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Polymer displacement in dye-affinity chromatography

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Abstract

Displacement of lactate dehydrogenase from dye-affinity matrices with poly(ethyleneimine) (PEI) was shown to be an effective elution strategy. It resulted in better recoveries and sharper elution profiles than traditional non-specific elution while the purification factors were unchanged. The elution is assumed to proceed via displacement of bound protein by PEI when the polymer binds to the dye-ligands. Complete elution of bound protein is a characteristic feature of such a mechanism. Hence displacement with PEI may be a promising strategy for eluting proteins with reported low recoveries in dye-affinity chromatography protocols.

1. Introduction

Dye-affinity chromatography has proven to be a convenient method of protein purification both in the laboratory and at a large scale [1,2]. It combines high specificity with robustness, simple coupling chemistry and cost efficiency [3–5]. Protein binding to the dye matrix is not the whole story. For a successful purification, the bound protein must be eluted with a high recovery and preferably in a small elution volume. Two strategies are traditionally used for protein elution from dye matrices. The first one is to reduce the binding efficiency of the protein to the dye ligands by eluting with a high-ionic-strength solution. This is called non-specific elution, and usually 1–2 *M* NaCl or KCl is used. The second strategy is to use a soluble ligand, which competes with the dye ligand for the same protein binding site. Usually millimolar solutions of nucleotides are used for such specific elution. Both methods are inefficient in many cases and recoveries of 40–60% are quite common in dye-affinity chromatography [6–11]. Champluvier and Kula [12] calculated an average recovery of about 60% for numerous purifications on dye-affinity matrices. Even recoveries as low as 20–30% are frequent, especially when specific elution has been used [6,13–17].

A new strategy for protein elution is to use an agent with stronger binding to the matrix than that of target protein. Binding of such an agent results in the displacement of bound protein and is therefore called displacement chromatography. Different proteins were displaced from ion-exchange matrices by polyelectrolytes, DEAE-dextran [18,19], dextran sulfate [18,19,21], poly(vinyl sulfonic acid) [22], CM-starch [23], chondroitin sulfate [20,24], low-molecular-mass compounds such as (ethyleneglycol-bis(β-amino ethylether-N,N,N′,N′-tetracetic acid) [25] or from hydrophobic matrices by poly(ethylene glycol) (PEG) [26,27]. To the best

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of our knowledge displacement has not been exploited in dye-affinity chromatography.

Strong interaction of poly(ethylene imine) (PEI) with triazine dyes was found during our study of dye interaction with different polymers [28]. The idea was to try this polymer for displacement of proteins bound to dye matrices in order to improve their recovery and elution profile.

2. Materials and methods

Lactate dehydrogenase (LDH) type XXX-S from porcine muscle, β-NADH, and bicinchonic acid solution were purchased from Sigma (St. Louis, MO, USA). PEI with molecular mass 35 000 ± 5000 was purchased from Serva (Heidelberg, Germany). PEIs with molecular masses 2000 and 700 were purchased from Aldrich (Steinheim, Germany). Poly(acrylic acid) sodium salt with molecular mass 170 000 was purchased from Fluka (Buchs, Switzerland). Sepharose CL 4B and Sepharose 4 Fast Flow were purchased from Pharmacia BioProcess Technology (Uppsala, Sweden). Cibacron Blue 3GA (CB), purchased from Sigma, and Procion Red HE-3B (PR), a gift from ICI (Manchester, UK), were used as received. The heterogeneous character of commercially available dye preparations is well known [29], nevertheless it is common to use triazine dyes in spectral studies without additional purification [30–33].

Blue Sepharose and Red Sepharose were synthesised by coupling CB or PR to Sepharose CL 4B according to [34]. Scarlet Sepharose was a generous gift from Professor R.K. Scopes (Centre for Protein Research and Enzyme Technology, La Trobe, Australia) and was synthesized by coupling of Procion Scarlet HGA to Sepharose CL-4B according to [34].

The spectral titration was performed at room temperature according to [30]. Sample and reference cuvettes each containing 2 ml of a dye solution in an appropriate buffer were placed in a double beam Shimadzu UV-260 spectrophotometer. Small volumes (1–10 μl) of PEI solution were added to the sample cuvette and equal volumes of buffer were added to the reference cuvette. The contents of the cuvettes were mixed and spectra in the region of 400–650 nm were registered.

Chromatographic procedures were done at room temperature. LDH and BSA were applied to the column in equilibrating buffer (20 m*M* Tris · HCl buffer pH 7.3) and eluted with either 1% PEI, or 1.5 *M* KCl, or 1 m*M* NADH + 10 m*M* oxamate in the same buffer. The Blue Sepharose and Fast Blue Sepharose columns were regenerated after PEI elution with 0.1 *M* glycine buffer pH 12, containing 1 *M* NaCl. The Red Sepharose column was regenerated by a pulse of equilibrating buffer and then with 0.1 *M* glycine buffer pH 12, containing 1 *M* NaCl and 1% polyacrylate. The columns were reequilibrated after regeneration in 20 m*M* Tris · HCl buffer pH 7.3.

Pork was purchased in a local shop and was homogenized in ice-cold 20 m*M* Tris · HCl buffer, pH 7.3 containing 1 m*M* EDTA (3 ml of buffer per g of muscle tissue). The homogenate was centrifuged at 16 000 *g* for 15 min to remove cell debris and the supernatant was filtered through Munktell Grade 3 filter paper (Grycksbo, Sweden) to remove traces of fat. The porcine muscle extract was kept frozen without any loss of LDH activity and was applied directly to the Red Sepharose column after thawing and filtering.

LDH activity was measured in the fractions according to a reported procedure [35]. Protein was determined according to the manufacturer's instruction for Bio-Rad protein assay (Bio-Rad Labs., Munich, Germany).

3. Results and discussion

Two triazine dyes, CB and PR were chosen for the study. PR is a bis-monochlorotriazine dye whereas CB is a monochlorotriazine dye (Fig. 1). The CB molecule contains three acidic sulfonate groups and four basic primary or secondary amino groups (ratio 3:4). The PR molecule contains six sulfonate groups and only four secondary amino groups (ratio 3:2) and bears

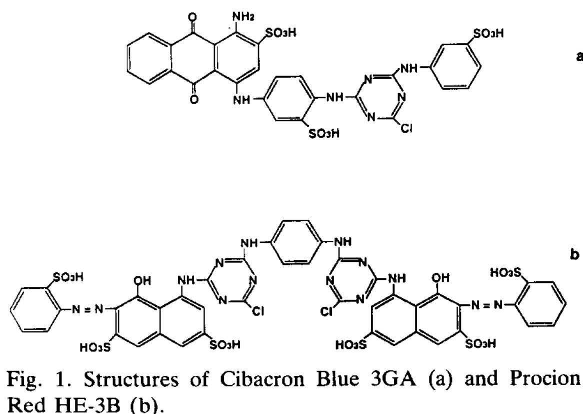

Fig. 1. Structures of Cibacron Blue 3GA (a) and Procion Red HE-3B (b).

higher total negative charge at neutral and basic conditions.

Both dyes interacted strongly with PEI at neutral conditions, resulting initially in a spectral change in the visible range and finally in precipitation of the dye–polymer complex. The difference spectra at low PEI concentration had a clear isobestic point indicating formation of soluble complexes (Fig. 2). At higher PEI concentrations this complex became insoluble and precipitated. Precipitation occurred at a polymer:CB ratio of 2:1 and a polymer:PR ratio of 6–7:1 (monomer units per dye molecule). It is reasonable that these complexes should precipitate when their total net charge is zero. Thus, the higher ratio in the case of PR is due to the more negative charge of this dye molecule, and more positive charges or more monomer units of PEI were needed to neutralize the charges.

Addition of excess polymer to the suspension of insoluble complex did not result in its dissolution. When an excess of PEI was added initially to the dye solution no precipitate formed. The difference spectrum of such soluble "PEI-saturated" complex (Fig. 2, dashed line) was quite close to that obtained just before precipitation starts and was independent of further increase in polymer concentration. The PEI–dye complex seemed to be kinetically stable. It precipitated at zero net charge and no redistribution of dye molecules between the precipitate and excessive polymer took place.

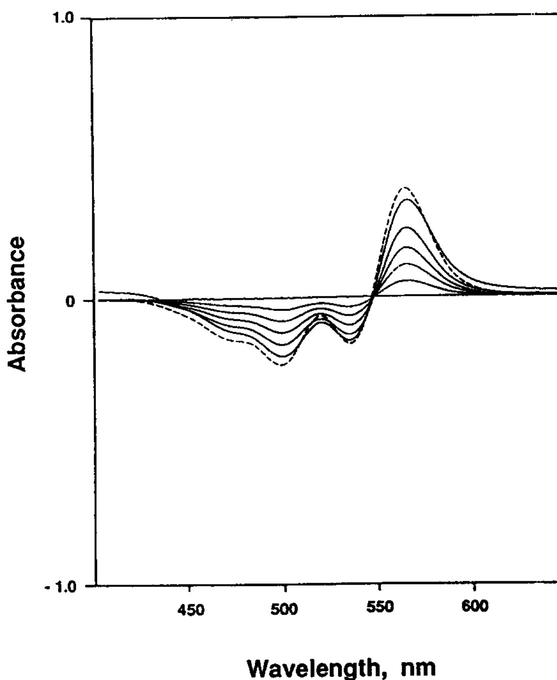

Fig. 2. Difference spectra obtained when Procion Red was titrated with increasing concentrations of PEI-35 000. Both sample and reference cuvette contained 2 ml of 0.05 mg/ml Procion Red in 20 m*M* Tris · HCl pH 7.3. Identical 1-μl portions of 0.25% PEI-35 000 solution in 20 m*M* Tris · HCl pH 7.3 and buffer were added in the reference and sample cuvette respectively. The dashed line was obtained when initially 10 μl of 10% PEI-35 000 solution in 20 m*M* Tris · HCl pH 7.3 were added to 2 ml of 0.05 mg/ml Procion Red in 20 m*M* Tris · HCl pH 7.3.

The application of the mathematical model previously developed [36] for calculation of constants for the binding of dyes with the soluble polymers, was prohibited by the precipitation of CB–PEI and PR–PEI complexes. Still some estimations of dye–PEI complex strength can be done on the basis of the amount of PEI needed for obtaining complete spectral changes. Using this scale, CB complexing with PEI at a pH around neutrality was about ten-fold more efficient than CB complexing with non-ionic polymer, poly(N-vinyl pyrrolidone). Dye interactions with PEI were also under experimental conditions used independent of the polymer molecular mass (700–35 000) resulting in nearly the

same difference spectra and the same polymer:dye ratio when precipitation took place.

The multipoint interaction of poly(N-vinyl pyrrolidone) and poly(vinyl alcohol) with dye ligands, coupled to the chromatographic matrix, was successfully exploited in polymer-shielded dye-affinity chromatography [28,36–40]. The interaction of poly(N-vinyl pyrrolidone) with dye ligands is strong enough to prevent the non-specific binding of proteins to the dye ligands. On the other hand this interaction is less efficient than specific binding of nucleotide-dependent enzymes to the dye ligands. Hence, these enzymes bind to the dye-affinity column pretreated with the polymer. The interaction of PEI with the dye ligands was much stronger than that of poly(N-vinyl pyrrolidone) and poly(vinyl alcohol). Thus, PEI could not be used for polymer shielding because it bound so strongly that it prevented specific binding to the column. In contrast, the strength of PEI binding can be exploited for the displacement of proteins bound to the dye-affinity column.

When coupled to the chromatographic matrix Sepharose CL-4B, both dyes, CB and PR, were able to bind pure LDH from porcine muscle. Efficient PEI binding to these dye ligands was used for the displacement of bound LDH. A 1% PEI solution in the same buffer used for LDH binding (20 m*M* Tris · HCl pH 7.3) proved to be an efficient eluent. Displacement of LDH with PEI resulted in quantitative recovery of the enzyme from both Blue Sepharose and Red Sepharose as well as from Scarlet Sepharose. LDH was displaced with 1% PEI and eluted as a sharp peak without tailing as usually observed during non-specific elution with high salt concentration. For instance, practically all LDH bound to Blue Sepharose was eluted with 1% PEI in 2.5-fold less volume than with 1.5 *M* KCl.

Fig. 3 presents LDH elution profiles using linear gradients of PEI-35 000, PEI-2000 and PEI-700. Unexpectedly, PEI-35 000 was a slightly weaker displacer than PEI-2000 and PEI-700, though all the polymers used displaced LDH efficiently from the Red Sepharose column. The difference in behaviour may be attributed to

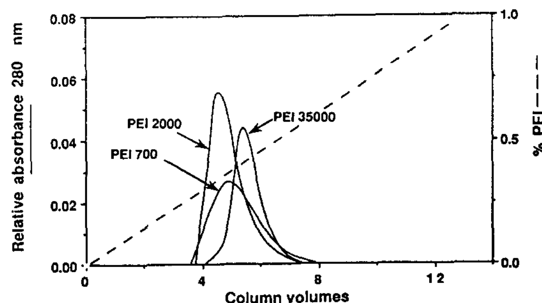

Fig. 3. LDH elution profile using a gradient of PEI. Experimental conditions: 1.5 × 1.0 cm I.D. Red Sepharose column. A 520-μl volume of pure LDH (1.1 mg/ml) was applied and eluted using a 0 to 1% linear PEI gradient at a flow-rate of 0.5 ml/min. The elution profiles are adjusted subtracting the contribution of PEI absorbance at 280 nm. Arrows indicate the molecular mass of PEI used.

some differences in polymer structure, e.g. degree of branching. The different PEI preparations are from different suppliers and sufficient documentation is not available to judge whether the preparations have the same degree of branching. On the other hand, LDH was not eluted with spermine, which can be regarded as a low-molecular-mass PEI analogue with degree of polymerization of 4. Thus, the polymeric nature of PEI and the possibility of multipoint interaction with the dye matrix was crucial for an efficient LDH elution.

Polymer bound strongly to the column after PEI elution and it was necessary to develop a proper column-regeneration protocol. Usually polycation displacers are washed out from cation exchangers with high-pH–high-salt buffer [18,19]. PEI ionization is suppressed at high pH and a high salt concentration decreases efficiency of ionic interaction. A 250-fold higher PEI concentration was required for complete "saturation" of spectral changes in CB solution at pH 12 (0.1 *M* glycine buffer, containing 1 *M* NaCl) comparing to that needed at neutral conditions. The PEI–CB complex was quite weak at these conditions and the polymer could be washed out from the column. Blue Sepharose column was successfully regenerated with 0.1 *M* glycine buffer pH 12 containing 1 *M* NaCl. On the contrary, the PR molecule bears more negative charges than the CB molecule and provides more oppor-

tunities for multipoint interactions with PEI. Even at these for complex formation unfavorable conditions, the PEI–PR complex is still strong enough resulting in significant spectral changes (data not shown). As a consequence the Red Sepharose column could not be regenerated with 0.1 *M* glycine buffer pH 12 containing 1 *M* NaCl.

It was necessary to make the mobile phase more attractive for PEI in order to remove it from the stationary phase. Addition of 1% polyacrylate (PAA) into the regeneration buffer was attempted. PAA is negatively charged at pH 12 and it does not bind to the negatively charged dye ligands, but is able to interact via hydrogen bonding with PEI, which is neutral at this pH. PEI-35 000 interacted strongly with PR even in the presence of 1% PAA, while the interaction of PEI-2000 with PR at these conditions was weaker. The interaction of PEI-700 with PR was abolished in the presence of 1% PAA (Fig. 4).The regeneration procedure using 1% PAA in 0.1 *M* glycine buffer pH 12 containing 1 *M* NaCl proved to be efficient after displacement of LDH with PEI-700 and PEI-2000 elution but not after displacement with PEI-35 000. A higher molecular mass of PEI results in more sites capable of interacting with dye ligands on the matrix. The polymer with higher molecular mass, PEI-35 000, binds to the dye matrix via a higher number of interactions and is therefore prevented from being washed out even by 1% PAA in 0.1 *M* glycine buffer pH 12 containing 1 *M* NaCl.

Two mechanisms can be assumed for the elution of LDH by PEI. According to the first mechanism the polymer binds to LDH thereby decreasing enzyme affinity towards the dye ligands. According to the second mechanisms. PEI binds to the dye ligands and thereby displaces LDH. PEI interacted strongly with dye ligands. The efficiency of these interactions is comparable to or even stronger than that of dye binding to LDH. PEI bound to the dye ligands prevented LDH from interaction with these ligands. LDH did not bind to the column after PEI elution and extensive washing with equilibrating buffer. A harsh regeneration procedure was required to remove bound PEI.

PEI interacted slightly with LDH, enzyme activity was decreased only 15–20% in the presence of 1% PEI. For comparison. LDH activity was decreased two-fold in 1.5 *M* KCl used for non-specific elution of enzyme. LDH was displaced with PEI and eluted as a concentrated solution, and usually only 10–100-μl samples

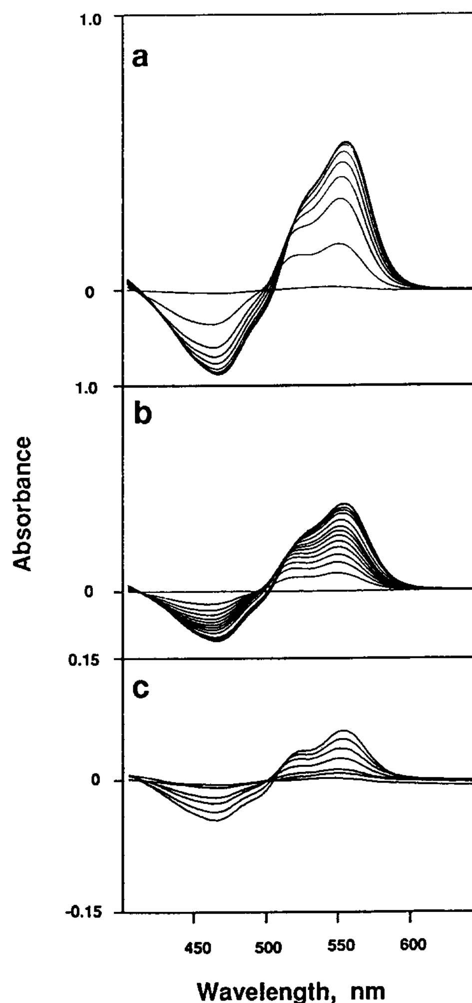

Fig. 4. Difference spectra obtained when Procion Red was titrated with increasing concentrations of (a) PEI-35 000, (b) PEI-2000 and (c) PEI-700. Both sample and reference cuvette contained 2 ml of 0.05 mg/ml Procion Red in 0.1 *M* glycine buffer pH 12, containing 1 *M* NaCl and 1% polyacrylate. Identical portions of buffer and of 10% PEI solution in 0.1 *M* glycine buffer pH 12, containing 1 *M* NaCl and 1% polyacrylate were added in the reference and sample cuvette respectively. Total 7×1 μl (a), 14×1 μl (b) and $1 + 1 + 5 + 5 + 10 + 10$ μl (c) aliquots of PEI solutions were added.

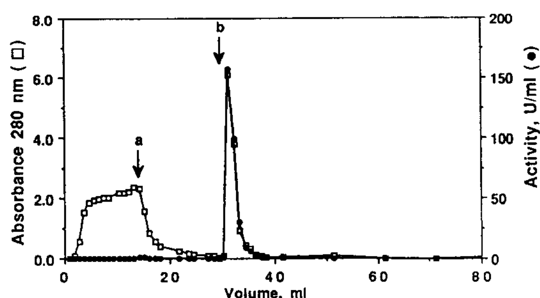

Fig. 5. LDH purification profile on Red Sepharose. Experimental conditions: 1.2 × 1.0 cm I.D.; flow-rate 0.5 ml/min. The crude extract was applied to a column until breakthrough. The arrows indicate: a = washing after breakthrough with 20 m*M* Tris · HCl, pH 7.3; b = elution with 1% PEI-700 in 20 m*M* Tris · HCl, pH 7.3. The column was regenerated with 1% polyacrylate in 0.1 *M* glycine buffer pH 12 containing 1 *M* NaCl and reequilibrated with 20 m*M* Tris · HCl buffer pH 7.3.

were used for assay of enzyme activity in a total assay volume of 1.5 ml. The 20–200 dilution of 1% PEI present in the eluent resulted in no measurable effect on LDH activity. Thus, we assume that LDH elution with PEI proceeds via the second mechanism, namely, via the displacement of enzyme by polymer due to the interaction of PEI with dye ligands.

Displacement with PEI-700 and subsequent column regeneration with 1% PAA in 0.1 *M* glycine buffer pH 12 containing 1 *M* NaCl was chosen for LDH purification from crude porcine muscle extract using Red Sepharose column. Crude extract was applied on a Red Sepharose column until breakthrough, the column was washed with buffer until no protein was eluted and LDH bound was eluted with 1% PEI-700 solution (Fig. 5).

Comparison with traditional strategies of LDH elution (Table 1) showed that displacement with PEI was more efficient than elution with 1.5 *M* KCl. It resulted in slightly better recoveries and sharper elution profiles. Displacement of LDH with PEI as well as 1.5 *M* KCl elution is non-specific and results in elution of all proteins bound on the column. On the other hand, specific elution proceeds via nucleotide competition with the dye ligand for the same protein sites. As a result, only proteins capable of nucleotide binding are eluted, the rest of the bound proteins should be eluted with high ionic strength to regenerate the column. Thus, the purification factors during specific elution with nucleotide (around 10) were higher than during both non-specific elution strategies (2.5–4). Though the structure of the dye resembles that of nucleotide, the dye is a pseudo-affinity ligand

Table 1
LDH purification on Red Sepharose with different elution strategies

Elution strategy	Capacity (units bound/ml matrix)	Recovery (%)	Elution volume (column volumes for 90% recovery)	Purification factor
PEI-700				
Run 1	320	100	3.0	4.0
Run 2	310	101	5.0	3.2
1.5 *M* KCl				
Run 1	310	81	[a]	2.5
Run 2	310	94	4.0	3.1
1 m*M* NADH + 10 m*M* oxamate				
Run 1	210	97	7.5	9.9
Run 2	270	92	10.0	9.5

Crude extract was applied until breakthrough at a flow-rate of 0.5 ml/min. The column was washed with 20 m*M* Tris · HCl buffer pH 7.3 until no protein was eluted and then eluted with either 1% PEI, or 1.5 *M* KCl, or 1 m*M* NADH + 10 m*M* oxamate, all in 20 m*M* Tris · HCl buffer pH 7.3.

[a] 81% recovery was obtained in 17.4 column volumes

and some relatively weak non-specific interactions of LDH with the dye are possible. These non-specific interactions resulted in broadened elution profiles. LDH was eluted specifically in 7–10 column volumes, while non-specifically it was eluted in 3–5 columns volumes.

Displacement chromatography has been regarded as a potentially very promising technique. Applications concerning the separation of small molecules have confirmed this statement, but it has been more difficult to attain all the advantages when separating crude protein mixtures. Only a few successful protein purifications using displacement chromatography have been reported [41]. A limiting factor has been that protein molecules are multifunctional and they interact with the support via a range of interactions. When using ion-exchange supports, there are many potential interactions that may take place and it is therefore difficult to obtain an idealized displacement behaviour. In theory it should be easier with hydrophobic sorbents since less potential interactions may occur, but the low solubility of highly hydrophobic displacers poses a problem. So far, a lot of effort has been invested but there are few successes in protein separation based on displacement chromatography.

The strategy adopted here is novel in the sense that conventional displacement is carried out, but the adsorption step has been chosen to include some more biospecificity than what is conventionally used. By means of this fewer potential interactions between the support and proteins are prevalent and the number of proteins binding to the adsorbent will be restricted. More efficient displacement steps may be achieved by this mode of operation.

4. Conclusions

Displacement of LDH from dye-affinity matrices with PEI was shown to be an effective elution strategy. It resulted in better recoveries and sharper elution profiles than traditional nonspecific elution with the same purification factors. The elution is supposed to proceed via displacement of bound protein by the polymer when the latter binds to the dye ligands. Complete elution of all bound protein is a characteristic feature of such a mechanism. Hence PEI displacement may be a promising strategy for eluting proteins with reported low recoveries in dye-affinity chromatography protocols.

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