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# Purification of lactic acid dehydrogenase from bovine heart crude extract by counter-current chromatography

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**Abstract**

To test the utility of counter-current chromatography in purifying proteins, lactic acid dehydrogenase (LDH) was extracted from a crude bovine heart filtrate using a cross-axis coil planet centrifuge. The purification was performed with several polymer phase systems composed of 16% (w/w) poly(ethylene glycol) (PEG) 1000–12.5% (w/w) potassium phosphate buffers and 4.4% (w/w) PEG 8000–7.0 (w/w) dextran T500 at pH values ranging from 6.5 to 11.0. The best purification was achieved using PEG 1000–potassium phosphate buffer system at pH 7.3 by eluting the upper phase at 1.0 ml/min. Fractions were analyzed by LDH enzymatic activity and sodium dodecyl sulfate slab gel electrophoresis (SDS-PAGE). The LDH was purified directly from bovine heart crude extract within 3 h. © 1997 Elsevier Science B.V.

*Keywords:* Lactic acid dehydrogenase; Enzymes

## 1. Introduction

Counter-current chromatography (CCC) is a form of liquid–liquid partition chromatography in which the stationary phase is retained in the column with the aid of a centrifugal force field. This chromatographic system eliminates various complications arising from the use of a solid support [1–3]. Among all the existing CCC systems, high-speed CCC is the most advanced form in terms of partition efficiency and separation time [4]. In the mid-1980s, the cross-axis coil planet centrifuge (CPC) was developed for performing high-speed CCC and its recent model has substantially improved the retention of the stationary phase of polar solvent systems including aqueous–aqueous polymer phase systems specifically designed for the separation of proteins [5–7]. This cross-axis CPC has been successfully used for the separation and purification of recombinant uridine phosphorylase from *Escherichia coli* lysate [8] and human serum lipoproteins [9–11]; all employ aqueous–aqueous polymer phase systems. All these works are, however, purifications of samples which contain relatively large quantities of target proteins. It is necessary to demonstrate the capability of the method to separate minute quantities of proteins from a crude biological sample.

In this paper, the CCC technique was applied to the purification of lactic acid dehydrogenase (LDH) from a bovine heart crude extract using two different

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types of polymer systems, one composed of 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate buffer and the other composed of 4.4% (w/w) PEG 8000–7.0% (w/w) dextran T500, at pH ranging from 6.5 to 11.0. The enzymatic activity was retained and measured in the LDH fractions, and the protein purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with coomassie brilliant blue staining [12].

## 2. Experimental

### 2.1. Apparatus

The cross-axis CPC has a unique feature among the CPC systems available in that it provides reliable retention of the stationary phase for viscous polymer phase systems. The detailed design of the cross-axis CPC was described earlier [13,14]. The present apparatus holds a pair of horizontal rotary shafts, symmetrically mounted one on each side of the rotary frame, at a distance of 10 cm from the centrifuge axis. A spool-shaped column holder is mounted on each rotary shaft at an off-center position 10 cm from its mid-point. The large multilayer coil separation column was prepared from a 2.6 mm I.D. polytetrafluoroethylene (PTFE) tube (Zeus Industrial Products, Raritan, NJ, USA) by winding it onto a 5.0 cm diameter holder hub, forming three layers of left-handed coils between a pair of flanges spaced 5 cm apart. A pair of columns mounted on the rotary frame were connected in series with a flow tube (PTFE, 0.85 mm I.D.) to give a total capacity of 165 ml. The speed of the apparatus is regulated at 500 rpm with a speed control unit (Bodine Electric, Chicago, IL, USA).

### 2.2. Reagents

LDH from a chicken heart (4000 IU/5 mg) was obtained from Oriental Yeast (Osaka, Japan) for the determination of the partition coefficient values. Dextran T500 (weight-average molecular mass, $M_r$= 500 000), poly(ethylene glycol) (PEG) 1000 (number-average molecular mass, $M_n$=950–1050), PEG 8000 ($M_n$=6000–7500), mono and dibasic potassium phosphates for the preparation of aqueous polymer two-phase systems were purchased from Kanto Chemicals (Tokyo, Japan). Acrylamide for electrophoresis was obtained from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were all of analytical reagent grade.

### 2.3. Preparation of bovine heart crude extract

About 40 g of bovine heart tissue was homogenized with 30 ml of ice-cold water in a household mixer for 10 min. The homogenate was centrifuged at 400 *g* for 30 min and the supernatant was collected and filtered using absorbent gauze to remove the cell debris. The filtrate was again filtered through a Millipore filter (pore size: 0.22 μm) (Nihon Millipore Kogyo K.K., Yonezawa, Japan) before loading onto the CCC column. The bovine heart crude extract thus obtained was stored at −20°C until use.

### 2.4. Preparation of PEG–potassium phosphate and PEG–dextran aqueous two-phase solvent systems

The PEG–phosphate polymer phase systems were prepared by dissolving 192 g of PEG 1000 and 150 g of potassium phosphate in water. The ratio of monobasic to dibasic potassium phosphates determines the pH of the solvent system and affects the partition coefficients of the proteins. These solutions form two layers; the upper layer is rich in PEG and the lower layer is rich in potassium phosphate.

The PEG 8000–dextran T500 systems are similarly prepared by dissolving 44 g of PEG 8000 and 70 g of dextran T500 in 886 g of 10 m*M* potassium phosphate buffer solution. The pH of the system is also adjusted by choosing the proper ratio between the monobasic and dibasic potassium phosphates. This two-phase system consists of the PEG-rich upper phase and dextran-rich lower phase. The solutions were thoroughly mixed in a separating funnel and allowed to settle into two clear layers before use.

### 2.5. Measurement of stationary phase retention in multilayer coil separation columns

In the cross-axis CPC, the effects of the internal diameter of the coiled tube and the number of coiled

layers around the column holder on the stationary phase retention were investigated using a typical polymer phase system for protein separation. In each experiment, the coil was first filled entirely with the upper stationary phase of the 16% (w/w) PEG 1000–12.5% (w/w) $K_2HPO_4$ two-phase system. Then the cross-axis CPC was rotated at 500 rpm while the lower mobile phase was pumped into the column at a flow-rate of 1.0 ml/min. Elution continued until the total elution volume exceeded the column capacity. Then the centrifuge was stopped and the column contents emptied into a graduated cylinder by connecting the inlet of the column to a pressured nitrogen line thus measuring the stationary phase volume to the total column capacity.

### 2.6. Determination of partition coefficient of lactic acid dehydrogenase

The partition coefficients of the LDH were determined at various concentrations, in the two-phase solvent systems composed of PEG 1000 and potassium phosphate dissolved in distilled water and PEG 8000 and dextran dissolved in 10 m*M* potassium phosphate buffers at various pH values. The enzyme solution containing 200 IU of LDH was partitioned in 3 ml of the polymer two-phase systems (1.5 ml of each phase). Aliquots of the upper and lower phases were diluted ten times with distilled water and LDH enzyme activities in both phases were determined with the method described by Babson and Phillips [15].

### 2.7. Counter-current chromatography of bovine heart crude extract

In each experiment, the CCC column was first entirely filled with the stationary phase, either upper or lower phase, followed by injection of the sample solution (a mixture of 3 ml of bovine heart crude extract and PEG, dextran or potassium phosphate to meet the composition of the two-phase solvent system used for the separation). Then the other phase was eluted through the column at the desired flow-rate while the apparatus was rotated at 500 rpm. The effluent from the outlet of the column was continuously monitored with an EYELA UV-9000 absorbance monitor (Tokyo Rikakikai, Tokyo, Japan) at 280 nm and fractionated into a Bio-Rad Model 2110 fraction collector (Bio-Rad Labs., Richmond, CA, USA).

### 2.8. Measurement of enzyme activity

LDH in the CCC fractions was determined by enzymatic analyses [15]. When lactic acid, the substrate of LDH, is oxidized to pyruvic acid by LDH in the CCC fractions, NAD (nicotinamide adenine dinucleotide) is reduced to NADH, which in turn reduces nitrotetrazolium blue in the reaction mixture to form diformazan in the presence of diaphorase. The blue color of this product was measured by absorbance at 560 nm. The LDH enzymatic activities in the CCC fractions were determined using the calibration curves constructed from the standard LDH solutions.

### 2.9. Characterization of lactic acid dehydrogenase and other proteins by SDS-PAGE

LDH in the CCC fractions was characterized by SDS-PAGE according to the method of Laemmli [12]. Gels containing 3% (w/v) (stacking gel) and 12% (w/v) (separation gel) were prepared from stock solutions of 30% (w/v) acrylamide and 0.8% (w/v) N,N′-methylene-bisacrylamide. A 5.5×10 cm separation gel and a 1.0×10 cm stacking gel, each 0.75 mm thick, were prepared between glass plates. A 5 ml volume of concentrated eluate was mixed with 95 ml of sample solution [a mixture of 0.025 *M* tris-(hydroxymethyl) aminomethane, 2% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 4% (w/v) glycerol and 0.01% (w/v) bromophenol blue (BPB)] and 10–20 ml of the sample solution was loaded over the stacking gel. Electrophoresis proceeded at a current of 10 mA until the BPB marker reached the bottom of the separation gel. The migrated LDH and proteins were stained for 5 min at room temperature with a staining solution composed of 0.25% (w/v) coomassie brilliant blue, 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was destained by washing in a mixture of 7.5% (v/v) acetic acid and 2.5% (v/v) methanol.

## 3. Results and discussion

### 3.1. Stationary phase retention

CCC is a support-free liquid–liquid partition chromatographic technique where the liquid stationary phase is retained in the column by the aid of a centrifugal force field. Therefore, good retention of the stationary phase is critical, and it is greatly affected by the internal diameter of the coiled tube. As mentioned elsewhere [6], even at a flow-rate of 1.0 ml/min, the cross-axis CPC holds the stationary phase near 50% of the total column capacity, promising an efficient peak resolution of LDH in a short elution time. In the present study, the percentage retention of the polymer phase system composed of 16% (w/w) PEG 1000–12.5% (w/w) $K_2HPO_4$ was examined in three columns with different internal diameters at a flow-rate of 1.0 ml/min.

Table 1 shows the dimensions of the different coaxial multilayer columns together with the retention of the PEG 1000-rich upper phase. No retention was observed in the small internal diameter coiled tube (0.9 mm I.D.) probably due to a strong wall interaction of the viscous solvent system. Increasing the internal diameter of the tube to 2.6 mm I.D. results in a high retention of the stationary phase. The retention further increases from 46.4 to 48.0% as the coil dimension was increased from single to triple-layers. On the basis of these findings, we selected the column of 2.6 mm I.D. tube wound into triple-layers onto the column holder.

Table 1
Dimensions of different coaxial multilayer columns used for lactic acid dehydrogenase purification

| Tube I.D. (mm) | Coil layers | Total length (m) | Column capacity (ml) | Stationary phase retention (%)[a] |
|---|---|---|---|---|
| 0.9 | 4 | 75 | 48 | 0 |
| 2.0 | 2 | 20 | 63 | 41.3 |
| 2.6 | 1 | 10 | 53 | 46.4 |
| 2.6 | 3 | 32 | 165 | 48.0 |

[a] Upper phase of 16% PEG 1000–12.5% potassium phosphate at pH 9.2. Lower phase mobile at flow-rate of 1.0 ml/min.

### 3.2. Partition coefficient of the standard LDH

Fig. 1 shows the partition coefficients of the standard LDH plotted against the pH of two different polymer phase systems: 4.4% (w/w) PEG 8000–7.0 (w/w) dextran T500–100 m*M* potassium phosphate buffer and 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate. The isoelectric point of LDH is p*I* 6.3, so the enzyme must have a net negative charge at pH 8.0. In both solvent systems, the partition coefficients of LDH are close to unity at pH from 7.0 to 8.0 but sharply increase at pH 8.0. Thus pH 7.3 was selected. LDH is more evenly distributed between the two phases in the PEG 1000–potassium phosphate system at pH 7.3 and in the PEG 8000–dextran T500 system at pH 7.5, respectively. Therefore, these two solvent systems were chosen for the LDH purification by CCC.

### 3.3. Counter-current chromatographic purification of LDH from bovine heart crude extract

Fig. 2 shows the elution profile of the bovine heart crude extract obtained by the cross-axis CPC using the 4.4% PEG 8000–7.0% dextran T500 aqueous polymer system at pH 7.5. The sample solution containing 3 g of the crude extract, 0.15 g of PEG 8000 and 0.26 g of dextran T500 was injected through the sample port. The separation was performed at 500 rpm at a flow-rate of 1.0 ml/min using the PEG-rich upper phase as the mobile phase.

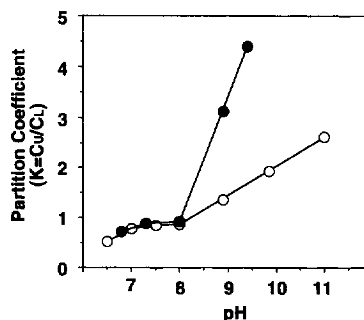


Fig. 1. Partition coefficient of lactic acid dehydrogenase in the poly(ethylene glycol) (PEG) 1000–potassium phosphate buffers (●) and PEG 8000–dextran T500 (○) polymer phase systems.

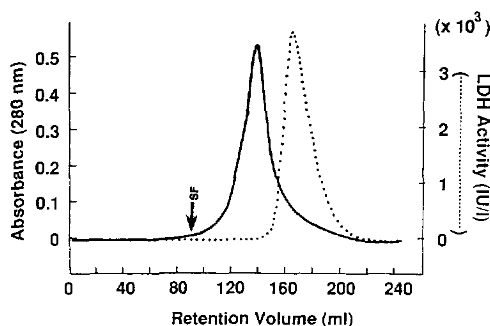


Fig. 2. Counter-current chromatography of bovine heart homogenate using the dextran–PEG system. Experimental conditions: apparatus: the XL cross-axis CPC with a pair of multilayer coils columns of 2.6 mm I.D. and 165 ml capacity; solvent system: 4.4% (w/w) PEG 8000–7.0% (w/w) dextran T500–100 m*M* potassium phosphate buffer at pH 7.5; stationary phase: dextran-rich lower phase; mobile phase: PEG 8000-rich upper phase; sample: bovine heart crude extract in 3 ml of the solvent system (1.5 ml each phase); flow-rate: 1.0 ml/min; revolution: 500 rpm; stationary phase retention: 19% of the total column capacity; SF=solvent front.

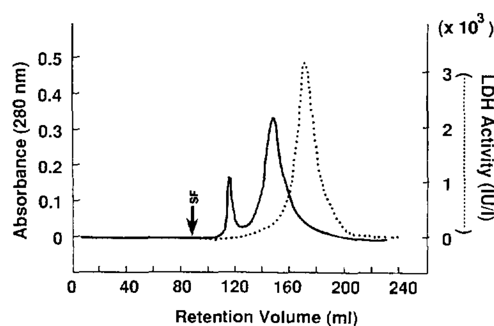


Fig. 3. Counter-current chromatography of bovine heart homogenate using PEG 1000–potassium phosphate system with the upper phase mobile. Experimental conditions: apparatus: the XL cross-axis CPC with a pair of multilayer coils columns of 2.6 mm I.D. and 165 ml capacity; solvent system: 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate at pH 7.3; stationary phase: potassium phosphate-rich lower phase; mobile phase: PEG 1000-rich upper phase; sample: bovine heart crude extract in 3 ml of the solvent system (1.5 ml each phase); flow-rate: 1.0 ml/min; revolution: 500 rpm; stationary phase retention: 45% of the total column capacity; SF=solvent front.

The solvent front emerged at the 19th fraction (95 ml). The absorbance of proteins at 280 nm (solid line) in the fractions and the LDH activity (dotted line) are plotted against the retention volume. The majority of protein mass was eluted in the fractions from 20 to 36 (from 100 ml to 180 ml retention volume), whereas the enzyme activity of the LDH was detected at the fractions from 30 to 40 (150 ml to 200 ml). The retention of the stationary dextran T500-rich lower phase after the purification was 19% of the total column capacity. The results indicates that the LDH fractions are overlapped with other proteins, and we concluded that the PEG 8000–dextran T500 polymer phase system is not suitable for the present purpose.

The other polymer phase systems, the PEG 1000–potassium phosphate buffers, were then tested for the purification of LDH from the same extract. Fig. 3 shows the chromatograms of the bovine heart crude extract obtained by the cross-axis CPC using the 16.0% (w/w) PEG 1000–12.5% (w/w) potassium phosphate at pH 7.3, where the potassium phosphate buffer-rich lower phase was used as the stationary phase. The separation was similarly performed at 500 rpm at a flow-rate of 1.0 ml/min using the PEG 1000-rich upper phase as the mobile phase. The solvent front emerged at the 18th fraction (90 ml). Two protein peaks are indicated by the solid line and the LDH activity by the dotted line. The majority of the protein mass is eluted immediately after the solvent front in the fractions 18–34 or 90–170 ml of the retention volume. The LDH activities were found in fractions from 28 (140 ml) to 40 (200 ml) and the second protein peak is seen to be partly overlapped with the LDH peak.

In order to further improve the results, the PEG 1000-rich upper phase was used for the stationary phase, and the potassium phosphate buffer-rich lower phase as the mobile phase. The chromatograms of the total proteins (solid line) and the LDH activity (dotted line) are shown in Fig. 4 where the elution profile of proteins show three main peaks. The proteins and LDH were eluted from the column in the reverse order of their partition coefficient values and LDH was well resolved from the proteins. The LDH is eluted between second and third peaks. The fractions corresponding to the LDH peak showed the LDH activity in about 50 ml of fractions. The fractions were analyzed by 12.0% (w/v) SDS-PAGE with coomassie brilliant blue staining (Fig. 5). This

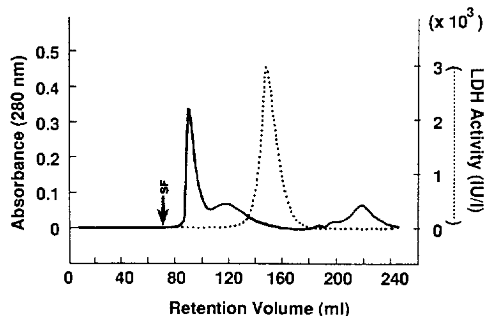


Fig. 4. Counter-current chromatography of bovine heart homogenate using the PEG 1000–potassium phosphate system with the lower phase mobile. Experimental conditions: apparatus: the XL cross-axis CPC with a pair of multilayer coils columns of 2.6 mm I.D. and 165 ml capacity; solvent system: 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate at pH 7.3; stationary phase: PEG 1000-rich upper phase; mobile phase: potassium phosphate-rich lower phase; sample: bovine heart crude extract in 3 ml of the solvent system (1.5 ml each phase); flow-rate: 1.0 ml/min; revolution: 500 rpm; stationary phase retention: 45% of the total column capacity; SF=solvent front.

indicates that the LDH is actually in the 140–170 ml fractions without detectable contamination of other proteins. Traditional purifications techniques using multi-step procedures – such as precipitation with ammonium sulfate, centrifugation, and dialysis – were very tedious and time-consuming. The use of the cross-axis CPC and the aqueous polymer phase presents a very simple method and LDH was purified within 3 h.

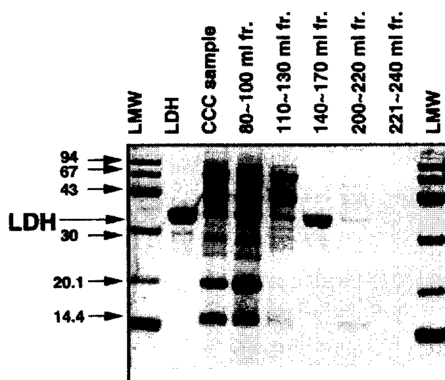


Fig. 5. SDS-PAGE profile of the fractions collected from counter-current chromatographic separation of lactic acid dehydrogenase from bovine heart crude extract.

## 4. Conclusions

The results of the above studies indicate that the purification of LDH from bovine heart crude extract could be performed in a one-step operation in the cross axis CPC with a PEG 1000–potassium phosphate system. The apparatus provides a satisfactory retention of the stationary phase at 1.0 ml/min, thus facilitating the use of polymer phase systems for the separation of various biopolymers. These results show that with relatively simple manipulation of several parameters (buffer, polymer molecular mass, rotation speed) CCC is well suited to the rapid purification of proteins from very crude extracts. Its ability to preserve enzymatic activity in the case of LDH is noteworthy and we expect that because of the absence of solid support surfaces used in other types of protein chromatography, activity will be preserved in other cases as well. Studies are underway to establish this point.

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