Journal of Chromatography, 266 (1983) 151–156
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 017

PREPARATIVE HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY

DAVID A. P. SMALL* and TONY ATKINSON
PHLS Centre for Applied Microbiology and Research, Porton Down, Nr. Salisbury, Wiltshire SP4 OJG (Great Britain)
and
CHRISTOPHER R. LOWE
Department of Biochemistry, University of Southampton, Southampton SO9 3TU (Great Britain)

SUMMARY

The reactive triazine dye, Procion Blue MX-R, has been covalently attached to preparative-grade silica and used for the large-scale purification of rabbit muscle lactate dehydrogenase by high-performance liquid affinity chromatography (HPLAC). Purified dye was coupled directly to glycol-silylated silica via the reactive triazine ring to yield an adsorbent containing 12 μmol dye/g silica. Essentially homogeneous lactate dehydrogenase in 80% overall yield was obtained from crude extracts. Thus this report demonstrates the potential for adapting the speed of operation and resolution shown for triazine dye-HPLAC in analytical applications to preparative protein purification.

INTRODUCTION

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 in recent years[1,2]. Furthermore it has also been shown that Cibacron Blue F3G-A can be applied to the resolution of enzymes and isoenzymes by high-performance liquid affinity chromatography (HPLAC)[3]. Subsequent studies have demonstrated that affinity ligands drawn from the whole class of triazine dyes can be used in this new chromatographic technique thus permitting a greater degree of latitude in the purification of particular proteins[4]. In addition, advances have been made in the application of agarose-immobilised triazine dyes to the large scale purification of enzymes. Thus 3-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30) and malate dehydrogenase (E.C. 1.1.1.37) were purified to homogeneity on a large scale involving sequential affinity chromatography on two triazine dye-Sepharose matrices with yields of 78% and 64%, respectively[2,5]. Major limitations in the use of agarose for preparative separations are low flow-rates, diminished peak resolution and bacterial degradation of the agarose backbone of the matrix resulting in ligand leakage.

Large-scale or preparative HPLAC would be essentially free of these problems.

0021-9673/83/$03.00

In particular preparative HPLAC would be of special value when the protein to be purified is unstable in crude extracts and rapid isolation is required. Many crude protein extracts may, for example, contain proteolytic enzymes which remain active during the course of conventional time-consuming chromatographic procedures. This report therefore examines the potential for adapting the speed of operation and resolution demonstrated for triazine dye-HPLAC in analytical applications to preparative protein purification.

EXPERIMENTAL

Chemicals

Preparative-grade silica (LiChrosorb Si 60, 40–63 μm) was obtained from E. Merck (Darmstadt, G.F.R.). HEPES (N-2-hydroxyethylpiperazine N′-2-ethanesulphonic acid), nicotamide–adenine dinucleotide, reduced (NADH), bovine serum albumin (fraction V), and crude lactate dehydrogenase [L-lactate: nicotinamide–adenine dinucleotide, oxidised (NAD^+) oxidoreductase, E.C. 1.1.1.27; rabbit muscle, Type I, 40–100 U/mg], were purchased from Sigma (London) (Poole, Great Britain). The organofunctional silane, γ-glycidoxypropyltrimethoxysilane was from Silor Labs. (Scotia, NY, U.S.A.). The dye Procion Blue MX-R (C.I. 61205) was a generous gift from Dr. C. V. Stead, I.C.I. Organics Division, Blakely, Great Britain.

Synthesis of Procion blue MX-R substituted silica

Preparative-grade silica (LiChrosorb Si 60, 40–63 μm) was epoxy-silylated with γ-glycidoxypropyltrimethoxysilane as described previously[3] and subsequently converted to the glycol form by heating the epoxy-silylated silica at 70°C in an aqueous solution adjusted to pH 3.5 with hydrochloric acid. Glycol-substituted silica (60 g dry weight) was added to 0.1 *M* $NaHCO_3$–Na_2CO_3 buffer pH 8.5 (200 ml) containing Procion Blue MX-R (3.2 g, 5 mmol). The resulting slurry was sonicated for 10 min and incubated overnight at 22°C with gentle agitation. The procion Blue MX-R silica adsorbent was exhaustively washed with water (5000 ml) until no blue dye was evident in the washings. The conjugate was washed with 1 *M* potassium chloride (1000 ml), water (1000 ml) and methanol–water (1:1) prior to drying under vacuum. The concentration of Procion Blue MX-R immobilised on the silica was determined by spectrophotometric analysis of alkaline hydrolysates as previously described[3]. Typically the immobilised ligand concentration was 12 μmol/g dry silica. The structure of silica-immobilized Procion Blue MX-R is shown in Fig. 1.

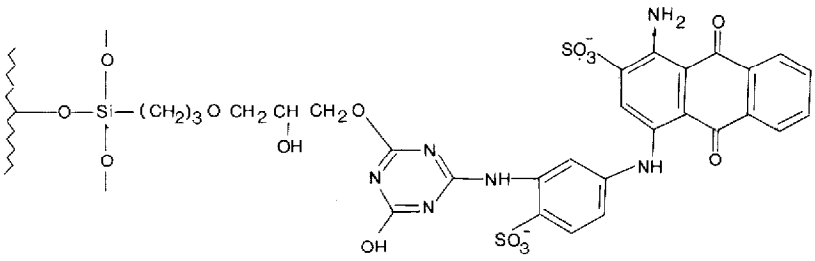

Fig. 1. The structure of Procion Blue MX-R attached directly via its triazine ring to glycol silylated silica.

Chromatographic procedures

The Procion Blue MX-R-silica adsorbent (60 g) was dry packed in a stainless-steel column (30 × 2.5 cm) busing a tap-and-fill technique[6]. The packed column was stored dry until required. All chromatographic procedures were performed at ambient temperature (20–22°C) with Waters Assoc. (Hartford, Northwich, Great Britain) high-performance liquid chromatographic equipment comprising a Model 6000 solvent metering pump, a Model 450 variable-wavelength detector (190–800 nm) and a Model U6K sample injector. Fractions were collected with a LKB Model 2070 Ultra-Rac II fraction collector.

Enzyme assay

Lactate dehydrogenase was assayed by following the oxidation of NADH by pyruvate at 340 nm and 30°C. The assay mixture contained in a total volume of 1 ml: potassium phosphate buffer pH 7.0, 50 μmol; 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 of NADH per min at 30°C.

Protein determination

Protein concentration were determined by using the Folin reagent with bovine serum albumin (fraction V) as the protein standard[8].

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed as described by Laemmli[9]. Densitometer scans of disc gels were obtained by scanning at 595 nm using a Gilford model 2600 microprocessor-controlled spectrophotometer, fitted with a Model 2520 gel scanner and coupled to a Hewlett-Packard 7225 A graphics plotter.

RESULTS AND DISCUSSION

The analytical application of Cibacron Blue F3G-A and Procion Blue MX-R silica HPLAC adsorbents for the purification of lactate dehydrogenase has been reported previously[3,4]. Both immobilised dyes act as effective affinity adsorbents for lactate dehydrogenase, although the synthesis of the Procion Blue MX-R adsorbent is achieved by a simpler and more direct route. Procion Blue MX-R reacts readily with the terminal diol functional group of glycol-silylated silica whereas Cibacron Blue F3G-A requires prior derivatisation with an aminohexyl spacer arm to facilitate covalent attachment to epoxy-silylated silica. Thus the use of the more reactive dichlorotriazinyl dye, where appropriate, greatly reduces the synthetic work rquired to produce a HPLAC adsorbent.

The large-scale purification of rabbit muscle lactate dehydrogenase from a crude extract (79 U/mg) was achieved on an adsorbent comprising Procion Blue MX-R immobilised on preparative-grade silica at a ligand concentration of 12 μmol/g. The preparative column was equilibrated with 10 m*M* HEPES pH 7.0 at room temperature at a flow-rate of 10 ml/min. An ammonium sulphate suspension of crude rabbit muscle lactate dehydrogenase (1 ml, 79 U/mg) was dialyzed against 10 m*M* HEPES buffer pH 7.0, and centrifuged (12,000 *g*) for 20 min to remove insoluble

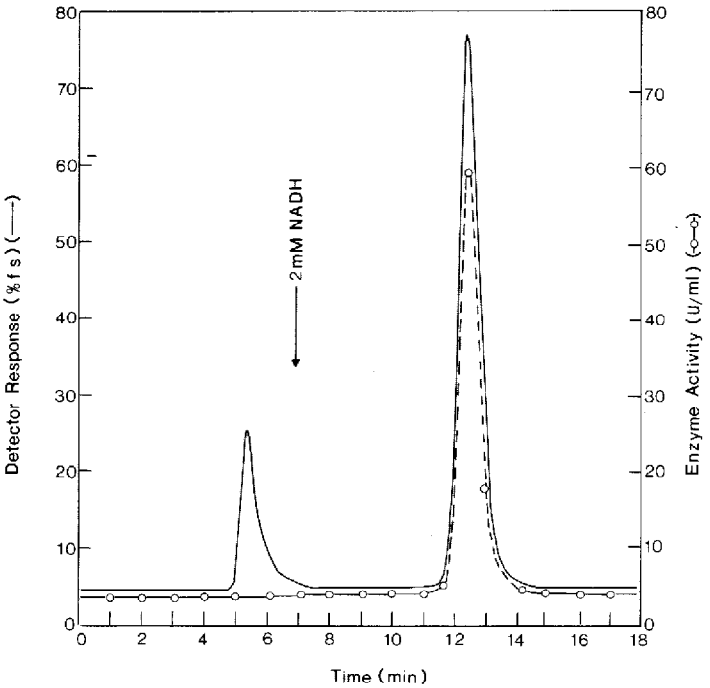

Fig. 2. Purification of rabbit muscle lactate dehydrogenase on a Procion Blue MX-R silica column. Crude enzyme extract in 10 m*M* HEPES pH 7.0 (1 ml, 13.9 mg, 79 U/mg) applied at time zero; temperature: 20–22°C; flow-rate: 10 ml/min; pressure: 3.5 MPa (500 p.s.i.); detector: 280 nm, 2.0 a.u.f.s.; eluent: 2 m*M* NADH (2 ml) in 10 m*M* HEPES pH 7.0 as indicated by the arrow. Full details of the enzyme assay procedure are given in the experimental section.

protein prior to injection. Fig. 2 shows the chromatographic profile obtained from the injection of a sample of the crude extract (1 ml; 13.9 mg protein; 79 U/mg). The void time at a flow-rate of 10 ml/min was 5 min as determined by applying an unretained protein such as bovine serum albumin (1 ml, 10 mg/ml) to the column[4]. Bound protein was biospecifically eluted by injecting a pulse (2 ml) of NADH (2 m*M*) onto the column. Eluates from the column were monitored at 280 nm and 5-ml fractions were collected for assay. The protein content of the biospecifically eluted fraction was determined by the Lowry method[8], as the absorbance at 280 nm of this peak was predominantly due to NADH. Table I summarizes the purification procedure. There

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

Stage	*Volume (ml)*	*Protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Purification (fold)*	*Yield (%)*
Crude	1.0	1104	79	1.0	100	100
NADH eluate from HPLAC column	5.0	2.2	880	400	5.1	80

is a 5.1-fold increase in specific activity of the biospecifically eluted lactate dehydrogenase compared to the crude extract with an overall yield of 80%.

The purity of the eluted enzyme was established by SDS polyacrylamide gel electrophoresis. Fig. 3 is a densitometer scan of (A) the applied crude enzyme and (B) the purified pooled fractions. A densitometer scan of pure rabbit muscle lactate dehydrogenase corresponds with the major peak shown in Fig. 3B. In addition to the observed increase in specific activity the purification procedure from relatively crude to essentially homogeneous lactate dehydrogenase has the advantage of taking no more than 15 min from the time of injection to the collection of eluted enzyme.

The capacity of the preparative dye-silica adsorbent was determined by frontal analysis. Thus, continuous injections (2 ml) of crude rabbit muscle lactate dehydrogenase (13.9 mg/ml, 79 U/mg) were applied to the column until enzyme activity was observed in the eluent. For a column containing 60 g of silica–Procion Blue MX-R adsorbent enzyme activity was observed after 25 ml of crude extract containing 348 mg total protein had been applied. Subsequent elution of bound enzyme with NADH

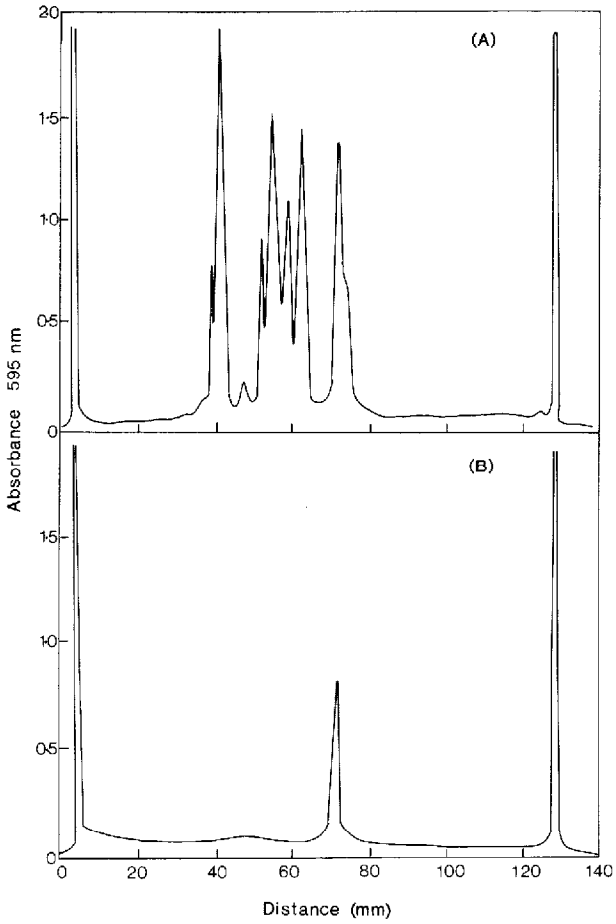

Fig. 3. Densitometer scans of SDS-polyacrylamide gels. (A) crude rabbit muscle lactate dehydrogenase applied to Procion Blue MX-R HPLAC column. (B) enzyme fraction eluted by 2 m*M* NADH.

resulted in the recovery of 22 mg of lactate dehydrogenase. This corresponds to a capacity of 0.33 mg pure enzyme per g silica.

The low capacity of Procion Blue MX-R-silica for lactate dehydrogenase almost certainly is a reflection of the pore diameter of the silica particles. The size of proteins and other molecules of interest for affinity chromatography varies within the range 25–250 Å. An enzyme such as lactate dehydrogenase (relative molecular mass, M_r = 142,000) has a diameter of approximately 70 Å (ref. 10). An analytical 10-μm silica matrix with a pore diameter of 60 Å interacts with such an enzyme predominately at its outer surface. For analytical HPLAC, this is not a major problem as the amounts applied to the column are frequently small and will not exceed the binding capacity of the column. However, to achieve high binding capacity for preparative purposes, there is an additional requirement for free interaction with the interior pore surfaces. Furthermore, the effect of the silanisation process on pore diameter must be taken into consideration. The treatment of silica with reagents such as γ-glycidoxypropyltrimethoxysilane effectively reduces the overall pore size and in some cases may block the pore entrance completely.

As high binding capacity is a prime objective in preparative protein separations by HPLAC there is a need to consider silica matrices with greater pore diameters, *e.g.* 300–500 Å. Such supports would allow free access for most proteins and at the same time maintain a reasonably high surface area, thus ensuring an adequate concentration of immobilised ligand. Despite these initial problems, this report demonstrates that preparative-scale HPLAC has great potential for the rapid isolation and resolution of proteins.

REFERENCES

1 P. D. G. Dean and D. H. Watson, *J. Chromatogr.*, 165 (1979) 301.
2 C. R. Lowe, D. A. P. Small and T. Atkinson, *Int. J. Biochem.*, 13 (1981) 33.
3 C. R. Lowe, M. Glad, P.-O. Larsson, S. Ohlson, D. A. P. Small, T. Atkinson and K. Mosbach, *J. Chromatogr.*, 215 (1981) 303.
4 D. A. P. Small, T. Atkinson and C. R. Lowe, *J. Chromatogr.*, 216 (1981) 175.
5 M. D. Scawen, J. Darbyshire, M. J. Harvey and T. Atkinson, *Biochem. J.*, 203 (1982) 699.
6 J. J. Kirkland, *J. Chromatogr. Sci.*, 10 (1972) 129.
7 H. U. Bergmeyer (Editor), *Methods of Enzymic Analysis*, Vol 2, Academic Press, New York, 1974, p. 574.
8 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
9 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
10 P.-O. Larsson, M. Glad, L. Hansson, M.-O. Mansson, S. Ohlson and K. Mosbach, *Advan. Chromatogr.*, 21 (1982) 1.