Theory and Practice in Affinity Techniques

P. V. SUNDARAM
F. ECKSTEIN

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Edited by

P. V. SUNDARAM

and

F. ECKSTEIN

Max-Planck-Institut
für Experimentelle Medizin
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Preface

During April 1978 we organised an International Symposium on 'Theory and Practice in Affinity Techniques' at the Max-Planck-Institut für experimentelle Medizin, Göttingen. The symposium covered some of the more recent developments in Affinity Chromatography and Affinity Labelling. Affinity chromatography has been a subject of intense activity in the last decade and has now become a powerful technique in biochemical research and preparative methods. There have been several international conferences on this subject and countless number of papers, review articles and books published, some of which are listed at the end of this book. Many of these conferences have dealt in great detail with the primary objectives of Affinity Chromatography namely that of isolation and purification of biologically active molecules such as enzymes, antibodies, antigens etc. This symposium was directed towards some of the more recent developments in the special applications of Affinity Chromatography and for this reason not all the specialists in the field were invited to the meeting.

There have not been many conferences dedicated solely to Affinity Labelling. It is nevertheless accepted as a very valuable technique in obtaining vital information about the active site of biopolymers. We were fortunate to have some of the leading workers in this field participate in this symposium.

Other areas of intense activity are Radioimmunoassay and Enzyme Immunoassay, the former having been under development by pioneers in the field such as Yalow, Berson and Ekin for the past 30 years. Attempts are being made to modify these assay techniques to solid phase systems. This development is certainly inspired by the growth in the field of Immobilized Enzymes in the last decade and a half. These two topics were also covered in the Symposium. However, since the contribution on Enzyme Immunoassay did not arrive on time, a chapter based on the lecture on that topic is not included herein.

This book is a result of the lectures given at this Symposium. However, it is not gathered together like a usual 'Proceedings' volume which often tends to be a collection of divergent material. Since the lectures covered a rather wide range of subjects it was considered essential that the volume be edited in a different manner. Thus we

have included information abstracted from the Round Table Discussion look ahead to the future prospects of improving various techniques and overcoming specific problems. A summary of the discussions held on some of the rather controversial questions in Affinity Chromatography is given at the end as an Editor's Note.

Chapter 6 is on an important aspect of Affinity Chromatography which did not come up for discussion, but upon careful scrutiny it was found to be less written about and discussed among all the publications available to date. Thus upon consultation with the participants it was considered worthwhile to include a chapter on 'Relative Reactivities of Functional Groups on Ligands in the synthesis of Affinity Columns'.

Although Affinity Labelling is recognized as an important technique to obtain information on active sites of biopolymers, it seems to be difficult to discuss the area in general terms in great detail as in the field of Affinity Chromatography. Various investigators use this technique with increasing success as exemplified by the excellent contributions by the various authors in this book.

In conclusion, it was the general consensus of the participants, that rapidly advancing fields of investigation such as Affinity Chromatography and Affinity Labelling should be the subject of periodic intensive discussion such as this Symposium held at Göttingen.

We wish to thank the Max-Planck-Gesellschaft for the generous financial support and other facilities in organizing this symposium. We also thank Messers Pharmacia International, LKB Product Bromma and Syva International Palo Alto, for their financial contributions towards the Symposium.

Göttingen
July 1, 1978

P.V. Sundaram
F. Eckstein

Contents

Interfacial salting out and the ligand induced solubility shift: Another affinity technique in purification of proteins

Friedrich von der Haar

Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Göttingen, Germany.

The results presented in this article were obtained during our studies on tRNA, aminoacyl-tRNA synthetase interaction. At the beginning of these studies the methodology for purification of this group of proteins was only very poorly developed. Usually three months purification work was followed by one month investigation with the isolated material. Consequently the attempt was to reverse this time schedule into the opposite or an even better relation. During these studies it became apparent that the underlying principles of purification proved to be useful for proteins in general though the bulk of the data were obtained with aminoacyl-tRNA synthetases.

Before I describe the salting out procedure, I would like to recall several experimental results obtained by the method we called "affinity elution" [1,2], since these results are directly related to the data presented later. Affinity elution involves the adsorbtion of a protein mixture to an ion exchanger in an unspecific way followed by the specific release of the desired protein with a specific ligand. The reason for the desorption of a specific protein is that the protein ligand complex is unable to bind to the ion exchanger whereas the free protein binds. tRNA proved to be especially useful in eluting aminoacyl-tRNA synthetase from a cation exchanger. Obviously, the many negative charges of the phosphodiester backbone of the tRNA change the net positive charge of the free enzyme in such a way that the complex cannot bind further to the negatively charged cation exchanger.

Surprisingly this procedure was much less specific than expected in view of the extremely specific aminoacylation of the individual tRNA [3]. Figure 1 demonstrates that down to a very low concentration the non-cognate $tRNA^{Val}$ is as efficient as the cognate $tRNA^{Phe}$

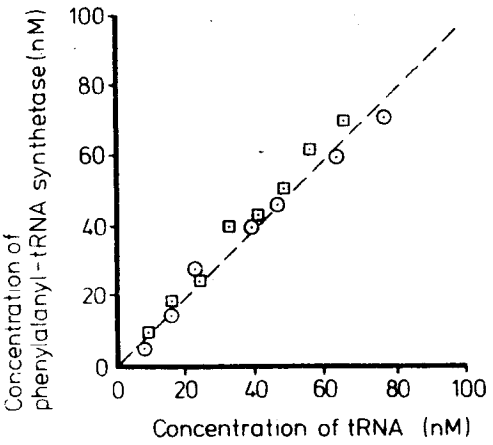

Fig. 1: Affinity elution of phenylalanyl-tRNA synthetase with cognate $tRNA^{Phe}$–C–C–A⊙ and non-cognate $tRNA^{Val}$–C–C–A⊡. Homogeneous phenylalanyl-tRNA synthetase is adsorbed on to a 1 ml phosphocellulose column equilibrated with 0.03 M potassium phosphate buffer at pH 6.0. The column is successively washed with tRNA solutions of increasing concentration. The enzyme concentration eluted with each wash is determined in the aminoacylation assay. Down to the lowest concentration measurable 1 molecule of cognate as well as non-cognate tRNA elutes one enzyme molecule.

in eluting purified phenylalanyl-tRNA synthetase from phosphocellulose. One tRNA-molecule is always able to elute one enzyme molecule from the resin.

Specificity on purification of protein mixtures arises from the fact [3] that cognate $tRNA^{Phe}$ binds slightly better than the non-cognate $tRNA^{Val}$ and hence the [E^{Phe}·$tRNA^{Phe}$] complex is eluted first followed by other enzymes as demonstrated in Figure 2. In practice it turned out that for these reasons affinity elution gave much better results with partially purified enzyme fractions than with crude extracts.

In view of the above an alternative purification procedure was desirable which should exhibit the following characteristics:

(a) Like the modern ion exchange resins it should permit the processing of large amounts of protein in a simple and reproducible manner

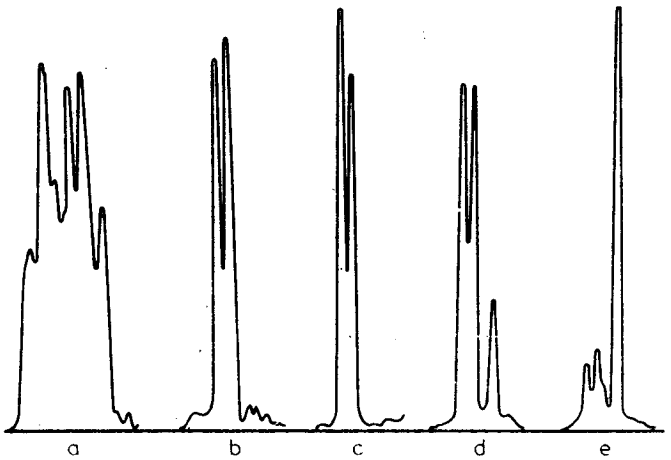

Fig. 2: Dodecylsulfate gel electrophoresis of proteins eluted during affinity elution. 280 mg protein of a partially purified phenylalanyl-tRNA synthetase fraction were adsorbed onto a 2.4 x 11 cm phosphocellulose column. The column was washed with buffered $tRNA^{Phe}$ solution (0.5 A_{260}-units/ml). 7 ml fractions were collected and aliquots of the total protein applied to the column (a) as well as of the first four fractions (b-e) were subjected to gel electrophoresis. Fractions one and two show the two bands of homogenous phenylalanyl-tRNA synthetase. Beginning with fraction 3 a new band appears which is the predominant protein in the fourth fraction.

yielding purification factors of 10 – 50 by simple stepwise elution techniques.

(b) The principle of separation, however, should be completely independent of ionic interactions in order to be a real alternative to ion exchange chromatography.

(c) It should be applicable to proteins in general.

It may appear that hydrophobic chromatography may satisfy several of these requirements. However, hydrophobic chromatography as described by Shaltiel [4], e.g. using low salt concentrations, has the disadvantage that it works only for few enzymes and the procedure has to be optimized in each individual case.

There is, however, a modification of hydrophobic chromatography, which lacks these disadvantages [5-8]. At high salt concentration slightly below that needed to precipitate a protein out of solution, one can salt the protein out on the surface of the so-called amphiphilic gels. These are hydrophilic gels of the Sepharose type which are substituted with lipophilic groups. If the salt concentration is subsequently reduced, the individual proteins can be released from the resin by the process of salting in.

The binding forces retaining the protein on the column are attributed to weak hydrophobic interactions which are stabilized by the high salt concentration. This interpretation is puzzling since all proteins investigated seem to exhibit this phenomenon, whereas not all proteins, whose tertiary structure is known, show accessible hydrophobic regions on the surface [5-8]. Furthermore the salt concentration needed for salting in of the individual proteins from the resin parallels their solubility in salt solution [5-8] rather than the number of accessible hydrophobic areas on their surface [9]. Hence the question is whether the binding forces are really of a hydrophobic nature or whether the binding process is due to other forces. From the arguments given here it appears, that binding should at least not primarily be due to hydrophobic forces. If this is so, then an analogous salting out should take place on an unsubstituted hydrophilic gel like Sepharose 4B itself.

Salting out on unsubstituted Sepharose 4B

There is a single report in the literature that an attempt to salt proteins out on the surface of Sepharose 4B failed [10]. However, the experimental evidence given in that report is not very strong and it could be shown experimentally, that under proper conditions proteins can in fact be salted out on unsubstituted hydrophilic gels [11].

Phenylalanyl-tRNA synthetase (E.C. 6.1.1.20) can be precipitated out of solution at an ammonium sulfate concentration above 50% saturation. On passing a protein fraction enriched in phenylalanyl-tRNA synthetase and saturated to 47% with ammonium sulfate over a Sepharose 4B column, which is equilibrated with the same salt concentration, the protein is nearly quantitatively adsorbed to the column. During this procedure the originally transparent gel becomes progressively opaque due to the binding of protein [11]. From the opaque area and the amount of protein adsorbed the capacity of the column is calculated to be 40-45 mg of protein per ml bed volume, which is quite high and permits the processing of large amounts of protein. The adsorbed proteins can be individually salted in by running a reverse ammonium sulphate gradient. As seen from Figure 3 the protein in general as well as the enzyme activities tested are very well resolved.

Probable binding process

A few remarks regarding the probable binding process may be helpful

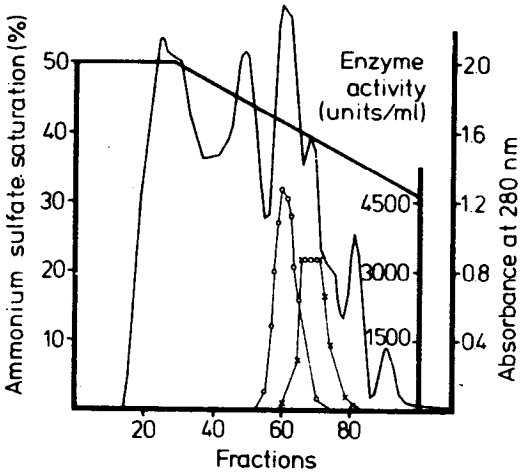

Figure 3: Fractional salting in of phenylalanyl-tRNA synthetase (0-0) and isoleucyl-tRNA synthetase (x-x) with ammonium sulfate. Absorbance at 280 nm. Note, that the decrease in salt concentration is 1.75% relative saturation per 100 ml gradient volume. If it is reduced to 1.25% decrease in saturation per 100 ml gradient volume the enzymatic activities tested are separated almost quantitatively.

in order to further optimize the procedure in a rational way. Since in contrast to the amphiphilic gels there are no hydrophobic groups on Sepharose 4B one cannot invoke hydrophobic interaction in binding as the exclusive determinating factor. One alternative which remains is that on interaction with the surface of the resin the solubility of the protein is reduced. How can this happen? It seems reasonable to speculate that the hydroxyl groups of the sugar moieties of the gel replace water molecules of the hydration sphere of the protein. As a consequence the solubility of the protein is reduced and it is forced to settle out on the resin. The interpretation agrees with results obtained by Wilchek and Miron [12].

From the capacity of the gel one can roughly estimate that a monomolecular film of protein is built up. This may explain the high resolution obtained, which exceeds precipitation out of solution by a factor of 5-8. The intermolecular protein interactions, which occur during precipitation out of solution and cause precipitation across a broad salt concentration are probably prevented in the film formation. Hence at any region of the gel surface an ideal or nearly ideal solubility equilibrium is established.

Mevarech *et al.* [13] independently found that they could salt out proteins from an extreme halophile bacterium on Sepharose 4B. In

view of the reports [10] that proteins from other sources should not bind to unsubstituted Sepharose 4B they speculated about some special properties of proteins from extreme halophiles such as for instance the extraordinarily high concentration of negatively charged aminoacids. In the light of our results there is nothing special about the halophile in this respect. It just reflects the behaviour of proteins in general. This justifies further careful thought about this type of interaction not only from the point of view of purification of proteins but also with reference to the structural organization of proteins within the living cell.

The procedure

To perform the whole purification one must first fix the protein to the resin. We found three useful ways of achieving this:

(a) The salt concentration at which the protein to be purified starts to precipitate out of solution is determined. The protein solution is adjusted to a salt concentration just below that needed for precipitation out of solution. A column of an appropriate size is filled with Sepharose 4B and equilibrated with a buffer containing the same salt concentration as that of the protein solution. One or two column volumes are enough for equilibration of the column. The protein solution is then poured over this column. In this way large volumes of solution can be processed and the procedure can even be used to concentrate dilute protein solutions. Half of the maximum capacity of the column can be successfully used for satisfactory purification. Since the maximum capacity is 40-45 mg per ml bed volume the column size should be 1 ml bed volume for 20 mg of protein to be bound. If the column is loaded to a higher extent the elution peak tends to be broadened, hence the resolution is less definitive. If, however, the main objective is processing of large amounts of protein then even up to 30-35 mg of protein may be applied per ml bed volume and a reasonable resolution is still obtained.

(b) If the solubility of the protein in salt solution is not known a batchwise procedure may be used. Protein and resin are mixed together and salt is added to such a concentration until all the protein from the supernatant is bound. The resin is poured into a column and the salt gradient can be started.

(c) A third variation is useful, if one has a concentrated solution of small amounts of protein. In this case the column is equilibrated with a salt solution sufficiently concentrated to achieve binding. To the protein solution is added saturated salt solution until the first turbidity just appears. Then the solution is poured over the column. Since the gel in the column is equilibrated with high salt concentration

the protein solution is concentrated during its passage and a point is reached where the protein precipitates. The bed volume in this case should be at least ten times that of the protein solution applied.

In order to elute the protein a linear gradient with decreasing salt concentration is successfully used. Working with ammonium sulfate we found a decrease of 1.25% in saturation per 100 ml gradient volume appropriate, which is equal to a decrease of about 5 mmoles per 100 ml.

Under these conditions about 90% of a particular protein is eluted within 250 ml. Hence two proteins are separated from each other to the same extent if they are salted in at a 3-3.5% difference in salt saturation.

In certain cases it may be advantageous to run a steeper gradient and to collect smaller fractions in order to obtain a good resolution. However, in order to avoid inhomogenities because of the relatively large density differences of the salt solutions the gradient should not be too steep.

After running the gradient and determining the position of the enzyme by the usual tests one has to recover the protein. If the protein concentration is greater than 0.3 mg/ml, the easiest way is to add an equal volume of saturated salt solution to the protein solution. By this procedure the salt concentration is increased to such an extent that the protein precipitates out of the solution and can be collected by centrifugation. Alternatively saturated salt solution is added to such an extent that a slight turbidity appears. The solution is then passed over a small salt equilibrated Sepharose 4B column and the adsorbed protein is released from it by a stepwise decreased salt concentration to below that needed for adsorption. By this procedure even a very dilute solution can be concentrated. To regenerate the column it is washed with 5% buffered ammonium sulfate solution in order to release any residual protein. It can then be equilibrated with the desired salt concentration and can be reused 10 times or more.

Variables

Since the separation process is based on solubility all the variables originally developed for precipitation of proteins out of solution may be useful [14]. These are in particular temperature, pH and different types of salt. Table 1 summarizes some results obtained by variation of these parameters.

Whereas with ammonium sulfate only a slight dependence on variation of pH is observed, with potassium phosphate at pH 7.0 a much higher concentration for salting out is needed than at pH 9.4. This indicates that the doubly charged phosphate ion, the predominant

form at pH 9.4, is much more efficient in salting out than the singly charged phosphate ion, which is predominant at pH 7.0.

TABLE 1

Effects of Salt and pH Variation during Salting Out of Crude Extract of Phenylalanyl-tRNA Synthetase from Baker's Yeast

	Total Protein mg	Spec. Activity units/mg	Total Activity units	Purification	Yield
I Crude Extract	70 000	1.6	114 000	1	100
II 47% $(NH_4)_2SO_4$ at saturation	38 200	2.9	113 000	1.8	99
III Salting Out with $(NH_4)_2SO_4$ at pH 7.5	3 500	17.3	60 000	10.7	53
IV Salting Out with $(NH_4)_2SO_4$ at pH 5.5	1 570	48.7	70 000	30.2	67
V Salting Out with K_2HPO_4 at pH 9.4	594	45.5 [a] 104.0 [b]	27 000 62 000	65	54

a) Prior to removal of potassium phosphate.
b) After removal of potassium phosphate, 3 kg pressed yeast cells were disrupted and debris removed by centrifugation. Salting out (step III) was performed on a 500 ml Sepharose 4B column as described [11]. Rechromatography (step IV and V) was performed using a 100 ml Sepharose 4B column running a gradient from 50-25% ammonium sulfate (IV) or 1.8–0.8 M potassium phosphate (V) respectively.

Advantages

The method is applicable to proteins in general. It has been used for the purification of proteins from bacteria and yeasts (Dr. H. Sternbach, Dr. M. Sprinzl, Göttingen, private communication). It turned out to be especially useful in purification of toxins (Dr. C. Katsaras, Berlin, private communication) and separation of immunoglobins according to hapten specificity (Dr. G. Stöffler, Berlin, private communication). Since the separation process is principally different from ion exchange and gel permeation chromatography it is a real alternative if these methods fail.

The process is remarkably reproducible. If the protein source is identical the elution profiles look identical too. Once the position of an individual protein has been determined, it will appear always in the same position, hence it can be isolated with only very few tests.

Proteins turn out to be extremely stable in the high salt concentration used for the process. In particular protease attack is almost completely suppressed. This was especially useful for our work with

yeast enzymes where originally we had serious difficulties in overcoming the degradation by proteases. In contrast to ion exchange chromatography where proteases tend to smear out over the entire elution profile, in this procedure they appear as nicely resolved peaks like all the other proteins. This is probably due to the fact that [protease·protein] complexes are dissociated at high salt concentrations. As a consequence the proteins to be purified are recovered in high yield.

Pitfalls

In some cases, especially with salt sensitive enzymes at low concentration, it may be difficult to localize a protein within the elution profile, because too much salt is introduced into the assay solution with the aliquots to be tested. Aliquots of the solution must then be desalted prior to assay.

In addition, in several instances we observed a more or less dramatic reversible denaturation of proteins. This leads to a partial loss in enzymic activity when tested immediately after chromatography. It returned, however, after prolonged dialysis against normal buffer table). In at least one case, alkaline phosphatase from E.coli, this behaviour could be rationalized. In this case we observed a loss of activity down to only a few per cent, which returned very slowly on dialysis. Phosphatase is a Zn^{++} enzyme and hence the high concentration of ammonium ions in the gradient might compete for the Zn^{++} ions resulting in loss of activity. Indeed the addition of a constant concentration of 10^{-4} M $ZnSO_4$ to the ammonium sulfate gradient prevented loss of activity completely.

The ligand induced solubility shift

The question arises whether the system can be modified in such a way that it is turned into a real affinity method. The answer is in principle 'yes'. In the salting out method as described an isolated protein fraction is characterized by *identical solubility* of all its components. If one could modify the solubility of just one of the protein components of a given protein fraction by a particular manipulation then the component should be shifted and appear either in front or behind the main peak on rechromatography. It is known from crystallography that a slight change in solubility is observed on complexation of enzyme with ligands. Since the resolution of the salting out method is 3-3.5% relative salt concentration, a solubility shift of 5% would be more than enough to achieve a useful further purification.

The complex of $tRNA^{Phe}$ with phenylalanyl-tRNA synthetase is salted in at about 5% lower salt concentration as compared to the

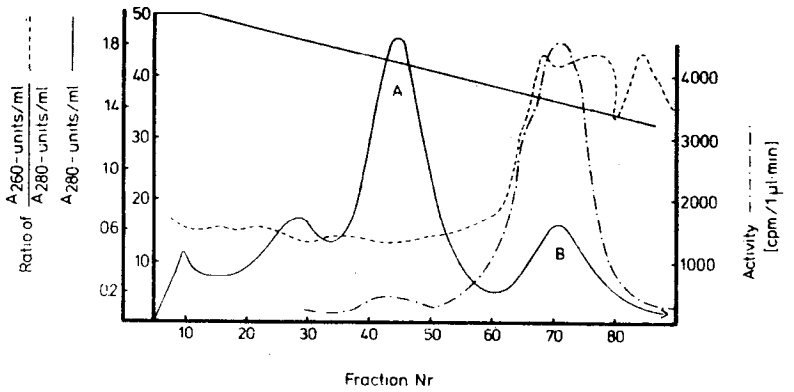

Fig. 4: $tRNA^{Phe}$ induced solubility shift of phenylalanyl-tRNA synthetase. The phenylalanyl-tRNA synthetase fraction obtained from a salting out chromatography (see figure 3) was mixed with 210 A_{260}-unit $tRNA^{Phe}$. The mixture was then rechromatographed running an identical gradient. Total protein was monitored by measuring the UV absorption at 280 nm. tRNA can be located due to its high value of the ratio of UV absorption at 260 and 280 nm. Phenylalanyl-tRNA synthetase was determined in the aminoacylation assay. The appearance of the phenylalanyl-tRNA synthetase is shifted due to the fact, that the solubility of the complex is lowered by about 6% as compared to the free enzyme.

free enzyme. Therefore the phenylalanyl-tRNA synthetase fraction obtained by a first salting out process in the absence of $tRNA^{Phe}$ (Figure 3), is split into two main components on rechromatography in presence of $tRNA^{Phe}$ under otherwise identical conditions (Figure 4, peak A and B).

An enzymatic test shows, that no phenylalanyl-tRNA synthetase activity appears with peak A (Figure 4) where it was salted in in the absence of $tRNA^{Phe}$. Activity is completely shifted into the protein peak B that newly appears behind the main peak A. Analysis for tRNA indicates that part of it appears together with the second protein peak followed immediately by excess of free tRNA. If one works with an excess of enzyme over $tRNA^{Phe}$ then part of the activity appears in the first and part of it in the second peak. Analysis of enzyme to tRNA ratio shows that one enzyme molecule coelutes with two tRNA molecules. The phenylalanyl-tRNA synthetase in the second peak is almost homogenous.

Analogous experiments with isoleucyl-tRNA synthetase show that in this case an [enzyme·tRNA] complex can also be chromatographed. Because in this case enzyme and [enzyme·tRNA] complex are salted in at almost the same salt concentration, the complex is shifted from