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Temperature-induced displacement of proteins from dye-affinity columns using an immobilized polymeric displacer

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Abstract

Poly(N-vinylcaprolactam) (PVCL), a polymer with a cloud point of 38°C, was used for the polymer shielding of Blue Sepharose. The globule–coil transition of the polymer was exploited in a dye-affinity column for the displacement of bound lactate dehydrogenase (LDH); elution was carried out without any change in the buffer composition by changing the temperature of the column. LDH from porcine muscle was bound to the PVCL-shielded column at 40°C. At this temperature LDH could not be eluted from the column with 0.1 *M* KCl. The decrease in temperature to *ca.* 23°C (room temperature) resulted in LDH elution with 0.1 *M* KCl. Crude porcine muscle extract was applied on the PVCL-shielded column at 40°C and the foreign proteins were washed out with 0.1 *M* KCl at 40°C. The flow was then interrupted, the column was cooled to room temperature and virtually homogeneous LDH was eluted with the same buffer. The purification factor was 17 and the recovery of LDH was 90%. This appears to be the first reported successful enzyme purification in which a temperature shift was used as the only eluting factor without changing the buffer composition.

1. Introduction

Reversible phase transitions in polymer solutions and critical phenomena in polymer gels have recently attracted attention because of their scientific and technological potential. These transitions may be initiated by small changes of temperature, pH, ionic strength, light, electric field or solvent composition [1]. Temperature change seems to be one of the most attractive triggers of such transitions. By using temperature-responsive polymers solutions can be manipulated without the addition of any substances, hence eliminating their removal at a later stage. In addition, the response of the system may be exploited many times simply by heating and cooling it repeatedly. The use of temperature-responsive polymers and non-ionic surfactants in biotechnology [2], as separation agents [3,4], for the preparation of biocatalysts [5], and for the design of intelligent materials [6] has been reviewed in detail during the last year.

Temperature-responsive polymers have serious potential in the development of chromatographic systems, as a small temperature shift can modify the binding and elution of target substances. One such attempt was made by Gewehr et al. [7]. Gel permeation chromatography was carried out using porous glass modified with a temperature-responsive polymer, poly(N-iso-

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propylacrylamide). The elution of dextrans of various molecular masses was strongly affected by the temperature due to a change in the effective pore size of the matrix. This can be attributed largely to the transition of polymer molecules from coils to globules on the surface of the pores of the glass beads.

Previously we have developed a concept of polymer-shielded dye-affinity chromatography [8,9]. Poly(N-vinyl pyrrolidone) (PVP) treatment of a Blue Sepharose column resulted in the binding of the polymer due to multi-point interaction with the dye ligands. The bound polymer molecules significantly decreased both the adsorption of foreign proteins and non-specific binding of the target enzymes without seriously impairing enzyme interactions with the dye ligands via specific nucleotide binding sites. The realization of only specific interactions improved recoveries and elution efficiency. In other words, the bound polymer served as a lid, opening the ligand for strong specific interactions but preventing more weak non-specific interactions [8,9].

The use of a temperature-responsive polymer, poly(N-vinylcaprolactam) (PVCL), for shielding in dye-affinity chromatography was believed not only to decrease the non-specific interactions, but also to manage the specific interactions in response to temperature. This work was carried out with the idea of developing a system in which the globule–coil transition of the polymer caused by a temperature shift could be used for the displacement of bound proteins without changing the buffer composition, and hence as the only eluting factor.

2. Experimental

Lactate dehydrogenase type XXX-S from porcine muscle, β-NADH Grade III, and Cibacron Blue 3GA were purchased from Sigma (St. Louis, MO, USA). Cibacron Blue 3GA was used as such, its concentrations were determined using extinction 13 600 l mol^{-1} cm^{-1} [10]. Oxamic acid was purchased from BDH (Poole, UK) and a molar absorptivity of N-vinylcaprolactam from Polysciences (Warrington, PA, USA). Blue Sepharose was synthesized by coupling Cibacron Blue 3GA to Sepharose CL-4B according to Ref. [11]. The Cibacron Blue content, determined according to Ref. [12], was 4.2 μmol g^{-1} dry gel. PVCL was synthesized by radical bulk polymerization according to Ref. [13] and had a molecular mass of about 40 000 (determined from intrinsic viscosity using the equation $[\eta] = 3.5 \cdot 10^{-4} M_w^{0.57}$ [14]) and a cloud point of 38°C in 20 m*M* Tris–HCl buffer (pH 7.3) in the presence of 0.1 *M* KCl.

Ground pork was purchased in a local shop and homogenized in ice-cold 20 m*M* Tris–HCl buffer (pH 7.3) containing 0.1 *M* KCl (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 (Grycksbo, Sweden) 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 on the Blue Sepharose column.

All chromatographic experiments with Blue Sepharose were carried out using a thermostated 1.3×0.9 cm I.D. column at a flow-rate of 0.17 ml min^{-1}. All solutions introduced on to the column were in 20 m*M* Tris–HCl buffer (pH 7.3). The elution of LDH was performed with 0.1 or 1.5 *M* KCl. Elution by a temperature shift was carried out as follows: the sample was applied at 40°C and eluted with 0.1 *M* KCl at the same temperature (in the case of pure LDH, to show that the enzyme was not eluted at these conditions, or in the case of the porcine muscle extract, to wash out foreign proteins). Then the pump was stopped, the column was cooled to 25°C and elution was continued at this temperature with the same buffer. The porcine muscle extract was applied to the column until breakthrough of LDH was observed. The column was washed with 0.1 *M* KCl in buffer until no further protein (monitored by the absorbance at 280 nm) was detected in the eluate. Fractions of 10 min (with commercial LDH) or 5 min (with crude extract) were collected.

The PVCL shielding of columns was performed with an excess of 1% polymer solution (about 50 column volumes) followed by washing with 1.5 *M* KCl (pH 3.4) and re-equilibration of the column with an appropriate buffer.

The complex formation of PVCL with Cibacron Blue in solution was studied by differential spectroscopy using a Shimadzu UV-260 double-beam spectrophotometer as described in Ref. [15].

LDH activity was measured in the fractions according to a reported procedure [16]. The inhibition of the LDH reaction in NADH to NAD^+ direction by Cibacron Blue was performed at 25 and 40°C in 20 m*M* Tris–HCl (pH 7.3) in the presence and absence of 0.1 *M* KCl.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% gel was performed according to Ref. [17] using soybean trypsin inhibitor (M_r 21 500), carbonic anhydrase (M_r 31 000), ovalbumin (M_r 45 000), bovine serum albumin (M_r 66 200), and phosphorylase B (M_r 97 400) as standards.

3. Results and discussion

Fig. 1 presents elution profiles (with 0.1 *M* KCl followed by 1.5 *M* KCl) of pure LDH from untreated and PVCL-shielded columns at room temperature (ca. 23°C) and at 40°C. The elution profiles at room temperature and at 40°C from the untreated column were similar, suggesting that this temperature shift had not substantial effect on the behaviour of the untreated column. This behaviour was in complete agreement with the effect of temperature on the specific binding of Cibacron Blue to LDH, which was estimated using the competitive inhibition constants.

Cibacron Blue binding constants to LDH determined by different methods are virtually identical [18]. Moreover, the inhibition constant reflects specific binding of the Cibacron Blue ligand with an active site of LDH without a contribution of non-specific binding. Addition of 0.1 *M* KCl increased the inhibition constants (and hence decreased the efficiency of Cibacron Blue binding) from 0.1 to 0.65 μM at 23°C and

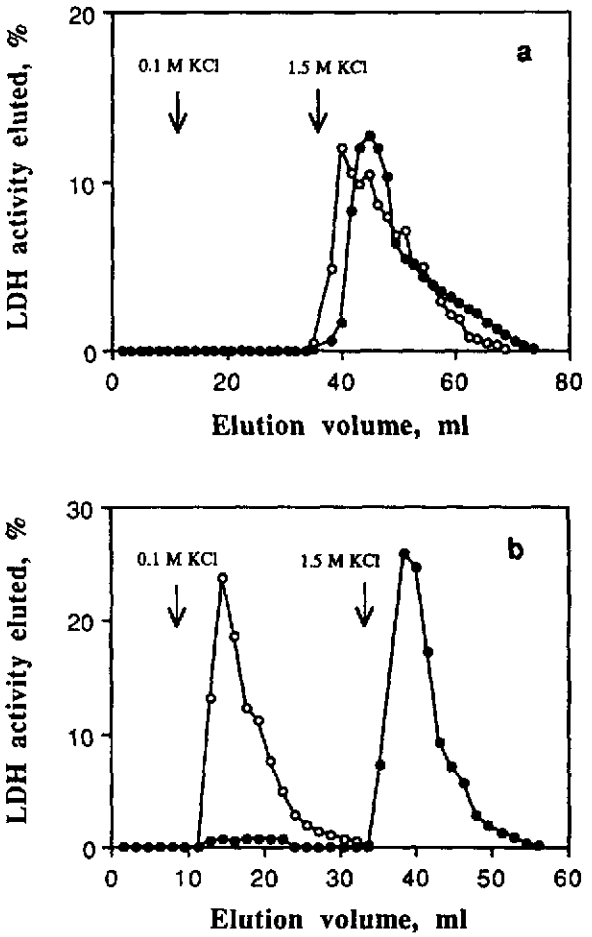

Fig. 1. Elution profile of LDH at (○) room temperature and (●) 40°C from (a) unmodified and (b) PVCL-shielded Blue Sepharose with 0.1 *M* KCl, followed by elution with 1.5 *M* KCl. Arrows indicate when elution with 0.1 *M* KCl and elution with 1.5 *M* KCl were begun. Experimental conditions: column, 1.3 × 0.9 cm I.D.; flow-rate, 0.17 ml min^{-1}; fractions collected every 10 min. The total amount of LDH eluted from the column was taken as 100% in all instances for the sake of comparison.

from 0.14 to 0.76 μM at 40°C. This small decrease in binding efficiency caused by 0.1 *M* KCl was not sufficient to elute LDH from untreated columns at either room temperature or 40°C.

The elution behaviour using the PVCL-shielded column was different from that using the unshielded column. PVCL shielding resulted in sharper elution profiles at 40°C, an observation in line with PVP shielding of Blue Sepha-

rose [8,9]. Most important was the temperature-induced change in the elution profiles. Whereas LDH was not eluted at all with 0.1 *M* KCl at 40°C, the enzyme was easily eluted with the same eluent from the PVCL-shielded column at room temperature. Hence the column behaviour was completely different at 23°C, a temperature below the cloud point of PVCL, and at 40°C, a temperature above the cloud point.

Fig. 2 presents the elution profiles of LDH from the PVCL-shielded column with 0.1 *M* KCl at different temperatures. The temperature was lowered to room temperature in the course of elution by interrupting the flow, cooling the column and continuing the elution again at room temperature. LDH is readily eluted with 0.1 *M* KCl at room temperature. Raising the temperature hindered elution, and virtually no enzyme was eluted at 40 and 45°C, the temperatures above the cloud point of PVCL. Cooling the column to room temperature resulted in elution of LDH with the same buffer. The buffer containing 0.1 *M* KCl is a much milder eluent, resulting in broader LDH elution profiles from polymer-shielded columns than those obtained by elution with 1.5 *M* KCl [9].

It is conceivable that PVCL binds to the matrix via multi-point interaction with Cibacron Blue ligands. In solution one polymer molecule of PVCL efficiently bound 7–8 molecules of Cibacron Blue with a binding constant of 1.8 μM. Owing to the multi-valent interaction of PVCL with Cibacron Blue ligands, the effective avidity of the polymer to the matrix is much higher than its affinity to the ligand.

Unfortunately, there is no convenient and sensitive method for the determination of PVCL. Hence it is difficult to determine amount of PVCL bound to the column. The binding of polymer to the column resulted in a decrease in the enzyme binding capacity of the column. This effect has been demonstrated and discussed in detail [8]. The dye-affinity column was treated with an excess of PVCL solution. Hence the polydispersity of the polymer bound to the column will be determined not only by the polydispersity of the applied PVCL but also by the relative avidity of polymer molecules with different molecular masses to the matrix.

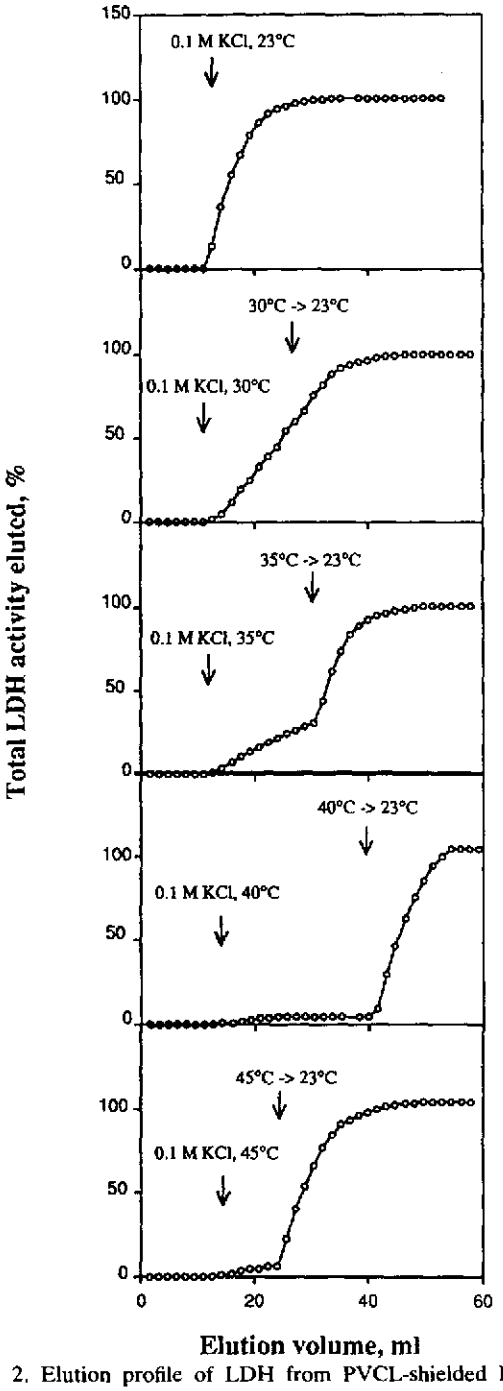

Fig. 2. Elution profile of LDH from PVCL-shielded Blue Sepharose with 0.1 *M* KCl at different temperatures. Arrows indicate when elution was interrupted, the column was cooled to room temperature and elution was continued at this temperature. Experimental conditions: column, 1.3 × 0.9 cm I.D.; flow-rate, 0.17 ml min^{-1}; fractions collected every 10 min. The total amount of LDH eluted from the column was taken as 100% in all instances for the sake of comparison.

The binding of Cibacron Blue to PVCL was independent of temperature at temperatures below the cloud point of PVCL where the polymer molecules were in the form of loose coils. When bound to the Cibacron Blue molecules coupled to the matrix, polymer molecules could efficiently compete with LDH for dye-ligands. The small decrease in LDH binding efficiency caused by the addition of 0.1 *M* KCl was sufficient to elute the enzyme from PVCL-shielded column at room temperature. The strong multi-point attachment of the PVCL to the matrix protects against complete removal of the polymer from the column. The PVCL-shielded column was used repeatedly for about ten runs without losing its temperature-responsive property. Above the transition temperature, PVCL molecules form compact globules striving for aggregation and formation of a separate phase. The polymer shielding decreased, so Cibacron Blue ligands were more available for both specific and non-specific interactions with LDH. Elution with 0.1 *M* KCl in that event was inefficient, and a more robust eluent was required. Above the transition temperature the PVCL-shielded column behaved similarly to the untreated column, although some effect of the polymer resulted in a sharper elution profile. Cooling of the PVCL-shielded column with bound LDH resulted in the transition of immobilized polymer molecules from globules to flexible coils. Owing to their relative flexibility, these coils could interact with more ligands than could compact polymer globules. This interaction of PVCL molecules is efficient enough to displace the LDH molecules bound to the Cibacron Blue ligands. Hence cooling the column resulted in displacement of bound LDH by immobilized polymer and hence in complete elution of enzyme without any changes in buffer composition.

The effect of temperature on the elution of different proteins is well documented in protein chromatography [7,19–26]. The increase in the affinity of monoclonal antibodies to oligosaccharides with decrease in temperature was used to optimize the resolution [24]. sAMP synthetase elutes in a sharper elution peak from a Cibacron Blue column at 20 than 4°C under otherwise identical conditions [25]. Binding of 6-phosphogluconate dehydrogenase from the thermophilic organism *Bacillus stearothermophilus* to Cibacron Blue and Procion Red HE 3B columns increases with increasing temperature from 5 to 50°C [26]. Nevertheless, the temperature was never a critical variable in binding or elution in dye-affinity chromatography [26].

The effect of temperature in hydrophobic chromatography is also not so drastic. In hydrophobic chromatography to alter the binding efficiency significantly by changing only the temperature requires the use of exotic conditions. We were able to find only one report of elution by means of a temperature shift [23]. A mixture of ovalbumin, catalase and chymotrypsinogen A was separated by hydrophobic chromatography on phenylbutylamine at sub-zero temperatures. At −15°C, ovalbumin and catalase were eluted with 30% ethylene glycol in a void volume, while chymotrypsinogen A remained bound to the column. An increase in temperature to 4°C resulted in the elution of chymotrypsinogen A with the same eluent. While low temperatures are healthy for enzyme purification, although the work at −15°C might be complicated, the use of 30% ethylene glycol is harmful to the activity of many enzymes. Certainly, the use of such procedures is restricted to analytical applications.

The drastic difference in the elution of LDH from the PVCL-shielded column at room temperature and 40°C can hardly be attributed to a simple decrease in efficiency of hydrophobic interaction. No LDH was eluted from the untreated Blue Sepharose column either at room temperature or at 40°C with 20% ethylene glycol (the use of higher ethylene glycol concentrations was prohibited by the progressive inactivation of LDH). Subsequent elution with 1.5 *M* KCl resulted in LDH recoveries of 71% and 48%, respectively. Hence the coil–globule transition of PVCL polymer bound to the Blue Sepharose induced by raising the temperature is the most reasonable explanation for the different column behaviours at room temperature and 40°C.

This property of a PVCL-shielded column was used for the development of a dye-affinity system where temperature was the only means of elution without changing the buffer composition.

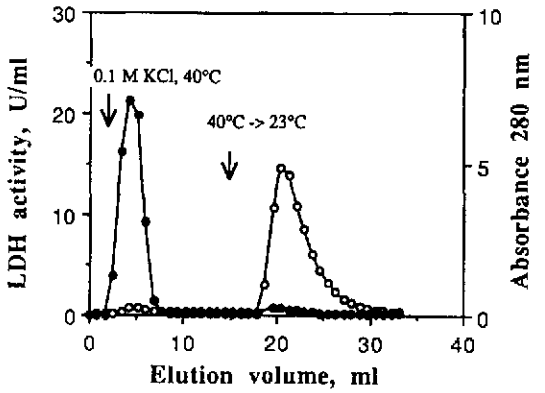

Fig. 3. Elution profile of (○) LDH activity and (●) protein with 0.1 *M* KCl from PVCL-shielded Blue Sepharose. The crude extract was applied on a column at 40°C. Arrows indicate when elution with 0.1 *M* KCl at 40°C was begun and when the column was cooled to room temperature and elution was continued at this temperature. Experimental conditions; column, 1.3 × 0.9 cm I.D.; flow-rate, 0.17 ml min^{-1}; fractions collected every 5 min.

Crude porcine muscle extract was applied to a column at 40°C until breakthrough, the foreign proteins were washed out with 0.1 *M* KCl at 40°C, the flow was interrupted, the column was cooled to room temperature and finally LDH was eluted with the same buffer (Fig. 3). The chromatographic procedure resulted in virtually homogeneous LDH (Fig. 4) with a purification factor of 17 and recovery of 90% of the enzyme.

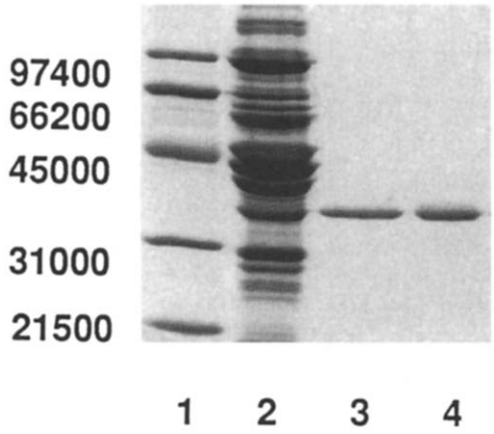

Fig. 4. SDS-PAGE patterns for LDH. Lanes: 1 = marker proteins; 2 = crude extract; 3 = LDH eluted after a temperature decrease from 40°C to room temperature (PVCL-shielded Blue Sepharose, 0.1 *M* KCl); 4 = commercial sample.

To the best of our knowledge, this is the first reported successful enzyme purification where the temperature shift is used as the only eluting factor without changing the buffer composition. PVCL, a polymer with a globule–coil transition temperature of 38°C, was used in this work. The properties of the polymer forced us to operate at a fairly high temperature (40°C) from the biotechnological point of view. Modern polymer chemistry can provide polymers with different transition temperatures, low enough to meet the requirements of biomolecule stability [2]. The use of a temperature shift as the only eluting factor in polymer-shielded dye-affinity chromatography is quite attractive. Relatively small temperature changes can be designed in a fast and convenient mode, replacing tedious procedures of column re-equilibration after elution buffer and purification of target enzyme from high salt concentrations used for elution in traditional dye-affinity chromatography.

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