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Cyclodextrin biospecific-like displacement in dye-affinity chromatography

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Abstract

Interactions between Cibacron Blue F3GA (CB F3GA), as a model of triazine dye, and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), as a model of cyclodextrin, were investigated by monitoring the spectral shift that accompanies the binding phenomena. Matrix analysis of the difference spectral titration of CB F3GA with HP-β-CD revealed only two absorbing species, indicating a host–guest ratio of 1:1. The dissociation constant for this HP-β-CD–CB F3GA complex, K_d, was found to be 0.43 m*M*. The data for HP-β-CD forming inclusion complexes with CB F3GA were used to develop the concept of competitive elution by inclusion complexes in dye-affinity chromatography. When this concept was applied to the elution of L-lactate dehydrogenase from a CB F3GA affinity matrix, it was shown to be an effective elution strategy. It provided a 15-fold purification factor with 89% recovery and sharp elution profile (0.8 column volumes for 80% recovery), which is as good as that obtained by specific elution with NADH (16-fold, 78% recovery and 1.8 column volumes). In addition, the new elution strategy showed a better purification factor and sharper elution profile than traditional non-specific elution with KCl (4.5-fold, and 1.4 column volumes). Hence, competitive elution by inclusion complexes may be a promising strategy for eluting proteins with high recoveries and purification factors in dye-affinity chromatography. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Affinity chromatography; Cyclodextrins; Cibacron Blue; Lactate dehydrogenase; Enzymes; Dyes; Triazines

1. Introduction

The low cost, general availability, ease of coupling to matrix materials via a stable linkage and characteristic spectral properties make the triazine dyes ideally suited to a number of preparative and analytical applications, including protein purification by affinity chromatography [1]. However, the choice of eluent is an important operational parameter in dye chromatography and is dictated largely by the system under investigation. In general, steps, pulses or gradients of salt [2], phosphate [3], coenzymes and nucleotides [4], polynucleotides [5], substrates [6], pH [7], polyelectrolytes [8], temperature [9], polyols, detergents or chaotropes have proven useful.

Cyclodextrins (CDs) are a group of naturally occurring cyclic oligosaccharides [10], with six, seven or eight glucose residues linked by α-(1→4) glycosidic bonds in a cylinder-shaped structure and

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are denominated as α-, β- and γ-cyclodextrins, respectively. The central cavity of a CD is hydrophobic and accommodates a wide range of guest molecules forming an inclusion complex [11–13]. Modified CDs have some advantages over parent CDs, including the fact that they are highly soluble both in water and in organic solvents. Hydroxypropyl-β-cyclodextrin (HP-β-CD), a modified CD is one of the most widely used because of its high solubility and low cost. Recently, our group have purified the L-(+)-lactate dehydrogenase of *Pediococcus acidilactici* (PA-L-LDH) to homogeneity by affinity chromatography on the triazine dye Procion Red MX-5B performing the elution step with 1 m*M* NADH [14]. In the progress of the PA-L-LDH characterization, it was found that Procion Red MX-5B causes inhibition of PA-L-LDH through competition with NADH but, when increasing concentrations of CDs were added in vitro to the reaction medium, a decrease in inhibition was observed. Since the structures of triazine dyes usually involve fused aromatic rings, we have supposed that the phenomenon observed is probably due to the complexation of triazine dyes into the hydrophobic cavity of the CDs. The intention of this work was to study the formation of inclusion complexes between HP-β-CD and Cibacron Blue (CB) F3GA to develop the new concept of competitive elution with inclusion complexes in dye-affinity chromatography and to apply it to the dye-affinity chromatography of PA-L-LDH.

2. Experimental

2.1. Materials

CB F3GA, pyruvate (sodium salt), NADH, lysozyme and bovine serum albumin (BSA) were purchased from Sigma (Madrid, Spain). Triton X-114 was obtained from Fluka (Madrid, Spain). Cyclodextrins were kindly supplied by Amaizo, American Maize-Products, Hammond, IN, USA. Bio-Rad protein assay was obtained from Bio-Rad Labs. (Munich, Germany). A Hi-Trap-Blue (5 ml) prepacked column was obtained from Pharmacia (Uppsala, Sweden). All other chemicals used were of analytical grade. CB F3GA was used as received. Although the heterogeneous character of commercially available dye preparations is well known [15], it is common to use triazine dyes in spectral studies without additional purification [8,16–20].

2.2. Difference spectroscopy

Difference spectroscopy was performed with a Uvikon 940 double-beam spectrophotometer (Kontron) using 50 m*M* potassium phosphate buffer, pH 7.0, at 25°C. The light path of the cuvettes was 1 cm and the spectra in the region 190–800 nm were recorded at a scan rate of 1000 nm/min. For the baseline the same amount of HP-β-CD was added to the reference and the sample cells. Then a new sample cuvette containing the same HP-β-CD final concentration (0–20 m*M*) plus a fixed concentration of CB F3GA (100 μ*M*) was placed in the sample cell and the difference spectra were recorded. The HP-β-CD–CB F3GA dissociation constant, K_d, was determined from the double reciprocal plot of $1/\Delta A_{620}$ versus 1/[HP-β-CD] and linear regression [21].

2.3. Preparation of cell free extract

Cell-free extract of *Pediococcus acidilactici* CECT98 (Spanish Type Culture Collection, Burjasot, Valencia, Spain) was prepared by a previously described procedure [14] involving both chemical treatment with lysozyme and phase separation of biomolecules in an aqueous two-phase system based on Triton X-114. The detergent-depleted phase was collected and used as cell-free extract for all chromatographic procedures.

2.4. Enzyme assay

Routine assays of LDH were performed at 37°C in reaction mixtures containing 50 m*M* potassium phosphate buffer, pH 7.0, 0.2 m*M* NADH, 10 m*M* pyruvate, and enzyme in a final volume of 1 ml. Initial velocities of pyruvate reduction were determined spectrophotometrically by measuring the decrease in absorbance at 340 nm resulting from NADH oxidation (ϵ_{340}=6220 M^{-1} cm^{-1}). One unit is defined as the amount of enzyme which catalyzes

the oxidation of 1 μmol of NADH/min under the assay conditions described.

2.5. Chromatographic procedures

The samples, 1 ml of the pure cell-free extract (600 units of LDH) or 1:5 diluted in 50 m*M* potassium phosphate buffer (120 units of LDH) were loaded at 1 ml/min on a Hi-Trap-Blue (5 ml) pre-packed column. The column was previously equilibrated with 50 m*M* potassium phosphate buffer, pH 7.0, at room temperature, using a fast protein liquid chromatography (FPLC) system (Äkta 10 purifier; Pharmacia). Then, the unbound protein was washed off the column with the same buffer at a flow-rate of 1 ml/min until the absorbance of the eluent at 280 nm had attained 0 again (≈30 ml). Then either 100, 50, or 25 m*M* HP-β-CD, or 1 *M* KCl, or 1 m*M* NADH in the same buffer were used for elution. Fractions (1 ml) were collected and assayed for both LDH activity and protein concentration. The column was regenerated according to the recommendations of the manufacturer, but in the case of HP-β-CD elution washing with ethanol 20% was necessary before the column-regeneration protocol was applied. The HP-β-CD–CB F3GA complex was quite weak in these conditions and the HP-β-CD could be washed out from the column.

2.6. Protein assay

The protein concentration was determined according to the manufacturer's instruction for Bio-Rad protein assay based on Bradford's method [22], using BSA as standard. When a standard curve with BSA, prepared using HP-β-CD as the diluent, was compared with a standard curve made by diluting BSA with water, a significant difference in dye color development was observed, although the linearity of the assay was not affected in the range of protein concentrations assayed. Therefore, the protein concentration in all fractions containing HP-β-CD was determined using a standard curve with BSA, which was prepared using HP-β-CD as the diluent at the same final concentration present in the assayed fractions.

3. Results

In order to choose a cyclodextrin as model for this study, different cyclodextrins were tested for their ability to form complexes with CB F3GA. Five cyclodextrins were tested by determining the decrease in the competitive inhibition of PA-L-LDH caused by CB F3GA. A decrease in the inhibition (expressed as relative catalytic rate) was observed with all cyclodextrins tested, except α-CD, which seems to have a too small hydrophobic cavity to interact with the dye. On the other hand, HP-β-CD almost led to a recovery of the initial relative catalytic rate, demonstrating its superior capacity to form complexes with CB F3GA than the other cyclodextrins tested. In addition, increasing concentrations of HP-β-CD (0–20 m*M* final concentration) in the reaction medium without CB F3GA neither activated nor inhibited the catalytic activity of PA-L-LDH (see Table 1). Thus, the apparent HP-β-CD mediated decrease in PA-L-LDH inhibition was apparently due to the complexation of CB F3GA into the hydrophobic cavity of the HP-β-CD. To test this hypothesis, CB F3GA was titrated with HP-β-CD (0–20 m*M* final concentration) and difference spectra were recorded. This process gave a clear isosbestic point at 671 nm, indicating the formation of soluble complexes (Fig. 1a). Matrix analysis of the difference spectra of Fig. 1a using the method of Coleman et al. [23] confirmed the presence of two related species: CB F3GA and HP-β-CD–CB F3GA (Fig. 1a, inset) and a host–guest ratio of 1:1 can be assumed. The difference spectra in the region of

Table 1
Effect of the addition of different cyclodextrins on the inhibition of PA-L-LDH[a]

Additive	Relative catalytic rate
None	100
50 μ*M* CB F3GA	61.6
20 m*M* HP-β-CD	100
50 μ*M* CB F3GA–20 m*M* HP-β-CD	96.6
50 μ*M* CB F3GA–20 m*M* α-CD	60.3
50 μ*M* CB F3GA–20 m*M* γ-CD	79.4
50 μ*M* CB F3GA–20 m*M* G_2-β-CD	84.0
50 μ*M* CB F3GA–20 m*M* methyl-β-CD	92.0

[a] The reaction media contained 0.2 m*M* NADH, 10 m*M* pyruvate, 50 μ*M* CB F3GA, 20 m*M* tested cyclodextrin, and enzyme, in 50 m*M* potassium phosphate buffer, pH 7.0 at 37°C.

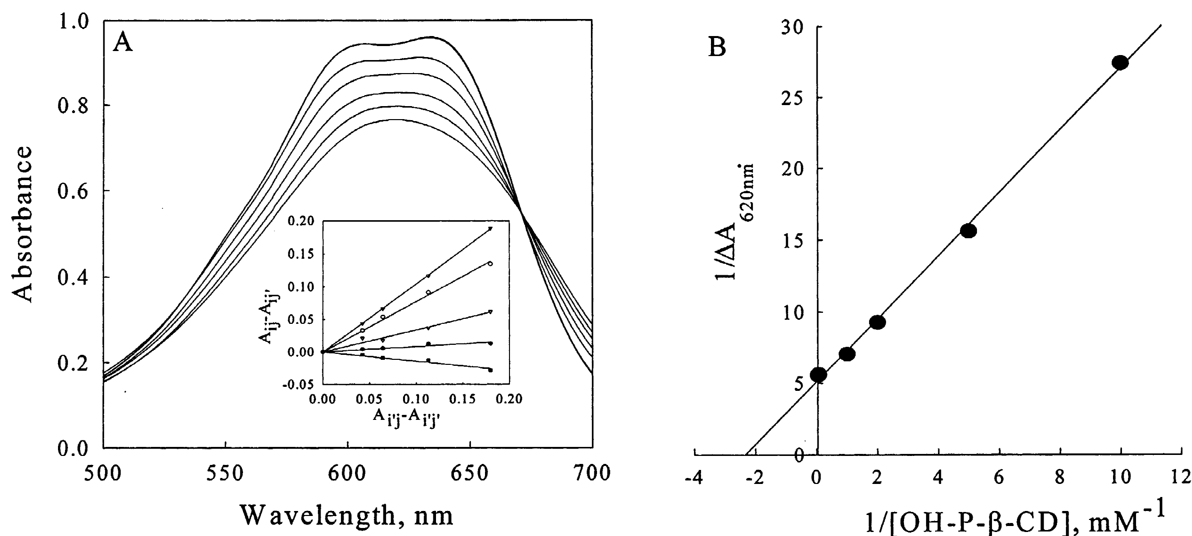

Fig. 1. (a) Difference spectral titration of CB F3GA with HP-β-CD. Difference spectra were recorded by the addition of increasing amounts of HP-β-CD (0–20 m*M* final concentrations) to CB F3GA (100 μ*M*) in 1 ml potassium phosphate buffer, pH 7.0. Inset, Coleman's graphical analysis for two absorbing species. In this analysis, A_{ij} is the absorbance at wavelength i obtained during tracing j: i'=620 nm, j'=first trace, i=● (500 nm), i=○ (570 nm), i=▼ (625 nm), i=△ (665 nm), and i=■ (745 nm). (b) K_d value was calculated by double reciprocal plot of $\Delta A_{620\ nm}$ vs. HP-β-CD concentration and regression analysis.

maximum absorption of the dye chromophore were analyzed in order to determine the HP-β-CD–CB F3GA dissociation constant using the double reciprocal plot of $1/\Delta A_{620\ nm}$ versus 1/[HP-β-CD] (mM^{-1}) [21] and regression analysis (Fig. 1b).By fitting the data by linear regression, a value of 0.43 m*M* was obtained for K_d. However, this interaction is less efficient than the binding of PA-L-LDH to CB F3GA since a K_i of 13 μ*M* was obtained (data not shown). This more than 30-fold difference in binding strength should be taken into account when performing the elution step.

Fig. 2 shows the specific elution profiles of PA-L-LDH from a cell-free extract (diluted 1:5) using three elution buffers with increasing concentrations of HP-β-CD (25, 50 and 100 m*M*). The cyclodextrin solution was applied after washing the unbound proteins out of the column (Fig. 2, arrows). The elution profile of PA-L-LDH activity was dependent on the HP-β-CD concentration, with quantitative elution in a small elution volume being observed at 100 m*M* HP-β-CD, representing a recovery of 99.7%, compared with the 75.1%, and 2.6% obtained with the lower cyclodextrin concentrations used. These two low recoveries were correlated with

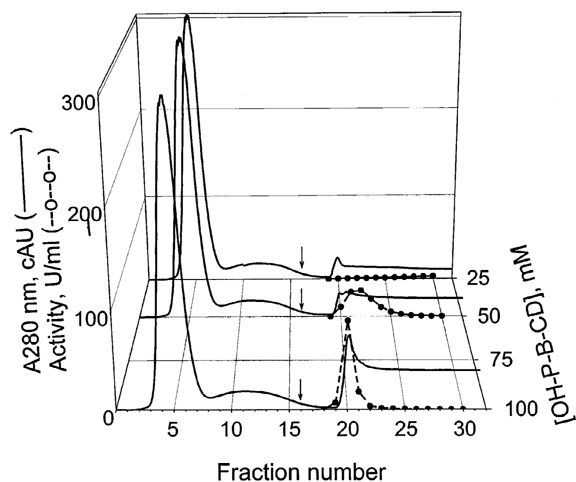

Fig. 2. L-LDH elution profiles using different HP-β-CD concentrations. Experimental conditions: 5 ml Hi-Trap-Blue column. A 1-ml volume of cell-free extract 1:5 diluted in 50 m*M* potassium phosphate buffer, pH 7.0 (120 units of L-LDH) was applied and eluted using 100, 50 and 25 m*M* HP-β-CD at a flow-rate of 1 ml/min. The arrows indicate the start of the elution step.

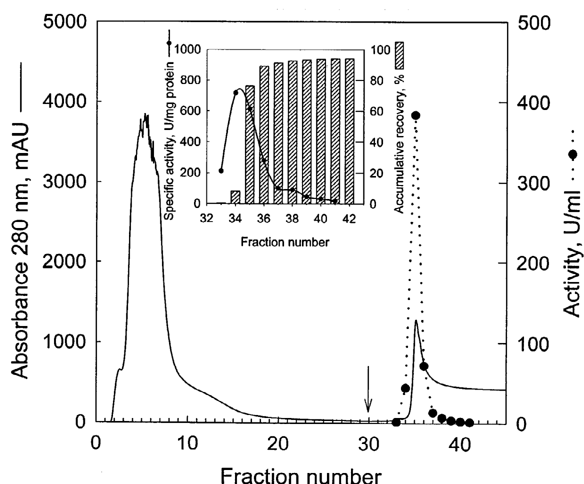

Fig. 3. L-LDH purification profiles on Hi-Trap-Blue column. A 1-ml volume of the cell-free extract containing 600 units of L-LDH was applied at a flow-rate of 1 ml/min. The column was washed with 50 m*M* potassium phosphate buffer, pH 7.0, at 25°C, until the absorbance of the eluent at 280 nm had attained 0 again (≈30 ml) and then eluted with 100 m*M* HP-β-CD in 50 m*M* potassium phosphate buffer, pH 7.0. The arrow indicates the start of the elution step. Inset: specific activity and accumulative recovery in each fraction with PA-L-LDH activity.

broadened elution profiles. The elution profile at 100 m*M* HP-β-CD cannot be attributed to a decrease in water activity caused by high cyclodextrin concentration since 0.7 *M* sucrose failed to elute PA-L-LDH. After elution of the enzyme, cyclodextrins were washed out with 20% (v/v) ethanol in water. Finally, the column was regenerated according to the recommendations of the manufacturer.

To check the efficiency of the competitive elution of PA-L-LDH with 100 m*M* HP-β-CD (Fig. 3), it was compared with the elution obtained using a biospecific (NADH) and a non-specific (KCl) ligand. Table 2 shows that competitive elution with 100 m*M* HP-β-CD was similar to the biospecific elution obtained with 1 m*M* NADH as regards the purification factor (≈15-fold) and more efficient than non-specific elution with 1 *M* KCl (≈4.5-fold). However, the recoveries were 10% higher with non-specific ligands (cyclodextrins and KCl) than with NADH. Thus, it seems that competitive elution of PA-L-LDH with HP-β-CD proceeds via a mechanism that shows specificity for some proteins, whereas non-specific elution with 1 *M* KCl results in the elution of a greater part of the proteins bound on the column, and specific elution with 1 m*M* NADH results only in the elution of proteins capable of nucleotide binding.

However, the elution volume (column volumes for 80% recovery) was smaller in the case of 100 m*M* HP-β-CD (Table 2 and Fig. 3, inset) compared with KCl and specially with NADH. In addition, the contribution of 1 m*M* NADH absorbance at 280 nm hides the peak caused by elution of PA-L-LDH (data not shown), whereas the moderate contribution of 100 m*M* HP-β-CD (less than a sixth that of 1 m*M* NADH) permitted fitting of the elution profile by

Table 2
PA-L-LDH purification on Hi-Trap-Blue with different elution strategies[a]

Elution strategy	Specific activity	Recovery (%)	Elution volume (column volumes for 80% recovery)	Purification factor
100 m*M* HP-β-CD				
Run 1	455	89	0.8	14.7
Run 2	448	87	0.9	14.2
1 m*M* NADH				
Run 1	503	78	1.8	16.2
Run 2	480	73	2.1	15.9
1 *M* KCl				
Run 1	141	90	1.4	4.5
Run 2	135	81	1.5	4.1

[a] A 1-ml volume of crude extract (600 units of PA-L-LDH) was applied at a flow-rate of 1 ml/min. The column was washed with 50 m*M* potassium phosphate buffer, pH 7.0 until no protein was eluted and then eluted with either 100 m*M* HP-β-CD, or 1 m*M* NADH, or 1 *M* KCl, all in 50 m*M* potassium phosphate buffer, pH 7.0.

subtracting the absorbance value at 280 nm recorded when the profile becomes a baseline (Fig. 3). Thus the elution profile obtained by competitive elution with HP-β-CD provides certain information that is not provided when elution is performed with NADH in the elution buffer. Cyclodextrin elution also showed good reproducibility compared with NADH and KCl (Table 2).

4. Discussion

In many cases affinity elution may result from competition at the protein's active site between the biospecific ligand (e.g., NADH) and the immobilized reactive dye (e.g., CB F3GA) [24,25]. This paper proposes a new concept, the competitive elution by inclusion complexes, which develop an inverse strategy. In this case, competition for the dye arises between the cyclodextrin added in the elution buffer and the target protein, resulting in desorption of the protein. Thus, after the binding stage most target protein (PA-L-LDH) is bound but a small proportion (2–10%) is unbound at any given instant. At the start of the elution stage, the cyclodextrin introduced into the buffer interacts with the covalently bound CB F3GA molecules to form inclusion complexes, so that target protein moves down the column. Further bound protein molecules dissociate from CB F3GA to restore the equilibrium between bound and unbound molecules. Then, the new CB F3GA molecules interact with the cyclodextrins to form new inclusion complexes and the unbound molecules of protein move away down the column. Finally the last molecules of target protein are removed from this portion of the column as cyclodextrin continues to permeate the column.

Competitive elution by inclusion complexes can be related with another elution strategy termed polymer displacement in dye-affinity chromatography [8]. In the latter case, the binding of poly-(ethylene imine) (PEI), a polyelectrolyte, to the immobilized reactive dye results in the displacement of bound proteins but displacement of L-LDH with PEI is non-specific and results in elution of all the proteins bound in the column. Another drawback is that PEI binds strongly to the column and relatively harsh conditions are necessary for column regeneration (0.1 *M* glycine buffer pH 12 containing 1 *M* NaCl and 1% polyacrylate). Therefore, the elution of bound protein by both polymer (PEI) displacement and competitive elution by inclusion complexes are based on the binding of either PEI or cyclodextrin to the dye–ligand. However, competitive elution by inclusion complexes has important advantages namely its greater specificity to desorb the target protein and a simple column-regeneration protocol with mild conditions [20% (v/v) ethanol in water]. In conclusion, affinity elution by cyclodextrins provides a similar purification factor to that obtained by biospecific affinity elution by NADH, and elution volumes (more recovery in fewer fractions) similar to those obtained with non-specific ligands (Table 2). Thus, this new method has the advantages of both biospecific and non-specific ligands, but also shares their disadvantages. For example, if two dehydrogenases are bound to the dye, both may be eluted together. However, cyclodextrins are cheap compared with biospecific ligands. In addition they are available commercially, and produce less absorption at 280 nm, clearly making them the best choice for large-scale purifications. Furthermore, they can be easily separated, if necessary, from the purified protein by dialysis or gel filtration.

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