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HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY OF PROTEINS ON CIBACRON BLUE F3G-A BONDED SILICA

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SUMMARY

The reactive triazine dye, Cibacron Blue F3G-A, has been covalently attached to microparticulate porous silica and used for the resolution of a number of complementary proteins by high-performance liquid affinity chromatography (HPLAC). Cibacron Blue F3G-A was converted to its 6-aminohexyl derivative by reaction with 1,6-diaminohexane and coupled to γ-glycidoxypropyltrimethoxysilane-activated porous silica (5 μm) to generate an adsorbent containing 5.5–6.7 μmol dye/g silica.

Cibacron Blue F3G-A silica columns, in conjunction with on-line monitoring of protein concentration and enzyme activity, may be exploited for the high speed fully automated resolution of dehydrogenases, isoenzymes, kinases and other proteins such as pancreatic ribonuclease A from simple or complex mixtures. The examples demonstrate the versatility of HPLAC on silica-immobilised Cibacron Blue F3G-A.

INTRODUCTION

The two chromatographic techniques of high-performance liquid chromatography (HPLC) and affinity chromatography have witnessed a spectacular development during the last few years and now occupy a central place in current separation technology for the analysis and purification of a plethora of biological substances[1–3]. More recently, the two techniques have been married to yield a new approach termed high-performance liquid affinity chromatography (HPLAC) which combines the inherent speed and resolving power of HPLC with the biological specificity of affinity

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chromatography[4]. The general versatility of the new technique has been amply demonstrated with the resolution of enzymes and isoenzymes, albumins, protein antigens, carbohydrates, nucleosides and nucleotides using immobilised group specific or general ligands, immunoadsorbents and boronic acid derivatives, respectively[4–6].

Cibacron Blue F3G-A, a reactive triazine-based textile dye whose chromophore comprises a sulphonated anthraquinone moiety, may be immobilised directly to agarose to yield a conjugate capable of purifying a wide range of proteins by affinity chromatography[2,7–9]. Thus, immobilised Cibacron Blue F3G-A appears to be a particularly effective adsorbent for the purification of pyridine nucleotide-dependent dehydrogenases, kinases, coenzyme A-dependent enzymes, hydrolases, acetyl-, phosphoribosyl- and amino-transferases, RNA and DNA nucleases, decarboxylases, sulphohydrolases, phosphorylase, myosin and a number of blood proteins including serum albumin, clotting factors, lipoproteins, complement proteins and interferon[9,10]. The basis for many of these selective interactions is believed to be that the sulphonated aromatic chromophore of Cibacron Blue F3G-A mimics the overall shape, charge and aromaticity of the naturally occurring anionic heterocycles[10,11]. Indeed, recent x-ray crystallographic studies on the binding of Cibacron Blue F3G-A to horse liver alcohol dehydrogenase have indicated remarkable similarities between chromophore and coenzyme binding[12].

The widespread applicability of immobilised Cibacron Blue F3G-A in "group specific" or "general ligand" affinity chromatography has prompted a study of its application as a ligand in HPLAC. The present report demonstrates the ability of microparticulate silica-bonded Cibacron Blue F3G-A to resolve, rapidly and selectively, complex mixtures of complementary proteins.

EXPERIMENTAL

Chemicals

Microparticulate porous silica (LiChrosorb Si 60, 5 μm) and 1,6-diaminohexane were obtained from E. Merck (Darmstadt, G.F.R.). Ribonuclease A (ribonucleate 3′-pyrimidino oligonucleotidohydrolase, EC 3.1.4.22; bovine pancreas, type 1-A, 50–75 Kunitz units/mg), ribonucleic acid (RNA, type II-S, sodium salt, torula yeast), cytidine 2′-monophosphate (2′-CMP, disodium salt), DL-lactic acid (sodium salt), bovine serum albumin (fraction V), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12; rabbit muscle, 70 U/mg), glucose-6-phosphate dehydrogenase (G6PDH) (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase, EC 1.1.1.49; torula yeast, type XI, 400 U/mg), glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NAD(P)^+$ 1-oxidoreductase, EC 1.1.1.49; *Leuconostoc mesenteroides*, Type XXI, 200–250 U/mg), alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1; type V, chicken intestine, 5 U/mg; type III, *E. coli*, 20–30 U/mg), NADH, $NADP^+$, NAD^+, ATP, lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27, rabbit muscle, type II, 400–900 U/mg) and Cibacron Blue F3G-A (Reactive Blue 2, Procion Blue H-B, C.I. 61211) were purchased from Sigma (St. Louis, MO, U.S.A.). Lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27, pig heart, 300 U/mg), alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1, horse liver, 2.7 U/mg), malate dehydrogenase [L-malate:

NAD^+ oxidoreductase, EC 1.1.1.37, pig heart (mitochondrial), 1200 U/mg] and 3-phosphoglycerate (trisodium salt) were supplied by Boehringer (Mannheim, G.F.R.). The organo-functional silane, γ-glycidoxypropyl-trimethoxysilane (Silane Z-6040) was from Dow Corning (Midland, MI, U.S.A.). All solvents and other chemicals were of analytical grade from commercial sources and were used without further purification.

Synthesis of 6-aminohexyl-Cibacron Blue F3G-A

Crude Cibacron Blue F3G-A (Reactive Blue 2; 1287 mg, 60% pure, 1 mmol) was dissolved in 20 ml water, 10 ml 1,6-diaminohexane (1 *M*, pH 10.0, 5 *M* HCl) added and the solution heated at 50°C for 1 h. The solution was added dropwise to 200 ml, 0.3 *M* HCl, incubated for 5 min at 20°C and the formed precipitate removed by centrifugation or filtration and thoroughly washed with 0.3 *M* HCl. The precipitate was washed thoroughly with acetone until the supernatant fraction or filtrate remained colourless and then with diethyl ether and finally dried in air to a deep blue powder. The resulting product, 6-aminohexyl-Cibacron Blue F3G-A, was >95% pure by thin-layer chromatography on silica (DC Fertigfolien F1500 LS 254 Kieselgel; Schleicher & Schüll, Dassel, G.F.R.; solvent system: butan-2-ol–propan-1-ol–ethyl ethanoate–water, 20:40:10:30, v/v; R_F 0.55) and by HPLC [C_{18} Hypersil 5 μm column; solvent: 22.5% (v/v) ethanol, 1% (v/v) triethylamine in water; operating pressure, 6.9 MPa (1000 p.s.i.)]. The product was positive to the 2,4,6-trinitrobenzene sulphonic acid test for primary amine[13] yielding a turquoise green compound of R_F 0.61 in the above silica thin-layer chromatography system.

Synthesis of Cibacron Blue F3G-A-substituted silica (Fig. 1)

Silica gel (LiChrosorb Si 60, 5 μm) was activated according to a previously published procedure[5] using γ-glycidoxypropyltrimethoxy silane. Epoxy-substituted silica (2 g dry weight) was added to 10 ml 0.1 *M* $NaHCO_3$–Na_2CO_3 buffer pH 8.6 containing 6-aminohexyl-Cibacron Blue F3G-A (180 mg, 0.21 mmol), the slurry sonicated for 10 min under reduced pressure and incubated overnight at 30°C with gentle

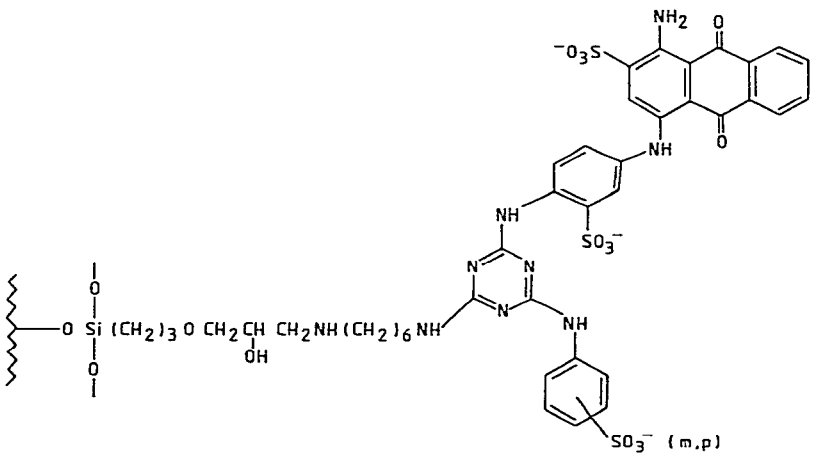

Fig. 1. Structure of the Cibacron Blue F3G-A bonded silica adsorbent.

agitation. The Cibacron Blue F3G-A silica was washed with water (500 ml), 1 *M* KCl (100 ml) and water (500 ml). The gel was sucked moist on a sintered funnel and the moist silica added to 10 m*M* HCl (50 ml). The slurry was heated to 75°C for 30 min to hydrolyse excess epoxy groups and washed with water (250 ml), 50% (v/v) aqueous methanol (200 ml), 100% methanol (200 ml) and diethyl ether (100 ml) prior to drying in air to a blue powder.

Determination of bound dye concentration

Weighed amounts of dry Cibacron Blue F3G-A silica (16–60 mg) were added to 1 *M* NaOH (5 ml) and heated to 60°C for 30 min to solubilise the gel. The resulting clear blue solution was diluted to approximately 25 ml with water, the pH adjusted to 7 with 1 *M* HCl, 1 *M* potassium phosphate buffer pH 7.5 (5 ml) added, the solution made up to 50 ml with water and the absorbance at 620 nm noted. The immobilised dye concentration, typically 5.5–6.7 μmol Cibacron Blue F3G-A/g dry weight silica, was calculated using a molar extinction coefficient of 13,600 l mol^{-1} cm^{-1} for Cibacron Blue F3G-A at 620 nm[9].

Chromatographic procedures

The Cibacron Blue F3G-A silica (approx. 1.2 g) was packed in "316" stainless-steel columns (100 × 5 mm I.D., total volume 2.0 ml) with the upward slurry packing technique[14] in water–methanol (10:90, v/v) at 13.8 MPa (2000 p.s.i.).

All chromatographic procedures were performed at ambient temperature (20–22°C). The pumping system comprised an Altex Model 110A solvent metering pump (Altex, Berkeley, CA, U.S.A.) equipped with a pulse dampener. Ultraviolet (UV) detection was performed with a variable-wavelength detector (190–350 nm; Spectromonitor III, Laboratory Data Control, Riviera Beach, FL, U.S.A.) and sample injections were made with an Altex 210 valve injector (Altex).

On-line detection of enzyme activities

Enzymes eluted from the Cibacron Blue F3G-A silica HPLAC column were detected with an on-line detector system[15,16] comprising an Altex Model 110A reagent pump, post-column reactor equilibrated to 40 ± 0.1°C (or 20–22°C in the case of ribonuclease A) in a water bath and a UV–visible monitor (Spectromonitor II, Laboratory Data Control). The post-column reactor comprised a stainless-steel "316" column (100 × 5 mm I.D., total volume 2.0 ml) containing non-porous glass beads (150 μm) silanized as described previously[4,5] with the resulting epoxy groups hydrolysed to diols as described above.

The assay solution for the on-line detection of lactate dehydrogenase comprised either 0.4 *M* Tris–HCl pH 8.8 containing 0.75 *M* DL-lactate and 1 m*M* NAD^+ or 0.1 *M* potassium phosphate buffer pH 7.5 containing 1 m*M* sodium pyruvate and 0.32 m*M* NADH. The post-column reagent for the detection of alcohol dehydrogenase comprised Tris–HCl pH 8.8 (0.4 *M*) containing ethanol (0.43 *M*) and NAD^+ (1 m*M*) whilst the reagent for malate dehydrogenase contained potassium phosphate buffer pH 7.5 (0.1 *M*), oxaloacetate (0.20 m*M*) and NADH (0.32 m*M*), and the reagent for *L. mesenteroides* glucose-6-phosphate dehydrogenase contained potassium phosphate buffer pH 7.5 (0.1 *M*), D-glucose-6-phosphate (1.2 m*M*) and $NADP^+$ (0.4 m*M*).

The assay solution for the simultaneous detection of hexokinase and 3-phosphoglycerate kinase in column effluents was prepared in triethanolamine–HCl pH 8.0 (0.1 *M*) and contained ATP (0.41 m*M*), $NADP^+$ (0.31 m*M*), NADH (48 μM), D-glucose (0.125 *M*), 3-phosphoglycerate (5.0 m*M*), glucose-6-phosphate dehydrogenase (2.53 μg/ml; 1.0 U/ml) and glyceraldehyde-3-phosphate dehydrogenase (1.75 μg/ml; 0.12 U/ml). In each case enzyme activity was monitored by the change in absorbance at 340 nm.

Bovine pancreatic ribonuclease A activity was monitored by the decrease in absorbance at 305 nm following hydrolysis of RNA to oligonucleotides. The assay solution contained RNA (2.2 mg/ml) in sodium acetate buffer pH 5.0 (0.1 *M*). Alkaline phosphatase activity was monitored at 410 nm with assay medium comprising *p*-nitrophenylphosphate (disodium salt) (4 m*M*) in Tris–HCl pH 8.0 (0.25 *M*).

On-line detection of protein (280 nm) and enzyme activity (305, 340 or 410 nm, respectively) was continuously displayed on a two-channel recorder (Omniscribe 3500, Houston Instruments, Austin, TX, U.S.A. or Pharmacia REC-2, Pharmacia, Uppsala, Sweden).

Preparation of crude extracts

Bakers yeast (42 g) (Jästbolaget, Sollentuna, Sweden) was dried at 35°C for 24 h and then at 20°C for a further 72 h. Dibasic sodium phosphate (1 *M*; 100 ml) was added to the dry cells (12.8 g) and lysis allowed to occur at 30°C for 12 h under gentle agitation. The slurry was centrifuged (14,000 *g*, 20 min) and the clear supernatant used for injection without further treatment. The crude yeast supernatant was stored at 4°C.

Bovine pancreatic acetone powder (2 g, Sigma) was extracted with 10 ml H_2SO_4 (0.25 *M*) and after filtration the filtrate was used directly for analysis.

RESULTS AND DISCUSSION

Preparation of Cibacron Blue F3G-A-substituted silica

Early attempts to covalently attach Cibacron Blue F3G-A directly to surface silanol groups on porous silica proved unsuccessful and yielded ineffective adsorbents. However, preassembled triazine-substituted 6-aminohexyl-Cibacron Blue F3G-A can be efficiently coupled to epoxysilylated microparticulate silica to generate ligand substitutions in the range 5.5–6.7 μmol/g dry weight under the specified conditions. This approach to the synthesis of the HPLAC adsorbent has several advantages. Firstly, the degree of ligand substitution may be varied simply by incubating at different pH values, lengths of time or temperatures although some deterioration of the silica may be expected under harsher conditions than those reported here. The solubilisation of derivatised silica in alkaline solution represents a facile approach to the quantitation of bound ligand concentrations. Secondly, excess epoxy groups may be readily hydrolysed to diols without affecting the bound dye to generate gels which, provided the irrigating buffers are maintained at appropriate ionic strengths, display no significant non-specific adsorption of any of the proteins studied in this paper. Thirdly, limited studies in affinity chromatography with the triazine dye coupled via spacer molecules suggest increased selectivity over directly coupled dyes[17,18] and finally, the procedure permits prepurification of the Cibacron Blue

F3G-A-spacer arm conjugate prior to attachment to the silica gel. Fig. 1 illustrates the structure of the Cibacron Blue F3G-A silica and shows the polysulphonated aromatic character of the ligand and the comparatively long spacer molecule interposed between the silica surface and the dye.

Separation of dehydrogenases

Agarose-immobilised Cibacron Blue F3G-A has been exploited for the resolution and purification of a number of pyridine nucleotide-dependent dehydrogenases from several sources and tissues[7–10,17,19]. Similarly, silica-immobilised Cibacron Blue F3G-A equilibrated with 0.1 *M* potassium phosphate pH 7.5 quantitatively adsorbs pig heart and rabbit muscle lactate dehydrogenase, pig heart malate dehydrogenase and horse-liver alcohol dehydrogenase and retards *L. mesenteroides* glucose-6-phosphate dehydrogenase. The strength of adsorption of these enzymes appears to be weakened at lower ionic strengths of irrigating buffer, suggesting a significant contribution from hydrophobic forces in the interaction. The implication of hydrophobic forces in the interaction of proteins with immobilised dyes is well established in affinity chromatography[19]. Consequently, bound enzymes could not be released by raising the ionic strength as has been observed in the case of HPLAC on immobilised AMP[4], although prompt desorption could be effected biospecifically with NAD^+, NADH or appropriate ternary complexes. Fig. 2 illustrates the experimental set-up for the resolution of a synthetic mixture of bovine serum albumin, pig heart lactate dehydrogenase and horse liver alcohol dehydrogenase by HPLAC on silica-immobilised Cibacron Blue F3G-A with continuous on-line monitoring of protein concentration (280 nm) and enzyme activity (340 nm). Fig. 3 illustrates the sequential elution of the two dehydrogenases with 1 m*M* NAD^+, 1 m*M* NAD^+/0.1 m*M* pyrazole and 1

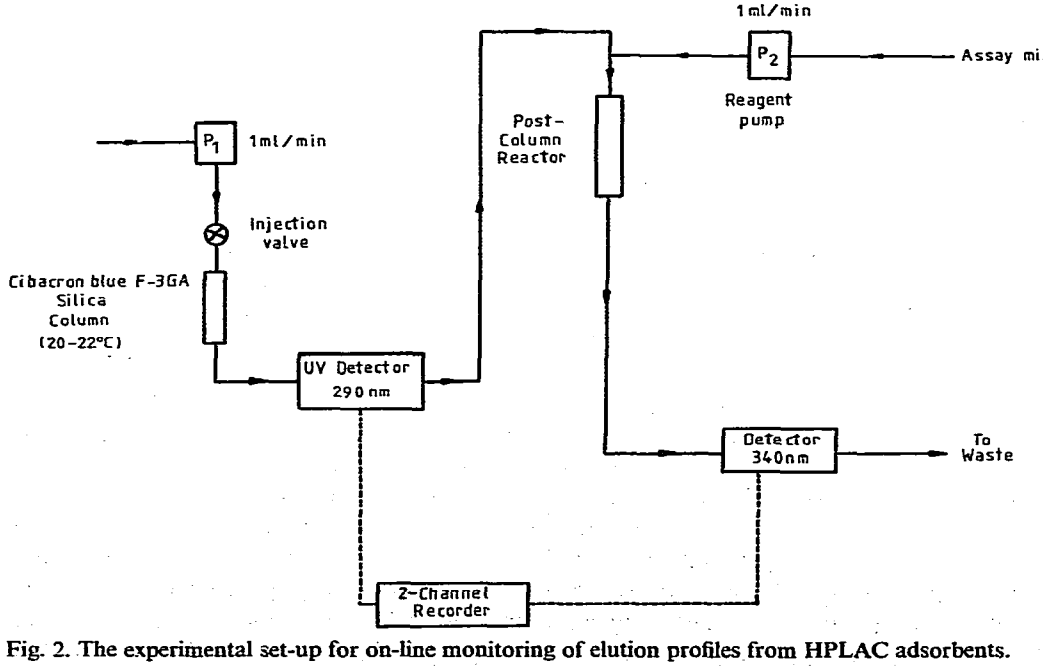

Fig. 2. The experimental set-up for on-line monitoring of elution profiles from HPLAC adsorbents.

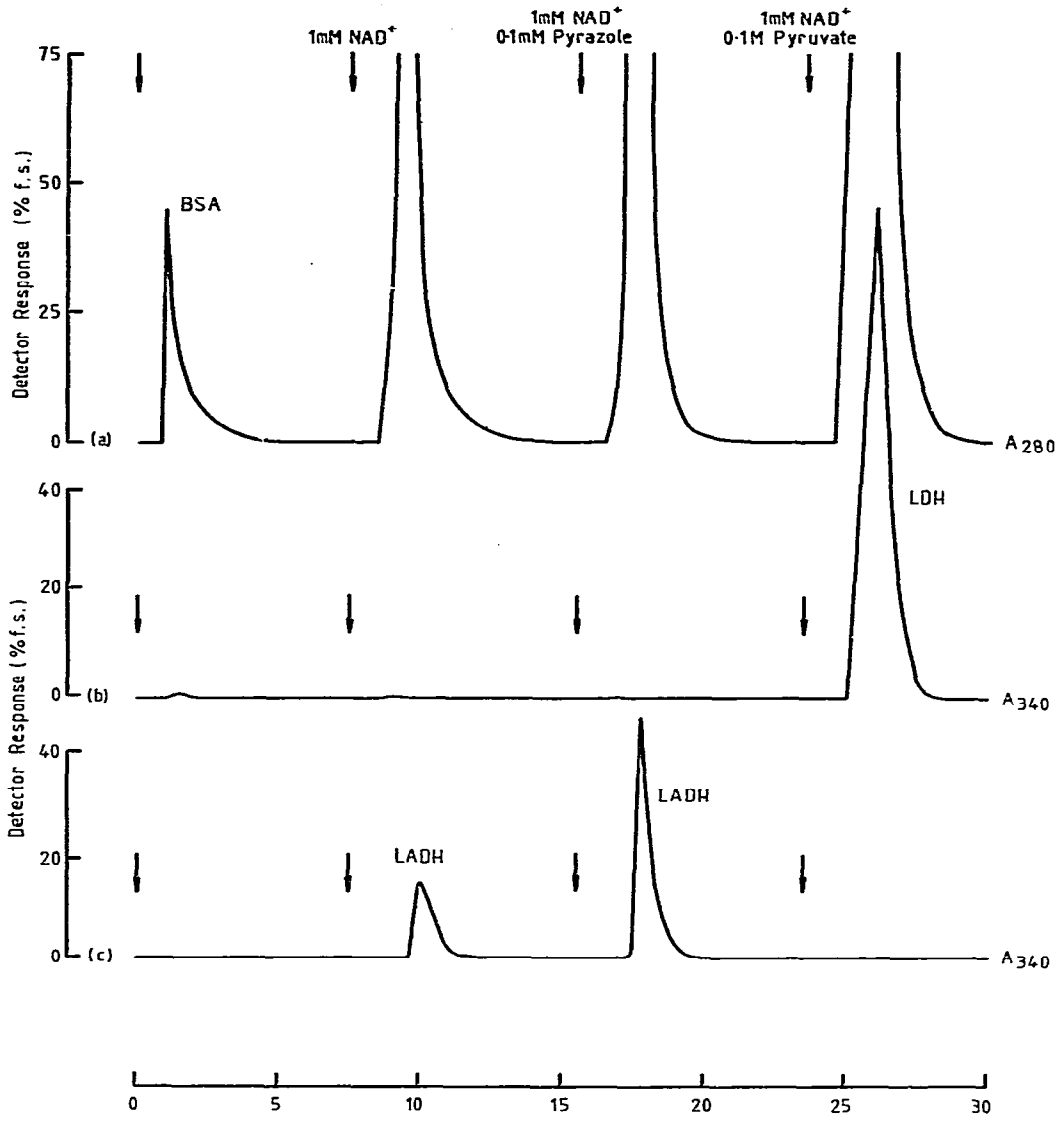

Fig. 3. Separation of bovine serum albumin (BSA), horse liver alcohol dehydrogenase (LADH) and pig heart lactate dehydrogenase (LDH) with ternary complex formation on a Cibacron Blue F3G-A silica column. Sample applied at first arrow: LADH (10 μg), LDH (5 μg) and BSA (30 μg) in 0.1 *M* potassium phosphate pH 7.5 (50 μl); column irrigant: 0.1 *M* potassium phosphate buffer pH 7.5; temperature of analytical column: 22°C; flow-rate (main pump and reagent pump): 1 ml/min; pressure: 8.3 MPa. 1200 p.s.i. (reagent pump); eluents: 500 μl, as indicated by the arrows; protein detector (280 nm). (a), 0.16 a.u.f.s., on-line detection of enzyme activity (340 nm); (b), 1.0 a.u.f.s., lactate dehydrogenase (H_4) and (c) alcohol dehydrogenase. Full details of the enzyme assay procedures are given in the experimental section.

m*M* NAD^+/0.1 *M* pyruvate. Fig. 3 represents a composite of two successive chromatographic runs with the UV monitor measuring protein concentration (Fig. 3a) and the post-column reactor set to monitor lactate dehydrogenase (Fig. 3b) and alcohol

dehydrogenase (Fig. 3c) activity. It is clear that both dehydrogenases are eluted with their specific ternary complexes, although some release of alcohol dehydrogenase was observed with the NAD^+ pulse[4].

Resolution of isoenzymes

The separation of the isoenzymes of lactate dehydrogenase has been achieved by conventional low-pressure affinity[20] or ion-exchange chromatography[21] and more recently by HPLC on DEAE-glycophase[15] and HPLAC on silica-immobilised AMP[4]. In the latter report, the high speed resolution of the two major isoenzymes, H_4 and M_4, was achieved with a NADH gradient with manual monitoring of enzyme activity and gradient profile. The present report demonstrates that the resolution of a similar synthetic mixture of bovine serum albumin, pig heart (H_4) and rabbit muscle (M_4) lactate dehydrogenase may be effected on an immobilised Cibacron Blue F3G-A HPLAC adsorbent with continuous on-line monitoring of protein concentration, gradient profile and isoenzyme activity. This approach to isoenzyme profiling considerably speeds up the overall time for analysis since it obviates the necessity for fraction collecting and manual enzyme assay. Fig. 4 illustrates the experimental set-up required for automatic recording of the entire chromatographic profile. The system automatically corrects for the increased absorbance in the post-column enzyme detector due to the NADH gradient. The effluent flow from the analytical HPLAC column is passed through a UV detector set at 290 nm to monitor unretarded protein and subsequently the gradient profile due to absorbance of NADH (ε 1400 l mol^{-1} cm^{-1} at 290 nm) before being split equally at a T-piece (Fig. 4). One half of the split flow passes on to the post-column reactor where, immediately prior to

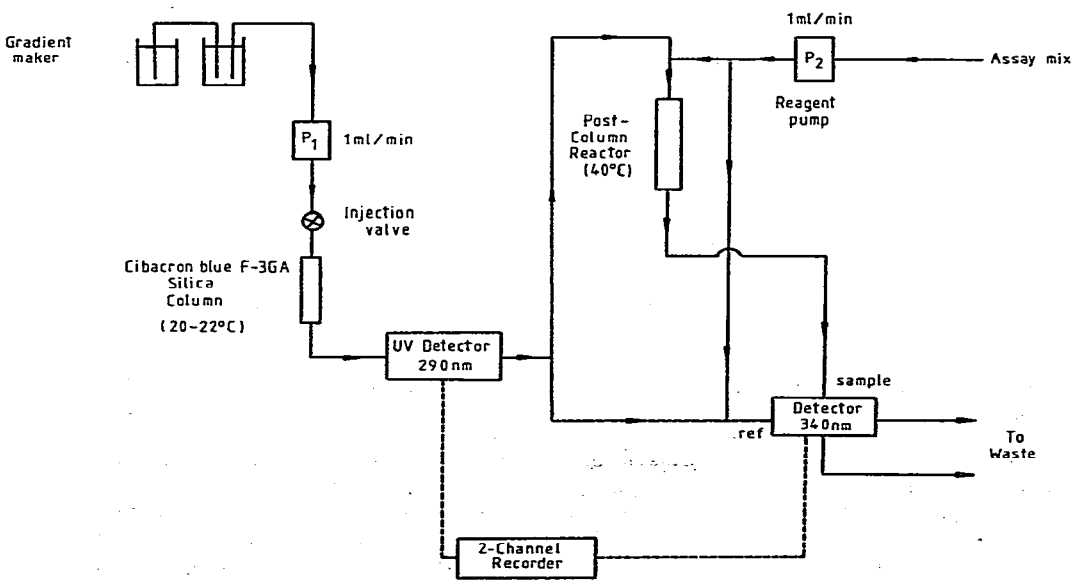

Fig. 4. Flow diagram for fully automated on-line detection of lactate dehydrogenase isoenzymes by NADH gradient elution from Cibacron Blue F3G-A silica. Main pump: P_1, 1 ml/min; reagent pump: P_2, 1 ml/min; UV detector, 290 nm; enzyme activity detector, 340 nm; post-column reactor temperature: 40 $\pm$ 0.1°C; pressures: P_1, 6.9 MPa, 1000 p.s.i.; P_2, 1.4 MPa, 200 p.s.i.

the column, it is mixed with half of the split flow from the reagent pump P_2 and the effluent from the post-column reactor flows directly through the sample cuvette of the enzyme activity detector set at 340 nm. The other half of the flow of assay mixture from the reagent pump is mixed with the effluent from the UV-detector, bypasses the post-column reactor and passes through the reference cuvette of the enzyme activity detector. Thus, when there is no enzyme activity in the effluent from the analytical HPLAC column, the system is fully compensated irrespective of the NADH gradient

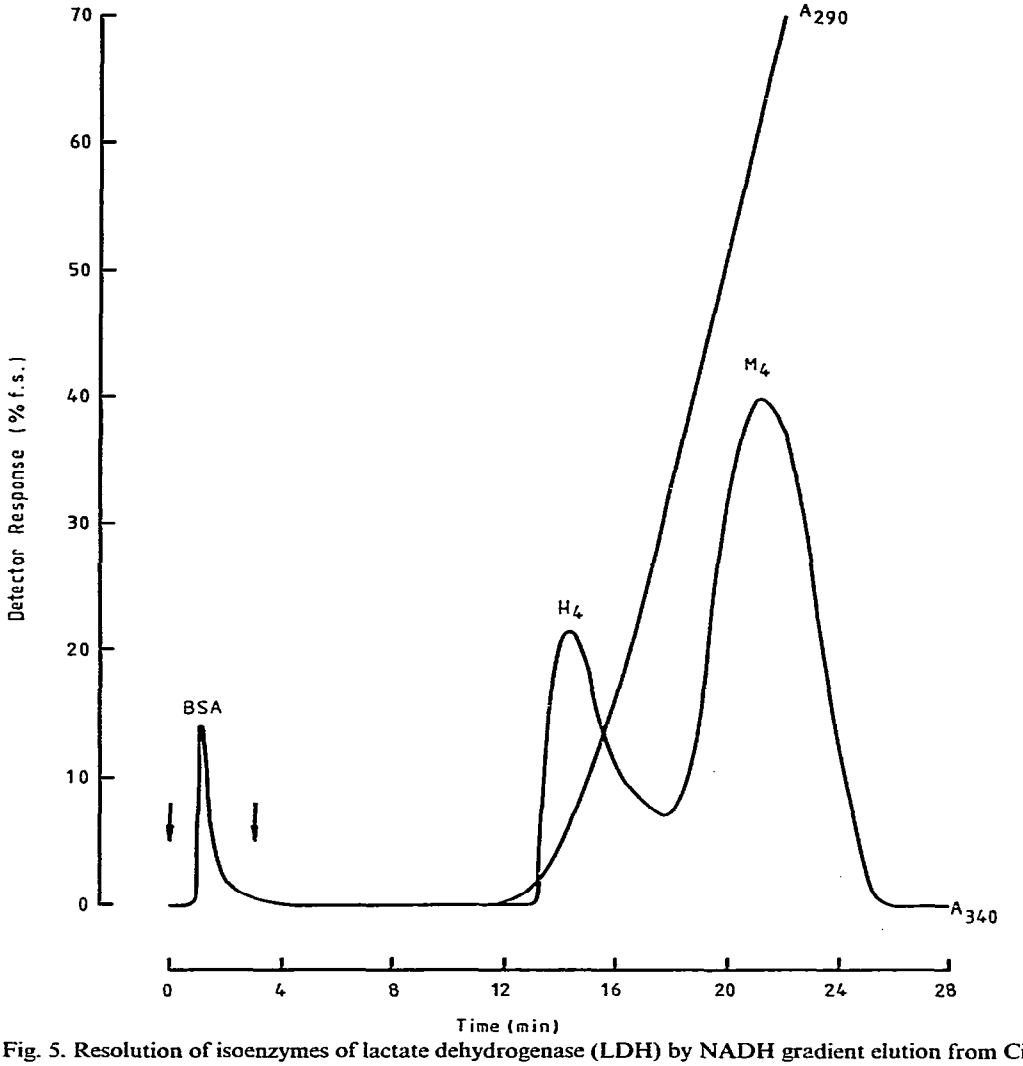

Fig. 5. Resolution of isoenzymes of lactate dehydrogenase (LDH) by NADH gradient elution from Cibacron Blue F3G-A silica. Sample applied at first arrow: LDH-H_4 (1.3 μg), LDH-M_4 (11.8 μg) and BSA (60 μg) in 0.1 *M* potassium phosphate buffer pH 7.5 (20 μl); column irrigant: 0.1 *M* potassium phosphate buffer pH 7.5; flow-rates: 1 ml/min, both pumps; pressure: P_1, 6.9 MPa, 1000 p.s.i.; P_2, 1.4 MPa, 200 p.s.i.; gradient commenced at second arrow: 0–4 m*M* NADH in 0.1 *M* potassium phosphate buffer pH 7.5, 20 ml mixing volume; temperature of analytical column: 22°C; post-column reactor temperature: 40 ± 0.1°C; UV detector (290 nm): 1.28 a.u.f.s.; enzyme activity detector (340 nm): 2.0 a.u.f.s.

profile and zero absorbance is recorded on the enzyme activity monitor. Slight adjustments to the flow-rates through the system could be achieved by crimping the tubing going to waste to ensure that this condition held at all times during the chromatographic run. In contrast, the presence of isoenzymes in the effluent from the post-column reactor reduce the absorbance in the sample cuvette and produce positive peaks on a two-channel recorder when the polarity of the signal is reversed.

Fig. 5 illustrates a typical fully automated chromatographic profile representing the NADH gradient elution of serum albumin and the two major isoenzymes of lactate dehydrogenase (H_4 and M_4) from an HPLAC adsorbent comprising silica-immobilised Cibacron Blue F3G-A. The complete chromatographic profile was recorded in less than 30 min, although with suitable adjustments of the flow-rate through the main pump (P_1) and the reagent pump (P_2) this could easily be reduced to 10 min or less.

Separation of kinases

The considerable advantage of Cibacron Blue F3G-A over other affinity ligands for HPLAC is that, in addition to specifically binding a wide range of dehydrogenases[7–10,17,19], it also displays affinity for a number of kinases[9,10]. Thus, purified or crude samples of yeast hexokinase (HK) or 3-phosphoglycerate kinase (PGK) may be separately or concurrently adsorbed to Cibacron Blue F3G-A silica in 0.1 *M* Tris–HCl pH 7.3 containing 0.5 m*M* EDTA, 5 m*M* $MgCl_2$ and 0.5 m*M* 2-mercaptoethanol and subsequently eluted by binary or ternary complex formation. Fig. 6 illustrates the chromatography of a crude yeast extract on Cibacron Blue F3G-A silica and subsequent pulse elution with MgATP, MgATP/D-glucose and MgATP/3-phosphoglycerate with simultaneous on-line detection of protein (280 nm) and enzyme activity (340 nm). The post-column detection of both kinases was accomplished with a "universal" assay medium comprising the reactions:

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{D-glucose-6-phosphate}$$

$$\text{D-Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

for hexokinase activity and the reactions:

$$\text{3-phosphoglycerate} + \text{ATP} \xrightarrow{\text{PGK}} \text{glycerate-1,3-bisphosphate} + \text{ADP}$$

$$\text{glycerate-1,3-bisphosphate} \xrightarrow{\text{G3PDH}} \text{glyceraldehyde-3-phosphate} +$$

$$+ \text{NADH} + \text{H}^+ \qquad\qquad \text{NAD}^+ + \text{phosphate}$$

for 3-phosphoglycerate kinase activity. It is evident that the presence of hexokinase in the column effluent produces an increase in absorbance at 340 nm whilst 3-phosphoglycerate kinase activity decreases the absorbance at 340 nm. It should be pointed out, however, that the specific elution method adopted here has some inherent disadvantages. For example, under certain conditions, a significant detector response

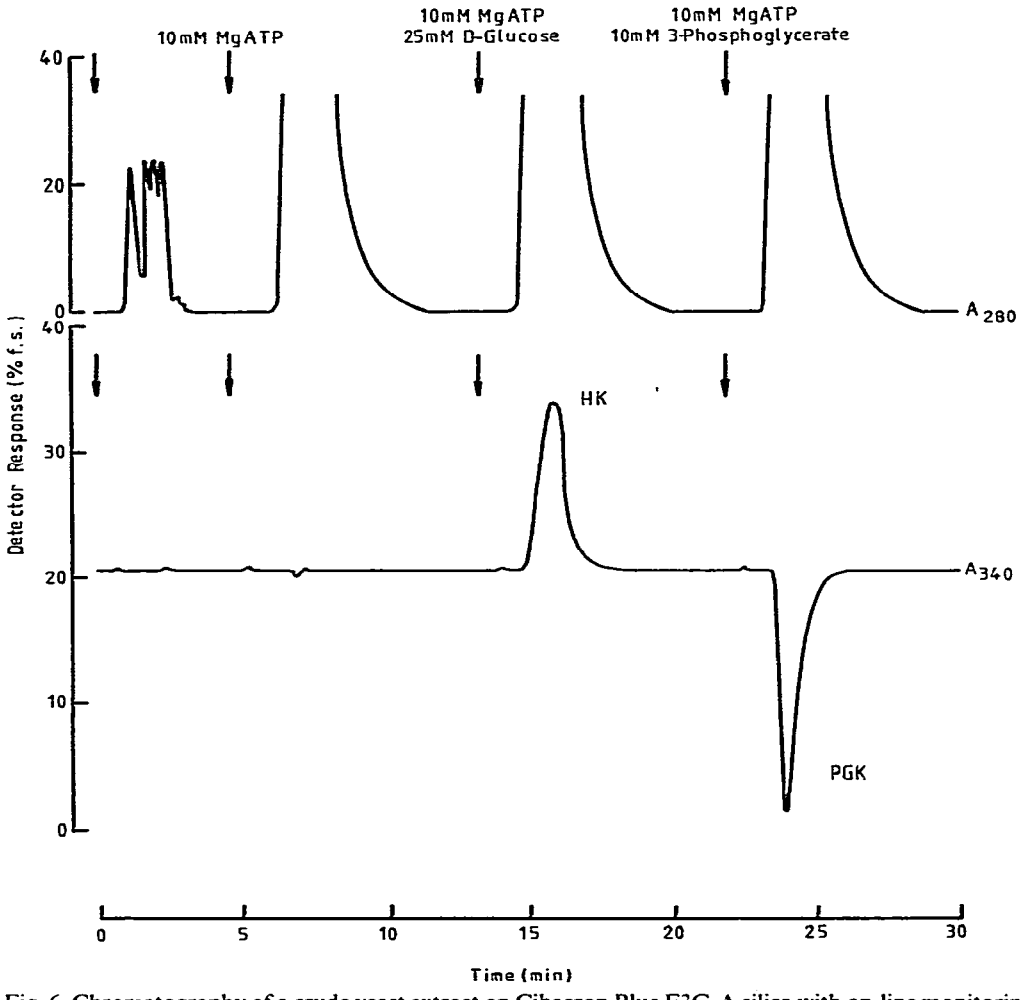

Fig. 6. Chromatography of a crude yeast extract on Cibacron Blue F3G-A silica with on-line monitoring of hexokinase (HK) and 3-phosphoglycerate kinase (PGK) activity. Sample applied at first arrow: crude yeast extract (2 μl); column irrigant: 0.1 *M* Tris–HCl buffer pH 7.3 containing 0.5 m*M* EDTA, 5 m*M* $MgCl_2$ and 0.5 m*M* 2-mercaptoethanol; flow-rates: 1 ml/min, both pumps; post-column reactor temperature: 40 ± 0.1°C; assay mix temperature: 0°C; temperature of analytical column: 22°C; eluents (400 μl): as indicated by the arrows; UV-detector (290 nm): 0.32 a.u.f.s.; enzyme activity detector: 0.5 a.u.f.s.

could be obtained despite the fact that only a small proportion of the bound enzyme was eluted. Hexokinase tightly bound to the immobilised Cibacron Blue F3G-A column catalysed the conversion of the substrate pulse into the primary product which subsequently caused the auxillary enzyme system to respond in the post-column reactor and thus produce a deflection in the 340 nm detector. The problem may be circumvented by exploiting other ternary complexes such as MgATP/D-mannose for the release of hexokinase activity from the adsorbent since, in this case, the primary product, D-mannose-6-phosphate, is not a substrate for the auxillary enzyme system.

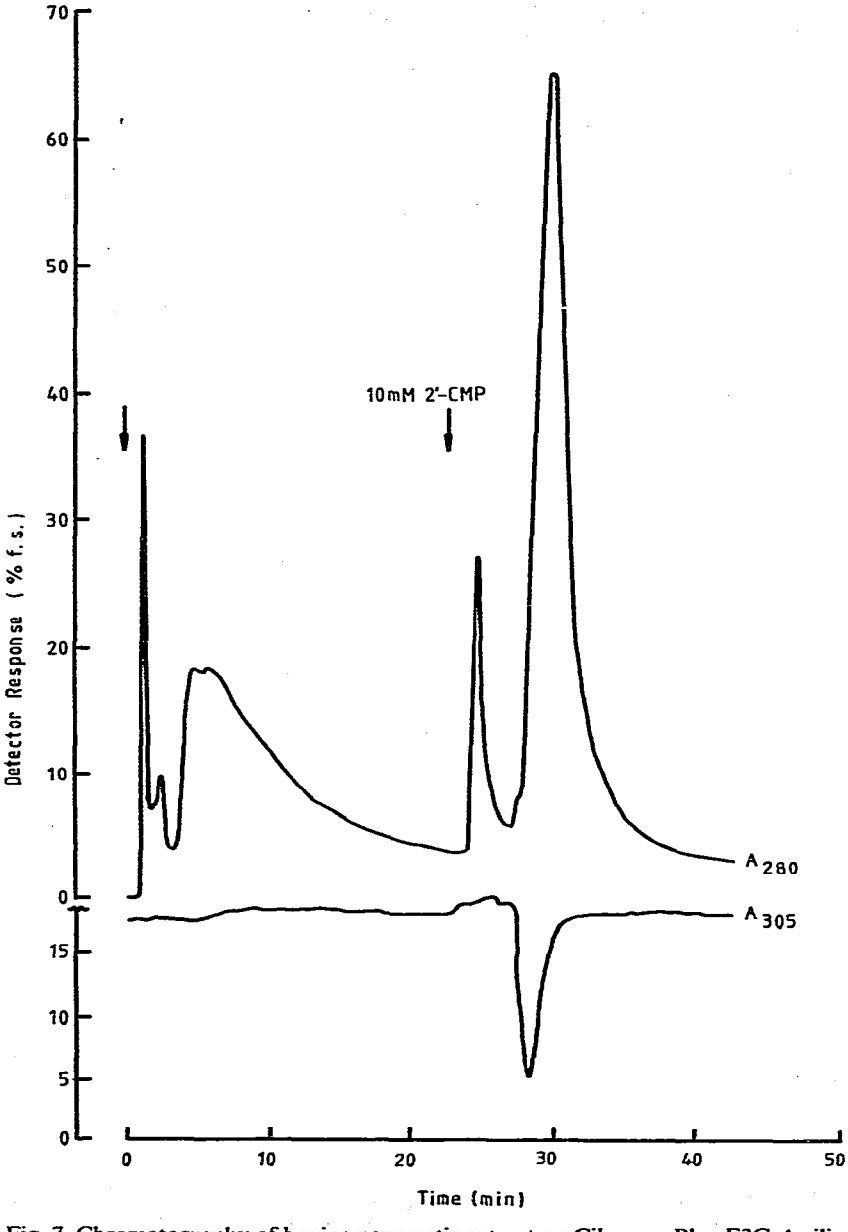

Fig. 7. Chromatography of bovine pancreatic extract on Cibacron Blue F3G-A silica with specific elution of ribonuclease A and on-line monitoring of enzyme activity. Sample applied at first arrow: simulated crude bovine pancreatic extract (50 μl) containing crystalline ribonuclease A (500 μg); column irrigant: 10 m*M* sodium acetate buffer pH 5.0; flow-rates: 1 ml/min (main pump), 2 ml/min (reagent pump); eluent: 10 m*M* 2'-CMP (50 μl); temperature of analytical column: 22°C; post-column reactor temperature: 22–24°C; UV detector (280 nm): 1.28 a.u.f.s.; enzyme activity detector (305 nm): 0.20 a.u.f.s.

The binding of other proteins

The great versatility of immobilised Cibacron Blue F3G-A as a chromatographic medium is further illustrated by the binding of bovine pancreatic ribonuclease A[7]. Fig. 7 illustrates the chromatography of a crude bovine pancreatic extract, spiked with additional crystalline bovine ribonuclease A, and subsequent biospecific elution with 10 m*M* 2′-CMP as indicated by the arrow. The activity of the enzyme was followed on-line by the hypochromicity at 305 nm resulting from hydrolysis of RNA to oligonucleotides.

CONCLUSIONS

Microparticulate silica-immobilised Cibacron Blue F3G-A is a highly versatile chromatographic adsorbent for HPLAC. This new stationary phase should find wide application in the resolution of enzymes and other proteins of clinical or diagnostic significance especially when coupled to the high speed fully automated chromatographic profiling system reported here. In the present authors' hands, the columns have behaved reliably and reproducibly without apparent loss of binding capacity for a continuous period of operation of several months. However, with samples comprising crude extracts, some fouling and clogging has been observed. Consequently the column may require rejuvenation by washing with 4 *M* urea or 0.1 *M* glycine–HCl pH 2.5 from time to time. Moreover, recoveries of enzyme activity (approx. 25%) are somewhat lower than on chromatography with the equivalent agarose-immobilised dye. Nevertheless, the authors are confident that the adsorbent will prove a useful addition to the HPLAC adsorbents already developed[4–6]. The decision whether to use immobilised pseudo-affinity ligands such as dyes or more biological molecules such as adenine coenzymes[2,3] in HPLAC systems would depend on the type of separation to be performed. Immobilised coenzymes are more specific, allow more quantitative recovery and permit model studies on enzyme–coenzyme interactions whereas immobilised Cibacron Blue F3G-A is more versatile and markedly cheaper.

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