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Interaction of Cibacron Blue with polymers: implications for polymer-shielded dye-affinity chromatography of phosphofructokinase from baker's yeast

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Abstract

Interactions between Cibacron Blue F3GA and water-soluble non-ionic polymers were investigated by monitoring the spectral shift that accompanies the binding phenomena. Polyvinylpyrrolidone (PVP) and poly(vinyl alcohol) were the only polymers among those tested found to interact effectively with the dye. The difference spectra for the PVP–dye complex was typical of "electrostatic interaction spectra" at low ionic strength and typical of "hydrophobic interaction spectra" in the presence of 1.5 *M* KCl. The binding constant and the number of binding sites per polymer molecule were calculated using the simplest model of independent binding sites. One dye molecule was bound by a PVP segment with a molecular mass of 1000–1300. Regardless of the size of the polymer molecules, the binding constants were in the micromolar range. Poly(vinyl alcohol) bound less efficiently to Cibacron Blue than PVP. One dye molecule was bound by a polymer segment with a molecular mass of about 10 000. The data on PVP complexing with Cibacron Blue were used to develop the concept of polymer-shielded dye-affinity chromatography. This concept was successfully applied to the chromatography of phosphofructokinase (EC 2.7.1.11) from baker's yeast. Specific elution of the bound enzyme from PVP-shielded column resulted in an efficient process with 27-fold purification.

1. Introduction

Affinity chromatography has traditionally been used in the last stages of purification processes owing to the high costs of matrices and ligands and instability of ligands towards degradative enzymes present in very crude extracts [1]. Recent efforts have been directed to the application of this high-resolution technique to the earlier stages of a purification scheme so as to reduce working volumes and processing times, thereby reducing the overall costs. One approach has been to replace the more expensive protein ligands with cheaper triazine dyes, which serve as group-specific ligands [2]. Purification of nucleotide-dependent enzymes such as dehydrogenases and kinases, and also non-nucleotide-dependent proteins such as bovine serum albumin (BSA) on dye-linked supports is well documented [3–11]. The commonly used triazine dyes provide possibilities for a range of different interactions between the protein and the dye. As

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a well defined biological affinity site is not always present, a number of non-specific binding interactions may occur. These interactions may lead to the irreversible binding of the protein, thereby obstructing the selectivity of the process [2].

The selectivity of affinity chromatographic procedures is governed in general by the ratio

$$\frac{\text{efficiency of specific interaction of the target protein with the ligand}}{\text{efficiency of non-specific interaction of foreign proteins with the ligand.}}$$

The use of more specific ligands increases selectivity owing to the increase of the numerator. Another way to improve the selectivity is to reduce the denominator, in other words, to reduce the non-specific interactions and to allow only specific interactions. This approach is also promising because it can improve the recovery of the target molecule. In fact, target molecules interact with the ligand-containing matrix via specific binding sites and non-specifically due to various comparatively weak hydrophobic and electrostatic interactions. Reduction of these non-specific interactions would probably improve the recovery, especially during specific elution of the target molecule. One possible way to reduce non-specific interactions is to block such sites on the ligand with an inert agent. This agent must bind to the ligand tightly enough to prevent the non-specific interactions but not strongly enough to disturb the specific interaction. The multipoint attachment of such an agent to numerous ligands allows it to stick constantly to the column and protects its displacement by the target protein, hence the agent must be of polymeric nature. This agent must not interact with the proteins in the sample. All these requirements of the blocking agent restrict its choice to water-soluble non-ionic polymers.

Previously we proposed the concept of polymer-shielded dye-affinity chromatography [12, 13]. Poly-N-vinylpyrrolidone (PVP) treatment of the column results in the binding of the polymer due to multi-point interaction with the dye ligands. The bound polymer molecules significantly decrease both adsorption of foreign proteins and non-specific binding of the target enzymes, while not seriously impairing enzyme interactions with the dye ligands via specific nucleotide binding sites. The realization of only specific interactions results in improved recoveries and elution efficiency.

The dye-affinity chromatography of phosphofructokinase (PFK) from baker's yeast (*Saccharomyces cerevisiae*) usually results in relatively low degrees of purification (3–13-fold) (see Table 1 and references therein). In the dye-affinity chromatography of other nucleotide-dependent enzymes, the degree of purification usually varies between 20- and 80-fold [11]. The

Table 1
Purification of yeast PFK by dye-affinity techniques using Cibacron Blue ligand

Matrix	Mode	Eluent	Yield (%)	Purification (-fold)	Ref.
Sephadex G-100	Batch adsorption	Ammonium sulfate	59	3.5	[37]
Sephadex G-200	Column chromatography	1.5 *M* ammonium sulfate	84	13.0	[36]
Sephadex G-200	Column chromatography	1.5 *M* ammonium sulfate	85	5.5	[36]
Sephadex G-200	Column chromatography	1.5 *M* ammonium sulfate	75	6.0	[36]
Sepharose CL6B	Column chromatography	1 *M* potassium chloride	91	–[a]	[38]
Sepharose CL6B	Column chromatography	10 m*M* ATP	69	–[a]	[38]
		1 m*M* ATP	26	–[a]	[38]
PEG-CB–Dextran	Affinity partitioning	0.34 *M* K_2HPO_4–0.37 *M* KH_2PO_4	75	9.0	[21]
Matrex Blue	Column chromatography	2 m*M* ATP	81	5.4	[39]
Sepharose CL4B	Column chromatography	50 m*M* ATP	56	27.0	This work

[a] With pure enzyme preparation.

intention of this work was to study the complexation of non-ionic water-soluble polymers with Cibacron Blue and to apply the concept of polymer shielding to the dye-affinity chromatography of PFK.

2. Theory

The simplest model of independent binding of n Cibacron Blue molecules to the polymer molecule was used, and it was treated according to a slightly modified procedure [14]. The observed absorbance, A, can be described mathematically by

$$A = E_{CB}[CB] + E_X[X] \quad (1)$$

where E_{CB} and E_X are molar absorptivities and [CB] and [X] are concentrations of uncomplexed and complexed Cibacron Blue, respectively. The path length was taken as 1 cm.

In the absence of polymer, the absorbance A' can be written as

$$A' = E_{CB}[CB]_0 \quad (2)$$

where the subscript zero indicates total concentration. Also,

$$[CB] + [X] = [CB]_0 \quad (3)$$

The change in absorbance, ΔA, due to the complexation of Cibacron Blue with the polymer may be written as

$$\Delta A = A - A' = (E_X - E_{CB})[X] \quad (4)$$

E_{CB} was determined by direct measurement of Cibacron Blue absorbance, and E_X was determined by measurement of Cibacron Blue absorbance in the presence of the excess of polymer.

The complex formation may be described as

$$[CB] + [\text{binding site}] \rightarrow [X]$$

where [binding site] is the concentration of free binding sites on the polymer molecule. The equilibrium constant taken as the dissociation constant will be

$$K = [CB][\text{binding site}]/[X] \quad (5)$$

The simplest assumption of independent binding of n Cibacron Blue molecules with one polymer molecule corresponds to

$$[\text{binding site}]_0 = n[\text{polymer}]_0 \quad (6)$$

From material balance,

$$[\text{binding site}] + [X] = [\text{binding site}]_0 \quad (7)$$

Substituting Eqs. 3, 6 and 7 in Eq. 5, we obtain

$$K = ([CB]_0 - [X])(n[\text{polymer}]_0 - [X])/[X] \quad (8)$$

Solving Eq. 8 for [X] gives the physically significant root

$$[X] = 0.5\{([CB]_0 + K + n[\text{polymer}]_0) - ([CB]_0 + K + n[\text{polymer}]_0)^2 - 4[CB]_0 n[\text{polymer}]_0\}^{1/2} \quad (9)$$

The experimental data obtained for [X] from Eq. 4 over a range of polymer concentrations were fitted to Eq. 9 allowing best-fit values of the parameters K and n. These best fit values were obtained by minimizing the sum of square deviations weighted with the squares of the values, allowing the K and n terms to float freely. A good fit of experimental values was obtained using only two variable, independent parameters. The use of any other combination of parameters significantly different from the best-fit values resulted in a curve clearly different from that obtained experimentally. This was clearly seen by the significant increase in the sum of square deviations.

3. Experimental

PVP with average molecular masses of 10 000 (PVP-10) and 40 000 (PVP-40), ATP, AMP, β-NADH, fructose-6-phosphate and bicinchonic acid solution were purchased from Sigma (St. Louis, MO, USA). Polyethylene glycol (PEG) with molecular mass 20 000, poly(vinyl alcohol) (PVA) with molecular mass 13 000, methylcellulose 15, hydroxymethylcellulose 20 and dextran F70 were purchased from Serva (Heidelberg, Germany). When PVA was dissolved some insoluble material was removed by centrifugation,

and the actual polymer content was determined on a dry mass basis. PEG 8000 was purchased from Union Carbide, Sepharose CL 4B from Pharmacia (Uppsala, Sweden) and fresh baker's yeast from a local supermarket. Cibacron Blue 3GA was purchased from Sigma and used as received. The heterogeneous character of commercially available dye preparations is well known [15]; nevertheless it is common to use Cibacron Blue in spectral studies without additional purification [14,16–18].

The spectral titration was performed at room temperature according to Ref. [14]. Sample and reference cuvettes each containing a solution of ca. 80 μM Cibacron Blue in 50 mM Tris (pH 8.0) were placed in a Shimadzu UV-260 double-beam spectrophotometer. Small volumes (1–10 μl) of 5% PVP 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 range 400–800 nm were registered (Figs. 1 and 2).

Blue Sepharose was synthesized by coupling Cibacron Blue 3GA to Sepharose CL 4B according to Ref. [19]. The Cibacron Blue content was determined according to Ref. [20] as 41.8 μmol/g dried gel, after drying the gel for 48 h at 80°C.

Yeast homogenate was prepared according to Ref. [21]. Fresh baker's yeast was homogenized with twice its mass of dry-ice in 100-g portions in a household blender. The resulting powder was layered on a tray and, after evaporation of the dry-ice, the liquified material was mixed with 0.05 M sodium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol, 0.2 mM EDTA and 0.5 mM phenylmethanesulfonyl fluoride (PMSF). It was centrifuged at 16 000 g for 15 min and the supernatant was subjected to fractional precipitation with PEG 8000. The precipitate between 4 and 8% (w/w) was collected and dissolved in the extraction buffer. This preparation was either used immediately or frozen at −20°C. The frozen material was thawed and centrifuged at 3000 g and the supernatant was applied to the column.

Chromatographic procedures were performed at 4°C. The column dimensions were 8.0 cm × 2.0 cm I.D. and the flow-rate was 0.1 ml/min.

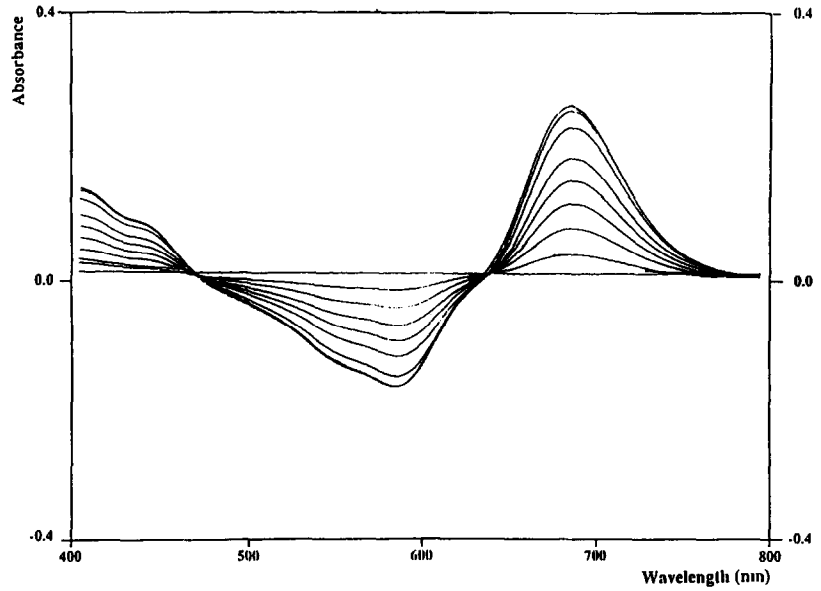

Fig. 1. Difference spectra obtained when Cibacron Blue was titrated with increasing concentrations of PVP-40. Both the sample and reference cuvettes contained 2 ml of 81 μM Cibacron Blue in 50 mM Tris (pH 8.0). Identical volumes of polymer [1.25 mM PVP-40 in 50 mM Tris (pH 8.0)] and buffer were added to the reference and sample cuvettes. The final concentration of PVP-40 in the sample cuvette was 0.6, 1.2, 1.9, 2.5, 3.1, 4.4, 5.6 and 6.8 μM.

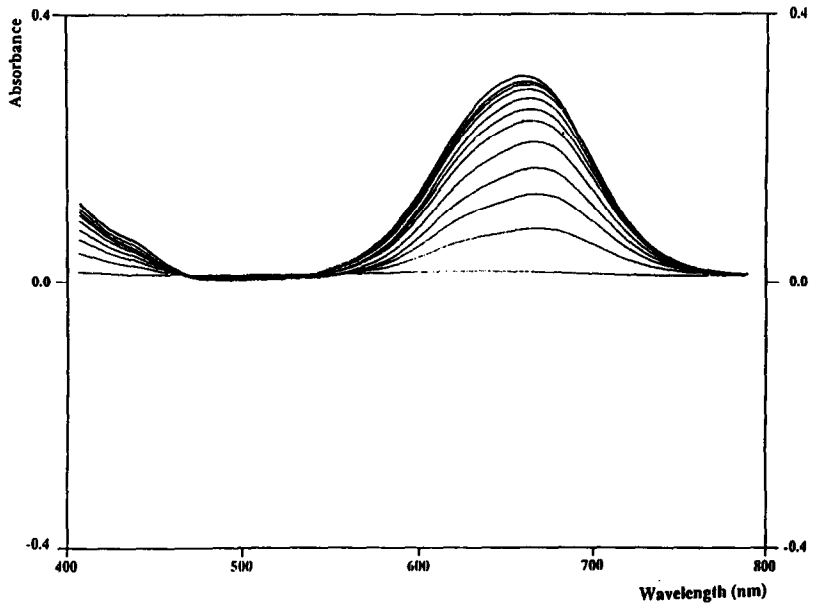

Fig. 2. Difference spectra obtained when Cibacron Blue was titrated with increasing concentrations of PVP-40 in the presence of salt. Both the sample and reference cuvettes contained 2 ml of 81 μM Cibacron Blue in 50 mM Tris (pH 8.0) containing 1.5 M KCl. Identical volumes of polymer [1.25 mM PVP-40 in 50 mM Tris (pH 8.0)] and buffer were added to the sample and reference cuvette. The final concentration of PVP-40 in the sample cuvette was 0.6, 1.2, 1.9, 2.5, 3.1, 3.7, 5.0, 6.2, 7.4 and 9.3 μM.

PVP shielding of the column was done by percolating a 1% PVP solution and washing the column with 1.5 M KCl (pH 3.4). The column was re-equilibrated with 0.05 M sodium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol, 0.2 mM EDTA and 0.5 mM PMSF before protein application; all solutions were subsequently introduced in the same buffer. Fractions were collected every 10 or 50 min. After application of the requisite amount of yeast extract, the column was washed extensively with buffer until the absorbance of the effluent at 280 nm was below 0.2. Specific elution was performed with ATP (0.05 M).

PFK activity was measured according to Ref. [22] using a Shimadzu UV-120-02 spectrophotometer. Protein concentration was determined by the BCA assay using the method given by the manufacturer of the reagent (Sigma procedure No. TPRO-562). The incubation time at 37°C was 60 min, and after cooling at room temperature for 10 minutes the absorbance of the samples was measured at 562 nm.

4. Results and discussion

Spectra of Cibacron Blue in the visible range are sensitive to the environment of the dye chromophore. The difference spectra according to Subramanian [16] can be classified as "electrostatic interaction spectra" (positive peak in the 690-nm region and negative double minima in the 630–585-nm region) and "hydrophobic interaction spectra" (positive peak at 655 nm, a shoulder at 610 nm and a small negative contribution below 550 nm). Using a difference spectroscopic technique we studied the interaction of Cibacron Blue with various non-ionic, water-soluble polymers. No appreciable interaction of the dye with dextran, polyacrylamide, hydroxyethylcellulose or PEG was observed. A very weak interaction of hydrophobic type was detected with methylcellulose (data not shown). Only PVP and PVA interacted with the dye. PVA interactions were weaker than interactions with PVP.

PVP interaction with Cibacron Blue resulted

in difference spectra with a peak at 680–690 nm, a minimum at 580–590 nm with a shoulder around 550 nm and an isosbestic point at 630–640 nm, indicating complex formation (Fig. 1). This type of difference spectra is typical of dye interaction with proteins, and can be classified as "electrostatic interaction spectra" according to Subramanian [16]. The difference spectra were significantly changed in the presence of 1.5 *M* KCl and characterized by a positive peak at 650–660 nm and a small negative contribution below 550 nm (Fig. 2). This spectrum type is similar to "hydrophobic interaction spectra" [16]. PVP is well known to complex both with negatively charged and hydrophobic substances [23]. The contribution of electrostatic interaction in the difference spectra was more pronounced at low ionic strength. The increase in ionic strength suppressed the electrostatic component and made the contribution of the hydrophobic interaction more pronounced.

The binding constant for PVP complexing with Cibacron Blue in solution was calculated as 2.1 μM (Fig. 3). A molecule of the polymer with a molecular mass of 40 000 contained 30 sites capable of binding Cibacron Blue ligands. For PVP-10 with a molecular mass of 10 000 the calculated value of the binding constant was 6.1 μM and the number of binding sites per polymer molecule was 8. Note should be taken that the calculated dependence of complex concentration on polymer concentration was very sensitive to the number of binding sites per polymer molecule. Even minimal changes in the number of binding sites resulted in significant changes of the binding curve (Fig. 3, dashed curves).

The dye ligand was bound by a polymer segment having a molecular mass of 1000–1300 irrespective of the size of the polymer molecule. This supported the assumption of independent binding sites and is in agreement with the direct measurement of I_3 and 1-anilinonaphthalene-8-sulfonate complexation with vinylpyrrolidone oligomers of different molecular masses. The complexation takes place only when the oligomer molecular mass is higher than 1500 [24].

Although the type of the difference spectra was changed with increase in ionic strength, the

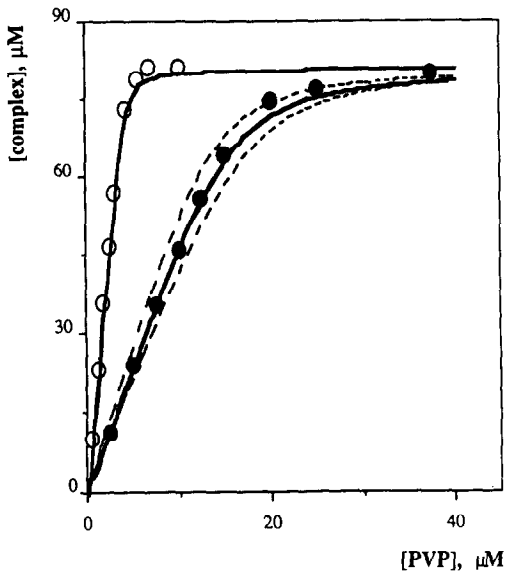

Fig. 3. PVP-40 (○) and PVP-10 (●) complexation with Cibacron Blue [81 μM Cibacron Blue, 50 m*M* Tris (pH 8.0)]. The open circle values were taken from Fig. 1 except the last point. The spectrum for this point and the one before it coincide. The values for E_{CB} and E_X at 680 nm are 6500 and 9600 l mol^{-1} cm^{-1}, respectively. The solid curves were calculated using the binding constants 2.1 and 6.1 μM and 30 and 8 binding sites for PVP-40 and PVP-10, respectively. The dashed curves were calculated using binding constant 6.1 μM and numbers of binding sites 7 (lower curve) and 9 (upper curve) for PVP-10.

binding constant of PVP-40 with Cibacron Blue was relatively insensitive to the high ionic strength. In the presence of 1.5 *M* KCl, which is frequently used as a non-specific eluent in affinity chromatography, the binding constant was 5.7 μM, the number of binding sites being practically the same, 32. A small regular shift of the wavelength at the maximum indicated a more complicated process of Cibacron Blue binding to the polymer under these conditions.

The interaction of PVA with Cibacron Blue was of the same type as for PVP: a positive maximum at 670–680 nm, an isosbestic point at 600–610 nm and a minimum at 570–580 nm with a shoulder around 540 nm. In contrast to PVP, the interaction with PVA was significantly weaker. The calculated value of the binding constant was 16.7 μM and the number of binding sites per polymer molecule was only 1.4. A

polymer segment with a molecular mass of about 10 000 complexed one Cibacron Blue molecule, ten times less than in the case of PVP. PVA with a molecular mass of 13 000 interacted with the dye nearly stoichiometrically. This polymer could not provide multi-point attachment when interacting with a Cibacron Blue-containing matrix, and for this reason it could not be used for polymer shielding in dye-affinity chromatography.

The interaction of Cibacron Blue with PVA reduces the access to the ligand in dye–PVA conjugates. That explains the previously reported result [25] that lactate dehydrogenase (LDH) from bovine heart was inhibited ten times less efficiently by Cibacron Blue–PVA conjugate than by Cibacron Blue–dextran conjugate, in which no interaction was detected between the dye and the polymer. The absence of a Cibacron Blue interaction with dextran and hence good access to the dye-ligand in such conjugates explains clearly the observation that the same values of the inhibition constants of lactate dehydrogenase by free and dextran-coupled triazine dyes are obtained [26]. The coupling of Cibacron Blue to Sepharose, which is carbohydrate in nature (as is dextran), does not alter the binding constant of LDH to the dye. The interaction of BSA with free and PEG-coupled Cibacron Blue is characterized by virtually the same half-saturation dye concentrations, whereas for PVA–Cibacron Blue it is about ten times higher [27]. The absence of a Cibacron Blue interaction with PEG is the reason for the extensive use of such conjugates in affinity partitioning [28]. Restricted access of the dye-ligand in the Cibacron Blue–PVA conjugates might be the reason for unsuccessful affinity precipitation of LDH using these conjugates [25,29].

Among the polymers studied, only PVP could bind efficiently and via multi-point attachment to the Cibacron Blue ligands of the Blue Sepharose and serve as an inert blocking agent of non-specific protein–dye interactions. The binding constant of one PVP segment with the dye ligand was in the micromolar range. The binding constants of Cibacron Blue to the nucleotide-binding sites of enzymes are in the same range or even lower [26,29–34]. Thus enzymes can successfully compete with PVP segments for the ligand. The non-specific interaction of the dye ligands with the proteins is much weaker and characterized by higher binding constants. For instance, binding of Cibacron Blue to bovine serum albumin, which is considered to take place due to non-specific hydrophobic interactions, is characterized by a binding constant estimated to be 35–85 μM [27]. The data on Cibacron Blue binding to cytochrome b_5 reductase can be rationalized assuming non-specific interaction with a binding constant of 85 μM together with specific binding (binding constant 1 μM) [35]. Hence the PVP segments, when bound to the dye-ligand, would protect it from relatively weak non-specific interactions with proteins. In other words, PVP molecules bound to the Blue Sepharose could serve as a lid, making the ligand available for strong specific interactions and protecting it from relatively weaker non-specific interactions. In dye affinity chromatography, retention of the target protein is governed by the sum of specific and non-specific interactions. When an affinity eluent is introduced, the specific interactions are minimized. If the non-specific interactions in the presence of affinity eluent are insufficient to retain the protein, it starts to move down the column. If the non-specific interactions are sufficiently strong, the protein remains bound and, as a result, the recovery is low [2]. Another consequence of the non-specific interactions is that even though they may be insufficient to retain the protein in the absence of the specific interactions, they may interact weakly with the protein as it moves down the column. These weak interactions will give rise to peak tailing and reduce the efficiency of the elution step. In the case of polymer treatment, it is assumed that the polymer prevents these non-specific interactions between the matrix and the target protein. As a result, only specific interactions occur. One consequence of this is an improved yield and elution efficiency. This forms the concept of polymer-shielded dye-affinity chromatography [12,13]. Table 2 summarizes the effects of polymer shielding. In all instances a decrease in capacity is seen with improved yields. There is a marginal

Table 2
Influence of PVP treatment in dye-affinity chromatography

System	Elution mode	Yield	Purification (-fold)	Efficiency	Capacity
Blue Sepharose–LDH	Non-specific	+	Marginal	+	–
	Specific	+	Marginal	+	–
Scarlet Sepharose–LDH	Non-specific	Marginal	+	+	–
	Specific	+	Marginal	Marginal	–
Scarlet Sepharose–sADH	Non-specific	Marginal	+	Marginal	–
	Specific	+	Marginal	+	–
Blue Sepharose–PFK	Specific	+	Marginal	+	–

The results are taken from Ref. [13] and this work; + indicates an increase; – indicates a decrease; marginal indicates marginal effects.

effect on the degree of purification fold, but the overall efficiency in terms of the elution volume is generally increased.

Like many other kinases, PFK has been shown to interact effectively with Cibacron Blue [36]. Binding is believed to occur at the ATP binding sites of the enzyme. A number of procedures that take advantage of this fact have been published for the purification of PFK on Cibacron Blue linked supports (Table 1). The degrees of purification achieved are significantly lower than those obtained in dye-affinity chromatography of other nucleotide-dependent enzymes, where they usually vary between 20- and 80-fold [11]. The low degrees of purification suggest strong non-specific interactions in the case of dye-affinity chromatography of PFK and makes this system suitable for evaluating the potential of polymer-shielded dye-affinity chromatography.

Specific elution of PFK (with its nucleotide substrate ATP) from an untreated and a PVP-shielded column is presented in Fig. 4 and Table 3. PVP shielding resulted in improved efficiency of the column. PFK concentration in the peak fractions was 5–6 times higher from the PVP-shielded column, and peak tailing, characteristic of the untreated column, was absent with the PVP-shielded column. PVP shielding also resulted in an improved PFK recovery, without significantly affecting the degree of purification (Table 3).

The purification factor obtained, 27, was significantly higher than that reported previously in Cibacron Blue-based chromatography of PFK [21,36–39], but the recovery of PFK by specific elution was lower. The high recovery reported

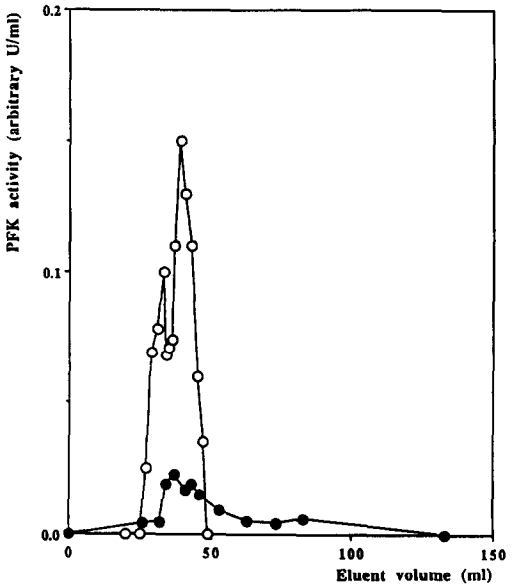

Fig. 4. Specific elution profile of PFK with 0.05 *M* ATP from untreated (●) and PVP-treated (○) Blue Sepharose column. Chromatographic procedures were performed at 4°C. Experimental conditions: 8.0 cm × 2.0 cm I.D. column; all solutions were introduced in 0.05 *M* sodium phosphate buffer (pH 7.0) containing 5 m*M* 2-mercaptoethanol, 0.2 m*M* EDTA and 0.5 m*M* PMSF; PVP treatment was done by percolating a 1% PVP solution through the column and then washing with 1.5 *M* KCl (pH 3.4); the column was re-equilibrated with 0.05 *M* sodium phosphate buffer (pH 7.0) containing 5 m*M* 2-mercaptoethanol, 0.2 m*M* EDTA and 0.5 m*M* PMSF; the flow-rate was 0.1 ml/min.

Table 3
Elution of PFK from Blue Sepharose

Column	Enzyme activity applied (U)	Enzyme activity bound (U)	Enzyme activity recovered			Specific activity of enzyme (U mg^{-1})		Purification (-fold)
			U	Volume (ml)	Yield (%)	Before affinity chromatography	After affinity chromatography	
Untreated	4.80	4.70	0.53	133	11.3	0.63	15.0	24.0
PVP-treated	4.30	2.30	1.30	47	56.0	0.60	16.0	27.0

Chromatographic procedures were performed at 4°C. The column dimensions were 8.0 cm × 2.0 cm I.D. and the flow-rate was 0.1 ml/min. The column was equilibrated before protein application with 0.05 *M* sodium phosphate buffer (pH 7.0) containing 5 m*M* 2-mercaptoethanol, 0.2 m*M* EDTA and 0.5 m*M* PMSF and all solutions were subsequently introduced in the same buffer. Fractions were collected every 10 or 50 min. After application of the requisite amount of yeast extract, the column was washed extensively with buffer until the absorbance 280 nm of the effluent was below 0.2. The specific elution was performed with ATP (0.05 *M*).

may be a result of the use of purified PFK, whereas crude yeast extract was used in these experiments.

The improvement of column efficiency and PFK recovery can be attributed to the PVP blockage of Cibacron Blue ligands capable of non-specific binding of PFK. The specific binding of PFK to the column was not seriously impaired. Some decrease in PFK binding by the PVP-shielded column was probably due to the decrease in non-specific PFK binding, which was much more significant with the untreated column.

Previously we have shown that PVP shielding results in improved efficiency of specific elution for systems such as porcine muscle lactate dehydrogenase–Blue Sepharose and secondary alcohol dehydrogenase from *Thermoanaerobium brockii*–Scarlet Sepharose. The present data provide a further example of the benefits of PVP shielding. These data, along with the results on the PVP binding to a Cibacron Blue, form a consistent concept of polymer-shielded dye affinity chromatography.

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