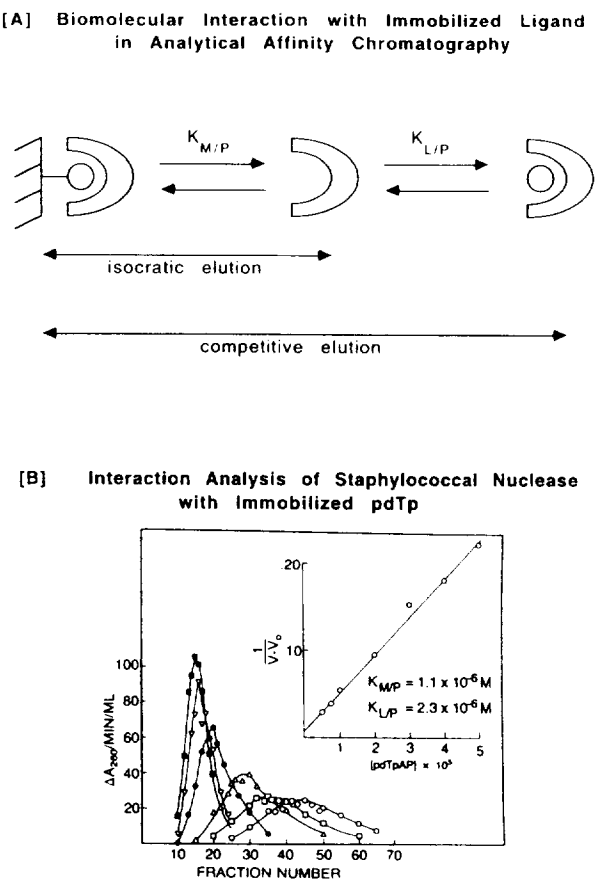


Fig. 10. Scheme and experimental results of analytical affinity chromatography. (A) Scheme comparing isocratic and competitive elution in affinity chromatography and the consequence of these features in affinity analysis. In isocratic elution, the analyte (here P for protein) associates reversibly with immobilized ligand (M for matrix) and the volume of elution increases as the magnitude of the equilibrium dissociation constant defined as $K_{M\cdot P}$ (for complex of M with P) decreases. In competitive elution, added soluble ligand (L) competes with immobilized ligand for binding to free analyte, so that the volume at which biomolecule elutes depends both on the relative affinities of the immobilized and soluble ligands, reflected in the respective dissociation constants $K_{M\cdot P}$ and $K_{L\cdot P}$ (for complex of L with P), and on the concentrations of these ligand species. (B) Zonal elution affinity chromatographic analysis of staphylococcal nuclease on the immobilized nucleotide ligand thymidine-3',5'-diphosphate. The concentrations of competing soluble thymidine diphosphate were: $0.5 \cdot 10^{-5}$ *M* (○), $0.75 \cdot 10^{-5}$ *M* (□), $1.0 \cdot 10^{-5}$ *M* (△), $2 \cdot 10^{-5}$ *M* (●), $3 \cdot 10^{-5}$ *M* (▽), and $4 \cdot 10^{-5}$ *M* (■). The inset shows the linearized plot of competitive elution data and the dissociation constant derived in the analysis. (Adapted from Dunn and Chaiken [85].)

immobilized ligand attached to a dextran layer on a gold sensor chip and detects the interaction of macromolecules passing over the chip through a flow cell by changes of refractive index at the gold surface using SPR [88–90]. The SPR biosensor is similar in concept to analytical affinity chromatography: both involve interaction analysis of mobile macromolecules flowing over surface-immobilized ligands. The SPR biosensor also provides some unique advantages. These include: (i) access to *on*- and *off*-rate analysis, thus providing more information for characterization and design; and (ii) analysis in real-time, thus promising the potential to stimulate an overall acceleration of molecular discovery. An evanescent wave biosensor for molecular recognition analysis, called IAsys^{TM}, has been introduced recently by Fisons [91,92]. In our own laboratories, we have used the optical biosensors to measure interaction properties of human interleukin 5 with its receptor [93] as well as antibody–antigen interactions [94,95]. It is worth adding that analysis of kinetic data obtained by optical biosensors remains a challenging area of ongoing research [96].

7. The future for molecular recognition in bioseparation

The results of the last 26 years since Cuatrecasas, Wilchek and Anfinsen first reported the purification of Staphylococcal nuclease using affinity chromatography [2] have validated the power built into biomolecular recognition for purification of macromolecules. This power has stimulated an explosion of affinity chromatographic applications. It also has stimulated methods to analyze macromolecular recognition, including both chromatographic and optical biosensor methods. The biosensor represents perhaps an instructive example of how molecular recognition can be adapted from chromatographic to non-chromatographic modes to allow new affinity methods development. Adaptation of Tag technology for plate assays and sensor techniques also is possible. In addition, the imminent promise of cheaper, more stable, en-

gineered antibody fragments (single chain FAbs, Fv's etc.) amenable to large-scale manufacture in bacterial systems may greatly extend their application into non-pharmaceutical separation applications such as detoxification of foodstuffs or decontamination of environmental pollutants. FAbs and Fv's presented on a filamentous phage surface can be used to screen for novel specificities (including from 'synthetic' combinatorial libraries) and for higher affinity (affinity maturation). Overall, it is tantalizing to envision a continued evolution of new affinity technologies which use the selectivity built into biomolecular recognition as a vehicle for purification, analysis, screening and other applications in separation sciences.

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