Journal of Chromatography A, 662 (1994) 27–35
Elsevier Science B.V., Amsterdam

CHROM. 25 645

Poly(N-vinylpyrrolidone) shielding of matrices for dye-affinity chromatography

Improved elution of lactate dehydrogenase from Blue Sepharose and secondary alcohol dehydrogenase from Scarlet Sepharose

Igor Yu. Galaev and Bo Mattiasson*

Department of Biotechnology, Chemical Centre, Lund University, P.O. Box 124, S-221 00 Lund (Sweden)

(First received August 5th, 1993; revised manuscript received October 4th, 1993)

ABSTRACT

Poly(N-vinylpyrrolidone) (PVP) shielding of Blue Sepharose and Scarlet Sepharose proved to be an efficient method for improving the process of purification of lactate dehydrogenase (LDH) from porcine muscle and secondary alcohol dehydrogenase (SADH) from *Thermoanaerobium brockii*, respectively. PVP shielding of Blue Sepharose resulted in improvement of the efficiency of either specific or non-specific elution of LDH. PVP protection of Scarlet Sepharose resulted in an improvement in the SADH recovery during specific elution and in an improvement in enzyme purity on non-specific elution. The dynamic capacities of the columns were in both cases decreased after PVP shielding. PVP shielding is considered to prevent the matrices from binding foreign proteins and from non-specific binding of nucleotide-dependent enzymes, while not seriously impairing the specific binding of these enzymes to the affinity matrices.

INTRODUCTION

The trend in protein purification is to use affinity-mediated separation fairly early in the purification work [1–3]. Earlier, affinity chromatography was often used towards the end of a tedious purification scheme when only minor contaminants with properties close to those of the target molecule were present. With the development towards the early application of affinity-mediated purification, some constraints are introduced. Stable, small ligands are preferred instead of proteinaceous ligands, even if the former are less specific [4]. This leads to group-specific isolation, and in many cases also some non-specific co-purification. The ligands belonging to this group of stable, small compounds are, *e.g.*, textile dyes [5–11], hydrophobic groups [12–15], chelating ligands [16,17] and boronates [18].

"Dye-affinity" chromatography using triazine dyes coupled to different matrices is widely used for the purification of dehydrogenases and kinases [5–11]. Traditionally these enzymes are eluted either specifically with nucleotide solutions or non-specifically with high salt concentrations [19,20]. Low or moderate recoveries (30–70%) of nucleotide-dependent enzymes by affinity chromatography using Cibacron Blue-substituted matrices [21–25] or Scarlet Red-substituted Sepharose [26] have been reported.

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E1089-I

Textile dyes have been described as having potential use in the purification of many different proteins [6–11,21–25]. The importance of this group of ligands has led to the development of a range of new synthetic dyes specially designed to become more specific [27].

An alternative mode of operation would be to try to manipulate the binding to the ligand and thereby to reduce the amount of non-specifically bound protein. From earlier model studies we have shown that additions of polymers such as poly(N-vinylpyrrolidone) (PVP) to Cibacron Blue formed complexes that shielded the dye from forming weak interactions with proteins. This resulted in an improved elution of lactate dehydrogenase from PVP-shielded Blue Sepharose. Both specific and non-specific elution were improved [28].

This work was carried out with the aim of investigating the possibility of exploiting the interaction between triazine dyes and PVP to improve the performance in the affinity-mediated separation of enzymes from crude homogenates with the idea that decreased non-specific binding would simplify the overall purification procedure.

EXPERIMENTAL

Lactate dehydrogenase type XXX-S from porcine muscle, β-NADH grade III, β-NADP sodium salt, Cibacron Blue 3GA and PVP K 26-35 with an average molecular mass of 40 000 were purchased form Sigma (St. Louis, MO, USA). Oxamic acid was purchased from BDH (Poole, UK). Blue Sepharose was synthesized by coupling Cibacron Blue 3GA to Sepharose CL-4B according to ref. 29. The Cibacron Blue content determined according to ref. 30 was 4.9 μmol per ml of swollen gel. Scarlet Sepharose was a generous gift from Professor R.K. Scopes (Centre for Protein and Enzyme Technology, La Trobe University, Australia) and was synthesized by coupling of Procion Scarlet H-2G to Sepharose CL-4B according to ref. 31.

Minced pork was purchased in a local shop and homogenized in ice-cold 20 m*M* Tris–HCl buffer (pH 7.3) containing 1 m*M* EDTA (10 ml of buffer per gram of muscle tissue). The homogenate was filtered through a synthetic fibre pad to remove larger particulate matter, centrifuged for 15 min to remove cell debris and the supernatant was filtered through Munktell filter-paper to remove traces of fat. The porcine muscle extract was kept frozen without any loss of LDH activity and was applied directly, after thawing and filtering, to the Blue Sepharose column. The crude extract had an activity of 64 U/ml and a protein content of 5.5 mg/ml (specific activity 11.6 U/mg protein).

The obligate anaerobic thermophilic organism *Thermoanaerobium brockii* was cultured in batch according to ref. 32. Cells were harvested by centrifugation and stored frozen at −18°C. Cells were not maintained under strictly anaerobic conditions during harvesting and storage. Extraction of cells was carried out by sonication in 20 m*M* morpholinopropanesulphonate buffer (pH 6.5) containing 30 m*M* NaCl and 2 m*M* $MgCl_2$ (MES buffer) (4 ml per gram wet mass of cells). Cell debris was removed by centrifugation and the supernatant was applied directly to the column. The crude extract had an activity of 49 U/ml and a protein content of 14.0 mg/ml (specific activity 3.5 U/mg protein).

Chromatographic experiments using Blue Sepharose

All chromatographic experiments with Blue Sepharose were carried out at room temperature using a 9.8 × 0.9 cm I.D. column at a flow-rate of 0.55 ml/min. All solutions introduced to the column were in 20 m*M* Tris–HCl buffer (pH 7.3). The porcine muscle extract was applied to the column until breakthrough of LDH (120–200 ml with the untreated column and 60–70 ml with the PVP-shielded column). The column was washed with buffer until no more protein (monitored as absorbance at 280 nm) was detected in the eluate. Non-specific elution of LDH was performed with 1.5 *M* KCl and specific elution was performed with 10 m*M* oxamate + 0.1 m*M* NADH. Fractions were collected every 20 min when eluted from the untreated column and every 5 min when eluted from the PVP-shielded column. The height equivalent to a theoretical plate (HETP) was calculated as $HETP = L/N$, where L is the bed length and N is the number

of theoretical plates calculated as $N = 5.54 (V/W_{1/2})^2$, where V is the elution volume and $W_{1/2}$ is the peak width at half-height.

Chromatographic experiments using Scarlet Sepharose

All chromatographic experiments with Scarlet Sepharose were carried out at room temperature using a 2.8 × 0.9 cm I.D. column at a flow-rate of 0.09 ml/min. For SADH elution all solutions introduced to the column were in MES buffer. The cell extract was applied to the column until breakthrough of SADH. The column was washed with buffer until no more protein (monitored as absorbance at 280 nm) was detected in the eluate. Non-specific elution of SADH was performed with 1.5 *M* KCl and specific elution with 0.5 m*M* NADP. For LDH elution all solutions introduced into the column were in 20 m*M* Tris–HCl buffer (pH 7.3). The porcine muscle extract was applied to the column until breakthrough of LDH. The column was washed with buffer until no more protein (monitored as absorbance at 280 nm) eluted. Non-specific elution of LDH was performed with 1.5 *M* KCl and specific elution with 10 m*M* oxamate + 0.1 m*M* NADH. Fractions were collected every 15 min.

The PVP shielding of the columns was performed with 1% PVP-40 000 solution followed by washing with 1.5 *M* KCl (pH 3.4) until no PVP was detected in the effluent. This was followed by re-equilibration of the column with an appropriate buffer.

LDH activity was measured in the fractions according to a reported procedure [33]. SADH activity was measured in the fractions according to ref. 26. Concentration of PVP was measured as the absorbance of a polymer–iodine complex at 480 nm, the complex being produced according to ref. 34. Protein was determined according to Lowry *et al.* [35].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% gel was performed according to ref. 36 using carbonic anhydrase (M_r 31 000), ovalbumin (M_r 45 000), bovine serum albumin (M_r 66 200) and phosphorylase B (M_r 92 500) as standards.

RESULTS AND DISCUSSION

Application of porcine muscle extract to a fresh Blue Sepharose column until breakthrough resulted in binding of LDH along with a significant amount of foreign proteins, which could not be eluted with the buffer. Non-specific elution with 1.5 *M* KCl resulted first in the elution of foreign proteins followed by LDH with a recovery of 76% as judged from activity measurements (Fig. 1 and Table I). The LDH recovery increased gradually to nearly 100% only after subsequent purification cycles with application of porcine muscle extract to the same column. Thus, pretreatment of Blue Sepharose with the homogenate resulted in masking of sites capable of non-specific irreversible binding of LDH. The same effect occurred during the specific elution of LDH with 0.1 m*M* NADH + 10 m*M* oxamate, but in contrast to non-specific elution foreign proteins adsorbed on the column were not eluted during specific elution. After specific elution the column needed to be regenerated; foreign proteins could be eluted with 1.5 *M* KCl (Fig. 2).

The PVP shielding of the column resulted in a

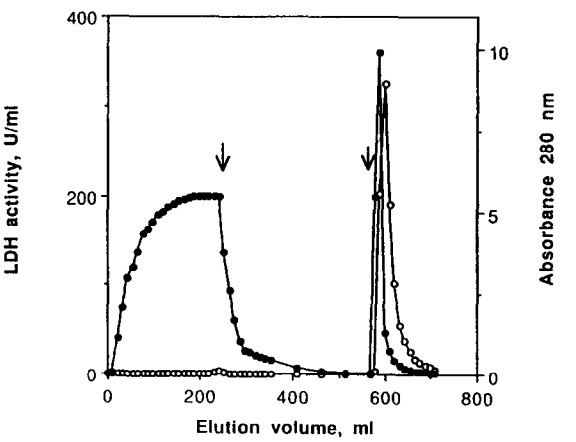

Fig. 1. Elution profile of (○) LDH activity and (●) protein with 1.5 *M* KCl from unmodified Blue Sepharose. Arrows indicate when washing with buffer and elution with 1.5 *M* KCl were begun. Experimental conditions: 9.8 × 0.9 cm I.D. column; the porcine muscle extract was applied to the column until breakthrough of LDH; the column was washed with 20 m*M* Tris–HCl buffer (pH 7.3) until no more protein (monitored as absorbance at 280 nm) eluted; LDH was eluted at a flow-rate of 0.55 ml/min with 1.5 *M* KCl; fractions were collected every 20 min.

TABLE I

LDH PREPARATIONS PRODUCED BY DIFFERENT ELUTION MODES

Material	Mode of chromatography	Enzyme bound (U)	Enzyme eluted (U)	Elution volume (ml)	Activity[a] (U/ml)	Protein[a] (mg/ml)	Specific activity[a] (U/mg)	Recovery (%)	Purification (-fold)[a]
Unmodified Blue Sepharose[b]	Non-specific elution	7120	5410	126.5	325	1.5	216	76	19
	Specific elution	12 100	5590	110	499	2.1	237	46	20
PVP-treated Blue Sepharose[c]	Non-specific elution	4200	4040	11	1160	5.2	223	96	19
	Specific elution	3550	3480	13.8	777	3.8	204	98	18
Unmodified Scarlet Sepharose[b]	Non-specific elution	1360	1280	10.8	760	13.8	55	94	4.7
	Specific elution	903	650	6.8	260	1.6	162	72	14
PVP-treated Scarlet Sepharose[c]	Non-specific elution	616	665	5.4	540	4.3	125	108	11
	Specific elution	375	364	5.4	290	1.6	180	97	15
Commercial sample					597	3.1	192		
Crude extract					64	5.5	11.6		

[a] In the peak fraction.
[b] First run on the newly packed column.
[c] First run on the newly packed and PVP-shielded column.

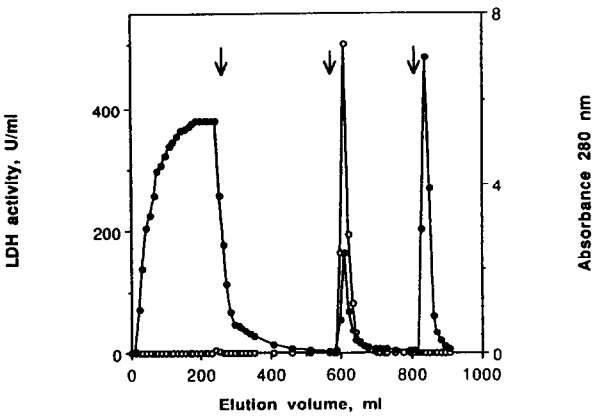

Fig. 2. Elution profile of (○) LDH activity and (●) protein from unmodified Blue Sepharose with 0.1 m*M* NADH + 10 m*M* oxamate, followed by elution with 1.5 *M* KCl. Arrows indicate when washing with buffer, elution with 0.1 m*M* NADH + 10 m*M* oxamate and elution with 1.5 *M* KCl were begun. Experimental conditions as in Fig. 1, except that LDH was eluted with 0.1 m*M* NADH + 10 m*M* oxamate.

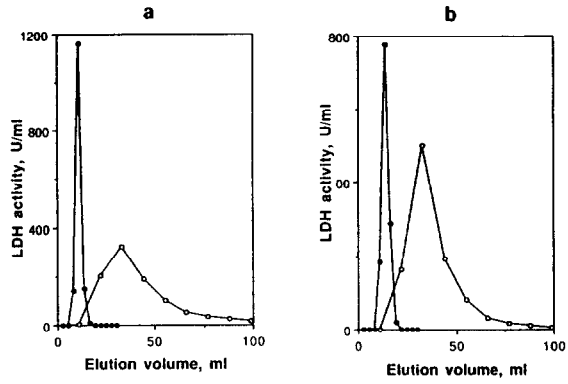

Fig. 3. Elution profile of LDH activity with (a) 1.5 *M* KCl and (b) 0.1 m*M* NADH + 10 m*M* oxamate from (○) unmodified and (●) PVP-treated Blue Sepharose. Experimental conditions: 9.8 × 0.9 cm I.D. column was treated with 1% PVP-40 000 solution followed by washing with 1.5 *M* KCl (pH 3.4) until no PVP was detected in the eluate, and re-equilibration with 20 m*M* Tris–HCl buffer (pH 7.3); the porcine muscle extract was applied to the column until breakthrough of LDH; the column was washed with buffer until no more protein (monitored as absorbance at 280 nm) eluted; LDH was eluted at a flow-rate of 0.55 ml/min with 1.5 *M* KCl or 10 m*M* oxamate + 0.1 m*M* NADH; fractions were collected every 20 min when eluted from the untreated column and every 5 min when eluted from the PVP-protected column. The total amount of LDH eluted from the column was taken as 100% in both instances for the sake of comparison.

significant decrease in binding of foreign proteins and in an improvement in the effectiveness of elution of LDH either specifically or non-specifically (Fig. 3). The decreased binding of foreign proteins with the PVP-shielded column eliminated the need for a regeneration step after the specific elution. The column could be used repeatedly after re-equilibration with buffer. The LDH recovery was about 100% even during the first run on a fresh Sepharose Blue column shielded with PVP. Thus, PVP blocked the sites to which LDH irreversibly bound. Some proteins from the homogenate played the same role during the first application of porcine muscle extract on to a Blue Sepharose column. It is preferable to use PVP to block binding sites on the matrix rather than an unidentified mixture of proteins from the homogenate. PVP is a cheap, stable, non-toxic and highly biocompatible polymer [37]. No polymer was detected in the eluate from the PVP-protected column during chromatography of LDH (the sensitivity of the method of PVP assay being 0.1 mg/ml).

PVP shielding resulted in a significant improvement in the effectiveness of the elution. The HETP for the untreated column was 1.3 cm (non-specific elution) and 0.47 cm (specific elution). PVP shielding decreased the HETP to 0.13 cm for both types of elution. LDH is eluted from the PVP-shielded column as a symmetrical peak and about 95% of eluted enzyme can be collected in a volume of 8 ml. LDH is eluted from an untreated column of the same size with significant tailing, and 95% of eluted LDH can be collected only in a volume of about 100 ml (Fig. 3). A volume decrease is preferable for the further downstream processing because the cost of the purification step is usually proportional to the feed concentration.

PVP shielding with a 1% polymer solution is a relatively inexpensive procedure, and considerably cheaper than, for instance, regeneration of the column with the same volume of 1.5 *M* KCl. The price of PVP-40 000 according to Sigma is $101.2/kg whereas that of KCL is $34.1/kg, but the latter is used at a ten times higher concentration (1.5 *M* KCl corresponds approximately to a 10% solution). Moreover, PVP adheres to the column and can be reused.

TABLE II

SADH PREPARATIONS PRODUCED BY DIFFERENT ELUTION MODES

Material	Mode of chromatography	Enzyme bound (U)	Enzyme eluted (U)	Elution volume (ml)	Activity[a] (U/ml)	Protein[a] (mg/ml)	Specific activity[a] (U/mg)	Recovery (%)	Purification (-fold)[a]
Unmodified Scarlet Sepharose[b]	Non-specific elution	1970	2130	8.1	308	6.1	50.5	108	14
	Specific elution	2525	1790	12.5	174	1.16	150	71	43
	Non-specific elution after specific		910	6.8	156	3.5	44.6	36	1
PVP-treated Scarlet Sepharose[c]	Non-specific elution	371	367	6.8	220	1.8	122	99	35
	Specific elution	240	224	8.1	81	0.5	162	93	46
	Non-specific elution after specific elution		19	6.8	7.8	0.6	13	8	3.7
Crude extract	Non-specific elution				49	14.0	3.5		

[a] In the peak fraction.
[b] First run on the newly packed column.
[c] First run on the newly packed and PVP-shielded column.

The results obtained correspond well with previous data on zonal elution of pure LDH from PVP-shielded Sepharose Blue [28]. PVP, owing to its high affinity to Cibacron Blue ligands, occupied sites capable of non-specific binding of foreign proteins and of irreversible binding of LDH and prevented this binding. LDH is considered to bind to PVP-protected Blue Sepharose only due to specific interactions of Cibacron Blue ligands with nucleotide binding sites of the enzyme. The number of sites suitable for specific binding is less than the total number of specific and non-specific LDH binding sites in unmodified Blue Sepharose. This resulted in a decrease in dynamic capacity from 1150–1950 U/ml swollen gel (230–400 U/mmol Cibacron Blue ligand) for the untreated column to 680–570 U/ml swollen gel (120–140 U/mmol Cibacron Blue ligand) for the PVP-shielded column.

Table I and Fig. 4 show virtually the same purity of LDH samples, obtained as the result of specific and non-specific elution from unmodified and PVP-shielded Blue Sepharose. In all cases, one step purification using dye-affinity chromatography resulted in highly purified preparations.

Application of a *Thermoanaerobium brockii* extract to a Scarlet Sepharose column resulted in adsorption of SADH along with significant amounts of foreign proteins. This behaviour resembled that of LDH binding to Blue Sepharose, but in contrast to the latter case foreign proteins were eluted simultaneously with SADH by 1.5 *M* KCl, resulting in a poor purification (Table II and Fig. 5, lane 2). Specific elution

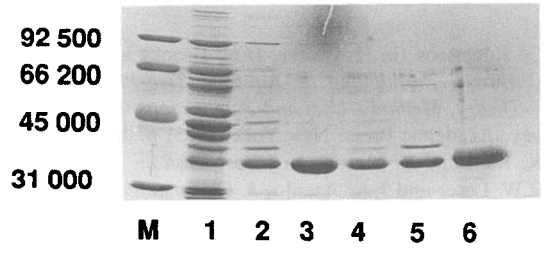

Fig. 4. SDS-PAGE patterns for LDH. Lanes: M = marker proteins; 1 = crude extract; 2 and 4 = elution with 1.5 *M* KCl from unmodified and PVP-treated Blue Sepharose, respectively; 3 and 5 = elution with 0.1 m*M* NADH + 10 m*M* oxamate from unmodified and PVP-protected Blue Sepharose, respectively; 6 = commercial sample.

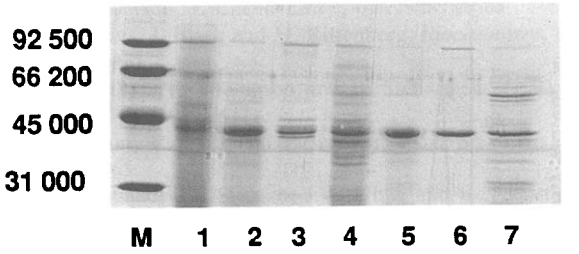

Fig. 5. SDS-PAGE patterns for SADH. Lanes: M = marker proteins; 1 = crude extract; 2 and 5 = elution with 1.5 *M* KCl from unmodified and PVP-treated Scarlet Sepharose, respectively; 3 and 6 = elution with 0.5 m*M* NADP from unmodified and PVP-protected Scarlet Sepharose, respectively; 4 and 7 = elution with 1.5 *M* KCl after elution with 0.5 m*M* NADP from unmodified and PVP-treated Scarlet Sepharose, respectively.

with 0.5 m*M* NADP resulted in more pure enzyme preparation with a recovery of 70%, and the remaining 30% of SADH activity could be eluted together with other adsorbed proteins by 1.5 *M* KCl as crude preparation (Table II and Fig. 5, lanes 3 and 4). The specific elution at lower loadings gave lower recoveries and in the zonal mode SADH was not eluted by 0.5 m*M* NADP at all. The strong binding of SADH to Scarlet Sepharose was also reported by Nagata *et al.* [26], who managed to obtain a 70% recovery only during overloading conditions; with conventional loading, elution with 0.5 m*M* NADP was not successful.

The PVP shielding of Scarlet Sepharose resulted in an improvement in the effectiveness of specific elution and purity of the enzyme preparation, the recovery being 93% (Fig. 5, lane 6, and Fig. 6). Again, as with Blue Sepharose, PVP shielding of Scarlet Sepharose prevented adsorption of foreign proteins and non-specific interaction of SADH with the matrix. The decreased adsorption of foreign proteins resulted in a reasonable purity of SADH even after non-specific elution from the PVP-treated column (Table II and Fig. 5, lane 5). The dynamic capacity of the column was decreased 5.3–10.5-fold after PVP protection.

The non-specific interactions seemed to be more pronounced with Scarlet Sepharose, which bound more foreign proteins than Blue Sepharose did, especially when porcine muscle extract was applied. LDH and SADH are eluted readily

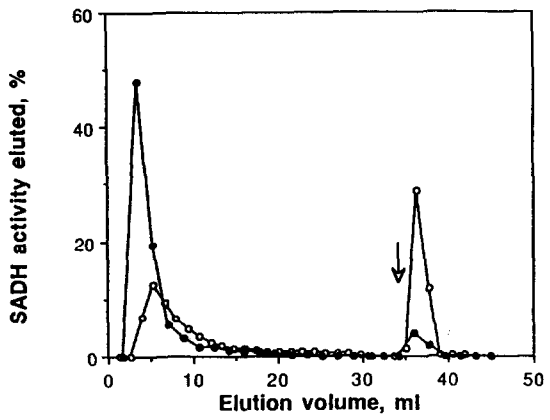

Fig. 6. Elution profile of SADH activity with 0.5 m*M* NADP from (○) unmodified and (●) PVP-treated Blue Sepharose followed by elution with 1.5 *M* KCl. The arrow indicates when elution with 1.5 *M* KCl was begun. Experimental conditions: 2.8 × 0.9 cm I.D. column was treated with 1% PVP-40 000 solution followed by washing with 1.5 *M* KCl (pH 3.4) until no PVP was detected in the eluate, and re-equilibration with 20 m*M* morpholinopropane sulphonate buffer (pH 6.5) containing 30 m*M* NaCl and 2 m*M* $MgCl_2$; the *Thermoanaerobium brockii* extract was applied to the column until breakthrough of SADH; the column was washed with buffer until no more protein (monitored as absorbance at 280 nm) eluted; SADH was eluted at a flow-rate of 0.09 ml/min with 0.5 m*M* NADP; fractions were collected every 15 min. The total amount of SADH eluted from the column was taken as 100% in both instances for the sake of comparison.

from Scarlet Sepharose with 1.5 *M* KCl along with foreign proteins. One could see a deep red ring of haeme-containing proteins on the Scarlet Sepharose column during KCl elution after application of porcine muscle extract, and the LDH-containing eluate fractions were coloured red. A high adsorption of foreign proteins resulted in poor purity of LDH preparations after dye-affinity chromatography on Scarlet Sepharose (Table I). Even in that case, PVP shielding of Scarlet Sepharose improved the purity of LDH preparations, although they had a lower specific activity compared with LDH preparations obtained after elution from untreated and PVP-treated Blue Sepharose.

Thus, PVP protection of dye-affinity matrices significantly decreased the adsorption of foreign proteins and also non-specific binding of the target molecules. The phenomena observed are interpreted as being the result of a shielding effect by PVP of the textile dye and also of non-specific sites of the support. PVP has affinity for the triazine dye, but when a dehydrogenase with higher affinity appears, displacement takes place. Owing to multi-point attachment, this displacement is local and the polymer stays on the column. The shielded ligand dye-affinity chromatographic process is characterized by improved elution effectiveness, facilitated column regeneration if specific elution is used, no decrease in the purity of enzyme preparations and a decrease in the dynamic capacity of the column. This concept of improving the performance of dye-affinity chromatography is now being further evaluated in our laboratory.

ACKNOWLEDGEMENTS

The support of the Swedish Royal Academy of Sciences (KVA), the National Swedish Board for Technical and Industrial Development (NUTEK), the Swedish Agency for Cooperation with Developing Countries (SAREC) and the Swedish Research Council for Engineering Sciences (TFR) is gratefully acknowledged. The authors thank Professor Robert K. Scopes for generously providing Scarlet Sepharose, Nora Perotti for the synthesis of Blue Sepharose, Dr. Olle Holst and Åsa Rosenlund for cultivation of cells, Eva Linné-Larsson for practical advice on electrophoresis and Dr. Rajni Kaul for linguistic advice.

REFERENCES

1 G. Johansson, in H. Walter, D.E. Brooks and D. Fisher (Editors), *Partitioning in Aqueous Two-Phase Systems —Theory, Methods, Uses and Applications to Biotechnology*, Academic Press, New York, 1985, p. 161.
2 R. Kaul and B. Mattiasson, *Bioseparation*, 3 (1992) 1.
3 R.W. Leser and J.A. Asenjo, *J. Chromatogr.*, 584 (1992) 43.
4 C.R. Lowe, in M.A. Vijayalakshmi and O. Bertrand (Editors), *Protein–Dye Interactions: Developments and Applications*, Elsevier Applied Science, London, 1989, p. 11.
5 F. Qadri, *Trends Biotechnol.*, 3 (1985) 7.
6 C.V. Stead, *Bioseparation*, 2 (1991) 129.
7 M.A. Vijayalakshmi, *Trends Biotechnol.*, 7 (1989) 71.

8 T. Makriyannis and Y.D. Clonis, *J. Chromatogr.*, 28 (1993) 179.
9 Y.D. Clonis, T. Atkinson C.J. Bruton and C.R. Lowe (Editors), *Reactive Dyes in Protein and Enzyme Technology*, Macmillan, Basingstoke, 1987.
10 Y.D. Clonis, in M.T.W. Hearn (Editor), *HPLC of Proteins and Polynucleotides*, VCH, New York, 1991, Ch. 13, p. 453.
11 Y.D. Clonis, *CRC Crit. Rev. Biotechnol.*, 7 (1988) 163.
12 A.J. Alpert, *J. Chromatogr.*, 359 (1986) 85.
13 Y. Guan, X.-Y. Wu, T.E. Treffry and T.H. Lilley, *Biotechnol. Bioeng.*, 40 (1992) 517.
14 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 298 (1984) 407.
15 Y.L.K. Sing, Y. Kroviarski, S. Cochet, D. Dhermy and O. Bertrand, *J. Chromatogr.*, 598 (1992) 181.
16 E. Sulkowski, *Trends Biotechnol.*, 3 (1985) 1.
17 R. Woker, B. Campluvier and M.-R. Kula, *J. Chromatogr.*, 584 (1992) 85.
18 P.D.G. Dean, F.A. Middle, C. Longstaff, A. Bannister and J.J. Dembinski, in I.M. Chaiken, M. Wilchek and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition*, Academic Press, Orlando, 1983, p. 433.
19 A.A. Glemza, B.B. Baskeviciute, V.LA. Kadusevicius, J.H.J. Pesliaskas and O.F. Sudziusviene, in M.A. Vijayalakshmi and O. Bertrand (Editors), *Protein–Dye Interactions: Developments and Applications*, Elsevier Applied Sci. Publ., London, 1989, p. 107.
20 R.K. Scopes, in M.A. Vijayalakshmi and O. Bertrand (Editors), *Protein–Dye Interactions: Developments and Applications*, Elsevier Applied Sci. Publ., London, 1989, p. 97.
21 A.J. Anderson, *J. Chem. Educ.*, 65 (1988) 901.
22 R. Harkins, J. Black and M. Rittenberg, *Biochemistry*, 16 (1977) 3831.
23 M. Morill, S.T. Thompson and E. Stellwagen, *J. Biol. Chem.*, 254 (1979) 4371.
24 B. Notton, R. Fido and E. Hewitt, *Plant Sci. Lett.*, 8 (1977) 165.
25 C.S. Ramadoss, J. Steczko, J.W. Uhlig and B. Axelrod, *Anal. Biochem.*, 130 (1983) 481.
26 Y. Nagata, K. Maeda and R.K. Scopes, *Bioseparation*, 2 (1992) 35.
27 C.R. Lowe, S.J. Burton, N.P. Burton, W.K. Alderton, J.M. Pitts and J.A. Thomas, *Trends Biotechnol.*, 10 (1992) 442.
28 I.Yu. Galaev and B. Mattiasson, *J. Chromatogr.*, 648 (1993) 367.
29 W. Heyns and P. De Moor, *Biochim. Biophys. Acta*, 358 (1974) 1.
30 G.K. Chambers, *Anal. Biochem*, 83 (1977) 551.
31 R.K. Scopes, *Anal. Biochem.*, 136 (1984) 525.
32 J.G. Zeicus, P.W. Hegge and M.A. Anderson, *Arch. Microbiol.*, 122 (1979) 41.
33 *Worthington Enzymes and Related Biochemicals*, Worthington Biochemical, Freehold, NJ, 1982, p. 109.
34 *European Pharmacopea*, Maisonneuve, Sainte-Ruffine, 1986, 2nd ed., Part I, p. 685-2.
35 O.H. Lowry, N.J. Rosebrough, A.L. Far and R.J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
36 U.K. Laemmli, *Nature*, 227 (1970) 680.
37 H.F. Mark, N.G. Gaylord and N.M. Bikales (Editors), *Encyclopedia of Polymer Science and Technology*, Vol. 14, Wiley, New York, 1971, p. 244.