Journal of Chromatography, 363 (1986) 31–36
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 599

Note

Process scale high-performance liquid affinity chromatography

Y. D. CLONIS
The Biotechnology Centre, University of Cambridge, Downing Street, Cambridge CB2 3EF (U.K.)
K. JONES
Phase Separations Ltd., Deeside Ind. Estate, Queensferry, Clwyd CH5 2LR (U.K.)
and
C. R. LOWE*
The Biotechnology Centre, University of Cambridge, Downing Street, Cambridge CB2 3EF (U.K.)

The applicability of triazine dyes as group-specific ligands in affinity chromatography has been well documented over many years. Different dyes immobilised to carbohydrate polymers have been employed to purify a plethora of proteins and enzymes both at the analytical [1–7] and preparative scales [2,5,7]. More recently, triazine dyes immobilised to bonded silica matrices have been used in high-performance liquid affinity chromatography (HPLAC) to isolate and purify a number of enzymes[8–10].

We now report preliminary details of the first application of process scale HPLAC using a 3.3-l column of silica-immobilised Procion Blue MX-R for the purification of rabbit muscle L-lactate dehydrogenase from a crude extract.

This system, in its non-optimised form, processes a minimum of 1.8 g crude protein per cycle time of approximately 1 h and affords enzyme of specific activity 450 U/mg in 46% overall yield with a 8.6-fold purification.

EXPERIMENTAL

Materials

γ-Glycidoxypropyltrimethoxysilane was purchased from Aldrich (U.K.); crude rabbit muscle L-lactate dehydrogenase (Type I, 40–100 U/mg) and sodium pyruvate were obtained from Sigma (U.K.); NADH was purchased from Boehringer (U.K.); Procion Blue MX-R was a gift from Dr. C. V. Stead (ICI Organics Division, Blackley, U.K.) and spherical silica (Spherisorb VLS; mean pore size 280 Å, mean particle diameter 20 μm) was a gift from Dr. K. Jones (Phase Separations, Clwyd, U.K.).

Synthesis of silica-immobilised Procion Blue MX-R

Spherical silica (2.5 kg) was suspended in a mixture of 16.7 l of 0.1 *M* sodium acetate buffer (pH 5.5) and γ-glycidoxypropyltrimethoxysilane (1.3 l) and heated at 90°C for 3–4 h under reduced pressure and gentle rotation. The epoxy-silica was washed with water (20 l) on a sintered funnel through a Whatman paper filter and then hydrolysed to the corresponding diol-silica at pH 3.0 (18 l of 1 m*M* hydrochloric acid) for 1 h at 90°C under reduced pressure and gentle rotation. The diol-silica was

0021-9673/86/$03.50

washed with water (20 l) and acetone (10 l) and dried under reduced pressure to yield diol-bonded silica containing 5.5% carbon. Procion Blue MX-R (130 g) in a solution of 0.1 *M* sodium bicarbonate (9.1 l) was introduced into a flask containing diol-silica (1.1 kg dry weight) and the suspension rotated under reduced pressure at 35–40°C for 22 h. The blue-silica was washed with water (10 l), 1 *M* potassium chloride solution (10 l) and water until the effluents were free of dye. The adsorbent was washed with acetone (20 l) and dried under reduced pressure to yield silica-immobilised Procion Blue MX-R containing 2.9 μmol dye per gram dry weight of silica. The molar extinction coefficient of Procion Blue MX-R was taken as 10 500 l mol^{-1} cm^{-1}. The procedure for the preparation of silica-immobilised Procion Blue MX-R is shown in Fig. 1.

Chromatographic procedures

The Procion Blue MX-R silica was packed with axial compression into a stainless-steel column with adjustable bed length [Chromatelf (Elf Solaize, St. Symphorien D'Ozou, France) system LC150 (Fig. 2)] to yield a 3.3-l dyed-silica HPLAC adsorbent of 15 × 18.8 cm bed dimensions.

A dialysed and filtered (Millipore, fibre glass) crude preparation of rabbit muscle L-lactate dehydrogenase (61 790 U; 1.8 g protein) in 100 ml of 30 m*M* sodium phosphate buffer (pH 7.0) was loaded onto the silica-immobilised Procion Blue MX-R column (15.0 × 18.8 cm, 3.3 l, 2.9 μmol dye per gram of silica) which had been equilibrated previously in 30 m*M* sodium phosphate buffer (pH 7.0). Unbound proteins were washed off with irrigating buffer until the adsorption at 280 nm was reduced to zero whence bound L-lactate dehydrogenase was eluted with a pulse of

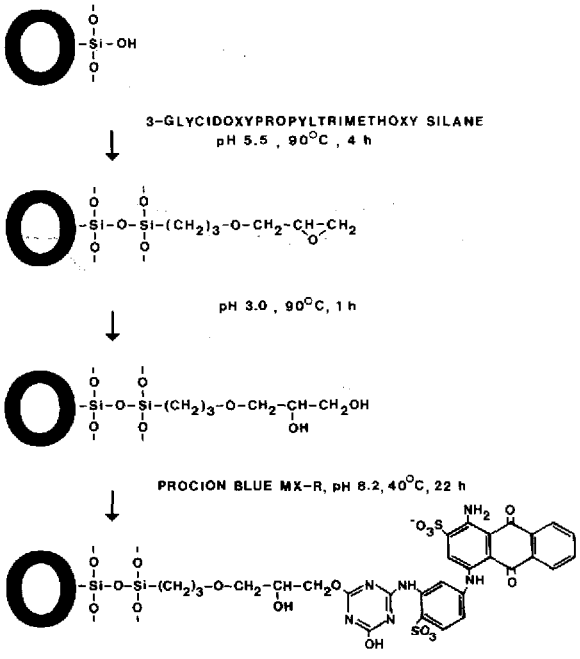

Fig. 1. Procedure for the preparation of silica-immobilised Procion Blue MX-R.

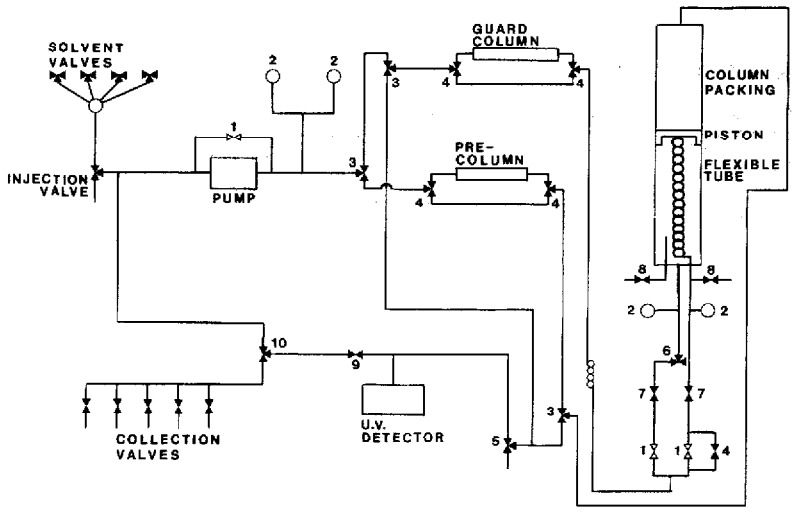

Fig. 2. Chromatelf LC150 System for process scale HPLAC. 1 = Check valve; 2 = pressure gauge; 3 = backflush valve; 4 = bypass valve; 5 = compressed air valve; 6 = purge compression valve; 7 = stop valve; 8 = purge air valve; 9 = needle valve; 10 = recycling valve.

NADH (7 m*M*) in the same buffer (10 ml), as shown in Fig. 3. Fractions (2 l) were collected at a flow-rate of 32.4 l/h. The protein content of each fraction was monitored by its absorbance at 280 nm whereas L-lactate dehydrogenase activity was monitored by following the oxidation of NADH by pyruvate at 340 nm. The molar extinction coefficient of NADH was taken as 6220 l mol^{-1} cm^{-1}.

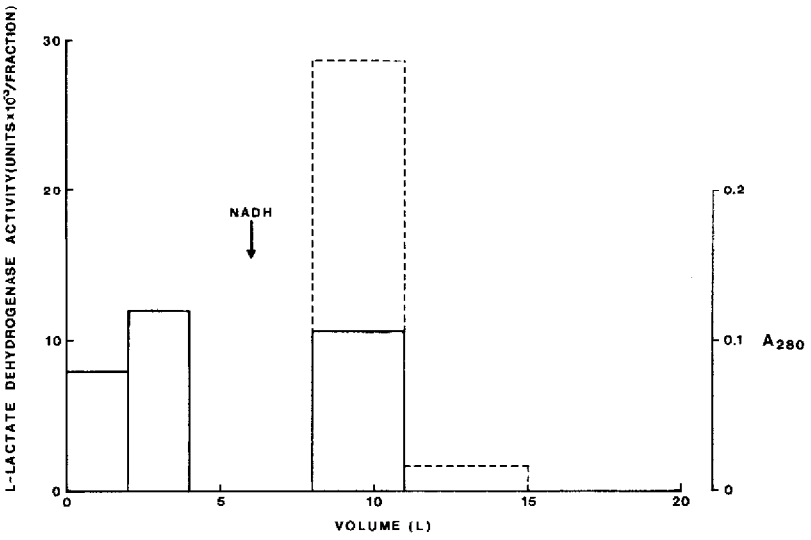

Fig. 3. Process scale purification of L-lactate dehydrogenase from a crude rabbit extract. ———, Absorbance at 280 nm; – – –, enzyme activity.

TABLE I

SUMMARY OF PURIFICATION OF L-LACTATE DEHYDROGENASE

	Volume (ml)	*Protein (mg)*	*Total activity* (U)*	*Specific activity* (U/mg)*	*Purification (fold)*	*Yield (%)*
Crude rabbit muscle preparation	100	1800	61 740	34.3	1	100
NADH eluate	3100	97	28 710	296 (450)**	8.6	46.5

* Enzyme assays were performed at 20°C unless stated otherwise.
** Specific activity at 37°C.

Assays

L-lactate dehydrogenase activity was monitored at 340 nm by the oxidation of NADH to NAD^+ with the concomitant conversion of pyruvate to lactate at pH 7.0[6]. Protein concentration was determined by the method of Bradford[11] using Coomassie Brilliant Blue G-250. Bovine serum albumin (fraction V) was used as the protein standard.

RESULTS AND DISCUSSION

The high reactivity of Procion Blue MX-R, a dichlorotriazinyl structural analogue of Cibacron Blue F3G-A, permitted efficient and facile dye coupling directly to diol-silica. This procedure circumvented the problems normally encountered with the immobilisation of monochlorotriazinyl dyes such as Cibacron Blue F3G-A and the Procion H-series[9]:

(i) low substitution when the dyes were coupled directly to diol-silica under mild alkaline conditions, and

(ii) conversion of the parent dye to the corresponding aminoalkyl dye prior to coupling to epoxy-silica.

The blue adsorbent was packed into a 2 m × 15 cm stainless-steel HPLC column with axial compression by exploiting the unique design of the Chromatelf system (Fig. 2). Packing was achieved via a mobile piston, with which the length of the column could be adjusted to afford any bed volume up to 23 l, but with a fixed diameter of 15 cm. Process scale HPLAC was assessed with a 3.3-l column of Procion Blue MX-R silica for the purification of rabbit muscle L-lactate dehydroghenase in one step from a crude rabbit muscle preparation. The adsorbent was loaded with a crude enzyme preparation (1.8 g protein, 61 740 U enzyme activity) at a flow-rate of 32.4 l/h (3.0 ml min^{-1} cm^{-2}). The majority of the inert proteins washed through unretarded whereas L-lactate dehydrogenase was bound and subsequently eluted specifically with a pulse of the competitive ligand, NADH (7 m*M*, 10 ml). This procedure afforded enzyme of specific activity 450 U/mg protein, purified 8.6-fold with an overall yield 46% (Table I). These results were obtained with a non-optimised system operating well below the theoretical capacity of the adsorbent. It is believed that the working capacity of this adsorbent, *i.e.* the maximum amount of total protein that could be loaded for which all the enzyme present is adsorbed, could be increased substantially on optimisation of the adsorbent and operational parameters. Nevertheless, the system described in this paper is capable of processing a minimum of 1.8 g of total protein per cycle (1 h), affording 97 mg of 8.6-fold purified L-lactate dehydrogenase. We believe that this report describes the first example of process scale HPLAC applied in enzyme purification technology on silica adsorbents.

ACKNOWLEDGEMENTS

The authors are indebted to Chromatelf (Centre de Recherche, Elf Solaize, BP22, F69360, St. Symphorien D'Ozou, France) for the loan of the Chromatelf LC150 chromatographic system and to Dr. D. Taylor for his assistance in locating the instrument in the Department of Chemistry, UMIST, Manchester, U.K.

REFERENCES

1 J. K. Baird, R. F. Sherwood, R. J. G. Carr and A. Atkinson, *FEBS Lett.*, 70 (1976) 61–66.
2 C. J. Bruton and T. Atkinson, *Nucl. Acids Res.*, 7 (1979) 1579–1591.
3 P. D. G. Dean and D. H. Watson, *J. Chromatogr.*, 165 (1979) 301–319.
4 C. R. Lowe, M. Hans, N. Spibey and W. T. Drabble, *Anal. Biochem.*, 104 (1980) 23–28.
5 Y. D. Clonis and C. R. Lowe, *Biochim. Biophys. Acta*, 659 (1981) 86–98.
6 Y. D. Clonis, *J. Chromatogr.*, 236 (1982) 69–80.
7 C. R. Lowe, in A. Wiseman (Editor), *Topics in Enzyme & Fermentation Biotechnology*, Vol. 9, Ellis Horwood, Chichester, U.K., 1984, pp. 78–161.
8 C. R. Lowe, M. Glad, P.-O. Larsson, S. Ohlson, D. A. P. Small, T. Atkinson and K. Mosbach, *J. Chromatogr.*, 215 (1981) 303–316.
9 D. A. P. Small, T. Atkinson and C. R. Lowe, *J. Chromatogr.*, 216 (1981) 175–190.
10 D. A. P. Small, T. Atkinson and C. R. Lowe, *J. Chromatogr.*, 266 (1983) 151–156.
11 M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.