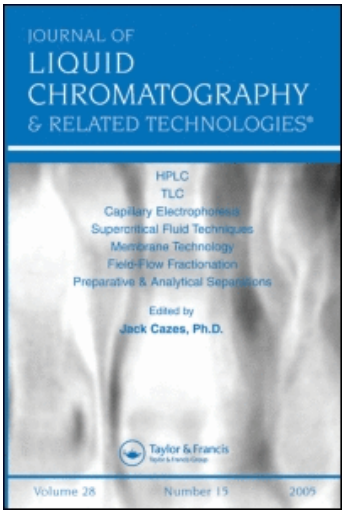

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PROTEIN SEPARATION BY CROSS-AXIS COIL PLANET CENTRIFUGE WITH TWO DIFFERENT POSITIONS OF ECCENTRIC COIL ASSEMBLIES USING POLYETHYLENE GLYCOL-DEXTRAN SOLVENT SYSTEM

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PROTEIN SEPARATION BY CROSS-AXIS COIL PLANET CENTRIFUGE WITH TWO DIFFERENT POSITIONS OF ECCENTRIC COIL ASSEMBLIES USING POLYETHYLENE GLYCOL-DEXTRAN SOLVENT SYSTEM

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ABSTRACT

Countercurrent chromatography (CCC) of proteins was performed by the cross-axis coil planet centrifuge (cross-axis CPC) using polyethylene glycol (PEG) - dextran biphasic systems. A pair of eccentric coil assemblies was mounted at two different positions (off-center and central) on the rotary frame. The best separation was obtained from a two-phase solvent system composed of 4.0% (w/w) PEG 8000 - 5.0% (w/w) dextran T500 in 5 mM potassium phosphate buffer (pH 7.0) and 3 M sodium chloride using the upper PEG-rich phase as the mobile phase. The off-center column position yielded substantially better peak resolution between lysozyme and myoglobin than

that obtained from the central position under otherwise identical conditions.

INTRODUCTION

The cross-axis coil planet centrifuge (cross-axis CPC)[1-5] has been developed for performing countercurrent chromatography (CCC) with low interfacial tension two-phase solvent systems which produce emulsification and carryover of the stationary phase in a multilayer coil of the type J high-speed CCC centrifuge. The apparatus has a unique mode of planetary motion such that the column holder rotates about its horizontal axis while revolving around the vertical axis of the centrifuge. This motion produces stable retention of the low interfacial tension solvent systems including viscous polymer phase systems.[6] It has been shown that the ratio between the revolution radius (X) and the lateral deviation (L) of the column is an important parameter for the retention of the stationary phase. The studies on various types of the cross-axis multilayer CPC including X,[1,2] XL,[7] XLL,[8] and XLLL,[9] indicated that increasing the ratio L/X improves the retention of the stationary phase by moderating the phase mixing. Consequently, these cross-axis CPCs were used for separating proteins according to their solubility in the polymer phase system.

Many proteins can be efficiently separated with a PEG-phosphate solvent system using the XL and XLL CPCs,[7,8] whereas, some proteins such as globulins and histones with a low solubility in the above high salt PEG system may be separated with a low salt PEG-dextran system using the XLLL or L CPC which can produce satisfactory retention of the highly viscous, extremely low interfacial tension polymer system.[9]

In this connection, we have developed a versatile design of the cross-axis CPC which can accommodate a pair of column holders in two different positions (L/X = 1.5 and X = 0), thus allowing the separation of proteins using both PEG-phosphate and PEG-dextran polymer phase systems.[10,11] Our recent studies revealed that high partition efficiency was attained with a PEG-phosphate solvent system in small diameter eccentric coil assemblies mounted in the off-center position[12] while the best separation of proteins in this column was achieved using a polymer phase system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate at 850 rpm.[13]

The present paper describes the protein separation with PEG-dextran solvent systems by our cross-axis CPC equipped with eccentric coil assemblies mounted at off-center (X-1.5L) and central (L) positions.

EXPERIMENTAL

Apparatus

The cross-axis CPC employed in the present studies was constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The design of the apparatus was previously described in detail,[10, 11] so only a brief description is given here. The apparatus induces a synchronous planetary motion of the column holder which rotates about its own horizontal axis and simultaneously revolves around the vertical axis of the centrifuge at the same angular velocity. A pair of column holders can be mounted at two different locations in either off-center (X-1.5L) or central (L) position, where X = 10 cm and L = 15 cm (X is the distance from the holder axis to the central axis of the centrifuge, and L, distance between the holder and the middle point of the rotary shaft).

Preparation of CCC Column

Separation columns used in the present studies were a pair of eccentric coil assemblies described earlier.[10,11,15] Each coil assembly was prepared by winding a single piece of 1 mm ID PTFE (polytetrafluoroethylene) tubing (Flon Kogyo Co., Tokyo, Japan) onto 7.6 cm long, 5 mm OD nylon pipes forming 20 units of serially connected left handed coils, which were then arranged around the holder with their axes parallel to the holder axis. A pair of identical coil assemblies was connected in series to obtain a total column capacity of 26.5 mL.

Reagents

Polyethylene glycol (PEG) 8000 (M.W. 8000), cytochrome C (horse heart), myoglobin (horse skeletal muscle), ovalbumin (chicken egg), hemoglobin (human), trypsinogen (bovine pancreas), apo-transferrin (bovine), carbonic anhydrase (bovine erythrocytes), trypsin inhibitor (soy bean), lactalbumin, α-chymotrypsinogen A (bovine pancreas), γ-globulins (human), and lysozyme (chicken egg) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Dextran T 500 (M.W. 500,000) was purchased from Pharmacia (Sollentuna, Sweden). Bovine serum albumin, dibasic potassium phosphate, monobasic potassium phosphate, and sodium chloride were obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of reagent grade.

Preparation of Polymer Phase Systems and Sample Solution

Two polymer phase systems were prepared – solvent system I: 4.4% (w/w) PEG 8000 - 7.0% (w/w) dextran T500 in 5 mM potassium phosphate buffer (pH 7.0); and solvent system II: 4.0% (w/w) PEG 8000 - 5.0% (w/w) dextran T500 in 5 mM potassium phosphate buffer (pH 7.0). Sodium chloride was added to each solvent system at various concentrations ranging from 1-3 M. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated after two clear layers formed.

Sample solutions were prepared by dissolving each sample mixture in 1 mL of the solvent consisting of equal volume of each phase.

Measurement of Partition Coefficients of Protein Samples

The partition coefficient of each protein was determined spectrophotometrically using a simple test tube procedure. Two milliliters of each phase of the equilibrated two-phase solvent system were delivered into a test tube to which 1 mg of the sample was added. The contents were thoroughly mixed and allowed to settle at room temperature. After the two clear layers were formed, a 1 mL aliquot of each phase was diluted with 2 mL of distilled water and the absorbance was measured at 280 nm using a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan).

The partition coefficient was obtained by dividing the absorbance value of the upper phase by that of the lower phase.

CCC Separation Procedure

For each separation, the coil was first completely filled with the stationary lower phase and the sample solution (ca 1 mL) was injected into the column. Then the mobile upper phase was pumped into the column using a reciprocating pump (Model KHU-W-52H, Kyowa Seimitsu Co., Tokyo, Japan) while the column was rotated at 800 rpm. The effluent from the outlet of the column was collected into test tubes at 0.4 mL/tube using a fraction collector (Model SF-200, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Collected CCC fractions were analyzed by adding 2.5 mL of distilled water to each tube and measuring the absorbance at 280 nm and 540 nm with a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan).

Evaluation of Partition Efficiency

Partition efficiencies of the protein separation were computed from the chromatogram and expressed in terms of theoretical plate number (N) and peak resolution (Rs) according to the method described earlier.[15]

RESULTS AND DISCUSSION

The polymer phase systems useful for partitioning proteins may be divided into two types, i.e., PEG-inorganic salt and PEG-dextran systems. As mentioned earlier, the PEG-salt system has relatively high interfacial tension and low viscosity hence producing an efficient separation for many proteins. In contrast the PEG-dextran system has high viscosity and extremely low interfacial tension which produce emulsification and retention problems in the HSCCC column. On the other hand, the PEG-dextran system provides high solubility for most proteins including those which are not well dissolved in the PEG-salt system due to its high salt concentration.

In the present studies, the protein samples were separated by cross-axis CPC using the PEG-dextran solvent systems. The separations were performed with a pair of eccentric coil assemblies at both off-center and central positions.

Table 1 shows partition coefficients of various proteins in two different solvent systems: solvent system I (Table 1) and solvent system II (Table 2). Increasing sodium chloride concentration to each solvent system increases the partition coefficient values of proteins up to 3 M or its saturation point. Among those proteins, lysozyme and myoglobin were selected as test samples for CCC separation since we have used these two proteins in our previous studies on PEG-phosphate solvent systems.[15] Separations were performed using the less viscous PEG-rich upper phase as a mobile phase to obtain better retention of the stationary phase.[10]

Figure 1 illustrates the CCC separation of lysozyme and myoglobin, each 10 mg, by the cross-axis CPC with the eccentric coil assemblies mounted at off-center position. Solvent system I was used for separation at various concentrations of sodium chloride as indicated. The best peak resolution was achieved at a 2 M sodium chloride concentration.

Figure 2 similarly illustrates the CCC separation of the same sample mixture with solvent system II by the cross-axis CPC using the same column at two different positions: off-center position (upper diagram) and central position (lower diagram). Increasing the sodium chloride concentration produced a better peak resolution. The results clearly indicate that the off-center column position gives substantially better peak resolution than the central position at a

Table 1

Partition Coefficients of Proteins in 4.4% (w/w) PEG 8000 - 7.0% (w/w) Dextran Solvent Systems*

PEG 8000 (%)	**4.4**	**4.4**	**4.4**	**4.4**	**4.4**
Dextran T500 (%)	**7.0**	**7.0**	**7.0**	**7.0**	**7.0**
KH_2PO_4 (M)	**---**	**0.005**	**0.005**	**0.005**	**0.005**
K_2HPO_4 (M)	**---**	**0.005**	**0.005**	**0.005**	**0.005**
NaCl (M)	**---**	**---**	**1**	**2**	**3**
Protein					
BSA (68,000)	0.27	0.54	0.27	0.27	0.89
Ovalbumin (45,000)	0.19	0.82	0.48	0.48	0.59
Cytochrome C (12,000)	0.40	0.31	0.73	0.78	0.67
Hemoglobin (67,000)	0.30	0.36	0.49	0.76	1.37
Myoglobin (17,000)	0.38	0.56	0.47	0.49	0.70
γ-Globulins	0.33	0.42	0.18	0.50	0.91
Trypsinogen (24,000)	0.51	0.57	1.35	1.87	2.17
Trypsin Inhibitor (20,100)	0.24	1.29	1.27	1.86	2.12
α-Chymotrypsinogen A (25,635)	0.76	0.84	1.03	1.89	2.71
apo-Transferrin	0.20	0.56	0.34	0.32	0.38
Carbonic Anhydrase (29,000)	0.56	0.58	0.66	0.63	0.98
Lactalbumin (14,200)	0.09	0.39	0.07	0.07	0.08
Lysozyme (14,300)	0.79	0.65	3.87	5.69	7.86

* Partition coefficients were calculated from the absorbance of the upper phase divided by that of lower phases.

Table 2

Partition Coefficients of Proteins in 4.0% (w/w) PEG 8000 - 5.0% (w/w) Dextran Solvent Systems*

PEG 8000 (%)	**4.0**	**4.0**	**4.0**	**4.0**	**4.0**
Dextran T500 (%)	**5.0**	**5.0**	**5.0**	**5.0**	**5.0**
KH_2PO_4 (M)	**---**	**0.005**	**0.005**	**0.005**	**0.005**
K_2HPO_4 (M)	**---**	**0.005**	**0.005**	**0.005**	**0.005**
NaCl (M)	**---**	**---**	**1**	**2**	**3**
Protein					
BSA (68,000)	0.08	0.77	0.32	0.49	0.81
Ovalbumin (45,000)	0.23	0.95	0.56	0.60	0.71
Cytochrome C (12,000)	0.50	0.53	0.68	0.78	0.69
Hemoglobin (67,000)	0.43	0.52	0.59	1.29	1.33
Myoglobin (17,000)	0.46	0.57	0.52	0.58	0.55
γ-Globulins	0.40	0.23	0.27	1.00	3.56
Trypsinogen (24,000)	0.45	0.59	0.90	1.52	1.87
Trypsin Inhibitor (20,100)	1.06	1.09	0.81	1.34	1.00
α-Chymotrypsinogen A (25,635)	0.59	1.02	1.51	1.88	1.52
apo-Transferrin	0.10	0.57	0.41	0.44	0.49
Carbonic Anhydrase (29,000)	0.56	0.85	0.76	0.82	1.03
Lactalbumin (14,200)	0.16	5.12	0.08	0.07	0.06
Lysozyme (14,300)	0.90	0.74	2.18	4.17	6.09

* Partition coefficients were calculated from the absorbance of the upper phase divided by that of lower phase.

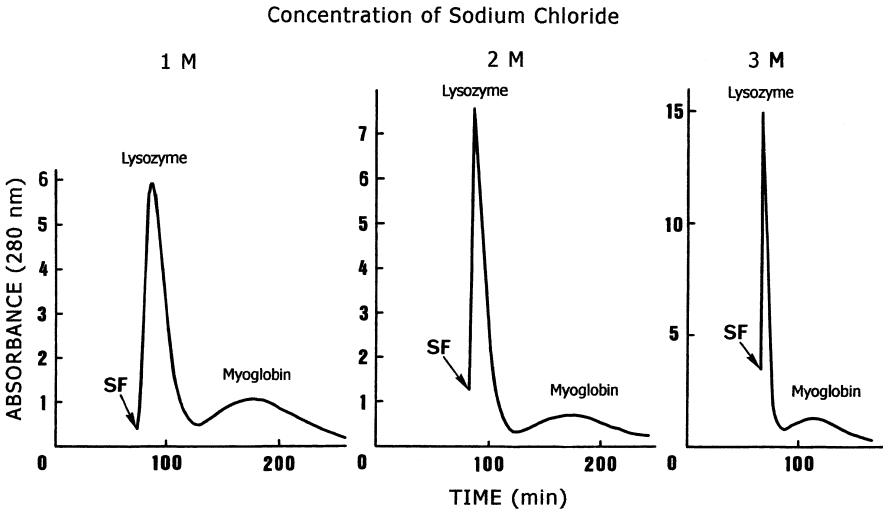

Figure 1. CCC separation of lysozyme and myoglobin by cross-axis CPC at off-center column position with solvent system I. Experimental conditions were as follows: column: a pair of eccentric coil assemblies, 1 mm ID with a total capacity of 26.5 mL; sample: lysozyme and myoglobin each 10 mg; solvent system: 4.4% (w/w) PEG 8000 - 7.0% (w/w) dextran T500 in 5 mM potassium phosphate buffer (pH 7.0) containing sodium chloride at 1 - 3 M concentrations; mobile phase: PEG-rich upper phase; flow rate: 0.2 mL/min; revolution: 800 rpm. SF = solvent front.

given salt concentration while, in both column positions, the 3 M salt concentration produced the best peak resolution.

Table 3 summarizes the analytical data for the present studies. The best separation evaluated by both peak resolution and partition efficiency was obtained from solvent system II at a 3 M salt concentration in the off-center column position, although, solvent system I also produced a similar peak resolution at 2 M sodium chloride concentration despite a lower retention of the stationary phase. The data also indicate that the retention of the stationary phase at the two different column positions is quite similar, except, at the low salt concentrations of 0 and 1 M. Inferior separations at low salt concentrations in both solvent systems are clearly due to the small separation factor between the two proteins (Table 1 and 2).

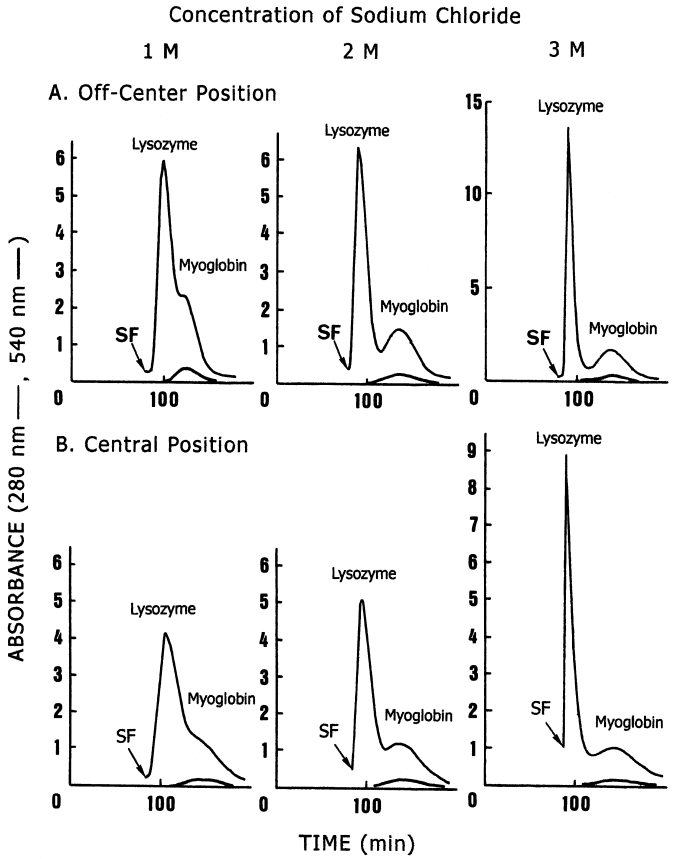

Figure 2. CCC separation of lysozyme and myoglobin by cross-axis CPC at off-center and central column positions with solvent system II. (A) Off-center column position; (B) Central column position. Experimental conditions were as follows: solvent system: 4.0% (w/w) PEG 8000 - 5.0% (w/w) dextran T500 in 5 mM potassium phosphate buffer (pH 7.0) containing sodium chloride at 1 - 3 M concentrations; Other conditions were same as those described in the Figure 1 caption. SF = solvent front.

These results, however, show that the PEG-dextran solvent system yields considerably lower peak resolution (Rs = 1.3) than that obtained by the PEG-phosphate solvent system (Rs = 1.8) under otherwise identical experimental conditions.[15]

Table 3

Analytical Values Obtained from Off-Center (X-1.5L) and Central (L) Positions with Two Different Solvent Systems

Aqueous Two-Phase Solvent System	Column Position	Concn. of NaCl (M)	Peak Resolution (Rs)	Theoretical Plate Number (TP)[a]	Retention of Stationary Phase (%)
4.4% (w/w) PEG 8000/ 7.0% (w/w) Dextran T500 in 5mM phosphate buffer (pH 7.0)	X-1.5L	0	N.S.	---	30.8
		1	1.1	114	29.5
		2	1.2	224	25.5
		3	0.9	684	28.8
4.0% (w/w) PEG 8000/ 5.0% (w/w) Dextran T500 in 5mM phosphate buffer (pH 7.0)	X-1.5L	0	N.S.	---	21.8
		1	N.S.	---	21.8
		2	1.0	173	30.9
		3	1.3	1006	29.6
	L	0	N.S.	---	30.9
		1	N.S.	---	29.5
		2	0.7	138	27.3
		3	0.9	486	30.3

Abbreviations: N.S. = not separated. [a] The values of the theoretical plate number was calculated from the lysozyme peak in the chromatogram.

CONCLUSIONS

The overall results of the present studies indicate that the cross-axis CPC can be used for protein separation with PEG-dextran polymer phase systems. Best separation may be obtained with the eccentric coil assemblies mounted at an off-center position and by eluting the column with the PEG-rich upper phase. Although the PEG-dextran system yields considerably lower peak resolution than the PEG-salt system, it can be more universally applied for the separation of proteins.

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REFERENCES

1. Y. Ito, Sep. Sci. Technol., **22**, 1971 (1987).

2. Y. Ito, Sep. Sci. Technol., **22**, 1989 (1987).

3. Y. Ito, in **Countercurrent Chromatography: Theory and Practice**, N. B. Mandava, Y. Ito, eds., Marcel Dekker, New York, 1988.

4. W. D. Conway, **Countercurrent Chromatography: Apparatus, Theory and Applications**, VCH, New York, 1990.

5. Y. Ito, in **High-Speed Countercurrent Chromatography**, Y. Ito, W. D. Conway, eds., Wiley-Interscience, New York, 1996.

6. P. Å. Albertsson, **Partition of Cell Particles and Macromolecules**, Wiley-Interscience, New York, 1986.

7. Y. Ito, H. Oka, J. Slemp, J. Chromatogr., **463**, 305 (1989).

8. Y. Ito, E. Kitazume, M. Bhatnagar, F. Trimble, J. Chromatogr., **538**, 59 (1991).

9. Y. Shibusawa, Y. Ito, J. Liq. Chromatogr., **15**, 2787 (1992).

10. K. Shinomiya, J.-M. Menet, H. M. Fales, Y. Ito, J. Chromatogr., **644**, 215 (1993).

11. K. Shinomiya, M. Muto, Y. Kabasawa, H. M. Fales, Y. Ito, J. Liq. Chrom.& Rel. Technol., **19**, 415 (1996).

12. K. Shinomiya, Y. Kabasawa, Y. Ito, J. Liq. Chrom. & Rel. Technol., **21**, 111 (1998).

13. K. Shinomiya, Y. Kabasawa, Y. Ito, J. Liq. Chrom. & Rel. Technol., **21**, 1727 (1998).

14. Y. Shibusawa, Y. Eriguchi, Y. Ito, J. Chromatogr. B, **696**, 25 (1997).

15. K. Shinomiya, Y. Kabasawa, Y. Ito, Prep. Biochem. & Biotechnol., **29**, 139 (1999).

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