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Affinity-repulsion chromatography

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ABSTRACT

Affinity-repulsion chromatography is a protein separation process in which the electrostatic charges present, or purposely introduced,on affinity matrices are exploited to allow the elution, by electrostatic repulsion, of proteins carrying electrostatic charges of the same sign as that of the matrix. In this process, proteins are loaded on the affinity matrix in a salt solution and eluted with low ionic strength solutions or deionized water. Examples of protein separations carried out by affinity-repulsion chromatography are presented.

INTRODUCTION

Affinity chromatography[1,2] has become established as one of the most powerful techniques for the isolation and purification of proteins. In this procedure, the protein to be purified from a mixture binds to one of its specific ligands immobilized on an insoluble matrix whereas the other proteins in the mixture do not. The protein retained on the matrix is thereby isolated from the other proteins and can be removed from the matrix by addition of its free ligand at concentrations that allow displacement from the immobilized ligand. As a result, the protein is recovered in solution as a complex with its free ligand, from which it can then be dissociated by dialysis or molecular sieving. Often the above procedure encounters difficulties when the protein binds too strongly to the affinity matrix. In such cases, the protein cannot be readily eluted from the matrix by the addition of its free ligand and more drastic elution conditions need to be applied. These include changes in pH, ionic strength, temperature and polarity of the eluting solvent (to decrease van der Waals interactions), the use of chaotropic salts such as guanidine hydrochloride or urea or the use of detergents which alter the structure of the protein, and the application of electrophoretic desorption[3,4]. The disadvantages of some of these elution conditions are their relatively low yields and the possible loss of biological activity of the eluted proteins.

In this paper, we discuss the principle of affinity-repulsion chromatography[5], which aims at easing the elution process, and describe some of its applications, advantages and disadvantages and consider its suitability as a general procedure for the adsorption–desorption of proteins.

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PRINCIPLE OF AFFINITY-REPULSION CHROMATOGRAPHY

The principle of the method (Fig. 1) is based on the fact that both the affinity chromatographic matrix and the proteins that interact with it possess electrostatic charges that can either repel or attract each other according to their sign and their respective distance. The strength of such electrostatic interactions is not substantial in solutions containing high salt concentrations, but can become considerable in deionized water. Accordingly, if a protein and the affinity matrix carry the same overall net electrostatic charge, the protein will be eluted by deionized water whenever the strength of the electrostatic repulsion between the protein and the matrix exceeds the attractive forces between the protein and the immobilized ligand. Such situations can be artificially devised by appropriate chemical modifications of the affinity matrix so as to increase the charge density and adjust the distance between the protein and the matrix.

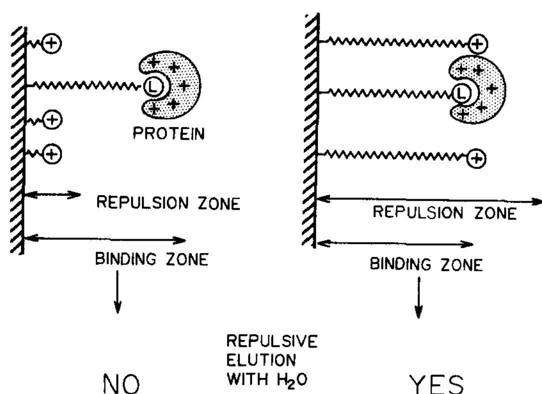

Fig. 1. Principle of affinity-repulsion chromatography. Successful dissociation of the positively charged protein from its immobilized ligand L on elution with deionized water depends on the distance separating the positive charges on the protein from those present on the charge spacers. On the left, the protein electrostatic charges are outside the repulsion zone created by the positive charge spacers and therefore the protein cannot be eluted by deionized water. On the right, the protein binding zone is within the charge repulsion zone and therefore the protein will be eluted either with deionized water only or with the free ligand in deionized water (for proteins with very high ligand binding affinity). From Teichberg *et al.*[5].

EXPERIMENTAL AND RESULTS

Fig. 2 shows the results of the application of peanut agglutinin in a 150 m*M* sodium chloride solution to a lactosyl-Sepharose column. The protein is eluted either with deionized water (upper panel) or lactose in 150 m*M* sodium chloride (lower panel). The elution of the protein with water demonstrates that the strength of the electrostatic repulsion between the lectin and the matrix is greater than the "attraction" between the lectin and its immobilized ligand.

Fig. 3 shows the affinity-repulsion chromatography of peanut agglutinin on a "native" unmodified galactosyl-Sepharose matrix (A and B) and on a negatively charged galactosyl-Sepharose matrix (C). In spite of the presence of these additional

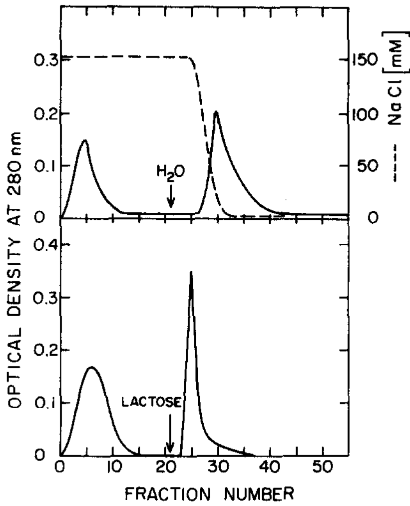

Fig. 2. Affinity chromatography of peanut agglutinin on a lactosyl-Sepharose matrix. The protein was applied in 150 mM NaCl and eluted either with deionized water (upper panel) or 300 mM lactose in 150 mM NaCl (lower panel). From Teichberg *et al.*[5].

negative charges on the matrix, the lectin is not eluted with deionized water more readily from this matrix than from the "native" unmodified matrix. This result indicates that the native Sepharose beads possess an adequate density of negative charges for the electrostatic repulsion of peanut agglutinin. In contrast to its behaviour on a negatively charged matrix, peanut agglutinin cannot be eluted with deionized

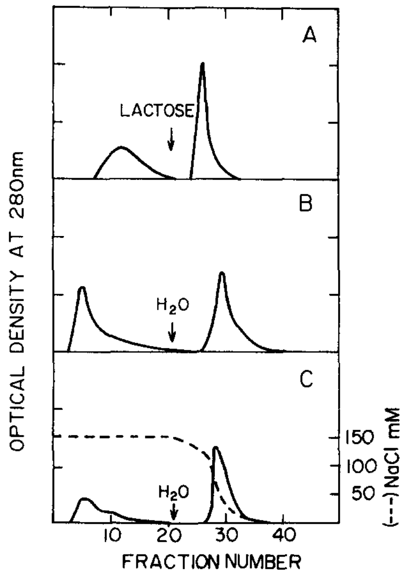

Fig. 3. Affinity chromatography of peanut agglutinin on a galactosyl-Sepharose matrix. A and B, "native" matrix; C, γ-aminobutyric acid-modified matrix. In all instances the protein was applied in 150 mM NaCl and eluted with either 300 mM lactose in water (A) or deionized water (B and C). From Teichberg *et al.*[5].

water when it is affinity chromatographed on a galactosyl-Sepharose matrix to which positively charged residues have been coupled (Fig. 4). However, in spite of this excess of positive charges on the matrix, the protein is eluted wih 300 m*M* lactose in deionized water indicating that the strength of the interaction of the lectin with the free saccharide ligand is stronger than the electrostatic attraction of the negatively charged lectin with the immobilized positive charges of the matrix, possibly because the latter charges are not localized at the optimum Coulombic distance.

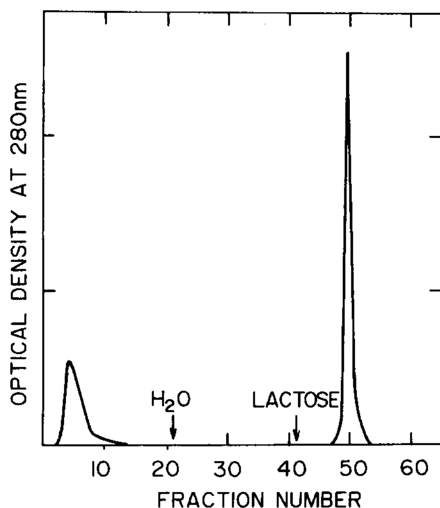

Fig. 4. Affinity chromatography of peanut agglutinin on a galactosyl-Sepharose matrix modified by conjugation with ethylenediamine residues. The protein was applied in 150 m*M* NaCl and eluted with 300 m*M* lactose in water. From Teichberg *et al.*[5].

Fig. 5 illustrates the affinity-repulsion chromatographic patterns of concanavalin A (Con A). Con A, loaded on a maltosyl-Sepharose matrix, cannot be eluted with either deionized water or methyl α-glucoside in water but only with a solution containing methyl α-glucoside, 150 m*M* sodium chloride, 1 m*M* calcium chloride and 1 m*M* manganese chloride (Fig. 5A). However, if Con A is applied on a maltosyl-Sepharose matrix to which ethylenediamine residues have been attached, the protein can be eluted with methyl α-glucoside in water and with deionized water (Fig. 5B and C).

Once applied to a more positive matrix, Con A is eluted with water (Fig. 5C), although the shape of the eluted protein peak is not symmetrical and its size is smaller than expected. The latter result indicates that some of the Con A applied has been retained on the column. Indeed, the application to the column of solutions at pH 8.0 and 3.5 allows the elution of two other protein peaks which, on neutralization, display a methyl α-glucoside specific agglutinin activity and fully account for the amount of Con A loaded on the column.

Interestingly, on rechromatography the protein peak eluted with the pH 3.5 solution emerges in its entirety with water elution at the position of the first peak. The

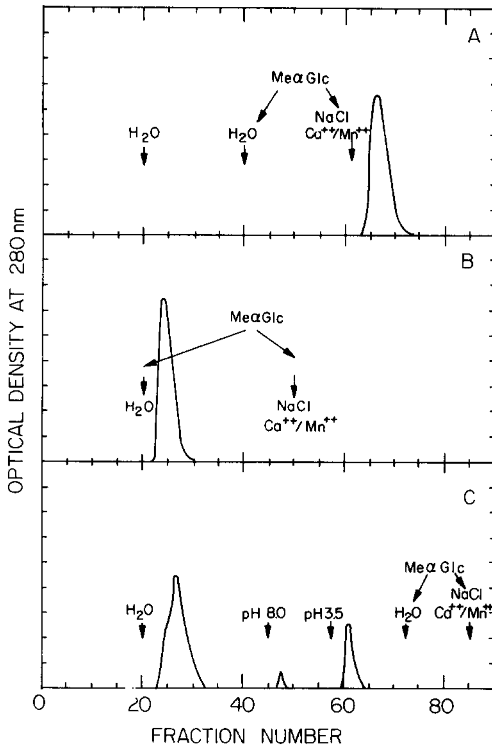

Fig. 5. Affinity chromatography of concanavalin A on a maltosyl-Sepharose matrix. A, "native" maltosyl-Sepharose matrix; B and C, maltosyl-Sepharose matrix modified by conjugation with ethylenediamine residues. Eluent applied at arrows: (A) deionized water, methyl α-glucoside (MeαGlc) in deionized water, methyl α-glucoside in 150 m*M* NaCl; (B) methyl α-glucoside in deionized water, methyl α-glucoside in 150 m*M* NaCl; (C) deionized water (pH 6.5), deionized water (pH 8.0), deionized water (pH 3.5), methyl α-glucoside in deionized water, methyl α-glucoside in 150 m*M* NaCl. All the protein peaks eluted in C display a mannose-specific haemagglutinin activity. From Teichberg *et al.*[5].

finding of two Con A species eluting at neutral and acidic pH is in line with literature data indicating that acidic monomers and neutral dimers of Con A coexist as a non-equilibrium mixture[6].

DISCUSSION

The separation and isolation of proteins by affinity chromatography is based on their reversible binding to specific ligands immobilized on insoluble matrices. Inherent to the technique are the following three requirements: (i) the protein to be isolated should bind in a reversible fashion to its immobilized ligand with an affinity such that it can be retained on the affinity matrix while the other proteins are washed away; (ii) the protein should interact with its immobilized ligand and with the matrix with an affinity allowing its elution, by the free ligand or by other non-denaturating agents; and (iii) accompanying proteins should not bind to the matrix but, if retained, should not be

eluted from it with the same elution conditions applied for the protein specifically bound to the affinity matrix.

As these requirements are not always fulfilled, it is essential to understand and to be in a position to control the forces involved in the specific and non-specific interactions taking place between proteins and affinity matrices.

For all practical purposes, the specific interactions are all those which allow the selective retention of the protein on the affinity matrix. They therefore involve not only the strictly specific non-covalent binding of the protein to its immobilized ligand but also the less specific (hydrophobic and ionic) interactions either with the spacer arm (used to increase the distance separating the matrix and the immobilized ligand so as to prevent steric interferences on binding to the latter) or with the charged residues on the matrix. If the sum of these interactions produces a free energy of binding above 12 kcal/mol (corresponding to a binding affinity above 10^{-9} M), the elution of the native protein from the column may prove difficult and denaturating conditions may be necessary to dissociate it from the matrix. In such cases, the efficacy of the free ligand to cause the dissociation of the protein from the affinity matrix often depends on the mangnitude not only of the strictly specific but also of the non-specific interactions.

By their very nature, the non-specific interactions can affect proteins in an indiscriminate manner and therefore, when they do modify the efficiency of the protein separation process, it is of importance to be able to minimize their effects. The use of buffers with low ionic strength is effective in reducing the amplitude of the hydrophobic interactions whereas buffers with high ionic strength decrease the ionic interactions.

Although the presence of charged groups on the affinity matrix may contribute in some circumstances to the establishment of undesirable non-specific interactions, these groups may play, in other circumstances, a crucial and decisive role either in the binding of a protein to the affinity matrix or in its elution. Indeed, the forces of attraction between electrostatic charges of opposite sign on the matrix and on the protein will increase the overall protein-binding affinity in all the cases when the interactions of the protein with its immobilized ligand are too weak to allow the desired retention on the affinity matrix[3]. In contrast, the forces of repulsion between electrostatic charges of similar sign on the affinity matrix and on the bound protein will facilitate the elution of the protein to the extent that deionized water can be used as the eluent.

We have termed the latter process "affinity-repulsion chromatography" and have established some experimental protocols permitting its successful application[5]. The following discussion deals with some of its theoretical and practical aspects.

The physico-chemical mechanism of affinity-repulsion chromatography is based on the fact that the electrostatic free energy of a charged protein in contact with charged residues on the affinity matrix can be larger than, or at least of the same order of magnitude as the free energy of binding of the protein to its immobilized ligand.

In the successful cases when lectins are eluted with water from charged affinity matrices, the values of the electrostatic free energy must exceed the values of the free energy of lectin–saccharide binding which are of the order of 8.0 kcal/mol (corresponding to a binding affinity of 10^{-6} M) or less[7].

The value of the electrostatic free energy estimated here on the basis of its

counterbalancing relation to the free energy of binding has to be compared with the values available in the literature from theoretical calculation or experimental studies.

Using the Debye–Hückel theory, Tanford[8] calculated the electrostatic free energy of a 40 000-dalton spherical protein in aqueous solutions of various ionic strengths. Depending on the number of net charges on the protein (10–40), the values of the electrostatic free energy obtained varied from 2.5 to 39.6 kcal/mol in a 150 m*M* salt solution and from 6.7 to 108 kcal/mol in a 1 m*M* salt solution. In spite of the simplifying assumptions made in these calculations, the derived values, although approximate, suggest that the electrostatic free energy of a protein can attain a considerable magnitude in deionized water.

Using an experimental approach, Scopes studied, at various pHs, the binding of proteins to carboxymethylcellulose and derived values of the free energy of interaction between single charges on the protein and charges on the matrix[3,9]. Values between 0.14 and 0.95 kcal/mol per charge were obtained, depending on the molecular size of the protein studied. The smaller proteins have the strongest interactions per charge, as is expected from their tighter packing into the pores of the matrix and the shorter average distance that separates the charges on the protein and those on the matrix. Scopes further estimated that a free energy of interaction of 0.5 kcal/mol corresponds to an average distance of 5 nm between charges. As this distance is large with respect to the dimensions of a protein, one can expect the free energy of interaction to reach values higher than 0.5 kcal/mol when the affinity matrix is conjugated with spacers carrying their charges at distances from the bound protein shorter than 5 nm.

If one takes all the above data at face value, it is clear that a relatively small number of electrostatic charges placed on the affinity matrix at optimum interacting distances from those of the bound protein could produce, in salt-free solutions, an electrostatic free energy matching or even in excess of the free energy resulting from the binding of most proteins to their ligands, including the biotin–avidin complex, which displays a free energy of binding of about 20 kcal/mol (corresponding to a binding affinity of about 10^{-15} *M*)!

Evidently, the efficiency of affinity-repulsion chromatography depends on the strength of the Coulombic repulsion (*i.e.*, on the density and distance) between the electrostatic charges on the protein and those on the matrix. The density of charges on the matrix and their distance from the bound protein can be practically controlled by conjugation with charged arms of appropriate length, which we refer to here as "charge spacers".

Some careful thought has to be given to the choice of the charge spacers because, like the ligand spacer arms, they ought not to be hydrophobic. The main reason for avoiding hydrophobic spacers relates to the fact that the conditions of application of affinity-repulsion chromatography are similar to those of hydrophobic chromatography[10,11].

In hydrophobic chromatography, as in affinity-repulsion chromatography, proteins are applied to the hydrophobic matrix in aqueous solutions of relatively high ionic strength, to favour hydrophobic interactions, while they are eluted in low ionic strength solutions.

There are, however, significant quantitative differences between the two chromatographic procedures. In affinity-repulsion chromatography, the proteins are applied to the affinity matrix in monovalent ion solutions at concentrations that ought

not to exceed 80 mM, or may not exceed 20 mM if a divalent ion at 1 mM is also included. In hydrophobic chromatography, the proteins are generally loaded onto the matrix in a 0.5–4 M salt solution and are eluted either by lowering the ionic strength or the polarity of the eluent or by including a detergent in the eluent. In spite of these differences, it is advisible not to use hydrophobic charge spacers so as to minimize the hydrophobic retention of proteins on the affinity matrix.

Does the charged matrix used as support for affinity-repulsion chromatography act also as an ion exchanger? It does indeed, but without affecting the specificity and efficiency of affinity-repulsion chromatography. Once loaded in a salt solution providing counter ions, proteins possessing a net charge similar to that of the charge spacers will not be retained on the affinity matrix (with the exception of the protein that binds to the immobilized ligand) whereas the other proteins will be. The use of deionized water as eluent will increase the ionic interactions of the latter proteins, which will therefore remain tightly bound to the matrix. However, it is possible that these proteins, although not eluted, will neutralize the charge spacers and prevent the latter from playing their expected role in the affinity-repulsion process. It is therefore important to wash the matrix, after protein loading, with a high ionic strength salt solution in order to eliminate by ion exchange all the bound proteins.

Advantages of affinity-repulsion chromatography

The elution process is carried out in low ionic strength solutions or in deionized water. The process is faster than conventional affinity chromatography since the protein eluted does not have to be dialysed in order to remove the eluting free ligand or salts. The process is cheap, as there is no need for eluting ligand. Proteins that cannot be eluted from an affinity matrix because of the strength of their binding to the affinity ligand can be eluted either with deionized water or by the free ligand in deionized water. The process allows the separation of some of the isoelectric forms of proteins.

Disadvantages of affinity-repulsion chromatography

Chemical modifications of the matrix have to be performed in order to introduce onto it appropriate charge spacers. Prior knowledge of the isoelectric points of the protein to be purified or of its behaviour on ion exchangers is required in order to select the appropriate charge spacers. Problems may be encountered in applying the standard procedures for ligand coupling because of the presence of additional sites of reaction. The chemical coupling of the charge spacers subsequent to ligand coupling may be difficult and may require protection of the ligand. The length of the charge spacers may need to be adjusted in order to optimize the efficacy of the elution process. The process may not be effective in all cases and in particular when the immobilized ligand is of proteineous nature or contains multiple charges of opposite sign. In such cases, the application of hypotonic solutions may increase the protein–ligand interactions rather than decrease them.

Affinity-repulsion chromatography as a general process

Several examples can be found in the literature in which either low ionic strength solutions or distilled water were used for the elution of antigens from immuno-adsorbents. Svensson *et al.*[12] were the first to apply a low ionic strength elution method in the purification of dipeptidyl-peptidase IV. Vidal *et al.*[13] reported that phospho-

enolpyruvate carboxylase could be eluted with distilled water from a specific immunoadsorbent. A generalization of the latter procedure was then attempted by Bureau and Daussant[14]. They tested six different proteins with their corresponding immobilized immune sera and in all instances achieved elution with water of the adsorbed proteins with an efficiency above 65%. Danielsen *et al.*[15] made similar observations with five different enzymes solubilized from microvillar membranes. In all the above instances, no explanation was provided as to the molecular mechanism of this elution technique. We term it, by analogy, immunoaffinity-repulsion chromatography.

The successful applications of affinity-repulsion chromatography clearly demonstrate that the presence of appropriate charges on the affinity matrix may contribute significantly to the protein desorption process. The interactions between lectins and immobilized electroneutral saccharides present an ideal case, but the success of immunoaffinity-repulsion chromatography indicate that dissociation of a protein–protein complex can also be achieved. Nevertheless, the disadvantages outlined above set clear limits to the field of application of affinity-repulsion chromatography. Hence it does not replace but extends the range of applications of affinity chromatography.

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