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Effect of synthetic polymers, poly(N-vinyl pyrrolidone) and poly(N-vinyl caprolactam), on elution of lactate dehydrogenase bound to Blue Sepharose

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ABSTRACT

The synthetic polymers poly(N-vinyl pyrrolidone) and poly(N-vinyl caprolactam) are shown to be more efficient eluting agents of porcine muscle lactate dehydrogenase bound to Blue Sepharose than traditionally used NADH and oxamate or high salt concentration. Preliminary treatment of the Blue Sepharose column with a polymer solution drastically improved the elution effectiveness of the column and the enzyme recovery during "specific elution" with 10 m*M* oxamate and 0.1 m*M* NADH and "unspecific elution" with 1.5 *M* KCl. The effect of the polymers may be attributed to their ability to selectively mask sites on the matrix that are not specific for the nucleotide-binding region of the enzyme, while not seriously impairing interaction with specific sites.

INTRODUCTION

The reactive dye, Cibacron Blue 3 GA, is widely used as a ligand for "dye-affinity", "group-specific affinity" or "pseudoaffinity" chromatography of various proteins, especially dehydrogenases and kinases [1–3]. The success of chromatographic separations on Blue Sepharose (Sepharose to which Cibacron Blue is covalently bound) stems largely from the apparent specific binding between the nucleotide binding sites of these enzymes and the dye ligand [4]. The Cibacron Blue ligand can interact (apart from with nucleotide binding sites) unspecifically with the protein molecule as a result of hydrophobic and ion-exchange interaction. This fact may conceivably reduce the recovery of such a protein by "specific" elution with nucleotide. Unexpectedly low recoveries of nucleotide-dependent proteins in affinity chromatography on Cibacron Blue-containing matrices have been described [5–9].

High salt concentrations reduce protein-dye interactions, and most proteins can be eluted "unspecifically" from dye-containing matrices by l-l.5 *M* NaCl or KC1 [10]. Unspecific elution is often more efficient than specific elution [11].

Previously, we found that the synthetic polymer poly(N-vinyl caprolactam) (PVCL) interacts efficiently with Cibacron Blue and that this interaction is strong enough to prevent "specific" binding of the dye to lactate dehydrogenase (LDH) from porcine muscle [12]. The purpose of this work was to use PVCL and the related polymer, poly(N-vinyl pyrrolidone) (PVP) , for the improvement of elution of LDH from Blue Sepharose.

EXPERIMENTAL

Chemicals

Lactate dehydrogenase type XXX-S from porcine muscle, β-NADH grade III and PVP K

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26-35 with an average molecular mass of 40 000 and K 12-18 with an average molecular mass of 10 000 were purchased from Sigma. Oxamic acid was purchased from BDH. PVCL was produced by radical polymerization in isopropanol using azo-bis-isobutyronitrile as initiator [13]. The molecular mass of PVCL, 80 000, was calculated from viscosimetric data [14]. LDH was dialysed before use. Blue Sepharose was synthesized according to ref. 15. The Cibacron Blue content determined according to ref. 16 was 4.9 μmol per ml of swollen gel.

Chromatography

All chromatographic analyses were done at room temperature using a 1.8 x 0.9 cm I.D. column. All solutions introduced to the column were in 20 m*M* Tris–HCl buffer, pH 7.3. LDH (110-140 U) was applied to the column in a volume of 1.6 ml. LDH was eluted at a flow-rate of 0.16 ml/min; fractions were collected every 20 min.

The polymer elution was performed with 0.2 or 1% PVP-40 000 and 1% PVP-10 000 and 1% PVCL. The PVP passivation of the column 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 eluent and re-equilibration with 20 m*M* Tris–HCl buffer, pH 7.3. The unspecific elution was performed with 1.5 *M* KCl; the specific elution was performed with 10 m*M* oxamate + 0.1 m*M* NADH.

LDH activity was measured in the fractions according to ref. 17. The concentration of PVP or PVCL was measured as absorption of polymer-iodine complex at 480 nm, the complex being produced according to ref. 18.

RESULTS AND DISCUSSION

The efficiency of eluting agents was tested for some different compounds. Fig. 1 shows that 1% PVP-40 000 or PVCL solution is a more efficient unspecific eluting agent than a pulse of 1.5 *M* KCl. In all three cases binding was >99% and recovery was about 75%. Elution of LDH began with the front of polymer in the effluent. Elution with a lower PVP concentration, 0.2%, or with

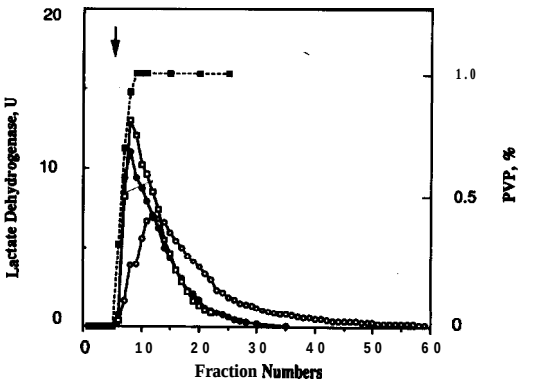

Fig. 1. LDH elution profile from Blue Sepharose with 1.5 *M* KCl (○), 1% PVP (●) and 1% PVCL (0). The arrow indicates when elution was begun. The polymer concentration in fractions eluted with 1% PVP is presented as ■ . Experimental conditions: 1.8 x 0.9 cm I.D. column; all solutions introduced to the column were in 20 m*M* Tris–HCl buffer, pH 7.3. Aliquots of 120 U (1.5 *M* KC1 and 1% PVP elution) or 135 U (1% PVCL elution) of LDH were applied to the column in a volume of 1.6 ml. LDH was eluted at a flow-rate of 0.16 ml/min. Fractions were collected every 20 min.

PVP of lower molecular mass (10 000) was less efficient (Fig. 2).

Thus, PVP and PVCL, owing to their strong interaction with Cibacron Blue, could displace LDH bound to Blue Sepharose. Neither polymer in 1% concentration had any effect on LDH

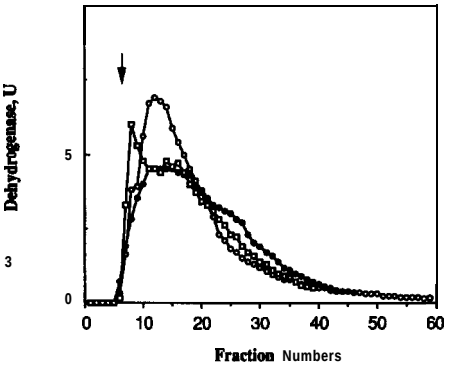

Fig. 2. LDH elution profile from Blue Sepharose with 1.5 M KC1 (0), 0.2% PVP (molecular mass 40000) (●) and 1% PVP (molecular mass 10 000) (0). The arrow indicates when elution was begun. Experimental conditions: 1.8 × 0.9 cm I.D. column; all solutions introduced to the column were in 20 m*M* Tris–HCl buffer, pH 7.3. Aliquots of 120 U of LDH were applied to the column in a volume of 1.6 ml. LDH was eluted at a flow-rate of 0.16 ml/min. Fractions were collected every 20 min.

activity. PVP is a commercially available, relatively cheap and biologically compatible polymer, and in some cases PVP elution might be a good alternative to specific elution with nucleotides or unspecific elution with high salt concentrations. PVP is widely used for plasma substitute production because the properties of the polymer solution resemble those of serum albumin [19].

Aqueous solutions of PVCL are characterized by a low critical solution temperature (LCST), and this property can be exploited for polymer recovery from the eluent [12]. On raising the temperature of aqueous solutions of PVCL to a point higher than the lower critical solution temperature (35–40°C), separation into two phases takes place. A polymer-enriched phase and an aqueous phase containing practically no polymer are formed. Both phases can be easily separated by low-speed centrifugation. This phase separation is reversible and PVCL readily dissolves in water on cooling [20,21]. LDH was completely recovered in the aqueous phase during the PVCL thermoprecipitation. The enzyme was stable enough at the temperatures at which phase separation took place [12].

The eluate after PVCL elution of LDH from the Blue Sepharose column was heated up to 45°C, which resulted in the precipitation of PVCL. The pellet was removed by centrifugation at 45°C. The supematant contained LDH in buffer solution with practically no polymer. The yield of LDH activity during this procedure was 85%. The PVCL pellet was resolubilized at room temperature with polymer recovery of 95–98%. The PVCL solution could be reused for LDH elution.

In Fig. 3 is shown the specific elution of LDH from Blue Sepharose using 10 m*M* oxamate + 0.1 m*M* NADH. After the pulse of specific eluent, a pulse of 1% PVP was added and more LDH was eluted. No LDH could be eluted by 10 m*M* oxamate + 0.1 m*M* NADH after PVP elution. This demonstrated that elution with PVP is efficient.

PVP bound strongly to the Blue Sepharose, and this resulted in a dramatic improvement in the elution effectiveness of the column during subsequent elution, either specific or unspecific

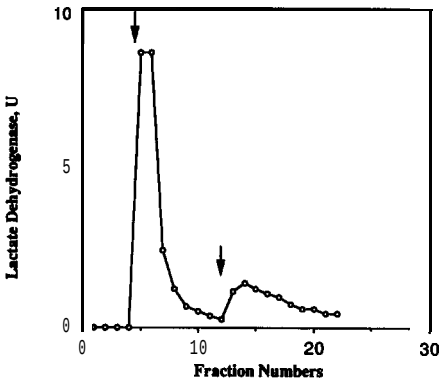

Fig. 3. LDH elution profile from Blue Sepharose with 10 m*M* oxamate and 0.1 m*M* NADH followed by 1% PVP elution. The arrows indicate when elution with 10 m*M* oxamate and 0.1 m*M* NADH and with 1% PVP was begun. Experimental conditions: 1.8 × 0.9 cm I.D. column; all solutions introduced to the column were in 20 m*M* Tris–HCl buffer, pH 7.3. 110 U of LDH were applied to the column in a volume of 1.6 ml. LDH was eluted at a flow-rate of 0.16 ml/min. Fractions were collected every 20 min.

(Fig. 4a and b, respectively). For comparison, LDH elution profiles from a polymer-untreated column under the same conditions are presented

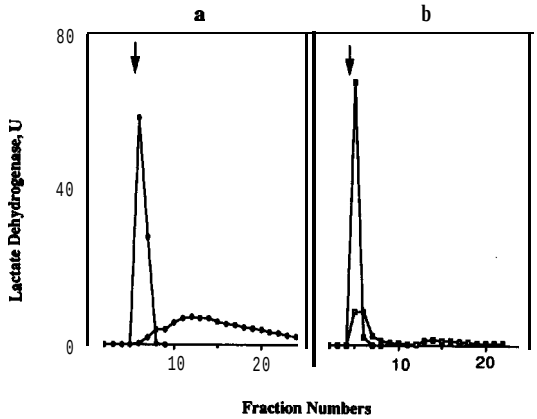

Fig. 4. LDH elution profile from PVP-treated Blue Sepharose with (a) 1.5 *M* KC1 (●) and (b) 10 m*M* oxamate and 0.1 m*M* NADH (■). The arrow indicates when elution was begun. For comparison, LDH elution profiles from an untreated column with (a) 1.5 *M* KC1 (0) and (b) 10 m*M* oxamate and 0.1 m*M* NADH (0) under the same conditions are presented. Experimental conditions: 1.8 × 0.9 cm I.D. The column was washed with 1% PVP solution and was then washed with 1.5 M KC1 until no PVP was detected in the eluent. Aliquots of 120 U of LDH were applied to the column in a volume of 1.6 ml. LDH was eluted at a flow-rate of 0.16 mllmin. Fractions were collected every 20 min.

TABLE I

EFFECT OF POLY(N-VINYL PYRROLIDONE) ON AFFINITY CHROMATOGRAPHY OF LACTATE DEHYDROGENASE ON BLUE SEPHAROSE

Experimental conditions are as described in the legend to Fig. 4.

Elution	LDH binding (%)		LDH recovery (%)	
	Untreated column	PVP-treated column	Untreated column	PVP-treated column
Unspecific	>99	>99	76	98
Specific	>99	>99	36	85

in Fig. 4a and b. Polymer treatment of the column improved LDH recovery, especially in the case of specific elution (Table I).

Coating with hydrophilic polymers has previously been used in chromatography of biomolecules either for imparting new properties to the matrix, e.g., formation of anion-exchange phases by cross-linking of polyimines with bifunctional glycidoxy compounds [22], or for decreasing unwanted interactions between protein and the matrix [23,24].

PVP can bind effectively various negatively charged dyes as a result of hydrophobic and ion-dipole interaction [19]. The affinity ligand used in this study, Cibacron Blue 3GA, is a dye of this type. PVP had no effect on LDH elution from unsubstituted Sepharose CL 4B and on peroxidase elution from concanavalin A–Sepharose (data not shown). We propose that the polymer bound tightly to the dye, each polymer molecule interacting with several Cibacron Blue molecules. This interaction was so efficient that PVP could not be completely eluted during re-equilibration of the column. PVP-ligand interaction is stronger than unspecific interaction of LDH with the ligand, but weaker than specific binding to Cibacron Blue ligand at the active site of the enzyme. The bound polymer rather prevented only weak unspecific interactions of LDH with the dye ligands. The realization of only specific interactions between the dye ligand and LDH resulted in an extremely sharp elution profile and improvement of enzyme recovery. After PVP passivation the column retained its high effectiveness of elution, at least in the course of three successive runs. The multisite attachment of PVP to the matrix is assumed to protect the polymer from complete replacement by LDH.

PVP treatment of the Blue Sepharose column thus eliminated many unspecific interactions. It was also noted that the binding capacity of the column was decreased by approximately a factor of two upon polymer treatment. These effects are now being studied in more detail.

There are thus several ways of eluting LDH bound to Blue Sepharose. Traditionally, KC1 has been used for unspecific elution and NADH + oxamate for specific elution. To these alternatives can now be added elution by certain polymers. It is still too early to say which method is ideal, since the model studies discussed in this paper all deal with pure enzyme preparations. Work is in progress in our laboratory to evaluate this new elution concept when isolating enzyme from crude homogenates. We hope to be able to report these experiments elsewhere.

A similar improvement in enzyme recovery after pretreatment with bovine serum albumin of a Blue Sepharose column was shown previously [8]. The authors ascribed the effect to the ability of albumin to selectively mask affinity sites on the matrix, which are not specific for the nucleotide-binding region of the enzyme, while not seriously impairing enzyme interaction with specific sites. This explanation seems to be reasonable also for PVP treatment of Blue Sepharose. The use of a synthetic biocompatible

polymer with low or no immunogenicity seems attractive as compared with adding new proteins to a purification system.

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