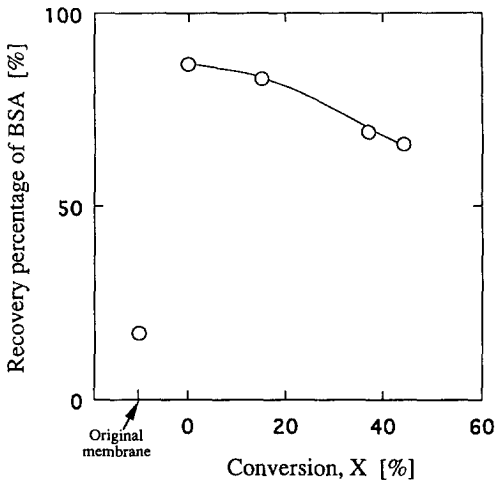

Fig. 8. Recovery of BSA with the Ph(X)-diol-C fibre as a function of conversion.

had a higher phenyl-group density. Rosengen et al. [35] have demonstrated that agarose-based beads with a higher density of hydrophobic groups such as octyl and phenyl groups exhibited a lower recovery of phycoerythrin as a model protein. The recovery of BSA with the Ph-diol-C fibre, 63–83%, was at the same level as that of commercially available HIC beads [TSK gel Phenyl-Toyopeal 650 (Tosoh) and Phenyl-Cellulofine S-type (Seikagaku Kogyo)] with an identical eluent, 53–92% [36,37].

The Ph(0)-diol-C fibre exhibited a recovery of 87% with a lower BSA binding capacity than the Ph(15–44)-diol-C fibre, whereas the original hollow-fibre membrane made of polyethylene exhibited a low recovery of 17%. This was due to non-selective adsorption, i.e., irreversible adsorption of BSA on to polyethylene.

4. Conclusion

A polymer chain containing both phenyl and diol groups as hydrophobic and hydrophilic groups, respectively, which was grafted on to the polyethylene hollow-fibre matrix, was demonstrated to provide adsorption space for hydrophobic interaction with a protein and to reduce the undesirable non-selective adsorption of the polyethylene matrix. The breakthrough curve was unchanged irrespective of the residence time of the bovine serum albumin (BSA) buffer solution containing 2 M $(NH_4)_2SO_4$ across the membrane in the dead-end mode because of negligible diffusional mass-transfer resistance. The membranes with a phenyl-group density of 0.6–1.6 mmol/g exhibited a BSA binding capacity of 30 mg/g, which indicated monolayer adsorption. The amount of BSA recovered by permeating an $(NH_4)_2SO_4$-free buffer through the membranes demonstrated that a higher density of phenyl groups decreased the recovery of BSA.

Symbols

a	cross-sectional area occupied by a BSA molecule (m^2)
a_v	specific surface area of the Ph-diol-C fibre (m^2/kg)
C	concentration of BSA in the effluent (mg/ml)
C_0	concentration of BSA in the feed (mg/ml)
d_g	degree of GMA grafting (%)
d_i	inner diameter of hollow-fibre membrane (cm)
d_o	outer diameter of hollow-fibre membrane (cm)
L	length of hollow-fibre membrane (cm)
M_r	molecular mass of BSA
N_A	Avogadro's number
Δp	permeation pressure (MPa)
q_0	amount of BSA adsorbed in equilibrium with C_0 (mg/g)
q_t	theoretical saturation capacity of BSA on the Ph-diol-C fibre (mg/g)
t_r	residence time (s)
V	effluent volume (ml)
V_s	effluent volume when C reaches C_0 (ml)
W_0	mass of the original membrane (g)
W_1	mass of the GMA-grafted membrane (g)
W_2	mass of the Ph(X)-diol-C fibre (g)
X	molar conversion of epoxy groups into phenyl groups (%)
ε	porosity of the Ph-diol-C fibre

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Biomimetic dye affinity chromatography for the purification of bovine heart lactate dehydrogenase

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Abstract

Three biomimetic dye ligands bearing as a triazine-linked terminal moiety a carboxylated structure, which mimics substrates and inhibitors of L-lactate dehydrogenase (LDH), were immobilized on cross-linked agarose Ultrogel A6R. These biomimetic dyes are purpose-designed analogues of commercial monochlorotriazine Cibacron Blue 3GA (CB3GA) and parent dichlorotriazine Vilmafix Blue A-R (VBAR). The corresponding biomimetic adsorbents, along with non-biomimetic adsorbents bearing CB3GA and VBAR, were evaluated for their ability to purify LDH from bovine heart crude extract. When compared with non-biomimetic adsorbents, all biomimetic adsorbents exhibited a higher purifying ability. Further, one immobilized biomimetic dye, bearing mercaptopyruvic acid as biomimetic moiety, displayed the highest purifying ability. The concentration of immobilized dye affected both the capacity and the purifying ability of the affinity column, exhibiting an optimum value 2.2 μmol dye/g moist gel. This affinity adsorbent was exploited for the purification of LDH from bovine heart in a two-step procedure. The procedure consisted in a biomimetic dye affinity chromatography step (NAD^+/sulphite elution, 25-fold purification, 64% step yield), followed by DEAE-agarose ion-exchange chromatography (1.4-fold purification, 78% step yield). The purified enzyme exhibited a specific activity of ca. 480 u/mg at 25°C (content of impurities: pyruvate kinase and glutamic–oxaloacetic transaminase were not detected; malate dehydrogenase, 0.01%), compared with ca. 250 u/mg of commercial bovine heart LDH (malate dehydrogenase, 0.05%) suitable for analytical purposes.

1. Introduction

Downstream processing is regarded as a key factor for the successful commercialization of high-purity proteins. Affinity chromatography [1–5], although a relatively expensive technique, is present in the production line of several high-purity products, e.g., therapeutic, molecular biology, analytical and diagnostic proteins. Reactive triazine dyes are robust affinity ligands promising for industrial-scale bioprocesses, and their immobilized forms are exploited in downstream processing [5–8]. Dyes offer clear advantages over biological ligands [4,5,7,8]; however, the main drawback of dye molecules appears to be their moderate, in general, selectivity for target proteins. Attempts to tackle this problem were realized through the biomimetic dye concept [9,10], according to which the presence of a purpose-designed biomimetic moiety on the parent dye can lead to a new dye mimicking natural ligands of the target protein. The purpose-de-

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signed biomimetic dyes are expected to exhibit increased affinity and purifying ability for target proteins. Earlier studies confirmed the effectiveness of this approach both, in terms of measured K_D values of free biomimetic dyes with formate dehydrogenase [10], oxalate oxidase [11], oxalate decarboxylase [11] and oxaloacetate decarboxylase [12], and in the purification of alcohol dehydrogenase [9], trypsin [13], alkaline phosphatase [14,15] and formate dehydrogenase [16] on immobilized biomimetic dyes.

The present biomimetic dye ligands are analogues of commercial monochlorotriazine Cibacron Blue 3GA (CB3GA) and parent dichlorotriazine Vilmafix Blue A-R (VBAR), and exhibit as the biomimetic moiety, linked on the chlorotriazine ring, an α-keto acid structure. Therefore, the biomimetic moiety mimics natural substrates and inhibitors of L-lactate dehydrogenase (LDH), e.g., pyruvate, 2-oxobutyrate, oxamate and oxalate. The corresponding biomimetic affinity adsorbents (BM) are expected to show increased purifying ability for LDH. The target enzyme is a tetramer of $M_r \approx 140\,000$, with H_4 and H_3M being the dominant components of heart LDH [17]. Lactate dehydrogenase has attracted attention as an auxiliary or indicator enzyme in the determination of several metabolites and enzymes [17]. However, the enzyme from bovine heart has attracted considerably less attention. Therefore, a simple and effective method for the purification of bovine heart LDH, based on biomimetic dye affinity chromatography, would be both interesting and useful.

2. Experimental

2.1. Materials

Reduced β-nicotinamide adenine dinucleotide (NADH) (disodium salt, ca. 100%), NAD^+ (free acid, ca. 98%), pyruvate monosodium salt and crystalline bovine serum albumin (fraction V) were obtained from Boehringer (Mannheim, Germany). DEAE-Sepharose CL6B, lipophilic Sephadex LH-20, 4-morpholinepropanesulfonic acid (MOPS) and CB3GA were obtained from Sigma. The cross-linked beaded agarose gel Ultrogel A6R was a much appreciated gift from BioSepra.

2.2. Synthesis of biomimetic dye ligands and dye adsorbents

Biomimetic dyes (Table 1, structures 1–3) were synthesized following the method of Labrou and Clonis [10]. Dye purification was performed on a lipophilic Sephadex LH-20 column according to published procedures [10,18]. Immobilization of dye ligands on cross-linked agarose (adsorbents were stored as moist gels in 20% methanol at 4°C) and determination of immobilized dye concentrations by the acid hydrolysis method were performed as described previously [16].

2.3. Assay of enzyme activity and protein

LDH assays were performed at 25°C according to a published method [19] using a Hitachi U-2000 double-beam UV–Vis spectrophotometer equipped with a thermostated cell holder (10 mm path length). One unit of enzyme activity is defined as the amount that catalyses the conversion of 1 μmol of pyruvate to L-lactate per minute. Protein concentration was determined by the method of Bradford [20], using bovine serum albumin (fraction V) as standard.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli [21] on a 1.5 mm thick vertical slab gel (14×16 cm), containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. The samples, after appropriate incubation, were applied to the wells and run at a current of 30 mA per gel for 4 h. Protein bands were stained with Coomassie Blue R-250.

2.5. Preparation of bovine heart extract

The tissue was kept at −20°C for up to 6 months. Frozen tissue was cut into small pieces

and frozen again at −20°C (15 g), before potassium phosphate buffer [30 ml, 50 m*M*, pH 7.0, containing 0.2% (v/v) β-mercaptoethanol (β-MeSH)] was added (4°C). The tissue was disintegrated in a blender (total 2 min, breaking every 15 s), centrifuged (14 000 *g*, 30 min) and the supernatant (ca. 30 ml) was dialysed overnight against 150 volumes of MOPS–NaOH buffer (20 m*M*, pH 7.0), containing 0.2% (v/v) β-MeSH. The dialysate was filtered through a cellulose membrane filter (Millipore, 0.45 μm pore size) and contained, typically, 13.5 units LDH/mg protein (yield 300 units LDH/g tissue).

2.6. Evaluation procedure for dye adsorbents for LDH binding from bovine heart extract. Effect of immobilized dye concentration on protein binding

All procedures were performed at 4°C. Lactate dehydrogenase binding was assessed using analytical columns each packed with 0.6 ml of dye adsorbent. Columns were equilibrated with 20 m*M* MOPS–NaOH buffer (pH 7.0), containing 0.2% (v/v) β-MeSH. Bovine heart extract (1360 μl, 204 units LDH, 15.1 mg protein) was loaded on the dye adsorbent and the flow was stopped for 15 min. The column was then washed with equilibration buffer (10 ml) before bound LDH was eluted with 5 ml of buffer containing either KCl (1 *M*) or NADH (5 m*M*). Fractions (5 ml) were collected and assayed for LDH activity and protein (Bradford method [20]).

2.7. Purification of LDH from bovine heart extract on mercaptopyruvic-VBAR-Ultrogel A6R biomimetic adsorbent and DEAE-Sepharose CL6B anion exchanger

All procedures were performed at 4°C.

Step 1: affinity chromatography on mercaptopyruvic-VBAR-Ultrogel A6R biomimetic adsorbent

Dialysed tissue extract (2370 μl, 26.3 mg protein, 356 units LDH) was applied to a column of mercaptopyruvic-VBAR-Ultrogel A6R (1 ml, 2.2 μmol dye/g moist gel; Table 1, structure 1), which was previously equilibrated with 20 m*M* MOPS–NaOH buffer (pH 7.0) containing 0.2% (v/v) β-MeSH. Non-adsorbed protein was washed off with 23 ml of equilibration buffer followed by 2 × 16.5 ml of buffer containing 10 and 20 m*M* KCl, respectively. Bound LDH was eluted with equilibration buffer (3 ml) containing 0.1 m*M* NAD^+ and 1 m*M* sulfite. Collected fractions were assayed for LDH activity and protein (A_{280}, except for effluents containing NAD^+, where the protein was determined by the Bradford method [20]).

Step 2: ion-exchange chromatography on DEAE-Sepharose CL6B

The eluted LDH activity from the previous column (3 ml, 228 LDH units, 0.68 mg protein) was directly applied to a DEAE-Sepharose CL6B ion-exchanger column (1.6 × 1.5 cm I.D.) which was previously equilibrated with 20 m*M* MOPS–NaOH buffer (pH 7.0) containing 0.2% (v/v) β-MeSH. Non-adsorbed protein was washed off with 12 ml of equilibration buffer followed by 3 × 8 ml of buffer containing 50, 75 and 100 m*M* KCl, respectively. Bound LDH was finally eluted with equilibration buffer containing 200 m*M* KCl (5 ml). Collected fractions were assayed for LDH activity and protein (A_{280}).

2.8. Course of adsorption of bovine heart protein and LDH on mercaptopyruvic-VBAR-Ultrogel A6R biomimetic adsorbent

All procedures were performed at 4°C. Frontal analysis experiments were performed as follows: dialysed bovine heart extract [13.5 units LDH/mg protein, 150 units LDH/ml, 11.1 mg protein/ml in 20 m*M* MOPS–NaOH buffer (pH 7.0) containing 0.2% (v/v) β-MeSH] was continuously applied (2.0 cm/min) to the mercaptopyruvic-VBAR-Ultrogel A6R column (0.6 ml adsorbent, 2.2 μmol dye/g moist gel), which was previously equilibrated with the above buffer. The extract was applied until the LDH activity in the effluents had reached a constant maximum value.

Collected fractions (1 ml) were assayed for LDH activity and protein (A_{280}).

3. Results and discussion

The concept of purpose-designed biomimetic dye ligands was first introduced to solve problems associated with the moderate selectivity of commercial dyes and their adsorbents during protein binding [9,13–16]. Introducing an appropriate structural moiety on the parent dye molecule may lead to a new purpose-designed dye which mimics natural ligands of the target protein [9–12]. Therefore, the corresponding biomimetic affinity adsorbent is expected to show increased purifying ability for the target macromolecule [9,13–16].

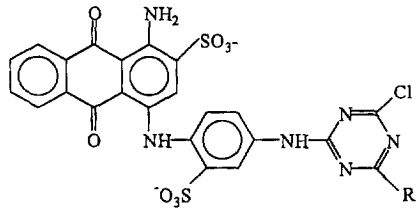
In this work the biomimetic dye concept was realized by designing three biomimetic dyes, analogues of the well known Cibacron Blue 3GA, bearing terminal α-keto acid functions (Table 1, structures 1–3). The terminal biomimetic structures were chosen to mimic the LDH substrate pyruvate (structure 1) and inhibitor oxamate (structure 2). Further, a biomimetic dye bearing an aromatic keto acid moiety (structure 3), along with non-biomimetic CB3GA and parent VBAR (structures 4 and 5, respectively) were evaluated. The 4-aminophenyloxanilic acid substituent (structure 3) was considered because of its keto acid function and hydrophobic aromatic ring. To this end, it is known that the LDH-inactivating potency of N-alkylmaleimides increases with increasing hydrophobicity and chain length of the maleimide [22]. Biomimetic moieties, analogues of lactic acid, were not considered since the substrate lactate shows a 22-fold higher K_m than pyruvate [17,23].

All adsorbents were substituted with dye ligand at the same level (2.0–2.2 μmol/g) and fell in the range used by most workers [16,24–30]. When comparing affinity adsorbents, synthesis-effected equal ligand substitution (not adjusted afterwards by appropriate dilution with unsubstituted gel) is an important but often overlooked prerequisite [31–33]. Wide variations in immobilized ligand concentration are undesirable because the interpretation of results regarding adsorbent behaviour can be unreliable.

In order to reveal the most effective dye adsorbent for purifying LDH, all dye adsorbents were evaluated for their purifying ability. Dialysed crude extract [204 LDH units, 15.1 mg protein, in 20 m*M* MOPS–NaOH (pH 7.0), containing 0.2% (v/v) β-MeSH] was loaded on each dye adsorbent. The column was washed with buffer before eluted with either KCl (1 *M*) or NADH (5 m*M*). Salt elution leads to protein desorption, and therefore the technique reveals the adsorbent selectivity during adsorption [14,15,27,34,35]. On the other hand, elution with substrate/cofactor provides information on the ability of the bound enzyme to desorb biospecifically, leaving unwanted proteins on the column [14,15,27,28,30,35,36]. Table 1 shows the purifying ability of dyed adsorbents during adsorption (KCl) and biospecific elution (NADH) of LDH activity, and also their capacity (units LDH/μmol immobilized dye). All three biomimetic-dyes (adsorbents BM 1–3) exhibited higher purifying ability and capacity than non-biomimetic structures (adsorbents 4 and 5), Further, the biomimetic dye bearing a terminal mercaptopyruvate linked to the triazine ring (adsorbent BM 1), exhibited the highest purifying ability (tenfold) and capacity (144 units/μmol dye), followed closely by the aromatic keto acid-substituted analogue (adsorbent BM 3; 9.1-fold and 136 units/μmol dye, respectively). On the basis of the above observations, adsorbent BM 1 was studied further and was eventually integrated in the purification protocol for LDH.

Prior to designing the purification protocol, we investigated the following factors: concentration of immobilized dye, pH and elution conditions. High concentrations of immobilized affinity ligand may lead to no binding, owing to steric effects caused by the ligand molecules, or even to non-specific binding [24]. The concentration employed here fell within the range used commonly [16,24–30] and near to its low end. Table 2 summarizes the results obtained when studying the effect of dye concentration on the purifying ability and capacity of the BM 1 adsorbent. A concentration of 2.2 μmol/g was found to be the

Table 1
Evaluation of dye adsorbents for binding LDH from bovine heart extract



No.	Immobilized dye (-R)	Capacity (u/μmol dye)	SA[a] (u/mg)		Purification (-fold)		Recovery[b] (%)	
			KCl	NADH	KCl	NADH	KCl	NADH
1	$-SCH_2COCOO^-$	144	73	135	5.4	10	96	95
2	$-HN(CH_2)_2NHCOCOO^-$	130	65	107	4.8	7.9	99	90
3	$-p$-HNBenzNHCOCOO$^-$	136	69	123	5.1	9.1	95	94
4	$-o$-HNBenzSO_3^- (CB3GA)	122	62	103	4.6	7.6	95	90
5	-Cl (VBAR)	125	55	87	4.1	6.4	93	90

Dialysed extract [204 units LDH, 15.1 mg protein, 20 m*M* MOPS–NaOH buffer, pH 7.0, containing 0.2% (v/v) β-MeSH] was applied on each dye adsorbent (0.6 ml). The adsorbent was washed with equilibration buffer (10 ml) prior to eluting bound proteins with 5ml of 1 *M* KCl or 5 m*M* NADH.
[a] Specific activity.
[b] Calculated on the basis of bound LDH units (100%).

optimum, since it ensured the highest purification (5.4-fold) along with a good capacity (144 units/μmol). The observed pattern of increasing capacity with decreasing ligand substitution (Table 2) has been reported before [30] and is probably due to the fact that as the concentration of immobilized ligand decreases, a larger proportion of immobilized dye molecules becomes available for protein binding (i.e., a smaller fraction of dye molecules is sterically hindered by bound proteins) [24].

Table 3 summarizes the effect of pH (during adsorption) and elution conditions on LDH chromatography. At neutral to slightly acidic pH, BM 1 displayed the same purifying ability (tenfold) and similar capacity (144 and 153 units/μmol, respectively), whereas a dramatic fall was observed under alkaline conditions [Table 3(a)]. This behaviour is in agreement with previous findings that acidic conditions facilitate protein binding to dye adsorbents [14–16,29,37,38]. Between the two acceptable pH values of 6.0 and

Table 2
Effect of concentration of immobilized dye on the chromatography of bovine heart LDH on mercaptopyruvate-VBAR-Ultrogel A6R

[Dye] (μmol/g)	Capacity (u/μmol dye)	SA (u/mg)	Purification (-fold)	Recovery (%)
0.8	214	65	4.8	96
2.2	144	73	5.4	96
2.9	93	62	4.6	88

Dialysed extract [204 units LDH, 15.1 mg protein, 20 m*M* MOPS–NaOH buffer, pH 7, containing 0.2% (v/v) β-MeSH] was applied on each adsorbent (0.6 ml). The absorbent was washed with equilibration buffer (10 ml) prior to eluting bound LDH with 1 *M* KCl (5 ml).

Table 3
Effect of pH and elution medium on the chromatography of bovine heart LDH on mercaptopyruvate-VBAR-Ultrogel A6R

Method	Capacity (u/μmol dye)	Washed LDH[a] (%)	SA (u/mg)	Purification (-fold)	Recovery[a] (%)
(a) Adsorption at					
pH 6.0	153	–	140	10.4	85
pH 7.0	144	–	135	10.0	95
pH 8.5	13.6	–	59	4.3	100
(b) Elution sequentially with (pH 7.0)					
10 *mM* KCl	–	5.7	205	15.2	
and 20 m*M* KCl	–	16.7	342	25.3	
and 40 m*M* KCl	–	41.7	305	22.6	95

(a) Effect of pH on protein adsorption: dialyzed extract (220 units LDH, 16.3 mg protein) was applied on the affinity adsorbent (0.6 ml), which was previously equilibrated in 20 m*M* MOPS–NaOH buffer, pH 6 or 7, or 20 m*M* Tris–HCl, pH 8.5, containing 0.2% (v/v) β-MeSH (4°C). After the adsorbent had been washed with equilibration buffer (10 ml), bound LDH was eluted with 5 m*M* NADH (5 ml). (b) Effect of ionic strength (KCl) on the elution and purification of LDH: dialysed extract (220 units LDH, 16.3 mg protein) was applied on the affinity adsorbent (0.6 ml), which was previously equilibrated in 20 m*M* MOPS–NaOH buffer, pH 7, containing 0.2% (v/v) β-MeSH (4°C). Unbound proteins were washed off with equilibration buffer (15 ml) and the adsorbent was washed with 10 ml each of the media shown, prior to eluting LDH with 5 m*M* NADH (5 ml).
[a]Calculated on the basis of bound and non-washed LDH units (100%).

7.0, we finally chose pH 7.0 because at acidic pH the contaminating pyruvate kinase activity starts binding the dye column [29,30].

The conditions adopted for LDH elution from the BM 1 adsorbent were of particular importance. Prior to eluting LDH biospecifically with NADH, the column was washed with steps of increasing salt concentration (10–40 m*M* KCl) to remove unwanted bound proteins. During salt washing, increasing amounts of LDH were co-eluted, and the specific activity of the final product exhibited a maximum value [Table 3(b)]. Step washing with 10 m*M* KCl followed by 20 m*M* KCl was adopted as the optimum conditions, since it led to the highest purification (342 u/mg, after NADH elution) and a moderate loss of LDH in the washings. Although NADH was used as biospecific eluent (5 m*M*) during preliminary experiments for adsorbent evaluation (Tables 1 and 3), in the final purification protocol elution with a much more economical agent, NAD^+ (0.1 m*M*) and sulphite (1.0 m*M*), was preferred and it led to the same purification and recovery as with NADH.

Based on the above results, we designed a simple two-step purification protocol for bovine heart LDH. Non-pretreated dialysed tissue extract was applied directly on a BM 1 adsorbent (2.2 μmol/g). Unbound proteins were removed with equilibration buffer (20 m*M* MOPS–NaOH, pH 7.0), the column was washed with 10 and 20 m*M* KCl, prior to LDH activity being eluted with three-column volumes of 0.1 m*M* NAD^+ and 1 m*M* sulphite (Fig. 1). The eluted enzyme activity was then directly applied to a DEAE-Sepharose CL6B anion-exchange column. The column was washed with irrigating buffer followed by a low KCl concentration to remove some proteins, prior to the bound LDH activity being desorbed with 0.2 *M* potassium chloride.

The results obtained from a typical purification run are summarized in Table 4. We attempted to improve the purity of the final product by introducing inexpensive techniques used routinely by enzyme manufacturers (e.g., ammonium sulfate fractionation and anion-exchange chromatography) prior to affinity chromatography. Pretreatment of the starting tissue extract with 30–65% (w/v) $(NH_4)_2SO_4$ led to 2.5-fold step

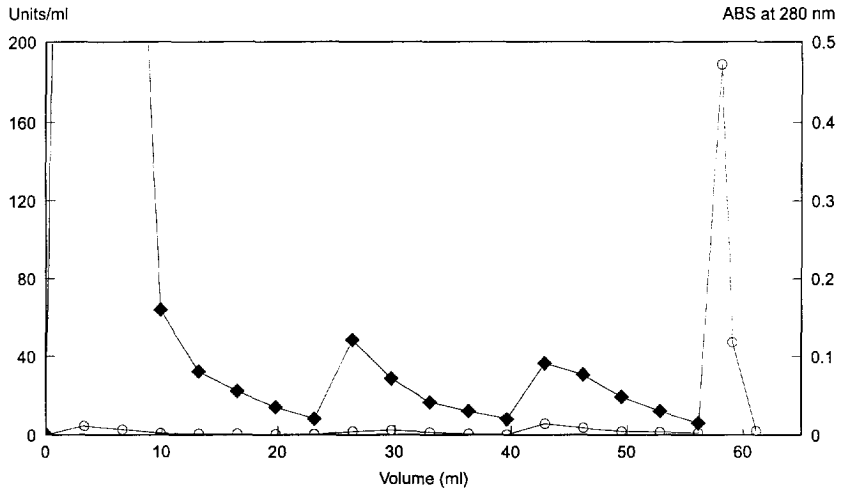


Fig. 1. Affinity chromatography of bovine heart extract on mercaptopyruvic-VBAR-Ultrogel A6R biomimetic adsorbent and the purification of LDH. Procedures were performed at 4°C. Dialysed cell-free extract (2370 μl, 26.3 mg protein, 356 units LDH) was applied at the biomimetic adsorbent (1 ml). Non-adsorbed protein was washed off with 23 ml of buffer followed by 2 × 16.5 ml of buffer containing 10 and 20 m*M* KCl. Bound LDH was eluted with equilibration buffer containing 0.1 m*M* NAD^+ and 1 m*M* sulfite (3 ml). Collected fractions were assayed for LDH (○) and protein (◆, A_{280} except for the fraction with NAD^+).

purification and 75% step recovery. However, when the pretreated extract was subsequently chromatographed on the BM 1 affinity adsorbent, the step purification on the affinity column remained unchanged (25-fold). Pretreatment of the starting extract by means of DEAE-cellulose anion-exchange chromatography (20 m*M* Tris–HCl, pH 7.4; desorption of bound LDH with 0.2 *M* KCl) led to 2.9-fold step purification and 71% step recovery. However, after the pretreated extract was chromatographed on a BM 1 adsorbent, the capacity of the affinity adsorbent was increased by 40% but the step purification remained approximately the same (≤26-fold). Therefore, since the purity of the final product could not be improved further, there was no need to perform any changes to the original protocol. Further, in order to demonstrate the superiority of biomimetic adsorbent BM 1 over the commercial dye, CB3GA bovine heart LDH was purified on immobilized CB3GA under the same conditions as for BM 1 (see legend to Fig. 1

Table 4
Summary of LDH purification from bovine heart extract

Step	Volume (ml)	Units (u)	Protein (mg)	SA (u/mg)	Purification (-fold)	Yield (%)
Crude extract	2.37	356	26.3	13.5	1	–
Step 1: Biomimetic dye chromatography (NAD^+–sulfite elution)	3.0	228	0.68	335	24.8	64
Step 2: Anion-exchange chromatography (KCl elution)	5.0	178	0.37	481	35.6	50

Procedures were performed at 4°C. The affinity adsorbent (1 ml) consisted of the ligand biomimetic mercaptopyruvate-VBAR immobilized on Ultrogel A6R (2.2 μmol dye/g moist gel). The agarose gel DEAE-Sepharose CL6B (1.6 × 1.5 cm I.D.) was employed as an anion exchanger. For experimental conditions, see text.

and Experimental). In this case both the specific activity and recovery of LDH were lower by 30% and 10%, respectively. Although such differences in adsorbent performance may numerically be regarded as undramatic, in practice, however, when the product purity approaches homogeneity, the above differences may prove to be significant and could mean an additional, third, step in the protocol had the biomimetic adsorbent not been used.

Upon protein chromatography with biomimetic adsorbent BM 1, we observed protein–protein competition phenomena, as became evident by frontal analysis experiments (Fig. 2). In an early phase (up to 4 ml of eluate), both total protein and LDH bound the adsorbent, although the former saturated the column faster. In the subsequent phase (4–8 ml of eluate), whereas adsorption of the total protein was accomplished, LDH kept binding the column. This observation indicates that protein–protein displacing phenomena operate during the binding process. Such phenomena have been observed before with dye ligand adsorbents [13,14,16,29].

The specific activity of our affinity-purified LDH, 335 u/mg (25°C) (step 1, Table 4), is substantially higher than for the respective commercial product suitable for analytical purposes (ca. 250 u/mg). However, in order to free LDH from remaining contaminating activity of malate dehydrogenase (MDH), it was necessary to add a second step to the purification protocol (step 2, Table 4), that of anion-exchange chromatography. Such a step does not affect significantly the speed and cost effectiveness of the total procedure, because the sample requires no dialysis prior to loading the adsorbent, and hence the LDH fraction obtained from the affinity adsorbent is applied directly to the anion exchanger. It is known that MDH, a nicotinamide nucleotide-dependent oxidoreductase, recognizes and binds anthraquinone dye ligands [6,39,40]. Further, the enzyme (mixture of mitochondrial and cytoplasmic activities) is present in

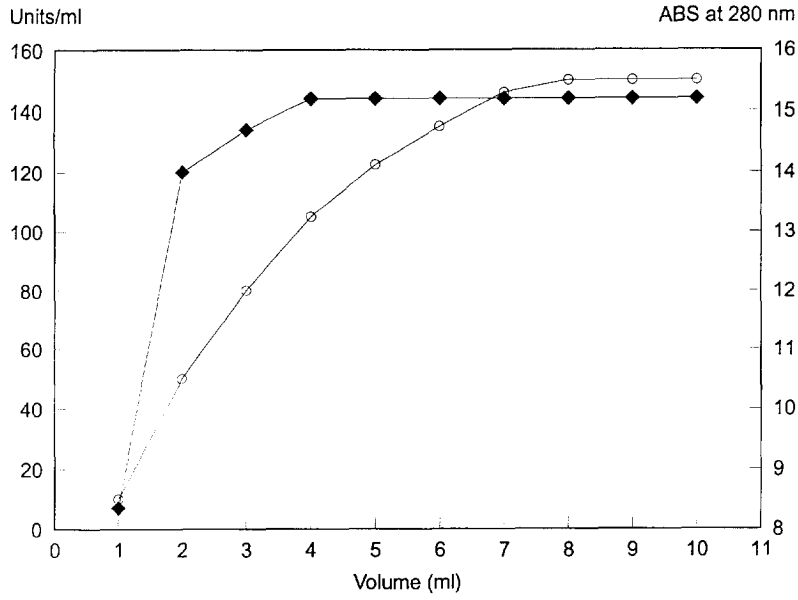


Fig. 2. Course of adsorption of bovine heart protein and LDH on mercaptopyruvic-VBAR-Ultrogel A6R biomimetic adsorbent. Procedures were performed at 4°C. A crude bovine heart extract [150 u LDH/ml, 11.1 mg protein/ml, in MOPS–NaOH buffer, 20 m*M*, pH 7.0, containing 0.2% (v/v) β-MeSH] was applied continuously to the biomimetic adsorbent (0.6 ml, 2.2 μmol/g wet gel) which was previously equilibrated in the same buffer. The application was stopped when the effluents had reached a constant activity of LDH. Collected fractions were assayed for LDH (○) and protein (◆, A_{280}).