Journal of Chromatography, 466 (1989) 319–329
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 189

AFFINITY CHROMATOGRAPHY OF BOVINE HEART LACTATE DEHYDROGENASE USING DYE LIGANDS LINKED DIRECTLY OR SPACER-MEDIATED TO BEAD CELLULOSE

M. NAUMANN, R. REUTER, P. METZ and G. KOPPERSCHLÄGER*
Institute of Biochemistry, Karl-Marx-University Leipzig, Liebigstrasse 16, Leipzig, 7010 (G.D.R.)
(First received May 26th, 1988; revised manuscript received October 25th, 1988)

SUMMARY

A number of reactive dyes coupled to bead cellulose directly or spacer-mediated has been investigated in respect of their interaction with lactate dehydrogenase (LDH; E.C. 1.1.1.28) from heart muscle. The Procion dyes Red HE-7B and Navy H-ER, as well as the Remazol dyes Brilliant Blue R and Brilliant Red 5-BN directly bound to bead cellulose provide high binding of LDH and the adsorbed enzyme is eluted specifically from these affinity adsorbents in high yield. In contrast, under the same conditions no binding of LDH has been found to the Procion dyes Green H-4G, Yellow HE-3G, Scarlet MX-G and Orange MX-G, although an opposite behaviour was expected from the results of affinity partitioning in aqueous two-phase systems. However, immobilizing these dyes via a spacer generated strong binding of the enzyme to the affinity adsorbent. The influence of the length of the spacer was studied in respect of the binding capacity and the yield of the enzyme specifically eluted. The applicability of Procion Scarlet MX-G-(diaminohexyl)-bead cellulose for the purification of LDH from muscle extract in one chromatographic step was demonstrated.

INTRODUCTION

The use of triazine dyes for the purification of proteins especially of enzymes by dye-ligand affinity chromatography is well established on the laboratory scale (for reviews see refs. 1 and 2). In the last decade, besides the reactive dyes Cibacron Blue F3G-A and Procion Red HE-3B, a series of other textile dyes were tested for their application as affinity ligands[3,4]. Supports of dyes frequently used are polysaccharides like agarose and diverse Sephadex types. Cellulose may also be a good support because reactive dyes were developed predominantly for the textile dyeing and printing industry. However, previous results showed that Cibacron Blue F3G-A bound to powered cellulose binds phosphofructokinase from yeast, but the enzyme was not eluted under different conditions[5]. The advantages of macroporous bead cellulose for dye-ligand affinity chromatography owing to its high porosity, sufficient rigidity and its low cost were first demonstrated by Mislovičová *et al.*[6].

In this study a commercially available macroporous bead cellulose has been

0021-9673/89/$03.50

tested for its usefulness as a matrix in dye-ligand affinity chromatography in respect of the adsorption and desorption behaviour of lactate dehydrogenase (LDH) from heart muscle. Particularly, the influence of the mode of coupling of the dyes on the properties of the resulting affinity adsorbent has been analyzed in more detail. Finally, a simple procedure for the purification of LDH to homogeneity was elaborated.

EXPERIMENTAL

Materials

Substrates and substances for buffers were obtained from Boehringer (Mannheim, F.R.G.), VEB Laborchemie (Apolda, G.D.R.) and VEB Arzneimittelwerk (Dresden, G.D.R.). All were of analytical reagent grade. The dyes of the Procion type (ICI, Manchester, U.K.), Cibacron Blue F3G-A (Ciba Geigy, Basle, Switzerland) and the Remazol dyes (Hoechst, Frankfurt/Main, F.R.G.) were gifts from VEB Chemisches Kombinat Bitterfeld (G.D.R.) and were used without further purification. Bead cellulose (sizes 80–200 and 200–400 μm) was a product from VEB Arzneimittelwerk (Leipzig, G.D.R.). Poly(ethylene glycol) (PEG 6000) was obtained from Serva (Heidelberg, F.R.G.), Dextran M 70 from VEB Serumwerke (Bernburg, G.D.R.). Cadoxen [tris(ethylenediamine) cadmium(II) hydroxide] was a gift from the Institute of Organic Chemistry of the Slovakian Academy of Sciences, Bratislava.

Methods

Preparation of lactate dehydrogenase. LDH was partially purified from bovine heart muscle as follows.

Extraction of minced muscle at room temperature with a ten-fold volume of distilled water containing 1 m*M* EDTA and 5 m*M* 2-mercaptoethanol. After centrifugation (9000 *g*, 30 min) the pH of the supernatant was lowered to 5.5 with 1 *M* H_3PO_4. The specific activity of LDH was 4–6 units/mg protein, where 1 unit is defined as the amount of enzyme which reduces 1.0 μmol pyruvate to lactate per minute.

Binding of LDH to DEAE-Sephadex A-50 equilibrated with 30 m*M* potassium phosphate buffer, pH 5.5, 1 m*M* EDTA, 5 m*M* 2-mercaptoethanol (buffer A). Ion-exchange chromatography was carried out in a batch procedure by stirring the gel with the supernatant (60 units of LDH per g of the exchanger) for about 30 min. In order to remove the unbound protein, the DEAE-cellulose was washed with buffer A (10-fold volume of the exchanger).

Elution of LDH from the ion exchanger by adding 0.1 *M* KCl to buffer A.

Precipitation of LDH by ammonium sulphate to 65% saturation at pH 7.4. The precipitate was suspended in buffer A, pH 7.4, saturated with ammonium sulphate and was designated as prepurified LDH (specific activity 30–70 units/mg protein).

Preparation of dye derivatives of bead cellulose. Commercial bead cellulose was prepared in a mixture of water and chlorobenzene. Before use the matrix was washed exhaustively with distilled water to remove traces of the organic solvent. The chlorotriazine dyes (listed in Table I) were covalently attached to the support according to the method of Lowe and Pearson[1]; 10 g of the moist gel cake were suspended with 45 ml of distilled water and 5 ml of the dye solution (20 mg per ml of distilled water). The suspension was gently stirred at 40–60°C for 30 min. Then NaCl

was added to a final concentration of 2% and the gel suspension was stirred 30 min prior adding solid Na_2CO_3 to a final concentration of 1%. The gel suspension was stirred again for 2 h. After keeping overnight at room temperature, the gel was exhaustively washed on a sintered glass funnel with distilled water, 0.1 *M* Na_2CO_3 and again distilled water until the final washing was colourless.

The Remazol dye derivatives of bead cellulose were prepared according to Mislovičová *et al.*[6]. A 10-g amount of cellulose (wet weight) was suspended in 10 ml of 0.25 *M* sodium hydroxide solution and after the addition of 100 mg of the dye the suspension was stirred at room temperature for 4 h. The product was washed thoroughly with distilled water and 0.1 *M* $NaHCO_3$ to remove the unbound dye. The affinity adsorbents were stored as suspensions in water at 4°C in the presence of 0.02% sodium azide. Before use they were exhaustively washed and equilibrated with the respective buffer.

Preparation of dye-(spacer)-bead cellulose. The preparation of the dye–spacer bead cellulose was carried out in three steps as summarized in Fig. 1.

Activation of the cellulose by N-chlorocarbonyloxy-5-norbornene-2,3-dicarboximide (Cl-CO-ONB)[7]: the cellulose (I) was transferred from water to acetone by stepwise washing with water–acetone mixtures (9:1, 7:3, 3:7, 1:9, v/v) and finally with dry acetone. A 10-g amount of this material was suspended in a solution of 8.3 m*M* Cl-CO-ONB in 10 ml of dry acetone. The suspension was gently tumbled for 16 h at room temperature. Then the acetone was sucked off and the resin was washed twice with dry acetone. The activated support (II) was stored in dry acetone.

Synthesis of dye-(spacer)-conjugate: 60 mmol of α,ω-diaminoalkane (III) were dissolved in 30 ml water and the solution was adjusted to pH 10 with 2 *M* HCl. A 3-mmol amount of monochloro- or dichlorotriazine dye (IV), dissolved in 30 ml water, was added dropwise and the reactants were stirred for 1 h at 50°C. After cooling to room temperature, the precipitate formed (V) was collected by filtration and dried. The structure of the dye–spacer conjugates was confirmed by ^1H NMR spectroscopy.

Coupling of dye-(spacer)-conjugate to activated bead cellulose: 500 mg of the dye–spacer conjugate (V) were dissolved in 30 ml of dimethyl sulphoxide (DMSO)–0.1 *M* $NaHCO_3$ (1:1, v/v). A 5-g amount of the activated bead cellulose swollen in water by stepwise treatment with acetone–water mixtures (9:1, 7:3, 3:7, 1:9, v/v) was suspended in 30 ml of ligand solution (V) and allowed to tumble for 16 h at 20°C. The reaction product (VI) was separated by filtration on a büchner funnel and washed with DMSO until the filtrate became colourless. Finally, the dye-(spacer)-bead cellulose was treated with 100 ml 0.1 *M* $NaHCO_3$, 100 ml water and stored at 4°C. For the preparation of dye-(spacer)-celluloses the following dyes were used: Cibacron Blue F3G-A and the Procion dyes Red HE-3B, Orange MX-G, Scarlet MX-G, Green H-4G and Yellow HE-3G (see also Fig. 1).

Determination of the degree of dye substitution. As already described for agarose matrices[1], the determination of the dye concentration bound to bead cellulose by acid hydrolysis was not successful. Therefore the affinity adsorbents (50–300 mg) with dyes directly linked to cellulose were dissolved in 2 ml of Cadoxen, which is a good solvent for bead cellulose as described by Gemeiner and Pasteka[8]. For a standard, 2–10 mg of the free dyes were also dissolved in 2 ml of Cadoxen in order to compensate changes in spectral properties of the dyes caused by the solvent. After dissolving the dye–cellulose, the absorbances of the solutions were measured immediately at λ_{max} using a Specord

M 40 spectrophotometer (VEB Carl-Zeiss, Jena, G.D.R.) and compared with the respective calibration graph for the standards. This procedure is common practice when either the structure of a dye is unknown or the molar absorbance of the dye is influenced by the solvent. The method cannot be applied for the determination of spacer-mediated dye substituents because these affinity matrices are insoluble in Cadoxen, probably due to some cross-links within the matrix generated by the activation procedure.

Affinity chromatography of LDH. All experiments were carried out in 20 m*M* potassium phosphate buffer, pH 7.0, 1 m*M* EDTA and 5 m*M* 2-mercaptoethanol (buffer B). The prepurified LDH was exhaustively dialyzed against buffer B. After centrifugation (20 000 *g*, 15 min) the enzyme was loaded onto a column (10 cm × 1 cm) packed with the affinity adsorbent. The resin was washed using a 12-fold column volume of buffer B to remove unbound protein. Then the adsorbed enzyme was eluted with buffer B containing either 1 *M* KCl or 0.05 m*M* oxidized nicotinamide–adenine dinucleotide (NAD^+) plus 1 m*M* sodium sulphite. Fractions of 2–5 ml were collected for assaying the LDH activity. The pooled enzyme was concentrated by ultrafiltration using collodium bags (Sartorius, Göttingen, F.R.G.). The affinity columns were regenerated by treatment with 0.1 *M* NaOH, water and buffer B.

Partition of LDH in two-phase systems. The partition of LDH in aqueous two-phase systems was performed as described previously[9] with minor modifications. The two-phase systems were prepared from aqueous stock solutions of PEG 6000 (40%, w/w), Dextran M 70 (20%, w/w) and 0.1 *M* triethanolamine–HCl buffer, pH 7.0, 50 m*M* 2-mercaptoethanol and 10 m*M* EDTA by weighing the respective amounts so that samples of 4 g contain finally 5.5% PEG 6000, 8.25% Dextran M 70 in 25 m*M* triethanolamine–HCl buffer, pH 7.0, and the respective amount of the enzyme. In the case of affinity partitioning, 2% of the total PEG was replaced by dye-liganded PEG 6000. The enzyme (0.01–0.05 ml) was dialyzed for 2 h against 200 ml of 25 m*M* triethanolamine–HCl buffer, pH 7.0, to remove the ammonium sulphate. A 1–2 μl volume of the dialyzed LDH containing 3–6 units of enzyme activity was added to each tube cooled to 0°C. The mixture was kept for 30 min at 0°C, mixed again for 15 s and centrifuged at 2000 *g* for 2 min at 0°C. For the enzyme assay, appropriate volumes of each of the phases were carefully removed. The partition coefficient, *K*, is defined as the ratio of the enzyme concentration in the upper and in the lower phases determined by the activity in both phases. The term $\Delta \log K$ is the difference between the log *K* value of a dye–PEG-containing system and that of a system without dye–PEG.

Assay conditions. The activities of LDH and malate dehydrogenase (MDH, E.C. 1.1.1.37) were measured spectrophotometrically at 340 nm using the following conditions: potassium phosphate buffer, pH 7.0, 0.8 m*M* pyruvate and 0.8 m*M* oxaloacetate, respectively, and 0.2 m*M* $NADH_2$.

Protein was assayed according to Bradford[10] with dried human albumin as a standard.

RESULTS AND DISCUSSION

A number of triazine dyes directly bound to bead cellulose have been tested for their affinity to LDH from bovine heart muscle. If the original muscle extract was applied to a limited amount of the affinity adsorbent, MDH was predominantly bound

due to its higher quantity in the extract in comparison to LDH. For example, Procion Navy H-ER-bead cellulose binds about 150 units of MDH and only 25 units of LDH per gram (wet weight). The competition of the two enzymes for the dye ligands is one of the reasons why various dye–bead celluloses exhibiting high binding ability for purified LDH do not efffectively adsorb the enzyme from the crude extract. In order to avoid this competition, LDH was prepurified to 30 to 70 units/mg of protein (see Methods) containing only 1.5% of MDH activity relative to the activity of LDH.

In Table I the binding capacities of various dye–bead celluloses for LDH, the eluted activity and the recovery of the enzyme are summarized. The variation of the amount of LDH bound to one type of dye–cellulose depends on the specific activity of the LDH used. The higher the specific activity the higher is the amount of LDH adsorbed and *vice versa*. The results show that Cibacron Blue F3G-A, Procion Red HE-7B, Procion Yellow HE-4R and Procion Navy H-ER are suitable affinity ligands for LDH. The various adsorbents show only small differences in respect to the recovery and the purification of the enzyme. The specific activity of the LDH was about 200–300 units/mg.

TABLE I

BINDING CAPACITIES OF DYE DERIVATIVES OF BEAD CELLULOSE AND RECOVERY OF ELUTED LACTATE DEHYDROGENASE

The binding capacity of dye–bead cellulose was determined using LDH prepurified by DEAE-Sephadex chromatography (specific activity 30–70 units/mg). Columns (10 cm × 1 cm) containing 5 g of the respective dye–cellulose were loaded with an excess of LDH (about 400 units/g cellulose) at 10°C. The amount of activity adsorbed (defined as 100%) was calculated from the difference between the total activity of LDH loaded onto the column and the unbound one determined in the breakthrough fraction and the wash pool. The purification factor was calculated from the increase in the specific activity of the enzyme. The degree of dye substitution of dye cellulose was determined after dissolving the adsorbents in cadoxen (see *Methods*).

Dye	*Degree of dye substitution [mg dye/g cellulose (wet weight)]*	*Binding capacity of the dye–bead cellulose for LDH [units/g cellulose (wet weight)]*	*LDH activity eluted in the main fractions (%)*	*Purification factor*
Cibacron Blue F3G-A	2.0	200–245	55–65	3–4
Procion dyes				
Red HE-3B	2.0	10–30	70	
Red HE-7B	5.7	190–270	75–90	n.d.
Navy H-ER	3.7	140–200	80–90	4–5
Brown HE-G	n.d.[a]	70–150	50–75	5–6
Yellow HE-4R	3.2	100–250	70	3
Scarlet MX-G	3.8	No binding	–	3–4
Orange MX-G	1.2	No binding	–	–
Green H-4G	2.0	No binding	–	–
Yellow HE-3G	1.3	No binding	–	–
Remazol dyes				
Brilliant Blue R	20.0	100–250	80–90	3–5
Brilliant Red 5 BN	n.d.	100–300	70–90	3–5

[a] n.d. = Not determined.

Immobilized Procion Red HE-3B known as a preferential adsorbent of $NADP^+$-linked dehydrogenases[12] had the lowest binding capacity for the LDH.

The two Remazol dye–bead celluloses successfully used in the purification of rat liver LDH[6] provided high binding also for the enzyme from heart muscle. Furthermore, these affinity adsorbents showed a recovery of 90–100% of the adsorbed enzyme and the LDH appeared in a small volume of the eluate.

Table I also shows that the Procion dyes Scarlet MX-G, Orange MX-G, Green H-4G and Yellow HE-3G, when directly bound to bead cellulose, are incapable of interacting with LDH. This result was not expected because affinity partitioning of pure LDH in aqueous two-phase systems using triazine dye-substituted poly(ethylene glycol) showed that the enzyme binds also to Procion Green H-4G and Procion Orange MX-G[9]. Moreover, Procion Scarlet MX-G and Procion Orange MX-G were found to interact specifically with pig heart LDH[11].

In order to rationalize these contradictory results, the affinity of prepurified LDH from heart muscle to diverse triazine dyes was screened by means of affinity partitioning in aqueous two-phase systems (Table II). Here, the $\Delta \log K$ values, as a measure of the strength of interaction of the enzyme with dyes[9], are shown. Values higher than one found under the experimental conditions indicate sufficient interaction of the dye molecule with the enzyme. This holds for most of the dye stuffs except Procion Scarlet MX-G and Procion Orange MX-G which exhibit weaker interactions.

Dyes listed in group 1 of Table II are able to interact with LDH if they are coupled directly to the matrix. In group 2 those dyes are listed which did not exert affinity to LDH when coupled directly to cellulose (see Table I). This was not expected, particularly for Procion Green H-4G and Procion Yellow HE-3G where no significant difference in the Δ log K values was observed compared with the dyes in group 1.

One possibility for the lack in affinity of the immobilized dyes of group 2 for LDH may be a steric hindrance to the dye–enzyme interaction caused by the matrix. Therefore the dyes in group 2 of Table II were coupled to bead cellulose via a spacer. The length of the spacer was varied between two and six carbon atoms using diamino-ethane, -butane and -hexane (see Fig. 1).

TABLE II

INTERACTION OF HEART MUSCLE LDH WITH DIFFERENT TRIAZINE DYES STUDIED BY MEANS OF AFFINITY PARTITIONING

The two-phase systems contained 8.25% Dextran M 70 and 5.5% PEG 6000. In the case of affinity partitioning, 2% of the total PEG was replaced by dye-liganded PEG. For further experimental details see *Methods*. The term $\Delta \log K$ is the difference between the log K values of a two-phase system with and without dye–PEG. In group 1 triazine dyes are listed which show binding of LDH when they are directly coupled to bead cellulose. In group 2 dyes are listed which do not interact with LDH when they are directly bound to the matrix.

Dye	*Δlog K*	*Dye*	*Δlog K*
Group 1		*Group 2*	
Procion Red HE-3B	1.62	Procion Green H-4G	1.69
Procion Red HE-7B	1.54	Procion Yellow HE-3G	1.86
Procion Yellow HE-4R	1.92	Procion Scarlet MX-G	0.97
Procion Navy H-ER	1.82	Procion Orange MX-G	0.94
Cibacron Blue F3G-A	1.33		

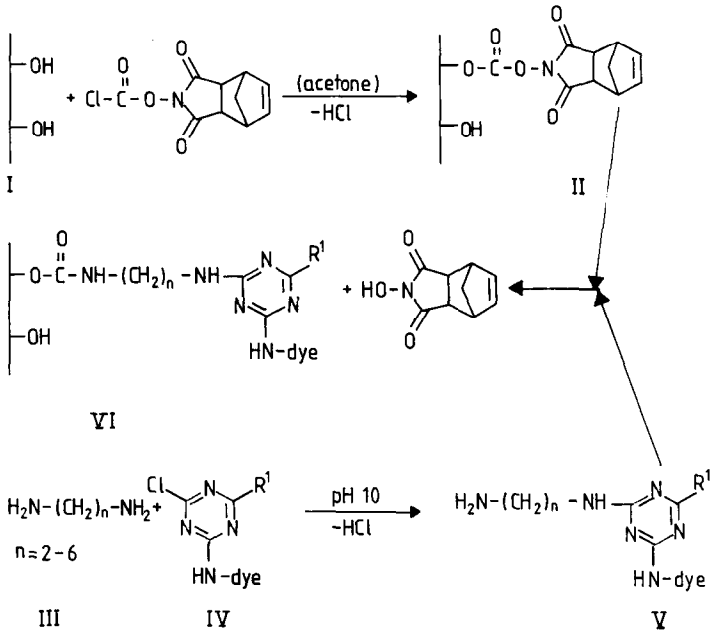

Fig. 1. Preparation of dye–spacer bead cellulose (VI) using activated bead cellulose (II) and the aminoalkyl dye (V) (see text).

In Table III the binding capacities of the adsorbents and the recoveries of the eluted enzymes are summarized. The introduction of a spacer enabled Procion Green H-4G, Procion Scarlet MX-G, Procion Orange MX-G and Procion Yellow HE-3G to bind LDH. In comparison with the affinity adsorbents involving directly attached dyes, some spacer-mediated affinity matrices provided higher binding capacity for LDH. This is not valid for the dyes Procion Red HE-3B and Cibacron Blue F3G-A, respectively. The apparent lower binding capacity of Procion Scarlet MX-G-(diaminoethyl)–bead cellulose and Procion Orange MX-G-(diaminohexyl)–bead cellulose for LDH is caused by the less specific activity of the enzyme used in the experiments.

The influence of the length of the spacer on the binding capacity and on the elution behaviour was studied in more detail using Procion Green H-4G and Procion Scarlet MX-G as affinity ligands (Table III). In the case of Procion Green H-4G, 75–90% of the adsorbed enzyme was eluted by 1 *M* KCl. With NAD^+/sulphite, differences in the effectiveness of elution as a function of the length of the spacer becomes obvious: 80–90% of the adsorbed activity was eluted in a small volume from dye-(diaminohexyl)-bead cellulose. However, applying affinity cellulose with a shorter spacer length gave a significantly lower yield and the enzyme appeared as a broad peak. In the latter case a ten-fold increase in the effector concentration in the elution buffer yielded only a two-fold increase in the amount of LDH eluted. The results revealed that the longer the spacer the lower is the concentration of NAD^+ required for elution of the enzyme and the sharper is the elution profile (not shown). This behaviour has been confirmed with spacer-mediated Procion Scarlet MX-G.

TABLE III

BINDING CAPACITY AND ELUTION BEHAVIOUR OF DYE-(SPACER)-BEAD CELLULOSE TO LACTATE DEHYDROGENASE

LDH with two different specific activities of 70 or 30 units/mg (marked by *) were applied. The percentage of the eluted activity is related to the adsorbed activity (defined as 100%). The latter was calculated from the difference between the total activity of LDH loaded onto the column and the portion unbound after washing the column with buffer B. Further experimental details are described in *Methods*. Abbreviation: Dye-C_n-BC = Dye-NH$(CH_2)_n$NH–CO–O–bead cellulose.

Adsorbent (Dye-C_n-BC)	*Binding capacity (units/g wet adsorbent)*	*Percentage of eluted enzyme activity of LDH in presence of*	
		1 M KCl	*0.05 mM NAD^+/1 mM sulphite*
Procion Red HE-3B			
-C_6-BC	30–60	n.d.	29
Cibacron Blue F3G-A			
-C_6-BC	150	n.d.	47
Procion Green H-4G			
-C_2-BC	450–620	84	35
-C_4-BC	500–640	92	51
-C_6-BC	500–680	75	84
ProcionScarlet MX-G			
-C_2-BC	500–650	5–10	31
-C_2-BC	200–300*	n.d.	30
-C_6-BC	500–770	5–10	79
Procion Orange MX-G			
-C_6-BC	200*	2–5	87
Procion Yellow HE-3G			
-C_6-BC	520	n.d.	30

In Table III, differences between Procion Green H-4G and Procion Scarlet MX-G in respect of the conditions of elution of LDH become evident. Although 1 *M* KCl is able to desorb most of the enzyme from the affinity matrix of the Procion Green type, independent of the spacer length of the ligands, LDH was almost completely retained on Procion Scarlet MX-G-(spacer)-bead cellulose under the same conditions. Decreasing or increasing the concentration of KCl did not essentially improve the yield of the enzyme. However, the enzyme was eluted by applying NAD^+/sulphite as in the case of Procion Green H-4G-cellulose (Table III) and also with buffer B containing 50% ethylene glycol, in 70% yield (not shown). Similar results were obtained with Procion Orange MX-G coupled via an aminohexyl spacer to bead cellulose. This is not surprising because of the structural similarities between the dichlorotriazinyl dyes Procion Scarlet MX-G and Procion Orange MX-G as shown in Fig. 2.

The superior properties of Procion Scarlet MX-G-(spacer)-bead cellulose have been successfully exploited for the purification of LDH from muscle extract without prepurification as demonstrated in Table IV. After centrifugation of the extract (specific activity of LDH: 5.8 units/mg protein) the clear supernatant was loaded onto a column containing dye-liganded cellulose. Then the column was rinsed with buffer B containing 1 *M* KCl to wash off non-adsorbed and non-specifically bound protein, followed by a washing step with buffer B to remove the high salt concentration. After adding NAD^+/sulphite to the elution buffer, the enzyme was desorbed in 90% yield.

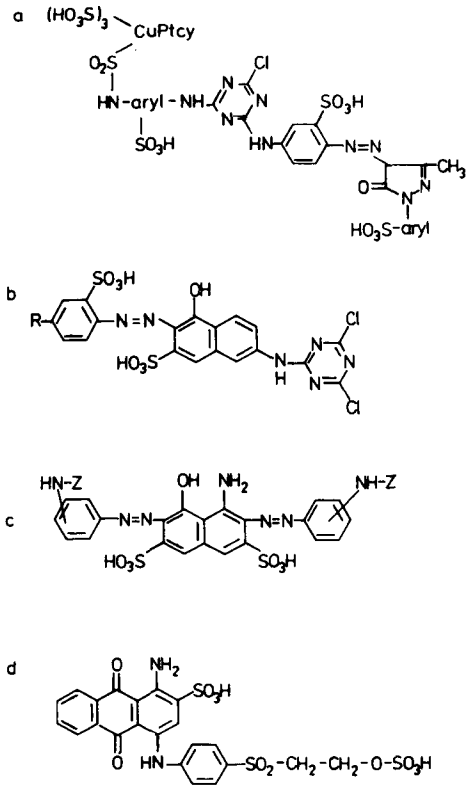

Fig. 2. Structures of reactive dyes. (a) Procion Green H-4G (Ptcy = phthalocyanine moiety); (b) Procion Orange MX-G (R = H) and Procion Scarlet MX-G (R = OCH_3); (c) Procion Navy H-ER (Z = halogenoheterocyclic reactive group[3]; (d) Remazol Brillant Blue R. Beside the well known structures of Cibacron Blue F3G-A and Procion Red HE-3B[2], the structures of other dyes used have not yet been published.

TABLE IV

PURIFICATION OF LACTATE DEHYDROGENASE FROM HEART MUSCLE BY APPLYING PROCION SCARLET MX-G-(DIAMINOHEXYL)-BEAD CELLULOSE

Experimental conditions: extract obtained from 30 g of minced muscle was centrifuged (9000 *g*, 30 min) and the supernatant was loaded onto a column (20 cm × 2 cm) packed with 14 g of dye–cellulose. After washing with buffer B containing 1 *M* KCl, and buffer B without KCl, the enzyme was desorbed by NAD^+/sulphite in buffer B. The purification is relative to the specific activity of LDH in the extract of heart muscle and in the main fraction from dye–cellulose chromatography.

Step	*Volume (ml)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor*
Extract	47	1700	5.8	100	1
Dye–cellulose	14	891	200	52	34
Ultrafiltration	1.5	750	210	44	36

The main fractions contained 52% of the bound activity of LDH with a specific activity of 200 units/mg protein. In the course of this step the activity of MDH decreased from 250 to about 50% relative to the activity of LDH. The binding capacity of Procion Scarlet MX-G-(diaminohexyl)-bead cellulose for LDH from a crude muscle extract was determined to be 100–120 units/g wet weight. Starting this chromatography with prepurified LDH (see Methods), an enzyme with a specific activity of 300–400 units/mg and a contaminating activity of 0.1% MDH (relative to the activity of LDH) results.

In Fig. 3 the protein patterns before and after the dye chromatography starting with prepurified LDH (A) and with a crude muscle extract (B) have been monitored by sodium dodecyl sulphate (SDS)-polyacrylamide gel elctrophoresis. Beside the main band of LDH, a more rapidly migrating faint band appeared but to different extents in the two procedures. This is related to the difference in the specific activities of the two preparations.

The results of this study revealed that the common property of diverse triazine dyes immobilized on bead cellulose to bind LDH is caused by several interactions.

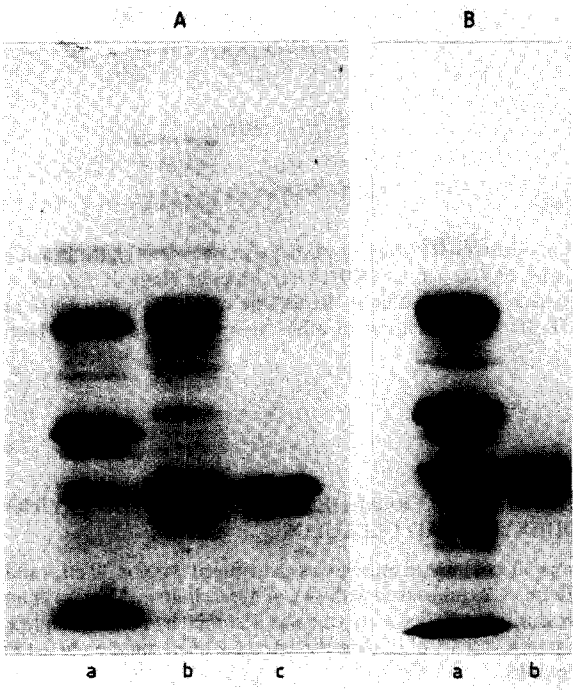

Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of LDH samples at different stages of purification. (A) (a) Extract of heart muscle; (b) sample after DEAE-Sephadex chromatography (prepurified LDH); (c) sample after chromatography on Procion Scarlet MX-G-(diaminohexyl)-bead cellulose using prepurified LDH. (B) (a) Extract of heart muscle; (b) sample after chromatography on Procion Scarlet MX-G-(diaminohexyl)-bead cellulose using extract of heart muscle. Contents of protein and specific activity of LDH, respectively: (A) lane (a) 0.1 mg (4 units/mg), (b) 0.24 mg (50 units/mg) and (c) 0.02 mg (380 units/mg); (B) lane (a) 0.06 mg (6 units/mg), (b) 0.01 mg (210 units/mg). The electrophoresis was performed according to Neville[13].

Beside binding forces which are created specifically due to the nature of the dyes and certain structural domains of the enzyme, the influence of the spacer and of the matrix is evident. Work is in progress using structurally related dyes of Procion Scarlet MX-G to study the significance of the spacer for the specificity of the interaction with LDH and other enzymes.

ACKNOWLEDGEMENTS

We gratefully acknowledge Professor Dr. E. Hofmann, Institute of Biochemistry, Karl-Marx-University Leipzig for fruitful discussions and critical reading of the manuscript. The authors are also indebted to VEB Chemiekombinet Bitterfeld, Coloristisches Labor for the generous gift of the reactive dyes and to VEB Arzneimittelwerk Leipzig for the samples of various bead celluloses. We also thank Mrs. E. Müller for carrying out the electrophoresis.

REFERENCES

1 C. R. Lowe and J. C. Pearson, *Methods Enzymol.*, 104 (1984) 97.
2 G. Kopperschläger, H.-J. Böhme and E. Hofmann, *Adv. Biochem. Eng.* (1982) 101.
3 C. V. Stead, *J. Tech. Biotechnol.*, 37 (1987) 55.
4 R. K. Scopes, *J. Chromatogr.*, 376 (1986) 131.
5 H.-J. Böhme, G. Kopperschläger, J. Schulz and E. Hofmann, *J. Chromatogr.*, 69 (1972) 209.
6 D. Mislovičová, P. Gemeiner, Ľ. Kuniak and J. Zemek, *J. Chromatogr.*, 194 (1980) 95.
7 P. Henklein, W. Büttner, M. Becker and C. Rupprich, *Eur. Pat. Appl.*, EP 0.134.041, A1 (1984).
8 P. Gemeiner and M. Pasteka, *Appl. Biochem. Technol.*, 8 (1983) 381.
9 G. Kopperschläger, G. Lorenz and E. Usbeck, *J. Chromatogr.*, 259 (1983) 97.
10 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
11 Y. D. Clonis and C. R. Lowe, *Biochem. J.*, 191 (1980) 247.
12 D. H. M. Harvey and P. D. G. Dean, *Biochem. J.*, 173 (1978) 591.
13 D. M. Neville, Jr., *J. Biol. Chem.*, 246 (1971) 6328.