ELSEVIER

Journal of Chromatography B, 776 (2002) 183–189

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

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

Yoichi Shibusawa[a,*], Naomi Misu[a], Heisaburo Shindo[a], Yoichiro Ito[b]

[a]*Department of Analytical Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

[b]*Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Received 12 February 2002; received in revised form 14 May 2002; accepted 14 May 2002

**Abstract**

pH-peak focusing counter-current chromatography (CCC) was applied to the purification of lactic acid dehydrogenase (LDH) from a crude bovine heart extract using a cross-axis coil planet centrifuge (CPC). The experiment was performed with two sets of polymer phase systems composed of 16% (w/w) polyethylene glycol (PEG) 1000–12.5% (w/w) potassium phosphate buffer and 15% (w/w) PEG 1540–15% (w/w) ammonium sulfate each at various pH values. The best result was achieved from the PEG 1540–ammonium sulfate polymer phase system by adding a retainer (10 m*M* acetic acid) to the upper stationary phase and an eluter (100 m*M* sodium hydroxide) to the lower mobile phase. At a flow-rate of 0.5 ml/min, LDH was eluted as a sharp peak which was well resolved from other proteins. Collected fractions were analyzed by the LDH enzymatic activity and by sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis to detect contaminated proteins. LDH was purified directly from crude bovine heart extract in a concentrated state.


*Keywords:* pH-peak focusing; Counter-current chromatography; Lactic acid dehydrogenase

## 1. Introduction

Counter-current chromatography (CCC) is a liquid–liquid partition chromatography free of solid support matrices [1–4] where the stationary phase is retained in the coiled column by the aid of unit gravity or a centrifugal force. Consequently, the system eliminates all complications, such as solute adsorption and denaturation caused by the solid supports. Among all the existing CCC systems, high-speed CCC is the most advanced form in terms of partition efficiency and the separation time. The cross-axis coil planet centrifuge (CPC) has been particularly developed for retaining large amounts of the stationary phase for low-interfacial-tension, viscous solvent systems effectively used for separation of polar compounds [5–7]. The cross-axis CPCs have been successfully used for the separation of cytochrome *c*, myoglobin, ovalbumin and hemoglobin [8], for the separation of basic histones from others

*Corresponding author. Tel.: +81-426-764-544; fax: +81-426-764-542.

*E-mail address:* sibusawa@ps.toyaku.ac.jp (Y. Shibusawa).

1570-0232/02/$ – see front matter 
PII: S1570-0232(02)00348-3

and of α-globulin and human serum albumin [9]. The method was applied to the purification of recombinant enzymes such as purine nucleoside phosphorylase [10], uridine phosphorylase [11] and ketosteroid isomerase [12] from *Escherichia coli* lysate, human lipoproteins from human serum [13–15], lactic acid dehydrogenase (LDH) from a crude bovine heart extract [16], proteins from chicken egg white [17,18] and cholinesterase from human serum [19] using polymer phase systems.

In the course of the CCC separation of protein mixture containing cytochrome *c*, myoglobin, ovalbumin and hemoglobin by the conventional CCC technique, we observed that the ovalbumin produced a very broad peak with a long elution time [8]. In order to improve the separation we applied the pH-peak focusing CCC techniques [20] which produced sharp elution peaks of cytochrome *c*, myoglobin and ovalbumin by eluting with the mobile phase at pH 6.8 [21]. A new CCC technique described here yields sharp elution peaks by manipulating the pH of both stationary and mobile phases. In the present studies, this technique was applied for the purification of LDH from a crude bovine heart extract using the cross-axis CPC.

## 2. Experimental

### *2.1. Apparatus*

The pH-peak focusing CCC of LDH was performed using type-XL cross axis CPC (X-axis CPC) shown in Fig. 1. The apparatus holds a pair of horizontal rotary shafts symmetrically, one on each side of the rotary frame, at a distance of 10 cm from the central axis of the centrifuge. A spool-shaped column holder is mounted on each rotary shaft at a lateral location 10 cm away from the midpoint. A large multilayer coil prepared from 2.6 mm I.D. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, USA) by winding it onto a 5 cm diameter holder hub making three layers of left-handed coils between a pair of flanges spaced 5 cm. A pair of columns was serially connected on the rotary frame using a flow tube (PTFE, 0.85 mm I.D.) to give a total column capacity of 165 ml. The revolution speed of the apparatus was regulated at 400 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. Polyethylene glycol (PEG) 1000 (average molecular mass $M_r$ 950–1050), 1540 ($M_r$ 1300–1600), mono- and dibasic potassium phosphates, ammonium sulfate for the preparation of aqueous polymer two-phase systems were purchased from Kanto Chemicals (Tokyo, Japan). Acrylamide for electrophoresis was obtained from Wako (Osaka, Japan). Other chemicals were all of analytical reagent grade.

### *2.3. Preparation of crude bovine heart 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 filtered with an absorbent gauze to remove the cell debris. The filtrate was again filtered through a Millipore filter (pore size: 0.22 μm) (Nihon Millipore Kogyo, Yonezawa, Japan) before loading into the CCC column. The crude bovine heart extract thus obtained was stored at −20 °C until use.

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

Two-phase solvent systems composed of 16.0% (w/w) PEG 1000 and 12.5% (w/w) potassium phosphate solutions were prepared by dissolving 320 g of PEG 1000, and 250 g of potassium phosphate in water to make the total mass of 2000 g. The desired pH of the solvent system was obtained by selecting a suitable ratio of monobasic potassium phosphates as shown in Table 1 where the settling times and the volume ratios between the upper and lower phases of the two-phase solvent systems are also indicated.

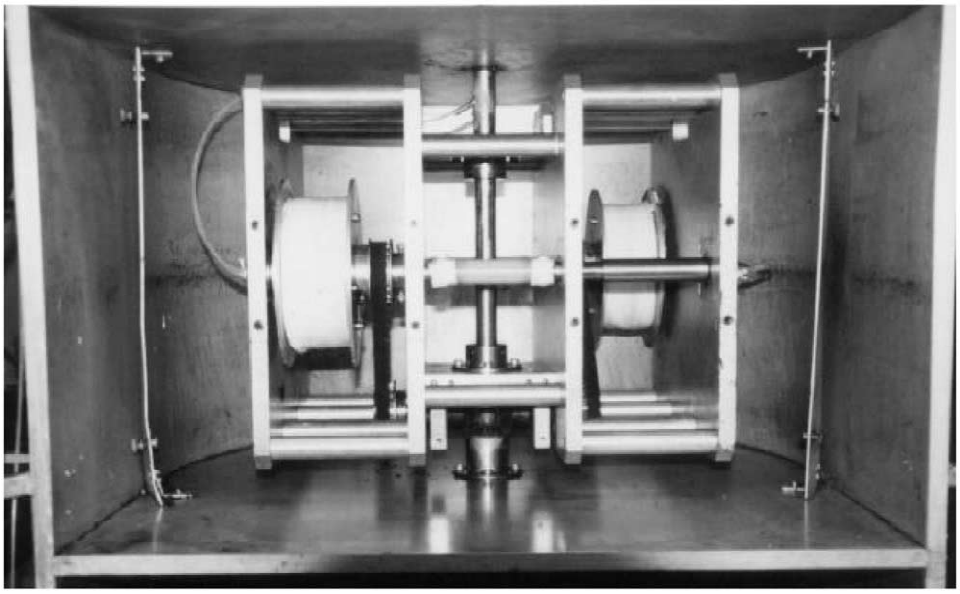

Fig. 1. Type-XL cross-axis coil planet centrifuge (CPC) equipped with a pair of multilayer coils connected in series.

The solvent mixture was throughly equilibrate in a separatory funnel at room temperature and the two phases were separated shortly before use.

### 2.5. Measurement of settling time

Using the above equilibrated aqueous two-phase systems, the settling time was measured as follows: a 2-ml volume of each phase was delivered into a 10-ml capacity graduated glass cylinder which was then sealed with a glass stopper. The contents was gently mixed by inverting the cylinder five times and the cylinder was immediately placed on a flat table to measure the time required for the mixture to settle into two clear layers. The experiment was repeated several times to obtain the mean value.

Table 1
Settling time and phase volume ratio of PEG-potassium phosphate at various pH

| Concentration (%, w/w) | | | pH | Settling time (sec) | Volume ratio (UP/LP) |
|---|---|---|---|---|---|
| PEG 1000 | $KH_2PO_4$ | $K_2HPO_4$ | | | |
| 16.0 | 6.25 | 6.25 | 6.8 | 120 | 1.22 |
| 16.0 | 4.2 | 8.3 | 7.3 | 114 | 1.00 |
| 16.0 | 2.1 | 10.4 | 8.0 | 112 | 0.88 |
| 16.0 | 0 | 12.5 | 9.2 | 96 | 0.82 |

### 2.6. Purification of LDH from crude bovine heart extract by conventional CCC

In each experiment, the CCC column was first entirely filled with the stationary phase, either upper or lower phase, and the sample solution (a mixture of 3 g of crude bovine heart extract with PEG and potassium phosphate to adjust its phase composition close to the two-phase solvent system used for the separation) was injected into the column using an EYELA type SV-6000 sample injector (Tokyo Rikakikai, Tokyo, Japan). Then the other phase was eluted through the column at a flow-rate of 0.5 ml/min while the apparatus was rotated at 400 rpm. The effluent from the outlet of the column was continuously monitored with an EYELA UV-9000 absorbance monitor (Tokyo Rikakikai) at 280 nm and fractionated into test tubes using an LKB 2112 Redirac fraction collector (LKB Instruments, Stockholm, Sweden).

### 2.7. pH-peak focusing CCC of crude bovine heart extract

The coil was first completely filled with the stationary upper phase of the solvent system composed of 16% (w/w) PEG 1000 and 12.5% (w/w) potassium phosphate at pH 9.2. The CCC sample solution was injected into the column using a sample injector. Then the lower phase of the above solvent system, with its pH adjusted to 6.8, 7.3 or 8.0, was pumped into the column at 0.5 ml/min while the apparatus was rotated at 400 rpm. The effluent from the outlet of the column was continuously monitored with the absorbance monitor and fractionated using a fraction collector. The pH of each fraction was manually determined by a Model MP 120 pH meter (Mettler, Greifensee, Switzerland).

An alternative pH-peak focusing CCC method was performed using both retainer acid and eluter base in the solvent system composed of 15% (w/w) PEG 1540–15% (w/w) ammonium sulfate. The coil was first completely filled with the stationary upper phase containing 10 m*M* acetic acid as a retainer to bring the pH to 4.2. This was followed by injection of CCC sample through the sample port. Then the lower phase of the above solvent system containing 100 m*M* sodium hydroxide as an eluter was pumped into the column at 0.5 ml/min while the apparatus was rotated at 400 rpm. The effluent was monitored and fractionated in a similar fashion. The pH of each fraction was manually determined by using a pH meter.

### 2.8. Measurement of LDH enzyme activity in CCC fractions

LDH in the CCC fractions was determined by enzymatic analyses [22]. When lactic acid, the substrate of LDH, is oxidized to pyruvic acid by LDH in the CCC fraction, nicotinamide adenine dinucleotide (NAD) 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 solution.

## 3. Results and discussion

### 3.1. Volume ratio and settling time of PEG–postassium phosphate two-phase systems

Table 1 lists the composition of four aqueous two-phase solvent systems together with their settling times and phase volume ratios. These polymer phase systems were composed of 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate at different pH values ranging from 6.8 to 9.2. In all these solvent systems, the upper phase is rich in PEG 1000 and the lower phase is rich in potassium phosphate. The settling time of these four aqueous polymer phase systems increases with increase the relative concentration of monobasic potassium phosphate. These four systems yield a desirable phase volume ratio near 1.0 so that either upper or lower phase can be chosen as the mobile phase without excessive waste of the solvent system. The ratio of 0.8 indicates the volume of the upper phase is 80% of that of the lower phase and the ratio of 1.22 indicates the volume of the lower phase is 82% of that of the upper phase.

### 3.2. Purification of LDH from crude bovine heart extract by conventional CCC

Fig. 2 shows the elution profile of the crude bovine heart extract obtained by the cross-axis CPC using the 16% PEG 1000–12.5% potassium phosphate buffer aqueous polymer phase system at pH 9.2. The sample solution containing 3 g of the crude extract, 0.67 g of PEG 1000 and 0.52 g of dibasic potassium phosphate was injected through the sample port. The separation was performed at 400 rpm at a flow-rate of 0.5 ml/min using the potassium phosphate-rich lower phase as the mobile phase. The solvent front emerged at the 22nd fraction (66 ml). The absorbance of proteins at 280 nm (open circle) in each fraction and its LDH activity (closed circle) are plotted against the retention volume. The enzyme activity of the LDH was detected at the very broad peak with the retention volume ranging from 125 to 320 ml. Almost all the other proteins in the crude bovine heart extract were contaminated in the LDH fractions, so this method is unsuitable for the isolation of LDH.

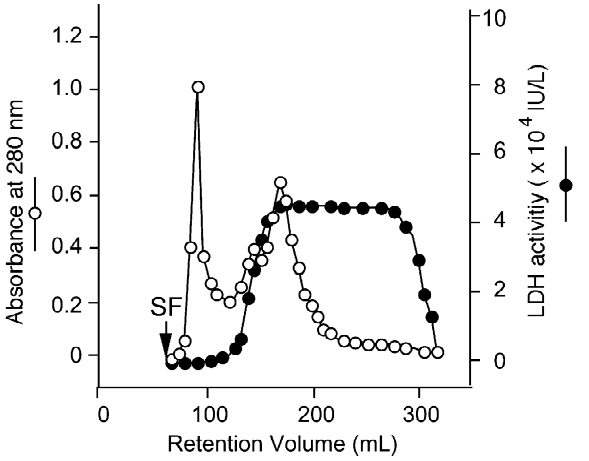


Fig. 2. Conventional counter-current chromatographic purification of lactic acid dehydrogenase from crude bovine heart extract. Experimental conditions: apparatus: the XL cross-axis CPC with a pair of multilayer coil columns of 2.6 mm I.D. and 165 ml capacity; solvent system: 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate at pH 9.2; stationary phase: PEG 1000-rich upper phase; mobile phase: potassium phosphate-rich lower phase; sample: a mixture of 3 g of bovine heart crude extract, 0.67 g of PEG 1000 and 0.52 g of dibasic potassium phosphate; flow-rate: 0.5 ml/min; revolution: 400 rpm; stationary phase retention: 60% of the total column capacity; SF=solvent front.

### *3.3. pH-peak focusing CCC without using retainer acid and elution base*

In a previous report on CCC separation of cytochrome *c*, myoglobin and ovalbumin [21], the pH of the mobile phase showed very important effects on the separation. When the PEG-rich upper phase at pH 9.2 was used as a stationary phase and the protein mixture was eluted with the mobile phase with its pH adjusted to pH 6.8, three proteins were well resolved at the point where a pH gradient was shifted from 9.2 to 6.8.

A new elution method, called pH-peak focusing CCC, can produce a sharp elution peak by manipulating the pH of both stationary phase and the mobile phases to shorten the separation. In the present study, the pH-peak focusing CCC technique was applied to the purification of LDH from the crude bovine heart extract.

Fig. 3A–C show three chromatograms obtained by pH-peak focusing CCC by changing the pH of the mobile phase (dotted line). The pH values of the

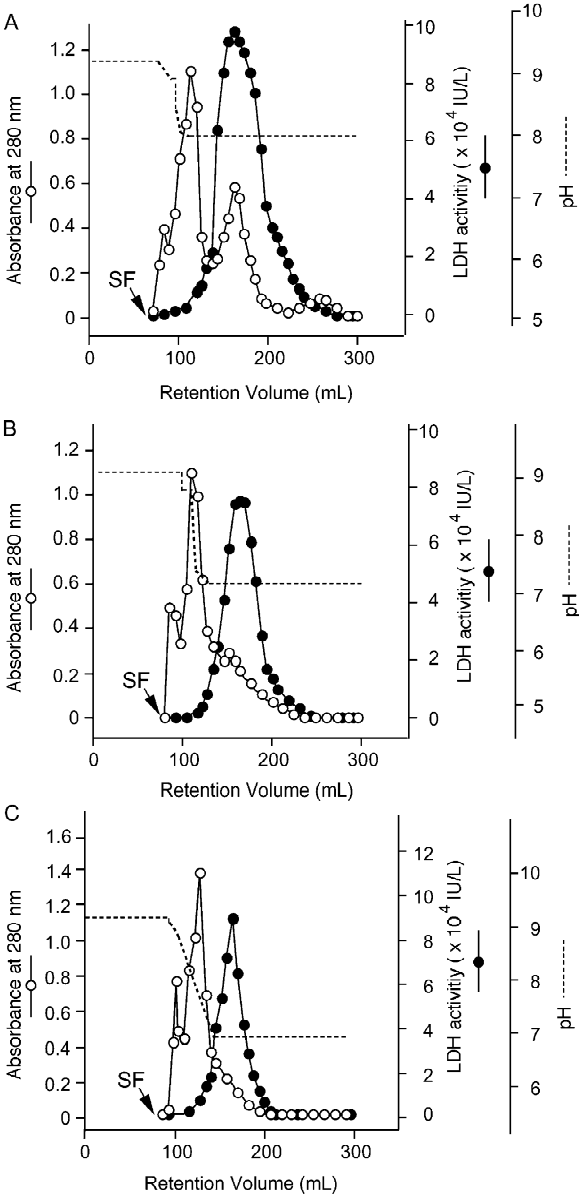


Fig. 3. pH-peak focusing CCC of crude bovine heart extract at different pH of the mobile phase. Experimental conditions: apparatus: the XL cross-axis CPC with a pair of multilayer coil columns of 2.6 mm I.D. and 165 ml capacity; solvent system: 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate; stationary phase: PEG 1000-rich upper phase at pH 9.2; mobile phase: potassium phosphate-rich lower phase at pH 8.0 (A), at pH 7.3 (B) and at pH 6.8 (C); sample: a mixture of 3 g of bovine heart crude extract, 0.67 g of PEG 1000 and 0.52 g of dibasic potassium phosphate; flow-rate: 0.5 ml/min; revolution: 400 rpm; stationary phase retention: 56.4% (A), 51.0% (B) and 47.3% (C) of the total column capacity; SF=solvent front.

fractions eluted from the column were measured by a pH meter. In these experiments, the CCC column was filled with the upper stationary phase of the 16% PEG 1000–12.5% potassium phosphate buffer at pH 9.2 followed by elution with the lower phase with different pH values 8.0 (A), 7.3 (B) and 6.8 (C). The pH of the final fraction of each experiment is same as that of the mobile phase. The lower aqueous stationary phase was slightly disturbed by the changing the pH of the mobile phase. When the column was eluted with the mobile phase of pH 8.0 (Fig. 3A), the LDH activity was detected in the retention volume from 100 to 250 ml with a shorter elution time compared with that of conventional CCC method (Fig. 2). However, LDH was eluted from the column (closed circle) still overlapping with the other proteins (open circle) in the crude extract. When the pH of the mobile phase was adjusted at pH 7.3 (Fig. 3B), the separation of LDH from other proteins was considerably improved. When the pH of the mobile phase was lowered to 6.8 (Fig. 3C), the separation of LDH was further improved, but the LDH fraction is still contaminated with other proteins. The improved separation of LDH in these experiments was apparently caused by shifting the second major protein peak toward the left by the lowered pH of the eluate while the LDH peak was less affected.

### *3.4. pH-peak focusing CCC using a retainer acid and a elute base*

In order to further improve the separation, the advanced pH-peak focusing CCC technique was tested using a retainer acid in the stationary phase and a eluent base in the mobile phase. For the purpose of making a greater change of the pH, phosphate in the solvent system was replaced by a neutral salt (ammonium sulfate) which gave a minimum buffering action. We selected the solvent composition of 15% (w/w) PEG 1540–15% (w/w) ammonium sulfate. The column was first filled with the upper phase containing 10 m*M* acetic acid as a retainer acid and the loaded sample solution was eluted with the lower phase containing 100 m*M* sodium hydroxide as an eluter base while other experimental conditions were the same as the previous experiments. In this CCC, there was no stationary phase disturbance. Fig. 4 shows the elution profile of crude bovine heart extract using this advanced pH-peak focusing CCC technique. Most proteins in the crude extract were eluted out before the retention volume of 160 ml, whereas the LDH peak was eluted between 150 and 220 ml with a sharpened front edge coincided with the sharp rise of the eluent pH from 2.0 to 7.2 as shown in Fig. 4. Sodium dodecyl sulfate (SDS)–gel electrophoresis revealed that the LDH fractions of the retention volume from 160 to 220 ml showed no contamination of other proteins in the crude extract (data not shown). In this pH-peak focusing CCC, it was impossible to separate different isoforms of LDH.

In dye–ligand affinity chromatography [23], 1,6-bisphosphate dependent lactic acid from *Streptococcus uberis* was purified and it's existence in different forms were investigated. Further, using a PEG 8000–dextran system, isoforms of pig LDH were separated into $H_4$ and $M_4$ by liquid–liquid partition chromatography [24]. In the pH-peak focusing CCC technique, isoforms of bovine heart LDH were not separated from each other.

The overall results of the above studies indicate that the elution of the LDH from the CCC column was greatly influenced by the pH of the mobile phase. The pH-peak focusing CCC technique using a retainer acid and a eluent base improved the separation of LDH from the proteins in the crude extract by shifting the protein peaks toward the left and sharpening the front edge of the LDH peak. The excellent purification of LDH from crude bovine heart extract was performed by the solvent system composed of 15% (w/w) PEG 1540–15% (w/w) ammonium sulfate by adding acetic acid to the upper stationary phase as a retainer and sodium hydroxide to the lower mobile phase as an eluter. This is the first report of the pH-peak focusing CCC techniques facilitates the rapid purification of target protein from a crude extract.

## Acknowledgements

The authors are indebted to Dr. Henry M. Fales for improving the manuscript with valuable suggestions.

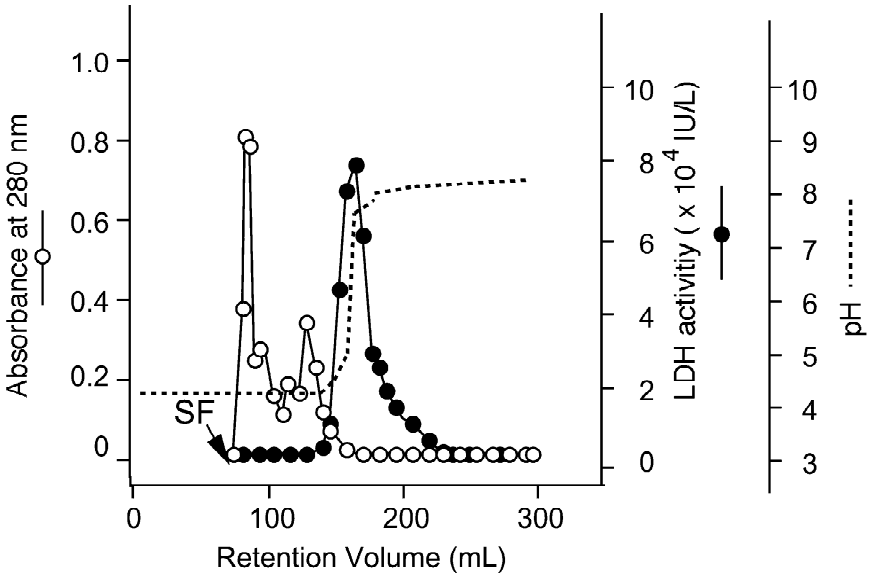


Fig. 4. pH-peak focusing CCC of crude bovine heart extract using a retainer acid and a elute base. Experimental conditions: apparatus: the XL cross-axis CPC with a pair of multilayer coil columns of 2.6 mm I.D. and 165 ml capacity; solvent system: 15% (w/w) PEG 1540–15% (w/w) ammonium sulfate; stationary phase: PEG 1540-rich upper phase containing 10 m*M* acetic acid as a retainer acid; mobile phase: ammonium sulfate-rich lower phase containing 100 m*M* sodium hydroxide as a elute base; sample: a mixture of 3 g of bovine heart crude extract, 0.67 g of PEG 1000 and 0.52 g of dibasic potassium phosphate; flow-rate: 0.5 ml/min; revolution: 400 rpm; stationary phase retention: 54.4% of the total column capacity; SF=solvent front.

## References


[1] Y. Ito, R.L. Bowman, Science 167 (1970) 281.
[2] Y. Ito, in: N.B. Mandava, Y. Ito (Eds.), Counter-Current Chromatography, Marcel Dekker, New York, 1988, p. 79, Chapter 3.
[3] W.D. Conway, Countercurrent Chromatography: Principle Apparatus and Applications, VCH, Weinheim, 1990.
[4] Y. Ito, in: Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography, Chemical Analysis Series, Vol. 132, Wiley-Interscience, 1996, p. 3, Chapter 1.
[5] Y. Ito, H. Oka, J.L. Slemp, J. Chromatogr. 463 (1989) 305.
[6] Y. Ito, E. Kitazume, M. Bhatnagar, J. Chromatogr. 538 (1991) 59.
[7] Y. Ito, E. Kitazume, J.L. Slemp, J. Chromatogr. 538 (1991) 81.
[8] Y. Shibusawa, Y. Ito, J. Chromatogr. 550 (1991) 695.
[9] Y. Shibusawa, Y. Ito, J. Liq. Chromatogr. 15 (1992) 2787.
[10] Y. Shibusawa, in: Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography, Chemical Analysis Series, Vol. 132, Wiley-Interscience, 1996, p. 385, Chapter 13.
[11] Y.W. Lee, Y. Shibusawa, F.T. Chen, J. Meyers, J.M. Schooler, Y. Ito, J. Liq. Chromatogr. 15 (1992) 2831.
[12] L. Qi, Y. Ma, Y. Ito, H.M. Fales, J. Liq. Chromatogr. 21 (1998) 83.
[13] Y. Shibusawa, Y. Ito, K. Ikewaki, D.J. Rader, B. Brewer, J. Chromatogr. 596 (1992).
[14] Y. Shibusawa, Y. Chiba, U. Matsumoto, Y. Ito, in: W.D. Conway, R.J. Petroski (Eds.), Modern Countercurrent Chromatography, ACS Monographs, American Chemical Society, Washington, DC, 1995, p. 19, Chapter 11.
[15] Y. Shibusawa, M. Mugiyama, U. Matsumoto, Y. Ito, J. Chromatogr. B 664 (1995).
[16] Y. Shibusawa, Y. Eriguchi, Y. Ito, J. Chromatogr. B 696 (1997) 25.
[17] Y. Shibusawa, S. Kihira, Y. Ito, J. Chromatogr. B 709 (1998) 301.
[18] Y. Shibusawa, S. Iino, H. Shindo, Y. Ito, J. Liq. Chromatogr. Rel. Technol. 24 (2001) 2007.
[19] Y. Shibusawa, T. Hosojima, N. Nakata, H. Shindo, Y. Ito, J. Liq. Chromatogr. Rel. Technol. 24 (2001) 1733.
[20] Y. Ito, in: Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography, Chemical Analysis Series, Vol. 132, Wiley-Interscience, 1996, p. 121, Chapter 6.
[21] Y. Shibusawa, M. Yamaguchi, Y. Ito, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 121.
[22] A.L. Babson, G.E. Phillips, Clin. Chim. Acta 12 (1965) 210.
[23] R.A. Williams, P. Andrews, Biochem. J. 236 (1986) 721.
[24] C. Wingren, V.-B. Hansson, Biochim. Biophys. Acta 1244 (1995) 209.