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Affinity partitioning of glucose-6-phosphate dehydrogenase and hexokinase in aqueous two-phase systems with free triazine dye ligands

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Abstract

The partitioning of glucose-6-phosphate dehydrogenase (G6PDH) (E.C. 1.1.1.49) and hexokinase (E.C. 2.7.1.1) in polyethylene glycol (PEG)–hydroxypropyl starch (PES) and PEG–phosphate aqueous two-phase systems was investigated with free triazine dyes, Cibacron Blue F3GA and Procion Red HE3B, as their affinity ligands. It was found that the free reactive triazine dyes, not bound to phase-forming polymers, preferentially partitioned in the top-PEG phase in the PEG–salt and PEG–PES systems. The effect of various parameters such as type and concentration of affinity ligands, pH of the system, molecular mass of PEG and phase composition on partitioning of the enzymes was estimated. Phosphate is a key factor affecting the enzyme partitioning in the PEG–PES system. Cibacron F3GA changed the partition coefficient of G6PDH from 0.73 to 1.59.

Keywords: Partitioning; Aqueous two-phase systems; Glucose-6-phosphate dehydrogenase; Hexokinase; Enzymes; Triazine dyes

1. Introduction

Aqueous two-phase systems (ATPSs) are finding increasing application in the recovery of proteins [1,2]. In order to improve the selectivity, affinity ligands were introduced into the aqueous two-phase systems. Affinity partitioning as currently practiced requires the ligand to be covalently attached to one of the phase-forming polymer components, thereby causing the ligand–polymer to partition predominantly into one of the phases. Although triazine dyes covalently coupled to polyethylene glycol (PEG) can be produced on a large scale, the process is complicated and requires a chromatographic step and several organic solvent extractions [3]. To simplify the affinity partitioning, Giuliano [4] used the free dyes, uncoupled to the phase-forming polymers, as affinity ligands for the partitioning of lysozyme in the polyvinylpyrrolidone–maltodextrin aqueous two-phase system. In the PEG–phosphate system, it was

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reported that free triazine dyes, partitioned predominantly into polymer phase, showed the affinity effect on some dehydrogenases and kinases [5,6]. Lin et al. [7,8] also used free triazine dyes as ligands to affinity extraction of lactate dehydrogenase in the PEG–hydroxypropyl starch (PES) aqueous two-phase system.

Glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme in the pentose phosphate pathway, is widely distributed in nature. It is a part of the antioxidant enzymatic system with an important role in tissue protection against the destructive action of oxygen free radicals [9,10]. Hexokinase (HK) is a key enzyme in carbohydrate metabolism. It has an important role in the control of the catabolic repression [11,12], as well as in the glucose uptake mechanism through the cytoplasmic membrane [13,14]. In addition to their importance in biochemical studies, G6PDH and HK present great interest as analytical reagents for the measurement of creatin-kinase activity, ATP and hexose concentrations [15].

In this paper, the partitioning of G6PDH and HK with free Cibacron Blue F3GA and Procion Red HE3B as affinity ligands in the PEG–PES and PEG–phosphate aqueous two-phase systems was investigated. A systematic study of the effects of various parameters, such as pH of the system, phase composition, molecular mass of PEG, and concentration of ligands on partition coefficients of the enzymes was carried out.

2. Experimental

2.1. Chemicals

PEG3000, PEG4000 and PEG6000 were purchased from LABSYNTH Produtos para Laboratorios. Hydroxypropyl starch (Reppal PES100) was a kind gift from Professor J.A. Teixeira (Departmento de Engenharia Biologica, Universidade do Minho, Portugal). Cibacron Blue F3GA and Procion Red HE3B were purchased from Sigma. G6PDH, HK, β-nicotinamide adenine dinucleotide phosphate (NADP), adenosine 5′-triphosphate (ATP), D-glucose-6-phosphate (G6P) and D-glucose were also from Sigma. All other reagents were analytical grade.

2.2. Aqueous two-phase systems

The systems were prepared from stock solutions: 50% (w/w) PEG, 30% (w/w) PES, 40% (w/w) ammonium sulfate and 40% (w/w) phosphate (mixture of NaH_2PO_4 and K_2HPO_4). The total mass was 3.0 g for polymer–polymer (PEG–PES), and 4.0 g for polymer–salt systems. The pH values of the systems in the experiments refer to the pH values of the mixed phosphate buffer stock solution. All concentrations were given in mass percentage except where otherwise indicated.

2.3. Partitioning experiments

For partitioning experiments, the enzymes and ligands were added to the systems. After 15 min of inversion mixing to ensure partition equilibrium, phase separation was accomplished by centrifugation at 2880 *g* for 5 min and samples were withdrawn for analysis. The experiments were conducted in duplicate at room temperature.

2.4. The preparation of derivatives of the dyes

According to Giuliano [4], the amino derivatives of chlorotriazine dyes (Cibacron Blue F3GA and Procion Red HE3B) were prepared as follows: 4 g of the dye was first dissolved in 50 ml of methanol followed by the addition of 20 ml of concentrated ammonium hydroxide. The solution was refluxed for 40 min. Solvents were recovered via rotary evaporation at reduced pressure (vacuum 60 kPa). The remaining solid was washed with 300 ml of acetone and dried for use. The acetone was removed by rotary evaporation.

2.5. The analysis

G6PDH activity was measured spectrophotometrically at 30 °C by following the rate of $NADP^+$ reduction at 340 nm in a coupled enzyme assay system. The assay mixture consisted of 50 m*M* Tris–HCl (pH 7.5), 5 m*M* $MgCl_2$, 0.5 m*M* $NADP^+$ and 10 m*M* glucose-6-phosphate. Hexokinase activity was determined spectrophotometrically by coupling the formation of glucose-6-phosphate from glucose to the reduction of $NADP^+$ with G6PDH.

The measurements were performed by following the increase rate of absorbance (340 nm) at 30 °C. The assay mixture consisted of 50 m*M* Tris–HCl (pH 7.5), 5 m*M* $MgCl_2$, 0.5 m*M* $NADP^+$, 50 m*M* glucose, 1.5 m*M* ATP and 1 U G6PDH. For both enzymes, one unit of enzyme activity was defined as the amount of enzyme required to form 1.0 μmol of NADPH/min with the substrate in excess.

The partition coefficient of the enzymes (K_e) was defined as the ratio between the enzyme activities in the top and bottom phases. For the determination of partition coefficient, the values of the enzyme activities were used without correction for the inhibition of dye ligands.

The concentration of the dye and the derivatives was determined photometrically at 615 nm for Cibacron Blue F3GA and its derivative, and 515 nm for Procion Red HE3B and its derivatives. The partition coefficient of the dyes (K_L) is defined as the ratio between the concentrations in the top and bottom phases.

The binding of a ligand to an enzyme was evaluated by inhibition constant, $K_{i,}$ which is the dissociation constant of enzyme–ligand complex. The value was determined by Lineweaver–Burk plot. Enzymatic assay was carried out in the corresponding equilibrated top and bottom phases.

3. Results and discussion

3.1. Partitioning of the dyes

In an affinity aqueous two-phase system, it is usually believed that the ligand must be covalently coupled to the target phase polymer, one of its purposes was to confine the ligand in one phase. To use uncoupled free dye molecules as ligand, their partition behaviors in the system should be clarified.

3.1.1. Partitioning in PEG–salt systems

In PEG–salt aqueous two-phase systems, the dyes partitioned predominantly to the top PEG phase. The effect of pH on the partitioning of Cibacron Blue F3GA and Procion Red HE3B in the PEG–phosphate system is presented in Table 1. With the increase of pH, the partition coefficient of both dyes increased. The effect of PEG molecular mass on the partition coefficient of Cibacron Blue F3GA and Procion Red HE3B in the PEG–ammonium sulfate system is shown in Table 2.

Table 1
The effect of pH on the partitioning of dyes in the PEG3000 (12.5%, w/w)–phosphate (10%, w/w) system

pH	K_L	
	Cibacron Blue F3GA	Procion Red HE3B
3.45	7.3	6.3
5.97	319	310
7.48	707	>1000
9.70	>1000	>1000

Table 2
The effect of PEG molecular mass on the partitioning of dyes in the PEG (12.5%, w/w)–ammonium sulfate (10%, w/w) system

Molecular mass	K_L	
	Cibacron Blue F3GA	Procion Red HE3B
3000	16.5	37.8
4000	27.6	133
6000	227	741

3.1.2. Partitioning in the PEG–PES system

In the PEG–PES system, although the partition coefficients are smaller than those in PEG–salt systems, most of the dyes still partitioned to the top PEG phase, which is the base for the affinity partitioning of the enzymes.

Fig. 1 shows the effect of pH on the partitioning of Cibacron Blue F3GA and Procion Red HE3B in the PEG–PES system. With the increase of pH, the partition coefficients of both dyes increased.

In aqueous two-phase systems, some salts have a strong effect on the partitioning behavior of substances. The influence of phosphate on the partitioning of Cibacron Blue F3GA and Procion Red HE3B in the PEG–PES system is presented in Table 3.

3.2. Effect of pH on the partitioning of enzymes

The effect of pH on the partitioning of G6PDH in the PEG6000–PES100 system without dye ligand is shown in Fig. 2 (curve 1). Without affinity ligand, the partition coefficient increased with the increase of system pH.

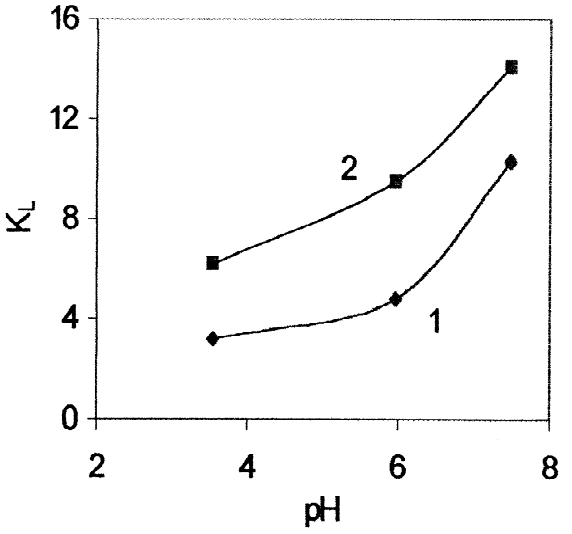

Fig. 1. The effect of pH on the partitioning of Cibacron Blue F3GA (1) and Procion Red HE3B (2) in the PEG3000 (8.3%, w/w)–PES100 (15%, w/w) system. Dye concentration (0.067%, w/w), phosphate buffer (2.67%, w/w).

For the charged proteins, Albertsson [1] developed the classical model for the electrochemical partitioning in ATPSs (Eq. (1)):

$$\ln K_p = \ln K_0 + (z_p F/RT)\Delta\varphi \quad (1)$$

Here K_p denotes the partition coefficient of protein, $(z_p F/RT)\Delta\varphi$ is the electrostatic term as a product of protein surface charge z_p and $\Delta\varphi$, the electrostatic potential difference between the two phases, and K_0 includes all the other factors affecting protein partitioning. With the increase of pH, the negative charges of G6PDH increased, and its partition coefficient was enhanced.

Table 3
The influence of phosphate on the partitioning of Cibacron Blue F3GA and Procion Red HE3B in the PEG3000–PES100 system[a]

$C_{phosphate}$	K_L	
(%, w/w)	Cibacron Blue F3GA	Procion Red HE3B
1.33	4.7	9.6
2.67	4.8	9.5
4.00	6.0	9.8
5.33	18.4	34

[a] PEG3000 (8.3%, w/w)–PES100 (15%, w/w) system, pH 5.97.

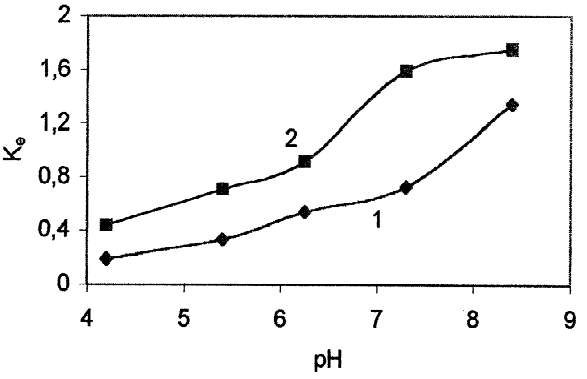

Fig. 2. Effect of pH on the partitioning of G6PDH in the PEG6000 (8.3%, w/w)–PES100 (15%, w/w) system. (1) Without blue dye, and (2) with 0.033% blue dye, 1.33% phosphate.

With the Cibacron Blue F3GA as affinity ligand, the affinity effect was observed and the partition coefficient increased also with the increase of system pH. Although the partition coefficient of dye ligand increased with the pH increase (Fig. 1), the affinity effect was not intensified.

3.3. Effect of phase composition on the partitioning of enzymes

The effect of PEG6000 concentration on the partitioning of G6PDH with and without ligand in the PEG6000–PES100 system is presented in Fig. 3.

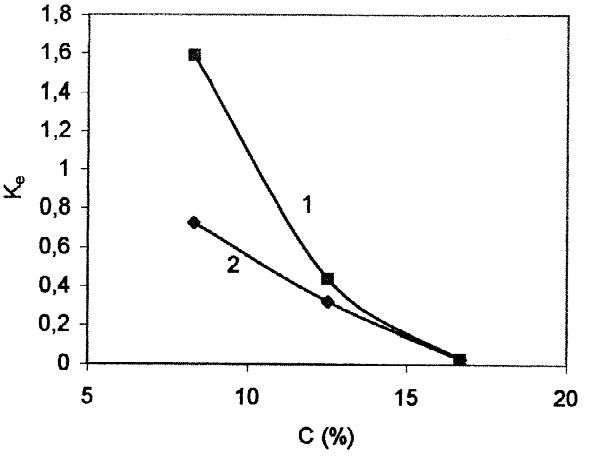

Fig. 3. Effect of PEG6000 concentration on the partitioning of G6PDH in the PEG6000–PES100 (15%, w/w) system. (1) With 0.033% blue dye, (2) without blue dye, 1.33% phosphate, pH 7.3.

Table 4
The effect of phase composition on the partitioning of Cibacron Blue F3GA and G6PDH

PEG6000[a]	K_L	φ[c]	PES100[b]	K_L	φ
8.3	11.0	2.18	12.5	9.07	1.78
12.5	17.2	1.38	15.0	11.0	2.18
16.7	22.7	0.96	20.0	15.4	1.24

[a] PEG6000–PES100 (15%, w/w) system; 1.33% phosphate, pH 7.3, 0.033% blue dye.
[b] PEG6000 (8.3%, w/w)–PES100 system; 1.33% phosphate, pH 7.3, 0.033% blue dye.
[c] Ratio of partition coefficient in the presence of dye to that in the absence of dye.

With the increase of PEG concentration, both of the partition coefficients of G6PDH with and without dye ligand decreased. Table 4 summarizes the effect of phase composition and blue dye on the partition behavior of G6PDH. Although the partition coefficient of the blue ligand increased with the increase of PEG concentration, the affinity effect diminished gradually (Fig. 3). The change of PES100 concentration showed a similar influence on the partitioning behavior of G6PDH (Fig. 4, Table 4).

3.4. Effect of phosphate concentration on the partitioning of enzymes

Fig. 5 indicates the effect of phosphate concentration on the partitioning of G6PDH in the PEG6000–PES100 system with and without blue dye ligand. The partition coefficient decreased with the increase of phosphate concentration, and is in accordance with the results of other researchers [16,17].

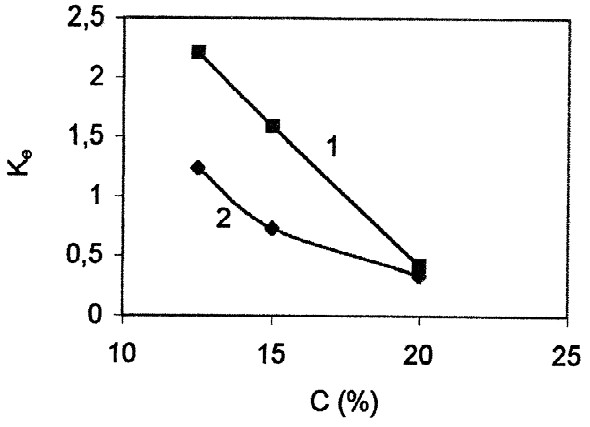

Fig. 4. Effect of PES100 concentration on the partitioning of G6PDH in the PEG6000 (8.3%, w/w)–PES100 system. (1) With 0.033% blue dye, (2) without blue dye, 1.33% phosphate, pH 7.3.

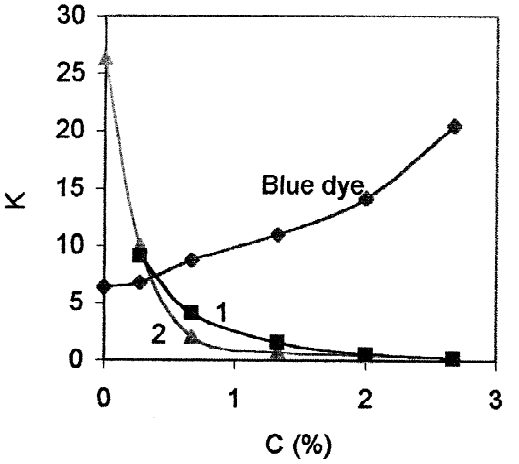

Fig. 5. Effect of phosphate concentration on the partitioning of G6PDH and Cibacron Blue F3GA in the PEG6000 (8.3%, w/w)–PES100 (15%, w/w) system. (1) With 0.033% blue dye, (2) without blue dye, pH 7.3. The point of 0% phosphate is a special point as a comparison.

With the low and high phosphate concentration, the ligand showed no affinity effect on the G6PDH. Only in the medium concentration range, could the enzyme be brought into the top-PEG phase by the blue dye ligand. With high phosphate concentration, although the partition coefficient of blue dye ligand was high enough for affinity partitioning, the interaction between the enzyme and ligand was eliminated by the shielding effect of the salt [5]. In the low phosphate concentration range, the partition coefficient of blue dye ligand was smaller than that of G6PDH, thus no affinity effect could be shown. Here, one should note that the phosphate had opposite effects on the same negatively charged small blue dye ligand molecule and bio-macromolecule G6PDH in the PEG–PES aqueous two-phase system.

Affinity partitioning means enhancement of selectivity, the change of the partition coefficient of the target substance with and without the affinity ligand. Generally, most negatively charged proteins partitioned to the top PEG phase in the PEG–PES aqueous two-phase system. In the presence of nega-

Table 5
The partitioning of G6PDH in the PEG3000 (12.5%, w/w)–phosphate (10%, w/w) system[a]

Dye	K_e
Cibacron Blue F3GA	0.0043
Procion Red HE3B	0.0040

[a] pH 7.48; 0.05% dyes.

tively charged phosphate, the proteins would be transferred to the bottom PES phase. With the selective ligand Cibacron Blue F3GA, G6PDH was transferred to the top PEG phase again by the affinity effect and the other proteins stayed in the bottom phase. Only in the medium phosphate concentration G6PDH was brought into the top PEG phase ($K_e > 1$) from the bottom PES phase ($K_e < 1$) by the blue dye ligand.

Table 5 lists the partition coefficient of G6PDH in the PEG3000–phosphate system in the presence of the dye ligands. In this extreme case, although the dyes were concentrated in the top PEG-rich phase (Table 1), the enzyme stayed in the bottom salt-rich phase. The dyes and the G6PDH could be separated in the PEG–phosphate system.

3.5. Effect of molecular mass on the partitioning of enzymes

The effect of PEG molecular mass on the partition coefficient of G6PDH and HK activity is shown in Fig. 6. Increase of PEG molecular mass brought about a reduction of partition coefficient of G6PDH and HK. This may be caused by the excluded volume effect of PEG [1,5], and by the different tie-line lengths of the systems.

Table 6 shows the effect of PEG molecular mass on the partition coefficient of G6PDH in the PEG–PES100 system with and without blue dye ligand. Although the partition coefficient is higher in the PEG3000 system, the enhancement by the dye ligand is very weak. In PEG6000 system, we obtained the largest increase of the partition coefficient and the G6PDH was transferred from the bottom PES phase ($K_e < 1$) to the top PEG phase ($K_e > 1$) by the affinity effect which might be useful in the selective extraction of the enzyme.

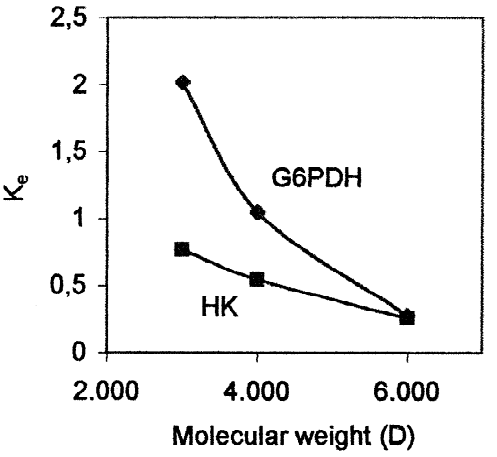

Fig. 6. Effect of PEG molecular mass on the partition coefficient of enzymes in the PEG (8.3%, w/w)–PES100 (15.0%, w/w) system. Phosphate (2.67%, w/w), pH 7.3.

3.6. Effect of ligand type and concentration on partitioning of enzymes

The triazine dyes may form covalent linkage with the proteins. To eliminate the possibility of covalent modification of HK or G6PDH, the reactive chlorine atom of the dyes could be removed by hydrolysis [18,19]. According to Giuliano [4], the triazine dyes were converted to their amino derivatives to prevent the formation of dye–protein covalent linkage.

Fig. 7 shows the effect of Cibacron Blue F3GA and its amino derivative concentration on the partitioning of G6PDH in the PEG6000–PES100 system. The blue dye and its derivatives have a similar effect on the partitioning of G6PDH. At high concentration of the blue dye ligand, the partition coefficient of

Table 6
The effect of PEG molecular mass on the partition coefficient of G6PDH in the PEG–PES100 system[a]

Molecular mass	K_e		ϕ[b]
	No dye	Cibacron Blue	
3000	4.46	4.96	1.11
6000	0.73	1.59	2.18
10 000	0.20	0.34	1.70

[a] PEG (8.3%, w/w)–PES100 (15.0%, w/w) system, 1.33% phosphate (buffer), 0.033% blue dye, pH 7.3.

[b] Ratio of partition coefficient in the presence of dye to that in the absence of dye.

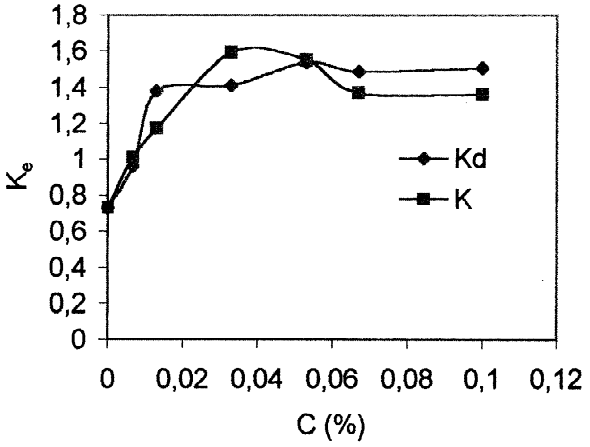

Fig. 7. Effect of blue dye (K) and dye derivative (K_d) concentration on the partitioning of G6PDH in the PEG6000 (8.3%, w/w)–PES100 (15%, w/w) system; 1.33% phosphate, pH 7.3.

G6PDH approaches a constant value. This is similar to what had been observed when dye–PEG [20] or dye–dextran [21] coupled ligands were used. Initially, when the ligand concentration is low, increasing the dye concentration makes more sites available for enzyme binding and the partition coefficients increased rapidly. However, when the dye concentration reached the stoichiometric quantity for a given enzyme concentration, a further increase in dye concentration has no effect on the partition coefficients.

The affinity effect of triazine dye ligands on the partitioning of G6PDH and HK in the PEG6000–PES100 system is summarized in Table 7. The two dye ligands could enhance the partition coefficient of G6PDH, and the effect of blue dye was stronger. For HK, both dyes did not show any enhancement of partition coefficient.

To elucidate the mechanism of affinity aqueous two-phase partitioning of proteins, Flanagan and Barondes [22] had proposed a thermodynamic model (Eq. (2)):

$$K = K_0(K_L K_{ib}/K_{it})^{\alpha} \quad (2)$$

where K and K_0 are the partition coefficients of proteins in the presence and absence of the ligand, respectively; K_L is the partition coefficient of the ligand polymer; K_{it} and K_{ib} denote the dissociation constants of the ligand with protein molecule in the top and bottom phase, respectively, and α is the number of ligand polymer molecules bound per protein molecule.

The binding strength of G6PDH to blue dye is similar (similar K_i) in the top and bottom phases (Table 8), but most of the dye partitioned in the top PEG phase ($K_L = 11.0$), so G6PDH was brought to the top phase by the ligand. Although the K_L of red dye is higher than that of blue dye, because its binding strength to G6PDH is stronger in bottom PES phase (smaller K_i), its affinity effect was weakened. The binding strength of HK to dye ligands is weaker than that of G6PDH (larger K_i value), so neither of the dyes showed enhancement of its partition coefficient.

Table 7
The effect of triazine reactive dyes on the partitioning of enzymes[a]

	K_L	K_e	
		G6PDH	HK
No dye		0.73	0.61
Cibacron Blue	11.0	1.59	0.57
Procion Red	25.7	0.92	0.46

[a] PEG6000 (8.3%, w/w)–PES100 (15%, w/w) system; 1.33% phosphate, pH 7.3, 0.033% dyes.

Table 8
Inhibition constants of G6PDH and HK by triazine dyes[a]

Enzyme	K_i (10^4 mM)			
	Cibacron Blue F3GA		Procion Red HE3B	
	In the presence of top phase	In the presence of bottom phase	In the presence of top phase	In the presence of bottom phase
G6PDH	7.94	6.02	2.49	0.67
HK	201	130	20.2	8.26

[a] In the top or bottom phase of the PEG6000 (8.3%, w/w)–PES100 (15%, w/w) system; 1.33% phosphate, pH 7.3.

4. Conclusion

The partitioning of glucose-6-phosphate dehydrogenase and hexokinase in PEG–PES and PEG–phosphate aqueous two-phase systems was investigated with free triazine dyes, Cibacron Blue F3GA and Procion Red HE3B, as their affinity ligands.

The dyes, not bound to phase-forming polymers, were concentrated in the top-PEG phase in PEG–phosphate and PEG–ammonium sulfate systems. In the PEG–PES system, although the partition coefficients are smaller than those in PEG–salt systems, most of the dyes still partitioned in the top PEG phase, which is the fundamental requirement for the affinity partitioning of the enzymes. Phosphate, with an opposite effect on the negatively charged small dye molecules and G6PDH, is a key factor affecting the enzyme partitioning in the PEG–PES free dye affinity system. It could direct the dye ligand to the top PEG phase which increases the partition coefficient of enzyme, but has the shielding effect on the interaction between ligand and enzyme that decreases the partition coefficient.

In the PEG–PES system, Cibacron Blue F3GA changed the partition coefficient of G6PDH from 0.73 to 1.59 with phosphate buffer solution. The enzyme was transferred from the bottom PES-rich phase to the top PEG-rich phase. Neither of the dyes showed enhancement of partition coefficient for hexokinase. In the PEG–phosphate system, the enzyme and dyes were concentrated in the top PEG and bottom phosphate phases, respectively. The enzyme in the top-PEG phase can be recovered if necessary from the second PEG–salt two-phase system.

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