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HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY OF ENZYMES ON SILICA-IMMOBILISED TRIAZINE DYES

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SUMMARY

A number of reactive triazine dyes, including Procion Blue MX-R, Procion Yellow H-A, Procion Green H-4G, Procion Red H-8BN, Procion Brown MX-5BR and Cibacron Blue F3G-A, have been covalently attached to microparticulate silica (10 μm) and used for the resolution of proteins by high-performance liquid affinity chromatography (HPLAC). Purified dyes were either converted to their 6-aminohexyl derivative by reaction with 1,6-diaminohexane and then coupled to γ-glycidoxypropyl-trimethoxysilane-activated silica or coupled directly to glycol-silylated-silica via the reactive triazine ring. These triazine dye–silica adsorbents have been used for the resolution of protein mixtures containing enzymes such as lactate dehydrogenase, hexokinase, alkaline phosphatase, carboxypeptidase G2 and L-tryptophanyl-tRNA synthetase. In addition, the effect of divalent metal ions such as Mg^{2+} and Zn^{2+} on promoting the adsorption of metalloenzymes to triazine dye adsorbents has been investigated. These examples illustrate the versatility of triazine dye HPLAC and further demonstrates the speed of analysis, resolution, ease of operation and selectivity of the technique.

INTRODUCTION

In the last few years the applicability of the triazine dye Cibacron Blue F3G-A as a group specific ligand for the affinity chromatography of a wide range of proteins has been amply documented[1,2]. More recently, however, a number of other immobilised triazine dyes, comprising chemically distinct chromophores, have become established as useful alternatives to Cibacron Blue F3G-A. Thus, Procion Red HE-3B was found to be especially useful in the purification of carboxypeptidase G2[3], lactate dehydrogenase, malate dehydrogenase, aldehyde reductase and 5,10-methylene tetrahydrofolate reductase[4,5] and for the resolution of $NADP^+$-dependent dehydrogenases[6]. Furthermore, IMP dehydrogenase has been purified from crude extracts of *E. coli* by chromatography on immobilised Procion Yellow MX-8G[7] and the

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specific activity of hexokinase from a crude yeast extract has been significantly enhanced by a metal ion-promoted interaction with immobilised Procion Green H-4G[8]. Finally, the interaction between thirteen aminoacyl tRNA synthetases and thirty-two immobilised Procion dyes has been systematically screened in the search for potential adsorbents for these enzymes[9]. Although most dyes were found to bind one or more enzymes, L-tryptophanyl-tRNA synthetase displayed an unusually strong affinity for immobilised Procion Brown MX-5BR and could subsequently be eluted biospecifically with L-tryptophan. These examples demonstrate quite clearly that Cibacron Blue F3G-A is not unique among the triazine dyes in its protein binding properties.

More recently, it has been demonstrated that Cibacron Blue F3G-A could be applied equally well to the resolution of enzymes and isoenzymes by high-performance liquid affinity chromatography (HPLAC)[10]. The 6-aminohexyl derivative of the triazine dye was covalently linked to epoxy-silylated microparticulate porous silica and the adsorbent subsequently exploited for the resolution of a number of enzymes in synthetic and crude mixtures. The present report examines further the potential applications of silica-immobilised triazine dyes other than Cibacron Blue F3G-A to the resolution of protein mixtures by HPLAC. In addition, the value of specific metal ion-mediated adsorption/desorption effects is evaluated as a means of enhancing the resolving power of the chromatographic procedures. Such metal ion-mediated effects on the interaction between proteins and immobilised triazine dyes has received little attention in classical affinity chromatography until very recently[8,11].

EXPERIMENTAL

Chemicals

Microparticulate porous silica (LiChrosorb Si 60, 10 μm) was obtained from E. Merck (Darmstadt, G.F.R.). Glycine, L-tyrosine, L-tryptophan, ethylenediaminetetraacetic acid (EDTA), disodium *p*-nitrophenyl phosphate, pyruvic acid (sodium salt), D-glucose, 1,6-diaminohexane, HEPES (N-2-hydroxyethylpiperazine N′-2-ethanesulphonic acid), $NADP^+$, NADH, ATP, ADP, AMP, GTP, NAD^+, Cibacron Blue F3G-A (reactive blue 2, C.I. 61211), alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1; calf intestinal mucosa, Type I-S, 2 U/mg), lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27; porcine heart, Type VI, 600 U/mg), hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1; yeast, Type C-300, 375 U/mg), bovine serum albumin (fraction V) and glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase, EC 1.1.1.49; torula yeast, Type VII, 300 U/mg) were purchased from Sigma (London) (Poole, Great Britain). The organo-functional silane, γ-glycidoxypropyltrimethoxysilane was from Silor Laboratories (Scotia, NY, U.S.A.) whilst the methotrexate (4-amino-N^{10}-methylpteroyl-glutamate) was obtained from American Cyanamid (Pearl River, NY, U.S.A.). L-Tryptophanyl-tRNA synthetase [L-tryptophan: tRNA ligase (AMP); EC 6.1.1.2., 800 U/mg] was purified by a previously published procedure[12] and was a generous gift from Dr. C. J. Bruton, Department of Biochemistry, Imperial College, London, Great Britain. The partially purified preparation of carboxypeptidase G2 from a *Pseudomonas sp.* (ATCC25301) was a gift from Dr. R. Sherwood, CAMR, Porton Down, Nr. Salisbury, Great Britain. The Procion dyes, Procion Blue MX-R (C.I. 61205), Procion Yellow H-A (C.I. 13245), Procion Green H-4G, Procion

Red H-8BN and Procion Brown MX-5BR were a generous and much appreciated gift from Dr. C. V. Stead, I.C.I. Organics Division, Blackley, Great Britain.

Synthesis of triazine dye-substituted silica adsorbents

The principal reactive component in commercial samples of the Procion dyes was purified by chromatography on Sephadex LH-20. Crude commercial dye (1 g) was dissolved in 60% (v/v) aqueous methanol and applied to a column of Sephadex LH-20 (39 × 2.5 cm) equilibrated with 60% (v/v) aqueous methanol at 20–22°C. Fractions (10 ml) were collected at a flow-rate of 3 ml/min and those containing the major component, pooled, concentrated by rotary evaporation under reduced pressure and lyophilised. The preparation of the 6-aminohexyl derivatives of the purified Procion dyes and their subsequent purification by precipitation in 0.3 *M* HCl and covalent attachment to microparticulate silica (LiChrosorb Si 60, 10 μm) epoxysilylated with γ-glycidoxypropyltrimethoxysilane was performed as described previously[10]. Alternative silica-immobilised triazine dye adsorbents were prepared by covalently anchoring the dyes through their reactive triazine rings to glycol-substituted silica. This form of the bonded phase was generated by performing the silylation reaction at pH 3.5[13]. Under these mildly acidic conditions used for the silylation of the support, the terminal oxirane is converted to a glycol during the coupling reaction. The triazine dyes were coupled to the glycol bonded phase under the same conditions described for the attachment of the 6-aminohexyl derivatives of the dyes to epoxysilylated silica[10]. Immobilised dye concentrations for both types of conjugate were determined by spectrophotometric analysis of alkaline hydrolysates as described previously[10] using the molar extinction coefficients and $\lambda_{max.}$ values given in Table I. Immobilised ligand concentrations in μmol/g dry weight silica are also given in Table I.

Chromatographic procedures

The triazine dye–silica conjugates (approx. 1.5 g) were packed in stainless-steel columns (100 × 4.5 mm I.D., total volume approx. 1.5 ml) with the upward slurry packing technique[10,14] in methanol at 20.7 MPa (3000 p.s.i.).

TABLE I

CHARACTERISTICS OF THE TRIAZINE DYES AND LIGAND SUBSTITUTIONS OF THE ADSORBENTS

Triazine dye	*Mol. wt.*	λ_{max} *(nm)*	ε_m *(l·mol⁻¹·cm⁻¹)*	*Ligand substitution (µmol/g dry wt. silica)*	
				6-Aminohexyl-derivative	*Directly linked to glycol-silylated silica*
Procion Yellow H-A	578.5	384	8900	3.1	1.8
Procion Brown MX-5BR	588.2	530	7780	—	1.5
Procion Red H-8BN	801.2	546	21300	4.8	2.6
Procion Blue MX-R	635.9	600	4100	—	3.0
Cibacron Blue F3G-A	773.5	615	7500	3.0	—
Procion Green H-4G	1760.1	676	56400	5.6	2.9

All chromatographic procedures were performed at ambient temperature (20–22°C) using Waters Assoc. (Hartford, Northwich, Great Britain) HPLC equipment comprising a Model 6000 solvent metering pump, a Model 450 variable wavelength detector (190–800 nm) and a Model U6K sample injector. Fractions (200–500 μl) were collected manually and assayed for enzyme activity.

Detection of enzyme activities

Carboxypeptidase G2 activity was determined by following the change in absorbance at 320 nm when methotrexate (4-amino-N^{10}-methylpteroylglutamate) is hydrolysed to 2,4-diamino-N^{10}-methylpteroate[15]. The standard assay medium contained, in a total volume of 1 ml: methotrexate, 60 nmol; Tris–HCl buffer pH 7.3, 100 μmol, and $ZnCl_2$, 200 nmol. One unit of enzyme activity is defined as the amount of enzyme catalysing the hydrolysis of 1 μmol methotrexate per min at 30°C. Yeast hexokinase activity was followed by the coupled reduction of $NADP^+$ with glucose-6-phosphate dehydrogenase at 340 nm and 30°C. The assay mixture contained the following in a total volume of 1 ml: Tris–HCl buffer pH 7.5, 0.1 mmol; D-glucose, 5 μmol; ATP, 3 μmol; $NADP^+$, 0.7 μmol; $MgCl_2$, 10 μmol; yeast glucose-6-phosphate dehydrogenase, 3 units. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol NADPH per min at 30°C. Alkaline phosphatase activity was monitored by following the production of *p*-nitrophenolate anion from *p*-nitrophenyl phosphate at pH 10.5 and 405 nm. The reaction mixture contained the following in a total volume of 1 ml: glycine–NaOH buffer pH 10.5, 0.1 mmol; $MgCl_2$, 1 μmol; $ZnCl_2$, 0.1 μmol; *p*-nitrophenylphosphate, 6 μmol. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol *p*-nitrophenolate anion per min at 30°C. Lactate dehydrogenase was assayed by following the oxidation of NADH by pyruvate at 340 nm and 30°C. The assay contained in a total volume of 1 ml: potassium phosphate buffer pH 7.0, 50 mmol; sodium pyruvate, 0.73 μmol; NADH 0.2 μmol. One unit of enzyme activity is defined as the amount of enzyme required to oxidise 1 μmol NADH per min at 30°C. L-Tryptophanyl-tRNA synthetase was assayed according to a previously published procedure[16]. All assay procedures were executed on a Beckman DU8 computing spectrophotometer.

RESULTS AND DISCUSSION

Preparation of triazine dye-substituted silica adsorbents

It has already been established that inorganic supports coated with a covalently-bonded organo-functional silane may be used for the HPLC of proteins, polynucleotides and polysaccharides[13]. Modification of the surface silanol groups of silica with γ-glycidoxypropyltrimethoxysilsane yields a neutral hydrophilic surface that neither adsorbs nor repels proteins and thus decreases the potential denaturation of sensitive biological substances by silica[13]. The inactivation of the silica surface is of particular importance in HPLAC where non-specific interactions between the matrix and protein should be minimised.

Table I shows that the 6-aminohexyl derivatives of triazine dyes can be efficiently coupled to epoxysilylated microparticulate silica to produce ligand concentrations in the range 3–6 μmol/g dry weight. The structure of a typical dye-silica conjugate comprising silica-immobilised 6-aminohexyl Procion Yellow H-A is illu-

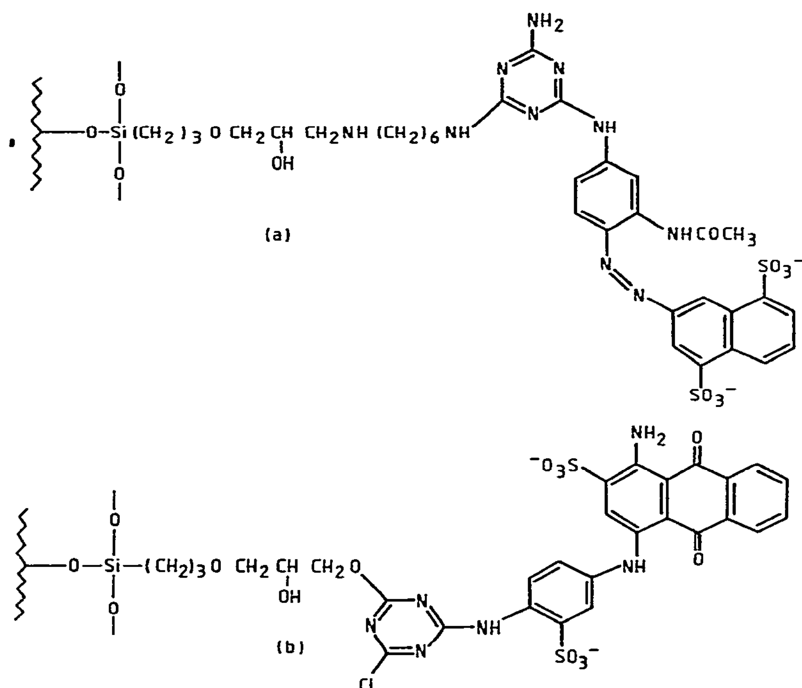

Fig. 1. The structure of (a) 6-aminohexyl-Procion Yellow H-A attached to epoxysilylated silica (10 μm), and (b) Procion Blue MX-R attached directly via its triazine ring to glycol-silylated silica.

strated in Fig. 1a. The alternative approach of coupling the dye via its reactive triazine ring directly to the glycol-silylated form of silica yields conjugates possessing a shorter propyl spacer arm but which still enable adequate interaction between the dye and its complementary protein. The structure of the conjugate produced on coupling Procion Blue MX-R directly to glycol-silylated silica is shown in Fig. 1B. In general, dye adsorbents prepared by this method have lower substitution levels of around 1.5–3.0 μmol/g dry weight silica (Table I), although this does not appear to adversely affect their ability to bind complementary proteins.

Effect of immobilisation chemistry on the chromatographic properties of HPLAC adsorbents

Lactate dehydrogenase was considered to be a suitable model protein for evaluating the 6-aminohexyl- and directly-linked triazine dye HPLAC adsorbents because its behaviour in conventional affinity chromatography and in silica-immobilised Cibacron Blue F3G-A HPLAC has been established[1,2,10]. However, in the latter technique, the strength of adsorption of lactate dehydrogenase was weaker in low ionic strength irrigating buffers, suggesting a significant contribution from hydrophobic forces in the interaction of proteins with immobilised Cibacron Blue F3G-A. This hydrophobic interaction is likely to be a consequence of the comparatively long polymethylene spacer molecule interposed between the silica surface and the Cibacron Blue F3G-A. In this case, the non-specific interaction can be circumvented by linking the dye directly to glycol-silylated silica through its triazine ring. Due to the

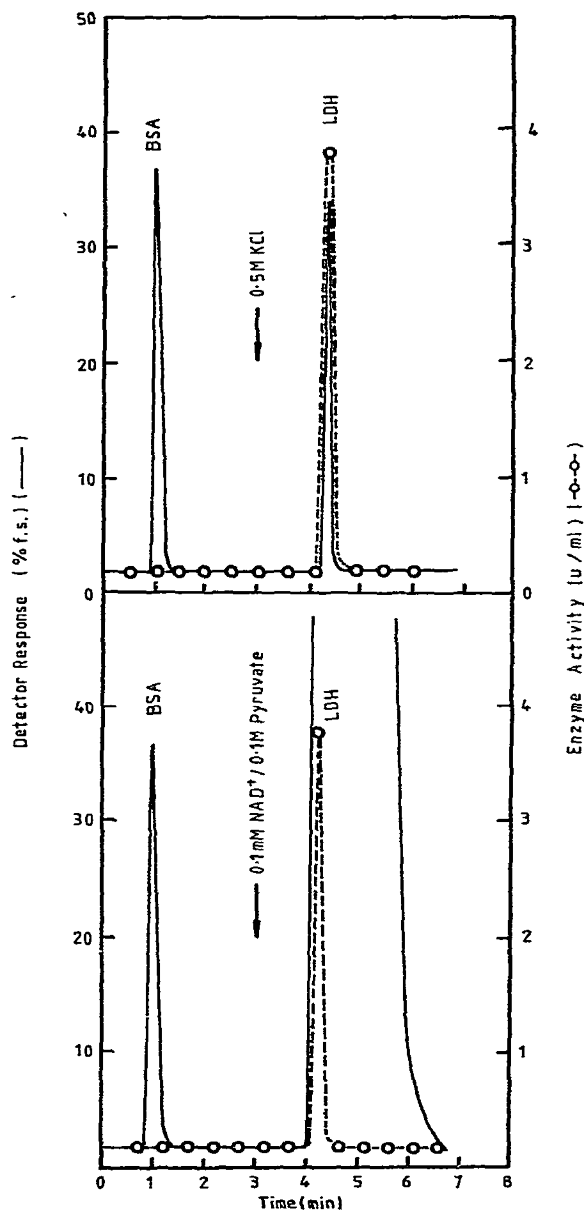

Fig. 2. Resolution of a mixture of bovine serum albumin (BSA) and pig heart lactate dehydrogenase (LDH) on a Procion Blue MX-R silica column. Sample applied at time zero: BSA (10 μg) and LDH (10 μg) in 25 m*M* potassium phosphate pH 7.0; temperature: 20–22°C; flow-rate: 1 ml/min; pressure: 2.8 MPa (400 p.s.i.); detector: 210 nm, 2.0 a.u.f.s.; eluents (a) 0.5 *M* KCl in 25 m*M* potassium phosphate pH 7.0 (200 μl); (b) 0.1 m*M* NAD^+–0.1 *M* pyruvate in 25 m*M* potassium phosphate pH 7.0 (200 μl). Full details of the enzyme assay procedures are given in the experimental section.

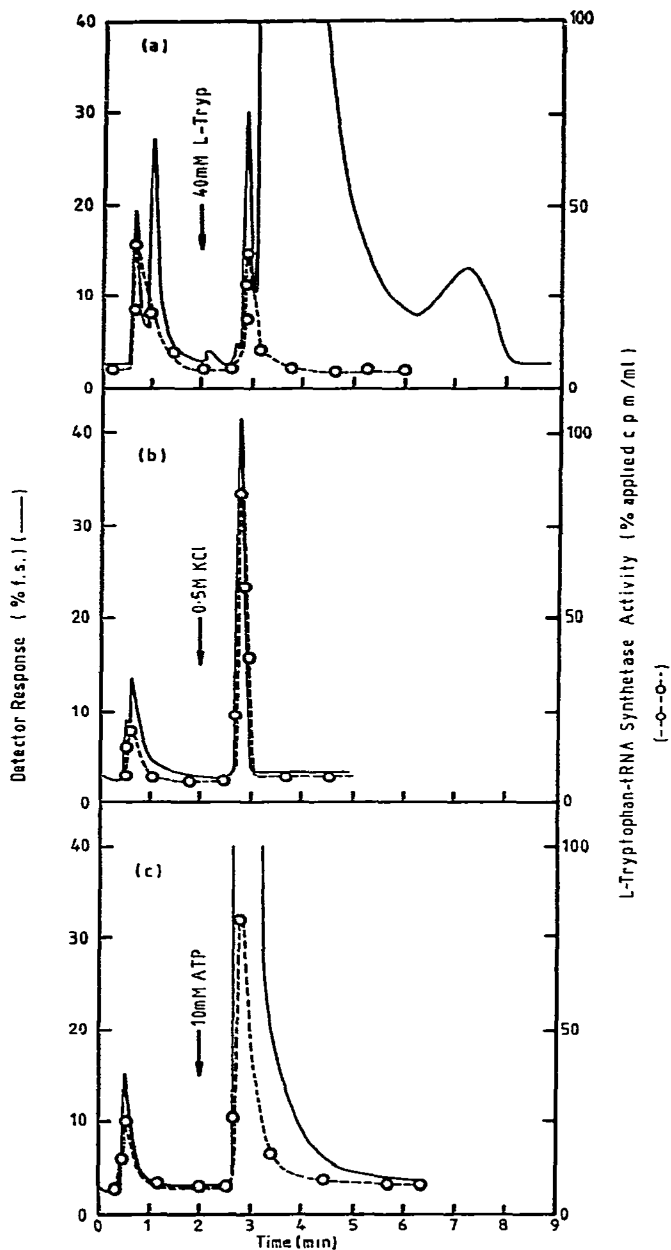

Fig. 3. HPLAC of L-tryptophanyl-tRNA synthetase on triazine dye-silica asorbents: (a) Cibacron Blue F3G-A, (b, c) Procion Brown MX-5BR. Sample applied at time zero: purified L-tryptophanyl-tRNA synthetase (20 μg) in 10 mM HEPES pH 7.0 (20 μl); column irrigant: 10 mM HEPES pH 7.0; temperature: 20–22°C; flow-rate: 2 ml/min; pressure: 3.5 MPa (500 p.s.i.); detector: 280 nm, 0.2 a.u.f.s.; eluents: 200 μl, as indicated by the arrows.

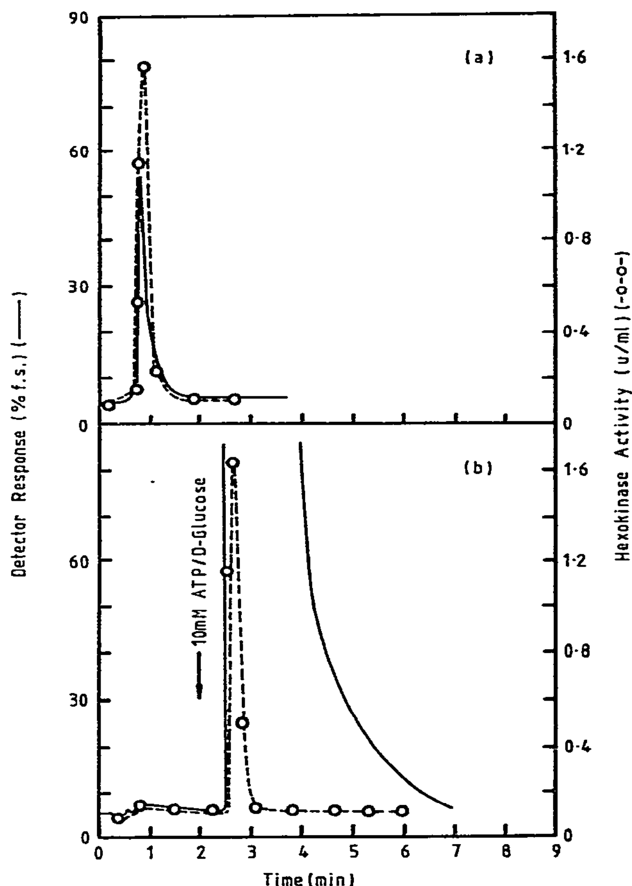

Fig. 4. Chromatography of yeast hexokinase on silica-immobilised Procion Green H-4G (a) in the absence of Mg^{2+} and (b) with 10 mM Mg^{2+} in the column irrigant. Sample applied at time zero: purified yeast hexokinase (10 μg, 160 U/mg) in 10 mM HEPES pH 7.0 (10 μl); column irrigant: 10 mM HEPES pH 7.0; temperature: 20–22°C; flow-rate; 2 ml/min; pressure: 2.8 MPa (400 p.s.i.); detector: 280 nm, 0.1 a.u.f.s.; eluent: 200 μl, as indicated by the arrow. Full details of the enzyme assay procedure are given in the experimental section.

low reactivity of the monochlorotriazinyl group of Cibacron Blue F3G-A, requiring reaction times of 48–60 h with the silica to achieve satisfactory ligand substitution, the more reactive and structurally similar dichlorotriazinyl dye Procion Blue MX-R, was used in these experiments. Fig. 2a illustrates the resolution of a synthetic mixture of bovine serum albumin (10 μg) and pig heart lactate dehydrogenase (10 μg) on glycol-silylated silica-immobilised Procion Blue MX-R. Unlike lactate dehydrogenase bound to silica-immobilised 6-aminohexyl-Cibacron Blue F3G-A[10], the enzyme could be quantitatively eluted from the Procion Blue MX-R adsorbent with a 200-μl pulse of 200 mM KCl, suggesting fewer non-specific hydrophobic interactions. Furthermore, lactate dehydrogenase activity could also be recovered from the Procion Blue MX-R adsorbent in good yield by biospecific ternary complex elution with a

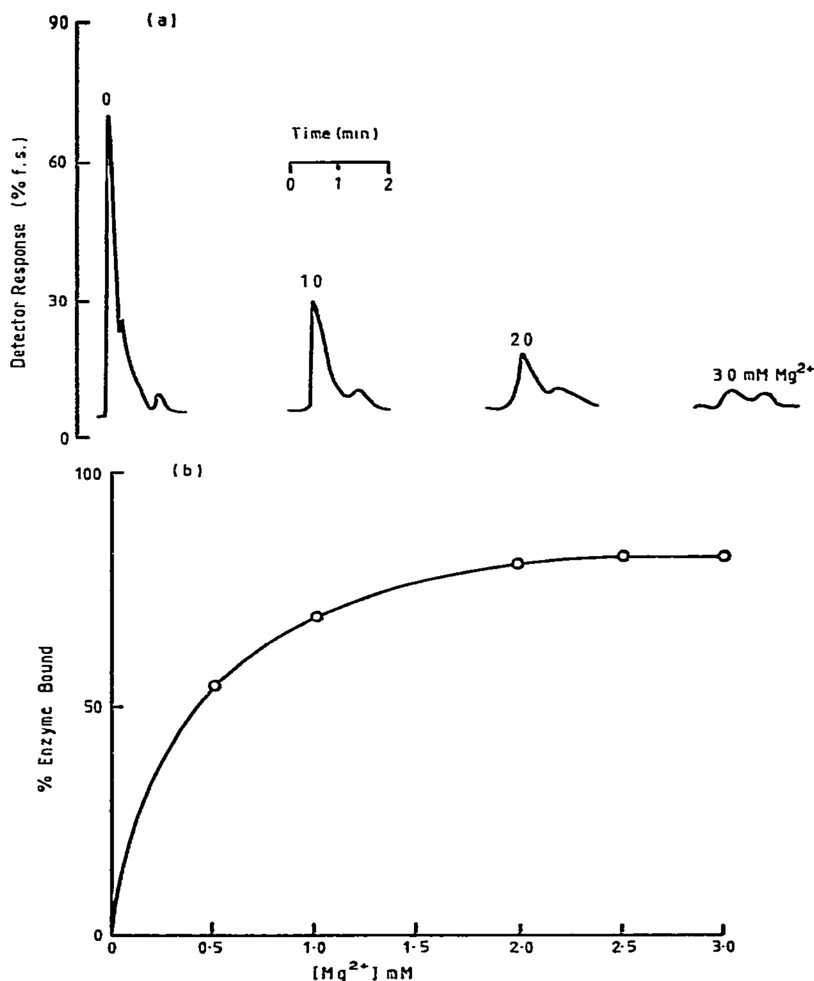

Fig. 5. The effect of Mg^{2+} on the binding of yeast hexokinase to Procion Green H-4G silica. (a) The appearance of the protein profile in the void volume at 0, 1.0, 2.0 and 3.0 m*M* Mg^{2+}, respectively, and (b) the percentage enzyme bound to the adsorbent as a function of Mg^{2+} concentration. Sample applied at time zero: purified yeast hexokinase (10 µg, 160 U/mg) in 10 m*M* HEPES pH 7.0 (10 µl); column irrigant: 10 m*M* HEPES pH 7.0 containing $MgCl_2$ at indicated concentrations; temperature: 20–22°C; flow-rate: 2 ml/min; pressure: 3.5 MPa (500 p.s.i.); detector: 280 nm, 0.1 a.u.f.s.

200-µl pulse of 0.1 m*M* NAD^+–0.1 *M* pyruvate. These observations suggest that the use of alternative dyes and/or coupling procedures may circumvent some of the problems associated with silica-immobilised 6-aminohexyl-Cibacron Blue F3G-A[10].

Selection of appropriate triazine dye adsorbents

The selection of an appropriate triazine dye for the HPLAC of a protein of interest is not an arbitary one and is based on information derived from purification methodology currently in use or from free solution studies[1–3,8,9]. For example, the

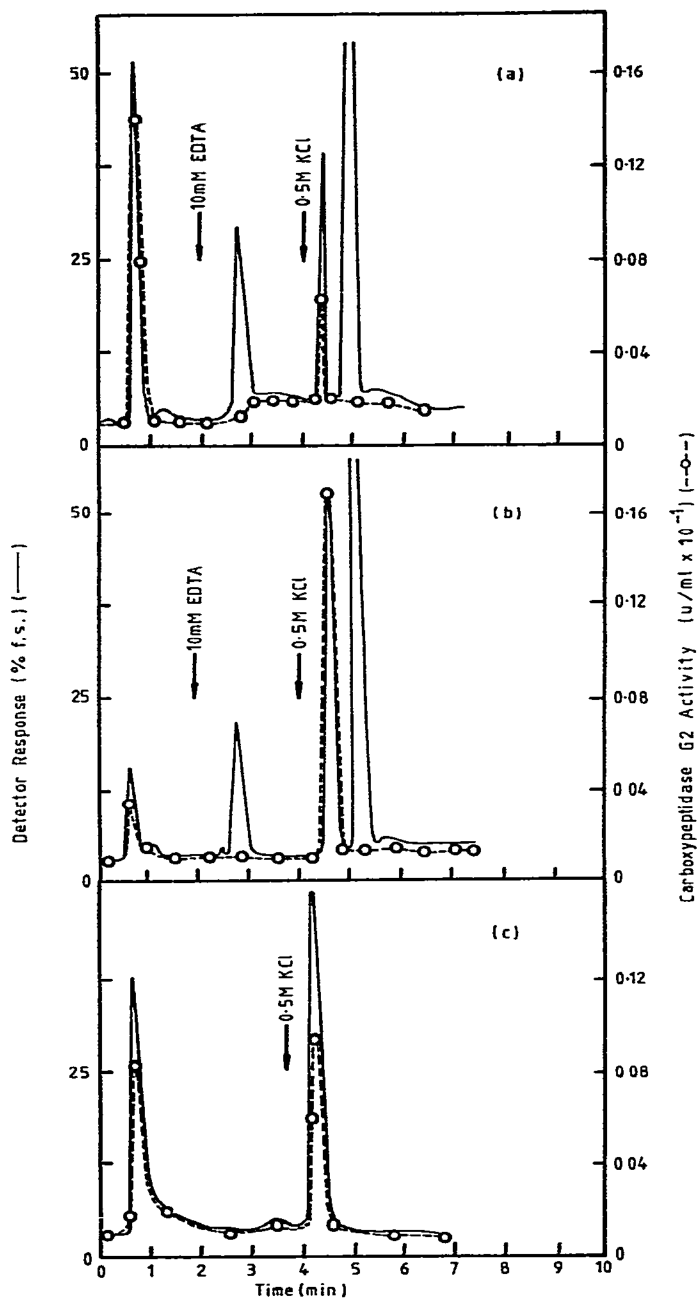

Fig. 6. HPLAC of carboxypeptidase G2 on triazine dye–silica adsorbents. (a, b) Procion Red H-8BN, (c) Cibacron Blue F3G-A. Sample applied at time zero: partially purified enzyme (196 μg, 10.8 U/mg) in 10 mM HEPES pH 7.0 (20 μl); column irrigant: 10 mM HEPES pH 7.0 $\pm$ 0.2 mM Zn^{2+}; temperature: 20–22°C; flow-rate: 2 ml/min; pressure: 3.5 MPa (500 p.s.i.); detector: 280 nm, 0.04 a.u.f.s.; eluents: 200 μl, as indicated by the arrows.

binding of thirteen aminoacyl-tRNA synthetases to thirty-two immobilised Procion dyes has been systematically investigated[9]. The reported purification of L-tryptophanyl-tRNA synthetase on immobilised Procion Brown MX-5BR, achieving a 137-fold purification and 88% yield, and the specific elution of this enzyme by its complementary substrate, L-tryptophan, made it an obvious candidate for study by HPLAC. Purified samples of L-tryptophanyl-tRNA synthetase from *B. stearothermophilus* were applied to HPLAC adsorbents comprising silica-immobilised 6-aminohexyl-Cibracon Blue F3G-A and Procion Brown MX-5BR directly linked to glycolsilylated silica, as shown in Fig. 3. Two serious drawbacks were encountered with the Cibacron Blue F3G-A adsorbent (Fig. 3a): firstly, synthetase activity was split into two unresolved peaks with 60% of active enzyme eluted in the void volume, and secondly, although enzyme was eluted by a pulse of 40 m*M* L-tryptophan, total recovery was only 50%. Irrigating the column with 0.5 *M* KCl failed to elute further enzyme activity. In contrast, almost quantitative binding of L-tryptophanyl-tRNA synthetase was observed when the enzyme (20 μg) was applied to the Procion Brown MX-5BR adsorbent. Bound enzyme could be quantitatively eluted with a step of 0.5 *M* KCl (Fig. 3b) or biospecifically with 10 m*M* ATP (Fig. 3c), 10 m*M* ADP, 10 m*M* GTP or 10 m*M* L-tryptophan. Elution could not be effected with AMP nor with non-complementary amino acids such as glycine or L-tyrosine.

Metal ion promoted adsorption to silica-immobilised triazine dyes

The resolution of hexokinase and 3-phosphoglycerate kinase from crude yeast extracts by HPLAC on silica-immobilised Cibacron Blue F3G-A has recently been reported[10]. More recent studies on the inactivation of yeast hexokinase by a number of reactive triazine dyes has established an apparently unique affinity of this enzyme for the dye Procion Green H-4G[8]. The inactivation of yeast hexokinase by Procion Green H-4G is competitively inhibited by substrates of both nucleotide and hexose classes and by Mg^{2+}. In the presence of 10 m*M* Mg^{2+}, the affinity of hexokinase for Procion Green H-4G is increased approximately 5-fold. This effect of Mg^{2+} has been used to promote the adsorption of the enzyme to agarose-immobilised Procion Green H-4G and thus lead to an improved purification protocol for this enzyme[8]. A similar Mg^{2+}-promoted adsorption of yeast hexokinase to silica-immobilised 6-aminohexyl-Procion Green H-4G has been established. Fig. 4a demonstrates that exclusion of Mg^{2+} from the irrigating buffer results in quantitative elution of the applied sample of yeast hexokinase in the void volume of the column. Inclusion of 6 m*M* Mg^{2+} in the column irrigants results in quantitative adsorption of the enzyme, with prompt elution being effected with a pulse of 10 m*M* ATP–10 m*M* D-glucose. Eluents comprising 10 m*M* ATP or 10 m*M* D-glucose alone resulted in only partial elution (approx. 35%) of the hexokinase activity. These observations are consistent with the inactivation studies and concur with the suggestion that dye binding may traverse both the nucleotide and sugar binding domains on the enzyme[8].

The role of Mg^{2+} ions in promoting the adsorption of hexokinase to immobilised Procion Green H-4G is unclear. Fig. 5 illustrates the effect of increasing the Mg^{2+} ion concentration in the column irrigants on the proportion of enzyme bound to the adsorbent. The hyperbolic response with saturation at higher Mg^{2+} ion concentrations suggests the formation of a dye–Mg^{2+}–enzyme ternary complex, not unlike that formed between the natural substrate, ATP, Mg^{2+} and the enzyme[17].

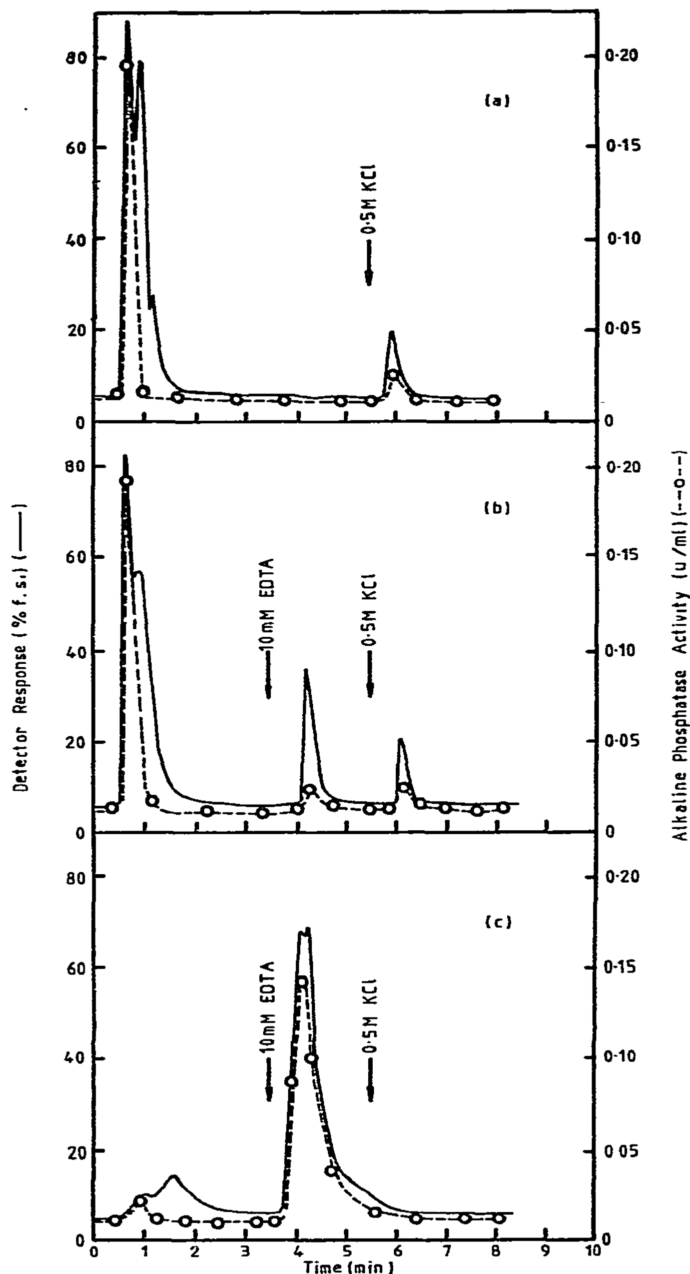

Fig. 7. Chromatography of calf intestinal alkaline phosphatase on silica-immobilised Procion Red H-8BN (a) and Procion Yellow H-A (b, c). Sample applied at time zero: crude preparation of calf intestinal alkaline phosphatase (250 μg, 2.13 U/mg) in 10 m*M* HEPES pH 7.0 (10 μl); column irrigant: 10 m*M* HEPES pH 7.0 $\pm$ 2.0 m*M* Zn^{2+}; temperature: 20–22°C; flow-rate: 2 ml/min; pressure: 3.5 MPa (500 p.s.i.); detector: 280 nm, 1.0 a.u.f.s.; eluents: 200 μl, as indicated by the arrows.

Interestingly, 20 m*M* Mg^{2+} has little effect on the binding of hexokinase to silica-immobilised Cibacron Blue F3G-A nor does it promote adsorption of the enzyme to an HPLAC adsorbent comprising 6-aminohexyl-Procion Yellow H-A attached to epoxysilylated silica. Furthermore, yeast hexokinase adsorbed to silica-immobilised Procion Green H-4G in the presence of Mg^{2+} may be eluted with Mg^{2+} free or EDTA-containing buffers.

The metal ion potentiated adsorption of enzymes to triazine dye HPLAC adsorbents appears to be a general phenomenon applicable to other systems. For example, the interaction of carboxypeptidase G2 and immobilised Procion Red H-8BN is known to be dependent on the presence of Zn^{2+} (0.2 m*M*) in the applied sample[11]. Fig. 6 illustrates the chromatography of a partially purified preparation of carboxypeptidase G2 on HPLAC adsorbents comprising 6-aminohexyl-Cibracon Blue F3G-A linked to epoxysilylated silica and Procion Red H-8BN directly linked to glycolsilylated silica. Fig. 6a shows that in the absence of Zn^{2+}, 60% of the applied enzyme elutes in the void volume of the Procion Red H-8BN adsorbent. Bound enzyme could not be released by a pulse of 10 m*M* EDTA but was subsequently eluted by a pulse 200 μl) of 0.5 *M* KCl. Fig. 6b shows that inclusion of 0.2 m*M* Zn^{2+} in the enzyme dialysate increased the observed binding of the enzyme to the Procion Red H-8BN adsorbent to 90%, with good recovery of enzyme activity being effected with a pulse of 0.5 *M* KCl. Chromatography on the Procion Red H-8BN adsorbent results in a significant increase (approx. 5-fold) in the specific activity of the recovered enzyme. In contrast, Fig. 6c shows that the presence of Zn^{2+} had no effect on the interaction of carboxypeptidase G2 with the Cibacron Blue F3G-A adsorbent and, despite the fact that 60% of the applied enzyme bound to this matrix, there was no enhancement in enzyme purity.

Calf intestinal alkaline phosphatase, like carboxypeptidase G2, is a Zn-metalloenzyme, but, unlike carboxypeptidase G2, does not bind to the Procion Red H-8BN adsorbent either in the presence or absence of Zn^{2+} (Fig. 7a). A systematic appraisal of a number of other triazine dye HPLAC columns produced essentially the same result with the exception of the Procion Yellow H-A adsorbent, comprising 6-aminohexyl-Procion Yellow H-A immobilised to epoxysilylated microparticulate silica (Fig. 1a). The effect of excluding or including 2 m*M* Zn^{2+} in the irrigating buffer on the chromatography of alkaline phosphatase is illustrated in Figs. 7b and 7c. In contrast to the Zn^{2+}-mediated behaviour of carboxypeptidase G2 on Procion Red H-8BN adsorbents, elution of alkaline phosphatase from the Procion Yellow H-A column could be effected with approximately 80% recovery with a 10 m*M* EDTA pulse. Determination of the optimum level of metal ion required to achieve quantitative binding of the applied enzyme is illustrated in Fig. 8.

The metal ion specificity

Whilst metal ion-mediated effects on the interaction between proteins and triazine dyes has received scant attention in the literature until recently[8,11], there is even less information on the specificity for the metal ion. Recent free solution studies on the interaction of yeast hexokinase with Procion Green H-4G have, however, demonstrated a marked specificity for Mg^{2+}, and to a lesser extent Ca^{2+} and Sr^{2+}, in enhancing the affinity between the dye and enzyme[8]. Divalent transition metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+} displayed no apparent effect on the dye–protein interaction.

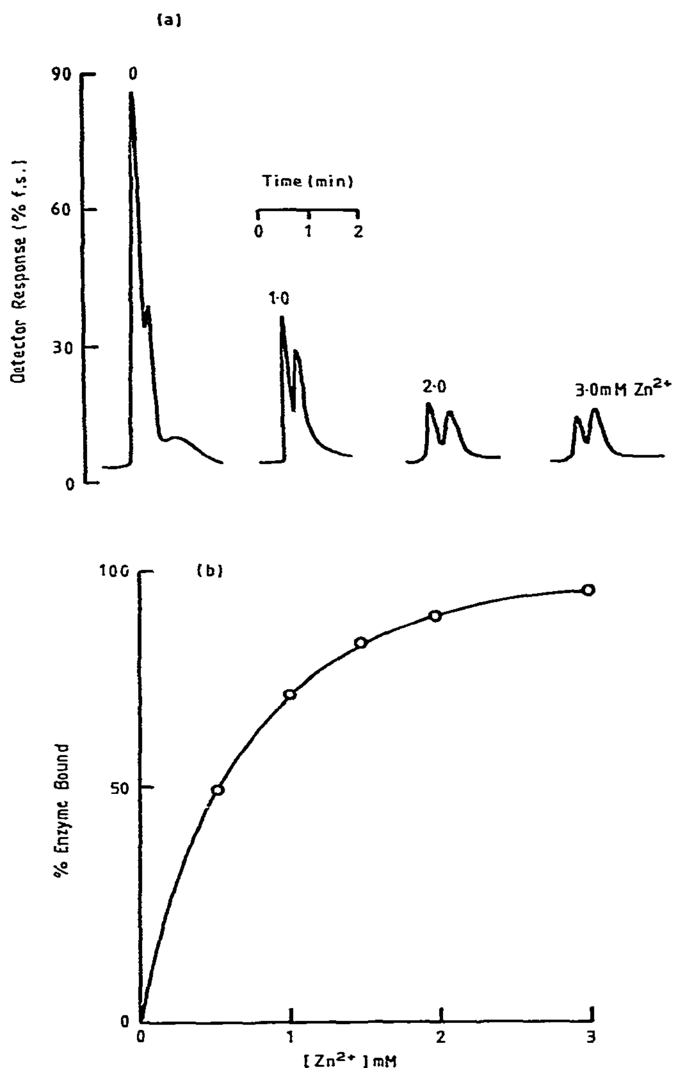

Fig. 8. The effect of Zn^{2+} on the binding of alkaline phosphatase to Procion Yellow H-A silica. (a) The appearance of the protein profile in the void volume at 0, 1.0, 2.0 and 3.0 m*M* Zn^{2+}, respectively, and (b) the percentage enzyme bound to the adsorbent as a function of Zn^{2+} concentration. Sample applied at time zero: crude calf intestinal alkaline phosphatase (250 μg, 2.13 U/mg) in 10 m*M* HEPES pH 7.0 (10 μl); column irrigant: 10 m*M* HEPES pH 7.0 containing $ZnCl_2$ at indicated concentrations; temperature: 20–22°C; flow-rate: 2 ml/min; pressure: 3.5 MPa (500 p.s.i.); detector: 280 nm, 1.0 a.u.f.s.

Similar effects can be observed on the specificity of metal ions in promoting adsorption to triazine–dye HPLAC adsorbents. Thus, for example, substituting other metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Ca^{2+}, K^{+} and Na^{+} for Zn^{2+} in the wash buffer, had no apparent effect on the binding of calf intestinal alkaline phosphatase to silica-immobilised Procion Yellow H-A. However, it is not clear at this

stage whether the specificity for Zn^{2+} in this case is a reflection of the fact that alkaline phosphatase is a constitutive Zn-metalloenzyme or whether these metal ion mediated effects are akin to metal chelate chromatography[18] and are therefore merely a reflection of the Irving–Williams series[19]. Nevertheless, it is clear that these effects warrant further investigation since they may represent a useful tool in the selective purification of individual proteins.

CONCLUSIONS

HPLAC is characterised by its high selectivity, resolving power and speed of separation[10,20]. In this study, the potential of the technique has been demonstrated with a variety of immobilised triazine dyes as affinity ligands. Our initial paper[10] concentrated on the application of Cibacron Blue F3G-A because of its dominant role as a pseudo-affinity ligand for the resolution of complex mixtures of dehydrogenases, kinases and other proteins. Indeed, Cibacron Blue F3G-A is such an avid binder of proteins thast it is rapidly assuming the role of a "universal" affinity ligand and we are confident that HPLAC on Cibacron Blue F3G-A silica will become an established procedure in the very near future. However, without undermining the usefulness of Cibacron Blue F3G-A we also wish to recommend the value of giving consideration to potential affinity ligands drawn from the whole class of triazine dyes[1–3]. The practical problems and expense of constructing numerous HPLAC columns has been largely circumvented by basing the selection of the appropriate dye on information already in the literature. This aspect has been most clearly demonstrated in the chromatography of yeast hexokinase on immobilised Procion Green H-4G and L-tryptophanyl-tRNA synthetase on immobilised Procion Brown MX-5BR.

The selection of dyes other than Cibacron Blue F3G-A for the preparation of HPLAC adsorbents has several advantages. For example, it permits a greater degree of latitude in the purification of a particular protein and has often led to improved resolution, recovery or purification of the complementary protein. Furthermore, the judicious exploitation of metal ion-promoted adsorption effects, which, under the conditions used in HPLAC, appear to be non-existent when Cibacron Blue F3G-A is the ligand, may have far reaching effects in the manipulation of chromatographic behaviour in these other triazine dye adsorbents. Such effects may find application where commercially important proteins and hormones are to be purified by HPLAC technology.

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