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STRATEGIES FOR ENZYME ISOLATION USING DYE-LIGAND AND RELATED ADSORBENTS

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SUMMARY

Approaches to the use of a large range of dye-ligand and similar adsorbents for protein isolation are described. The adsorbents behave in a similar fashion relative to each other with a variety of different applied protein mixtures, so a system of dividing the adsorbents into five groups according to their protein-binding ability has been adopted. In this way it becomes unnecessary to screen all dyes, and an ideal selection of dyes and conditions can quickly be achieved.

INTRODUCTION

Following the discovery that certain kinases were attracted to Blue Dextran [1, 2], the use of dyes as ligands in affinity chromatography has developed steadily. Yet it was a full decade before there were reports that dyes other than Cibacron Blue F3-GA had strong affinity for proteins [3]; since then it has become apparent that virtually any complex organic molecule will bind to proteins, and is potentially of value as an adsorbing ligand in chromatography. Dyestuffs are particularly useful because their structure is dictated by a requirement that they have an affinity for biological macromolecules; these structures usually involve fused aromatic rings, with charged groups to confer aqueous solubility, and a variety of side-groups which may make electrostatic, hydrogen-bond or hydrophobic interactions with the biological substrate. These are just the sort of interactions that are most important with proteins, so it is not surprising that dyes have become important adsorbent ligands in protein chromatography.

Reactive dyes were first introduced to overcome the problem of lack of fastness, the tendency for a cloth to lose its dye. Covalent bonds with the material ensure that the dyes remain attached in normal treatment. Such dyes are ideal for the protein isolation chemist, as they are not only excellent,

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selective adsorbents, but also have a built-in chemistry, which by-passes most of the tedious and hazardous reactions involved when coupling ligands to the matrix in the normal process of affinity chromatography.

A chromatographic procedure has two main potential steps in purifying a protein: at the adsorption stage and during the elution procedure. A large number of the reported usages of dye-ligand adsorbents have not optimised either of these steps. Only by screening a range of dye adsorbents can the best one be selected for the adsorption phase, and then it may be necessary to devise a two-column negative–positive system so that the column adsorbing the protein required does not bind too much other protein. This optimises the adsorption stage. For elution it is usual to employ a step-wise process, as gradients (especially in salt concentration) are often not very selective. Optimum elution is normally achieved by an affinity method in which the addition of a substrate or other specific ligand in the buffer causes displacement of the protein [4].

At the 4th International Symposium on Affinity Chromatography in Eindhoven, Hey et al. [5] demonstrated the use of a large number of dyes to select suitable adsorbents for purifying two dehydrogenases. Each scheme involved choosing a dye that bound much protein, but not the enzyme (the negative column) followed by a dye that bound the enzyme but not much protein (the positive column). Such is the selectivity of the interactions between the different dyes and various proteins that it is often possible to choose a negative column that binds more protein than the positive column, despite the affinity for the protein in question being the reverse. We and others have since developed a number of such procedures for the isolation of enzymes from bacteria [6–10] and from other sources; development of each procedure initially requires a screening process to choose the optimum dye-ligand adsorbents. This can be a time-consuming process with 60 or so dyes, and a plethora of possible buffer conditions. It has been necessary to adopt strategies to minimise the development work, without missing a potentially useful dye or elution scheme.

GENERAL PROCEDURES AND RESULTS

Matrix for coupling dyes

The majority of successful dye-ligand adsorbents have been made on an agarose matrix, with sufficiently large pore size to accommodate all but the largest proteins. Agarose gel beads of 4 or 6% solid content are most commonly used. Beads with smaller pore size have more rigidity and so can be operated at higher pressures, but larger proteins may be excluded from these beads. This can be operationally useful, if the size of the desired protein is known to be small; a gel exclusion step can then be combined with the dye-ligand chromatography. Matrix materials that are not sufficiently porous to accommodate proteins internally are not suitable even though they may take up dye very strongly. Most gel permeation bead materials made for protein separations are suitable (with the proviso that the pore size is large enough) and commercial materials such as Sephadex, Sephacryl and Sepharose (Pharmacia), Ultrogel and Trisacryl (LKB), Biogels (Bio-Rad Labs.) and Cellufines (Amicon) have all been successfully employed. We routinely use Sepharose CL-4B which has a

very open structure for large proteins but is cross-linked for rigidity. For small proteins (< 100 000 daltons) the cheaper Cellufine GC-700 has been satisfactory. Cellulose-derived matrices give a more permanent dye attachment; slow dye leakage can be a problem with some dichlorotriazinyl dyes on agarose.

Grouping of dyes

All dye adsorbents are prepared by a standard technique [11]. In addition, other complex chemicals can be attached to the matrix by standard affinity chromatography chemistry, and screened along with the dye adsorbents. They can be classified into five groups according to their ability to bind proteins in a simple buffer. We have used extracts of *Zymomonas mobilis*, yeast, rabbit muscle and rat liver, and based the classification of the dyes on the average results of these protein mixtures. Generally, a dye that falls into group N for one extract will be in group N for another, but sometimes in $N-1$ or $N+1$. Similarly when using different buffers for the same type of extract, even

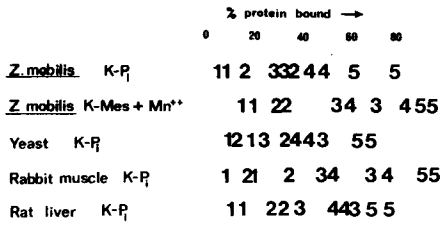

Fig. 1. Amounts of protein binding to dyes in groups 1—5. Group 1 dyes bind the least, group 5 the most protein. The percentage bound was determined from the difference between the amount applied (30 mg to 2-ml columns) and the non-adsorbed fraction washed through with 5 ml buffer (in each case pH = 6.5, ionic strength = 0.05—0.08). Group 1 columns were Yellow MX-8G and Blue MX-7RX; group 2 columns Red MX-5B and Blue MX-R; group 3 columns Red H-E3B and Blue MX-4GD; group 4 columns Red H-3B and Red 3-BA; group 5 columns Blue H-ERD and Red H-E7B. With the exception of Cibacron Red 3-BA, these were all I.C.I. Procion dyes; all were attached to Sepharose CL-4B by the method of Atkinson et al. [11].

TABLE I

GROUPING OF DYES

Group 1 dyes bind the least protein from crude extracts of tissues, and group 5 dyes the most. Actual groups may vary ± 1 with different types of extract. P = I.C.I. Procion; C = Ciba-Geigy Cibacron; R = Hoechst Remazol; D = Sandoz Drimarene. Not all of these dyes are still commercially available.

Group 1	Group 2	Group 3	Group 4	Group 5
P Blue MX-7RX	R Black GF	P Blue H-EG	P Black H-EXL	P Blue H-ERD
C Blue 2-RA	P Blue MX-R	P Blue H-EGN	P Blue H-GR	C Blue F-R
R Orange 3R	P Brown MX-GRN	P Blue H-4R	P Blue MX-G	P Brown H-5R
P Red MX-2B	C Brown 3-GRA	P Blue MX-3G	P Blue MX-4GD	P Green H-4G
P Rubine H-BN	P Navy H-4R	C Blue F3-GA	D Blue K-BL	P Green H-E4BD
P Turquoise H-A	P Orange MX-G	R Blue B	P Brown H-3R	P Navy H-ER
P Turquoise MX-G	R Orange FR	R Blue R	P Brown MX-5BR	P Red H-3B
C Turquoise 6-GE	P Red MX-5B	C Navy F-2R	P Orange H-ER	P Red H-8BN
R Violet R	P Scarlet MX-G	P Red H-E3B	P Orange MX-2R	P Red H-E7B
R Yellow GNL	P Scarlet MX-3G	P Rubine MX-B	P Red MX-7B	P Scarlet H-E3G
P Yellow H-A	C Turquoise GFP	P Scarlet H-2G	P Red MX-8B	P Yellow H-E3G
P Yellow MX-6G	C Yellow R-A	P Yellow H-E6R	C Red 3-BA	P Yellow H-E6G
P Yellow MX-8G	P Yellow MX-3R	P Yellow H-5G	P Violet H-3R	P Yellow H-E4R
	P Yellow MX-4R	P Yellow MX-R	P Yellow H-E6R	P Yellow MX-GR
		C Yellow 3-GP		

though the total amounts of protein binding may change, the relative amounts remain similar (Fig. 1). Thus a screening of 50 columns with *Z. mobilis* extract in 20 m*M* phosphate buffer, pH 6.5, resulted in group 1 columns binding 5—10%, and group 5 columns binding 45—60% of the protein. But in potassium—morpholinoethane sulphonate (Mes) buffer of similar ionic strength and pH, containing both Mg and Mn ions, group 1 columns bound 15—25% and group 5 columns 70—85% of the protein. This illustrates that not only do dyes behave consistently relative to each other, but also that the buffer composition can change the adsorptive properties considerably. The groups of some 70 dyes we have investigated are listed in Table I.

Choice of buffer

We normally use one of two standard buffers in the pH range 5.5—7; the lower pH maximises binding [12]; higher pH values may be more appropriate for some stronger-binding proteins. The normal approach is to screen at pH 6.0 in a phosphate buffer which has been made by lowering 30 m*M* dipotassium hydrogen phosphate with a mixture of 0.5 *M* succinic acid in 2 *M* acetic acid. This buffer system can be used at all pH values from 8 down to 4.5, retaining sufficient buffering power over the range. We also add 30 m*M* sodium chloride (to increase the ionic strength, thus lessening cation-exchange behaviour) and 2 m*M* magnesium chloride (to increase protein binding to dyes; also to stabilise some enzymes). This is a cheap buffer which can be used in large-scale methods. The other buffer consists of 10 m*M* potassium hydroxide adjusted to the pH with Mes, usually to pH 6.0 or 6.5, and including 30 m*M* sodium chloride and 2—5 m*M* magnesium chloride (and sometimes manganese chloride). This more expensive buffer is suited to laboratory scale processes; more proteins bind when using it than when using phosphate-containing buffers. It is mainly used in the circumstances when no dye is fully satisfactory in retaining the enzyme in question in phosphate buffers. For convenience of discussion, from here the word enzyme will be used for the protein being purified, even though it may not be an enzyme.

If after the initial screening it is found that the enzyme binds to all, or four out of the five groups of dyes, it may be desirable to choose conditions that are less favourable for protein binding. Both pH and ionic strength may be increased, and phosphate included, so that the enzyme wanted binds only to columns in the lower groups. Omission of divalent metal ions is also very effective in decreasing the amounts of protein binding. Most of the protein in the extract will then not bind, so even without a negative column a substantial purification can be achieved at the adsorption stage. At the other extreme it may be that the enzyme does not bind to any column in the initial screening. In that case, choice of a buffer to maximise protein binding is necessary, i.e. no phosphate, low pH and ionic strength, and divalent metal ions present [13]. If this succeeds in allowing the enzyme to bind to a group 5 column, a negative column in group 5 or 4 will be needed to maximise the purification at the adsorption stage. In general it may be stated that the stickier an enzyme is, the lower the group of dye needed in the purification scheme.

Loading and column capacity

Dye-ligand adsorbents have a quite high capacity for protein binding. It is fortunate that those dyes that bind most types of protein (i.e. group 5) also have the higher capacity for protein. As a result the amount of protein applied to dye columns can be the same for all groups (Table II). A group 1 column may be saturated with 3 mg/ml, achieved by adding 30 mg/ml with only 10% of the proteins binding. A group 5 column in the same conditions may not be saturated until 24 mg/ml, also achieved by adding 30 mg/ml, with 80% of the proteins binding. We usually screen with a loading of 15—20 mg/ml, which rarely amounts to overload, but saturates about half of the column. The loading is very critical with dye adsorbents; whereas overloading is self-explanatory, underloading compared with a previous run often results in difficulties in achieving elution by a scheme developed previously. Low loading of a column probably results in a bigger proportion of the proteins being held by the tighter affinity sites in the column (perhaps more than one dye molecule interacting with the protein), so it needs more vigorous conditions to elute the desired fraction. This feature cannot be overstressed; inconsistent loadings of protein is one of the main reasons for irreproducibility in dye-ligand procedures. Sometimes it has been found prudent to ignore all these rules. Using a group 4 or 5 dye with a strongly binding enzyme, it may be possible to apply huge amounts of extract; if the dye has enough affinity for the enzyme, it can select this enzyme and exclude most of the other proteins which would bind under normal loading regimes. It is a property of the enzyme itself that allows such an approach to succeed; notable examples are the isolation of yeast AMP kinase [14] and of *Alcaligenes eutrophus* lactate dehydrogenase [15], each of which has very high affinities for dyes in general.

TABLE II

SATURATION CAPACITIES AND PROTEIN BINDING OF SOME SELECTED DYE COLUMNS

Buffer: 20 m*M* potassium phosphate, pH 6.5. Saturation capacities were determined by adding 50 mg *Z. mobilis* extract to each 1 ml of column and measuring by difference the amount of protein bound. Normal percentage binding, on which groups were determined, involved adding 15 mg *Z. mobilis* extract to each 1 ml of column and measuring by difference the amount of protein bound. All the quantities are buffer-dependent. In some buffers, group 5 columns may bind up to 25 mg/ml.

Dye	Group	Percentage binding from 15 mg/ml	Amount binding from 50 mg/ml (mg)	Amount to add to achieve saturation (mg/ml)
Blue MX-R	2	11	3	27
Blue H-EG	2	13	3.5	27
Yellow H-5G	3	37	9.5	26
Yellow MX-3R	3	39	11	29
Blue F3-GA	4	44	14	32
Red 3-BA	4	42	14	32
Scarlet H-E3G	5	53	13	25
Green H-E4BD	5	55	13	24

Flow-rate

Flow-rates in protein chromatography are always a compromise between the theoretical ideal (very slow) and the practicalities of the system, which on a large scale introduce economics into the equation. Because proteins have low diffusion coefficients, linear diffusion is a negligible factor at flow-rates above about 1—2 cm/h [16]; dye-ligand columns are typically operated ten to twenty times faster than this. Full equilibration of the passing protein with the adsorbing sites in the beads is not achieved at this flow-rate, and the higher the flow-rate the less ideal is the column's operation. Loadings and flow-rates are inter-related, and it is important to keep them consistent. Small trial columns may flow rather fast under gravity, so when it comes to scaling up, different results at a lower linear flow-rate may be expected. We routinely operate at 20—30 cm/h at 20—25°C; lower rates (10—15 cm/h) are needed in the cold as diffusion coefficients are lower.

With labile enzymes speed can be a vital factor, so short, squat columns are often preferable to long, thin ones. As most applications are on—off procedures the extra chromatographic separation theoretically possible with a long column is not relevant; in any case, a large proportion of the adsorbent is loaded with protein at the start, so true chromatographic processes are not normally involved.

Screening procedure to select suitable dyes

Five dyes chosen at random from the five groups (the Amicon 5-dye screening kit is suitable) are set up in 2-ml columns and equilibrated with the chosen buffer. The amount of protein bound is determined by difference rather than by elution, as some group 5 columns bind protein very tenaciously. Similarly the amount of enzyme bound may be determined by difference, though it should be ascertained that adsorbed activity can subsequently be eluted. On the basis of this screening, at least two groups of dyes can be excluded from further considerations. For instance, if the enzyme binds to all

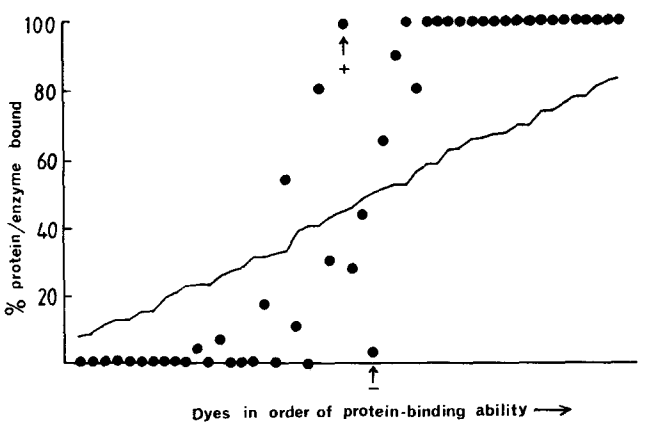

Fig. 2. Typical results for a total screening of a set of 49 dye columns, measuring percentage protein bound (solid line) and percentage enzyme bound (•). Dyes are arranged in order of percentage protein bound; the enzyme in this case was fructokinase EC 2.7.1.4 from *Z. mobilis*.

five dyes, then only group 1 needs to be investigated further (unless it is decided to change the buffer conditions: see above). If the enzyme binds to groups 5 and 4, partly 3, and not at all to group 2 or 1, then groups 1 and 5 may be discarded from further consideration. The object is to find two columns, one which binds as much protein as possible without binding the enzyme, the other which just holds onto the enzyme, and as little protein as possible, so that it may be eluted with only a slight change to the buffer conditions. In the latter example it may be possible to find a group 3 as a negative column, and a group 2 as positive (or groups 4 and 3, respectively). The next screening takes as many as possible of the columns around the adsorption point to build up a more detailed knowledge of the enzyme's behaviour. An example of a complete screen of 49 columns is shown in Fig. 2.

Having chosen both negative and positive columns, the next stage is to concentrate on the elution procedure. Five or more 2-ml positive columns are set up, and a larger negative column (10 ml or more) to provide non-adsorbed fraction for application to the 2-ml positive columns. Having bound the enzyme to each of the positive columns, various elution schemes are tried.

Elution procedures

Elution may be by either biospecific means or non-specific. If binding of the enzyme to the dye is significantly through the natural substrate's binding site, then addition of the substrate can cause displacement, so affinity elution is achieved. In practice this means that a small proportion of the enzyme must be desorbed (the partition coefficient, defined as the proportion adsorbed at any instant, should be < 1) so that it can bind the substrate; the equilibrium between adsorbed and desorbed is disturbed and more is desorbed (Fig. 3). In theory and in practice, for an effective elution, shifting the partition coefficient from around 0.95 to below 0.5 requires 0.5—1 column volumes of substrate at about ten times K_d (its dissociation constant) for a monovalent enzyme, but substantially less may be satisfactory for multimeric enzymes [17]. A small pulse of a much higher substrate concentration can be used. If attachment to the dye column is mainly through surfaces not involved with substrate binding, then affinity elution is unlikely to succeed. Substrates that themselves have some characteristics of the dyes (e.g. fused aromatic rings such as purines, negatively charged oxygens such as phosphates and carboxylates) are most likely to be effective in affinity elution. Transition-state analogues or reacting two-substrate mixtures may be most effective, even at quite low concentrations. With single substrate enzymes, e.g. lyases, reaction on the column making product may render the substrate ineffective; an inactive analogue or inhibitor is preferable (see, e.g., ref. 8).

If affinity elution is either ineffective or requires a reagent which is too costly to use on a larger scale, a non-specific procedure must be used. Stepwise processes include increasing pH (0.2—0.5 units at a time), increasing salt concentration, removal of divalent metal ions from buffer, addition of phosphate to buffer, or a combination of these. In some cases alteration of the hydrophobicity of the eluent, or a change in temperature may cause some elution. An illustration of some of these effects is shown in Fig. 4. Several of these can be tried simultaneously on a series of small positive columns as indicated previously.

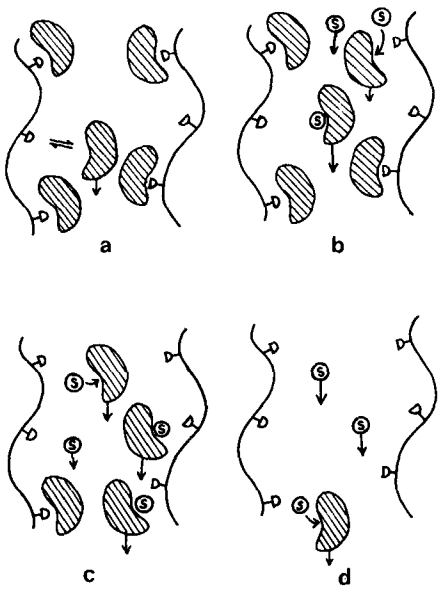

Fig. 3. Diagrammatic representation of affinity elution from dye column. D = Dye molecule; S = substrate/ligand for the protein (shaded). (a) Most protein molecules are bound but a small proportion (2—10%) is unbound at any given instant; (b) substrate introduced into the buffer at about ten times K_d interacts with the unbound protein molecules which then move down the column as protein—substrate complexes; (c) further bound protein molecules have dissociated to restore the equilibrium between bound and unbound molecules. These also pick up substrate molecules and are moved away down the column; (d) the last molecules are removed from this section as substrate continues to permeate the column.

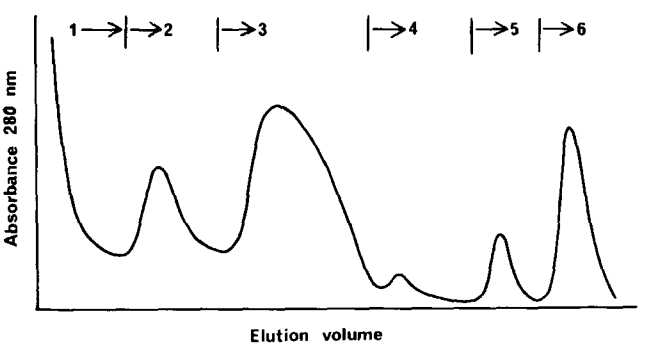

Fig. 4. Stepwise elution of proteins from a Procion Navy H-ER column. A 30-ml *Z. mobilis* extract (420 mg protein) was applied to a 24-ml column in a K—Mes buffer, pH 6.0, containing 2 m*M* magnesium chloride, total ionic strength (*I*) = 0.05. Stepwise changes in buffer were made as follows (percentage protein eluted between parentheses): step 1: K—Mes—Mg, pH 6.0, *I* = 0.05 (42%, non-adsorbed); step 2: K—Mes—Mg, pH 6.5, *I* = 0.05 (6%); step 3: K—Mes (no Mg), pH 6.5, *I* = 0.05 (10%); step 4: K—Mes—sodium chloride, pH 6.5, *I* = 0.10 (2%); step 5: potassium phosphate, pH 6.5, *I* = 0.10 (6%); step 6: potassium phosphate—sodium chloride, pH 6.5, *I* = 1.0 (19%); remaining on column: 15%. It will be noted that removal of Mg ions (step 3) resulted in a large peak of absorption, not all of which was due to protein. Very little protein was eluted by doubling the ionic strength (step 4). The protein peak eluted by a change to phosphate buffer (step 5) was not large; had the change to phosphate buffer been made at an earlier step it would have had more effect.

A gradient of eluting effector may be efficacious; particularly pH gradients in the 5.5—6.5 range. Over this range a considerable decrease in binding occurs; since only histidines in the proteins are titrating, it appears that dye—histidine (acid form) interactions are particularly important in binding the proteins.

CONCLUSION

Although dye-ligand chromatography has already achieved a large number of successes in enzyme and protein purification, the procedures have often not been optimised. In relatively few cases have a number of dyes been investigated to find the most suitable for the purpose at hand. This has been either through ignorance of the potential offered by dyes other than Cibacron Blue F3-GA, or because of the work involved in acquiring a range of adsorbents and screening them all before commencing the purification procedure proper. The availability of ready-made adsorbents has improved, and the dyes themselves have been made available through the generous co-operation of chemical companies and distributors. Once made, a set of dye adsorbents should last virtually indefinitely, though the storage conditions of the dyes themselves need careful attention. The tedious procedure of screening a large number of dyes each time a new enzyme is to be purified can be greatly improved by some empirical knowledge of the properties of the dyes, vis-a-vis protein binding, and grouping them accordingly. By adoption of standardised procedures, buffers and minimal screening strategies, we have found that dye-ligand procedures are not only quick to develop, but also offer the chance of a simple, rapid isolation, typically 20- to 80-fold purification with 80% recovery, from a crude extract without prior treatment. The column procedures are readily scaled up, and being economical are highly suited to large-scale commercial enzyme and protein production.

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