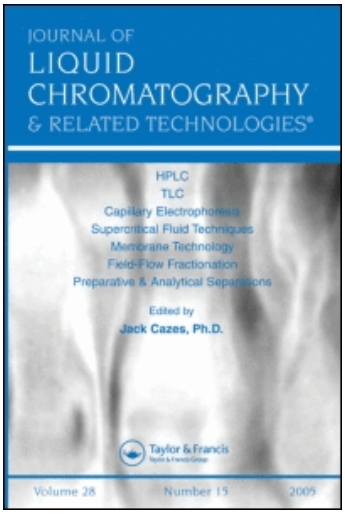

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PROTEIN SEPARATION BY CROSS-AXIS COIL PLANET CENTRIFUGE WITH SPIRAL COLUMN ASSEMBLIES

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ABSTRACT

A newly fabricated spiral column assembly was first applied to the countercurrent chromatographic separation of proteins using the cross-axis coil planet centrifuge (cross-axis CPC). The separation was performed by a set of stable proteins such as cytochrome C, myoglobin, and lysozyme with an aqueous–aqueous polymer phase solvent system composed of 12.5% (w/w) polyethylene glycol (PEG) 1000–12.5% (w/w) dibasic potassium phosphate. Three sets of left-handed single-layer spiral columns with different IDs (1.0, 1.5, and 2.0 mm) were employed to investigate the effect of column ID on partition efficiency at four different elution modes. The elution modes consisted of the

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combinations of the direction of revolution (P_I = counterclockwise; P_{II} = clockwise), the head–tail elution mode (H = head to tail; T = tail to head), and the inward–outward elution mode (I = inward; O = outward).

Among these experiments, the best separation of proteins was attained using a 1.5 mm-ID column assembly with the P_I-H-O elution mode of the lower phase mobile. The resolution between cytochrome C and myoglobin peaks was 0.9 and between myoglobin and lysozyme peaks was 1.0, while the retention of the stationary phase was 32.7%. In order to improve the partition efficiency, the four-layer spiral column assembly with 1.0 mm-ID column tubing was applied to the protein separation. The resolution between these peaks was improved to 1.1 and 1.2, respectively, while the stationary phase retention decreased to 20.9%. Further studies using a single-layer spiral coiled column also revealed the effective peak resolution of proteins, but with lower stationary phase retention. The overall results demonstrated the spiral column assembly was useful for the protein separation using the cross-axis CPC with the aqueous-aqueous polymer phase system.

INTRODUCTION

Countercurrent chromatography (CCC) has been widely used for the separation of various chemical compounds and synthetic products.[1–3] The most distinctive feature of this CCC method is a system based on liquid–liquid partition between two immiscible liquids, without a solid support that sometimes presents such problems as loss of samples by adsorption and chemical degradation of compounds. Among many CCC instruments developed in the past, both type-J multilayer coil planet centrifuge (type-J multilayer CPC) and cross-axis coil planet centrifuge (cross-axis CPC) have proven to be the most useful models.

The cross-axis CPC 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.[4,5] This motion provides excellent retention of the stationary phase, even for low-interfacial tension two-phase solvent systems, such as aqueous–aqueous polymer phase systems.

In order to obtain better partition efficiencies of the peak resolution, the following three different types of separation columns were designed for the cross-axis CPC: multilayer coil for preparative-scale separation, eccentric coil, and toroidal coil for analytical-scale separation. Our previous studies have demonstrated, that the cross-axis CPC equipped with these coil assemblies in the off-center

position was useful for the separation of proteins with aqueous–aqueous polymer phase systems by constructing the improved model in our laboratory.[6–10]

The present paper describes protein separation by the cross-axis CPC equipped with a newly fabricated spiral column assemblies, using an aqueous–aqueous polymer phase system composed of 12.5% (w/w) polyethylene glycol (PEG) 1000–12.5% (w/w) dibasic potassium phosphate. A set of standard protein samples was used for evaluating partition efficiency by varying ID and capacity of the coiled column. In the past, the single-layer spiral column was preliminarily studied to investigate the hydrodynamic behavior of solvent systems in type-J multilayer CPC as a simplified model coil, using organic–aqueous two-phase solvent systems.[11]

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[6–8] and a brief description is given here. The apparatus produces 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 accommodated 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, the distance between the holder and the middle point of the rotary shaft).

Preparation of Coiled Columns

The separation columns used in the present studies are summarized in Table 1. Three types of column configuration were employed. For the spiral column, three kinds of polytetrafluoroethylene (PTFE) tubing were chosen, 1 mm ID and 2 mm OD, 1.5 mm ID and 2.5 mm OD, and 2 mm ID and 3 mm OD. Each spiral column was prepared by winding the tubing tightly, left-handed, between a pair of closely spaced flanges mounted around the holder to make a single-layer flat spiral configuration. The spiral coiled column newly introduced in the present study (Column V in Table 1) was also prepared by winding a left-handed coiled tube, which was prepared by winding a 1 mm-ID PTFE tubing onto 1.25 m long, 5 mm-OD nylon pipe forming left-handed coils. In the spiral column assemblies, the orbital radius (R) is 10 cm, and the spiral ranges from an

Table 1. Countercurrent Chromatography Columns for Protein Separation Used in Present Studies

Column	PTFE Tubing	Turns	Length (m)	Capacity (mL)
		Spiral column (single layer)		
I.	2 mm ID, 3 mm OD	26	8.5	32.5
II.	1.5 mm ID, 2.5 mm OD	32	10.5	26.0
III.	1 mm ID, 2 mm OD	40	13.0	14.0
		Spiral column (four layers)		
IV.	1 mm ID, 2 mm OD	160	52.0	56.0
		Spiral coiled column (single layer)		
V.	1 mm ID, 2 mm OD	Two sets of four turns of toroidal coil made from 555 turns	2.5	23.5
		Helical column (single layer)		
VI.	1.5 mm ID, 2.5 mm OD	52	10.5	26.0

Each value is shown as a total amount of a pair of coil assemblies.

inner radius of 3.0 cm, $\beta = 0.3$ to an outer radius of 7.0 cm, $\beta = 0.7$, where $\beta = r/R$, r is the rotational radius (radius of the column holder) and R the revolution radius. The single-layer helical column (standard column of Column VI in Table 1) was further prepared by tightly winding a piece of PTFE tubing, 5.25 m long, 1.5 mm ID, and 2.5 mm OD, around the holder hub (6 cm diameter) forming left-handed uniform turns. In order to compare the partition efficiencies, this helical column was made from the same size of tubing as that of Column II in Table 1.

A pair of identical column assemblies was mounted on the rotary frame, one on each side, and serially connected with the flow tube.

Figure 1 shows the schematic drawing of the four-layer spiral column (A) and the single-layer spiral coiled column (B).

Reagents

Polyethylene glycol 1000 (M. W. 1000), cytochrome C (horse heart), myoglobin (horse skeletal muscle), and lysozyme (chicken egg) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dibasic potassium phosphate was obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of reagent grade.

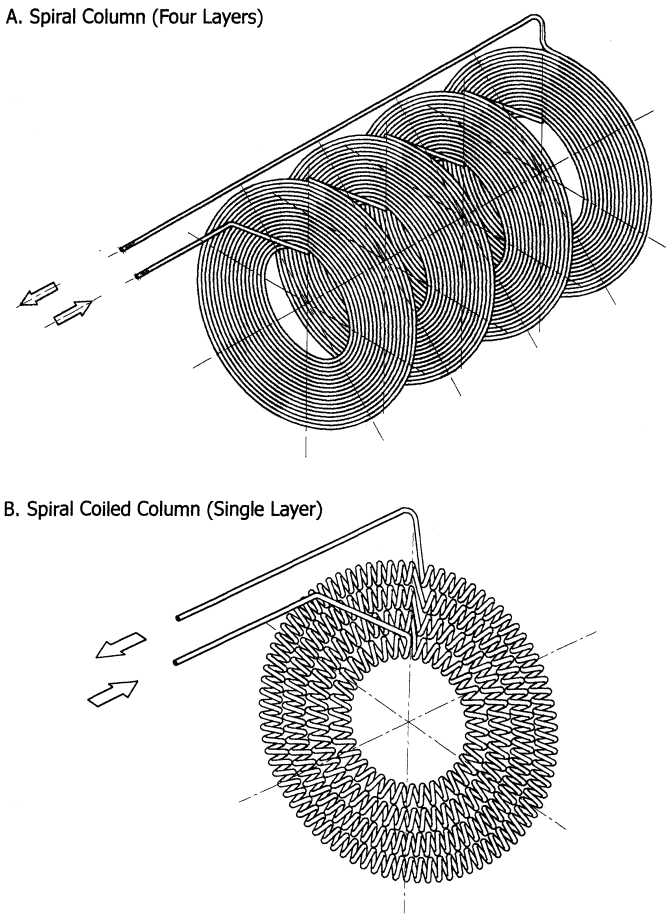

Figure 1. Schematic drawing of the four-layer spiral column (A) and the single-layer spiral coiled column (B).

Preparation of Aqueous–Aqueous Polymer Phase Systems and Sample Solutions

The polymer phase system was prepared by dissolving PEG 1000 and dibasic potassium phosphate (anhydrous) of each 125 g in 750 g of distilled water. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated after two clear layers were formed.

The sample solutions were prepared by dissolving each protein mixture in 0.5 mL of each phase of the two-phase solvent system used for separation.

CCC Separation Procedure

Each separation was initiated by completely filling the column with the stationary phase, followed by the injection of the sample solution (ca.1 mL) into the column inlet. Then, the mobile phase was pumped into the column using a reciprocating pump (Model LC-6A, Shimadzu Corporation, Kyoto, Japan), while the column was rotated at a revolution speed of 800 rpm. The effluent from 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

Each collected CCC fractions of proteins was diluted with 2.5 mL of distilled water and the absorbance was measured at 280 nm with a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan).

RESULTS AND DISCUSSION

Our previous studies demonstrated that the cross-axis CPC equipped with three different types of coiled columns, i.e., multilayer coil, eccentric coil, and toroidal coil assemblies was useful for the protein separation with aqueous–aqueous polymer phase systems.[6–10,12] In the present studies, a newly designed spiral column assembly was first introduced in a series of these experimental studies to investigate its performance.

The cross-axis CPC produces three-dimensional force fields that fluctuate during each revolution cycle.[13–15] In order to maximize the partition efficiency under the complex force field, a series of experiments may be required, both right-handed and left-handed coils using eight different elution modes. The elution modes consist of all possible combinations of the direction of revolution (P_I = counterclockwise; P_{II} = clockwise), the head–tail elution mode (H = head to tail; T = tail to head), and inward–outward elution mode (I = inward; O = outward). The inward–outward refers to the direction of the elution along the holder axis: "inward" is from peripheral toward the promixal, against the action of the centrifugal force field and "outward" is in the opposite direction. However, our previous studies revealed that the left-handed coils yielded better partition efficiencies than the right-handed coils.[6] Table 2 summarizes four elution modes of left-handed coils examined in the present studies.

Figure 2 illustrates the CCC chromatograms of proteins obtained by single-layer spiral column with 2.0 (upper side) and 1.5 (lower side) mm-ID column tubing, respectively, mounted at off-center position. The best separation in these

Table 2. Elution Modes of Left-Handed Coils Mounted on Cross-Axis CPC

Planetary Motion	Head–Tail Elution Mode	Inward–Outward Elution Mode	Combined Elution Mode	Mobile Phase
Counter clockwise (P_I)	Head → Tail (H)	Outward (O)	P_I-H-O	Lower phase
	Tail → Head (T)	Inward (I)	P_I-T-I	Upper phase
Clockwise (P_{II})	Tail → Head (T)	Outward (O)	P_{II}-T-O	Lower phase
	Head → Tail (H)	Inward (I)	P_{II}-H-I	Upper phase

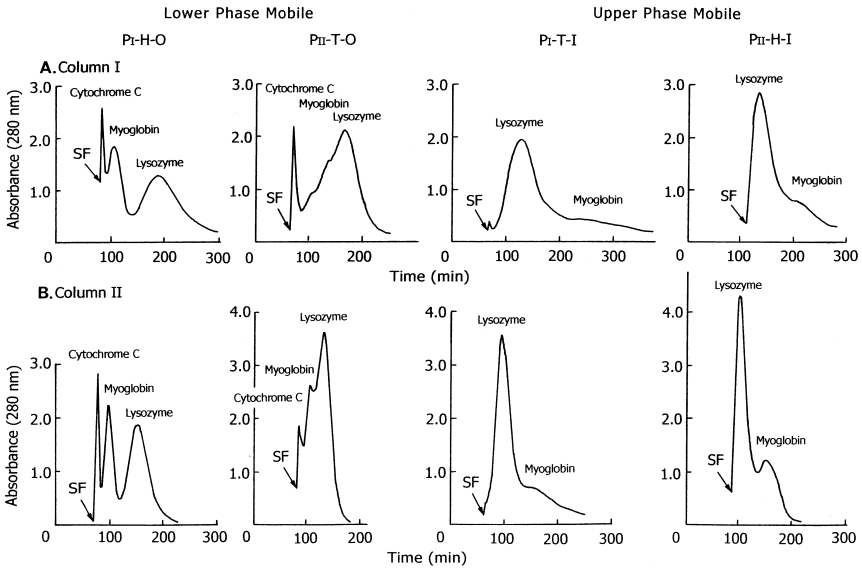

Figure 2. Countercurrent chromatography separations of proteins by the cross-axis CPC equipped with single-layer spiral column assemblies at off-center position. Experimental conditions: apparatus: cross-axis CPC equipped with single-layer spiral column assemblies, (A) 2.0 mm ID and 3.0 mm OD, and 32.5 mL capacity; (B) 1.5 mm ID and 2.5 mm OD, and 26.0 mL capacity; sample: cytochrome C (2.5 mg), myoglobin (8 mg) and lysozyme (10 mg) for lower phase mobile, myoglobin (8 mg) and lysozyme (10 mg) for upper phase mobile; solvent system: 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; stationary phase: upper phase for lower phase mobile, lower phase for upper phase mobile; flow rate: 0.2 mL/min; revolution: 800 rpm. SF = solvent front.

experiments was attained using a 1.5 mm-ID column assembly (Column II) with the P_I-H-O elution mode with lower phase mobile. The resolution between cytochrome C and myoglobin peaks was 0.9 and that between myoglobin and lysozyme peaks, 1.0, while the retention of the stationary phase was 32.7%. When using a 1.0 mm-ID column tubing of single-layer spiral coil (Column III), unsatisfactory peak resolution was obtained due to the lack of enough column volume.

At the central position of the single-layer spiral column with 1.5 mm-ID tubing (Column II), the resolution between cytochrome C and myoglobin peaks was decreased to 0.5 and that between myoglobin and lysozyme peaks, 0.8, while the stationary phase retention was increased to 36.0% as shown in Fig. 3. This result suggests that better peak resolution is achieved using the spiral column mounted at off-center position than at central position, where similar behavior was obtained by multilayer coil and eccentric coil in our previous studies.[6,12]

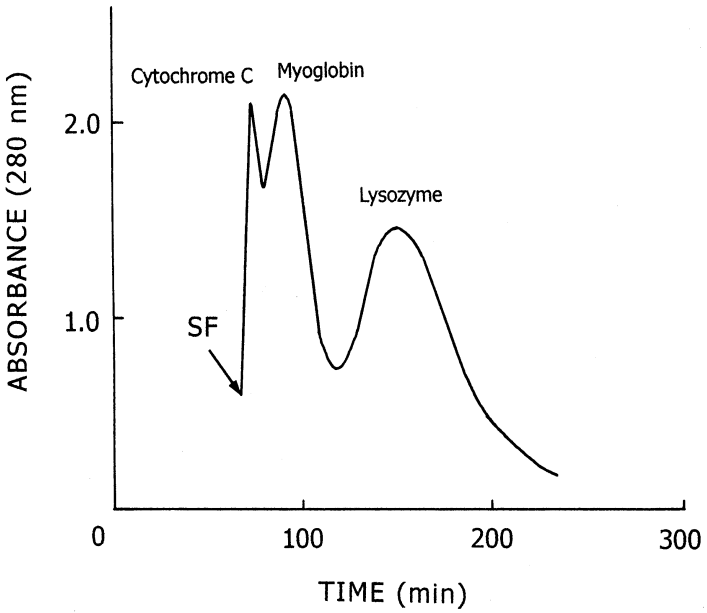

Figure 3. Countercurrent chromatography separation of proteins by the cross-axis CPC equipped with single-layer spiral column assemblies at central position. Experimental conditions: apparatus: cross-axis CPC equipped with single-layer spiral column assemblies, 1.5 mm ID and 2.5 mm OD, and 26.0 mL capacity; sample: cytochrome C (2.5 mg), myoglobin (8 mg) and lysozyme (10 mg); solvent system: 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase: lower phase; flow rate: 0.2 mL/min; revolution: 800 rpm. SF = solvent front.

In order to improve the partition efficiency of coiled columns, the four-layer spiral column assembly with 1.0 mm-ID tubing (Column IV) was applied for the protein separation, as illustrated in Fig. 4. The resolution between cytochrome C and myoglobin peaks was 1.1 and that between myoglobin and lysozyme peaks, 1.2, while the retention of the stationary phase was 20.9%. This result indicates that the spiral coil is useful for protein separation by selecting suitable ID PTFE tubing and enough column volume for separation.

Further experiments using a single-layer spiral coiled column, newly introduced in the present studies (Column V), were also examined at four different elution modes described above. Better peak resolution of proteins was also accomplished at the P_I-H-O elution mode as shown in Fig. 5, which had similar results as obtained by other spiral columns. However, the retention of the stationary phase was extremely lower than 12.8%, while the value of theoretical

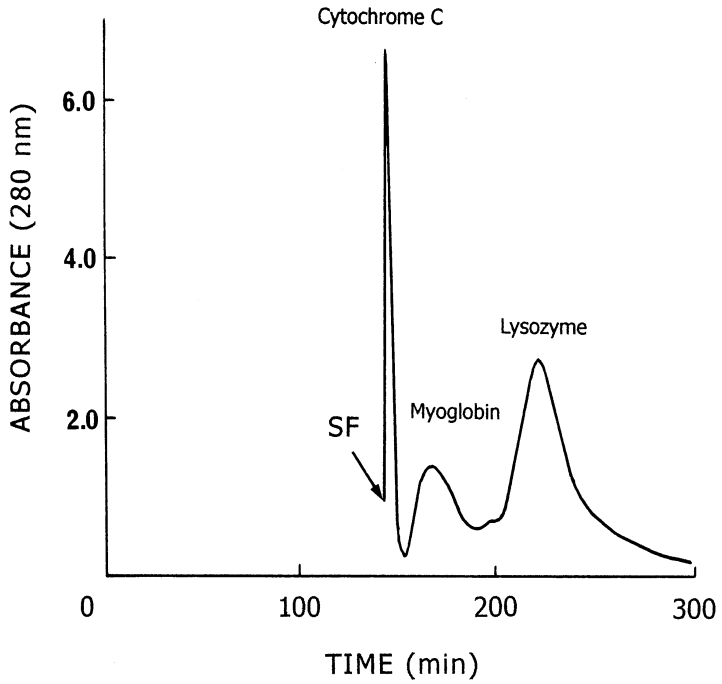

Figure 4. Countercurrent chromatography separation of proteins by the cross-axis CPC equipped with four-layer spiral column assemblies at off-center position. Experimental conditions: apparatus: cross-axis CPC equipped with four-layer spiral column assemblies, 1.0 mm ID and 2.0 mm OD, and 56.0 mL capacity. Other conditions were same as those described in the Fig. 3 captions. SF = solvent front.

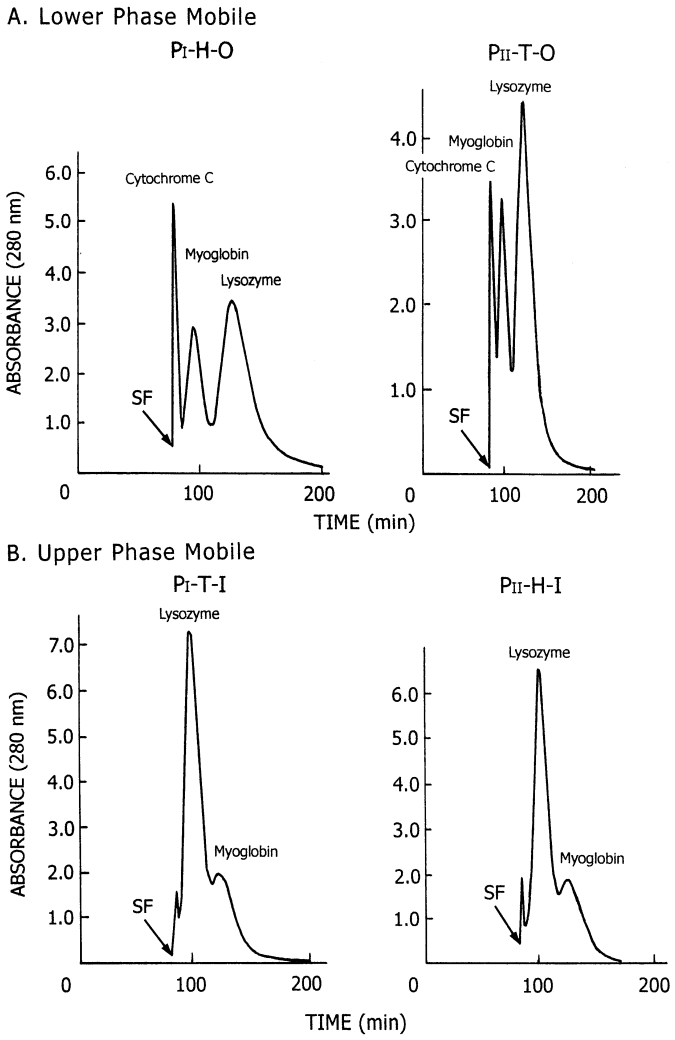

Figure 5. Countercurrent chromatography separations of proteins by the cross-axis CPC equipped with single-layer spiral coiled column assemblies at off-center position. Experimental conditions: apparatus: cross-axis CPC equipped with single-layer spiral coiled column assemblies, a single-layer of spiral coiled column consisted by four turns of toroidal coil with 555 turns of 1.0 mm ID PTFE tubing wound onto 125 cm long, 5 mm OD nylon pipe; sample: (A) cytochrome C (2.5 mg), myoglobin (8 mg) and lysozyme (10 mg); (B) myoglobin (8 mg) and lysozyme (10 mg); solvent system: 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase: (A) lower phase; (B) upper phase; flow rate: 0.2 mL/min; revolution: 800 rpm. SF = solvent front.

Table 3. Stationary Phase Retention on Protein Separation Using Cross-Axis CPC with Spiral Column and Helical Column Assemblies

	Stationary Phase Retention (%)				
	Lower Phase Mobile		Upper Phase Mobile		
Column	P_I-H-O	P_{II}-T-O	P_I-T-I	P_{II}-H-I	Reference
			Spiral column		
I.	42.2	10.8	49.2	18.5	Fig. 2 (upper side)
II.	32.7	34.6	34.6	25.6	Fig. 2 (lower side)
	36.0				Fig. 3 (central position)
III.	14.3				
IV.	20.9				Fig. 4
V.	12.8	6.4	21.3	18.3	Fig. 5
			Helical column (single layer)		
VI.	44.0	17.4	67.9	27.2	Fig. 6

Table 4. Analytical Values Obtained from CCC Separation of Proteins at P_I-H-O Elution Mode

	Theoretical Plates	Resolution (Rs)		Theoretical Plates/ Column Capacity	
Column	(N)	Cyt C/Myo	Myo/Lys	(N/mL)	Reference
			Spiral column		
I.	65	0.6	0.9	2.0	Fig. 2 (upper side)
II.	134	0.9	1.0	5.2	Fig. 2 (lower side)
	62	0.5	0.8	2.4	Fig. 3 (central position)
IV.	376	1.1	1.2	6.7	Fig. 4
V.	238	1.0	0.9	10.1	Fig. 5
			Helical column (single layer)		
VI.	85	0.9	1.0	3.3	Fig. 6

Abbreviations: Cyt C = Cytochrome C; Myo = Myoglobin; Lys = Lysozyme. The value of the theoretical plate number was calculated from the myoglobin peak in the chromatogram.

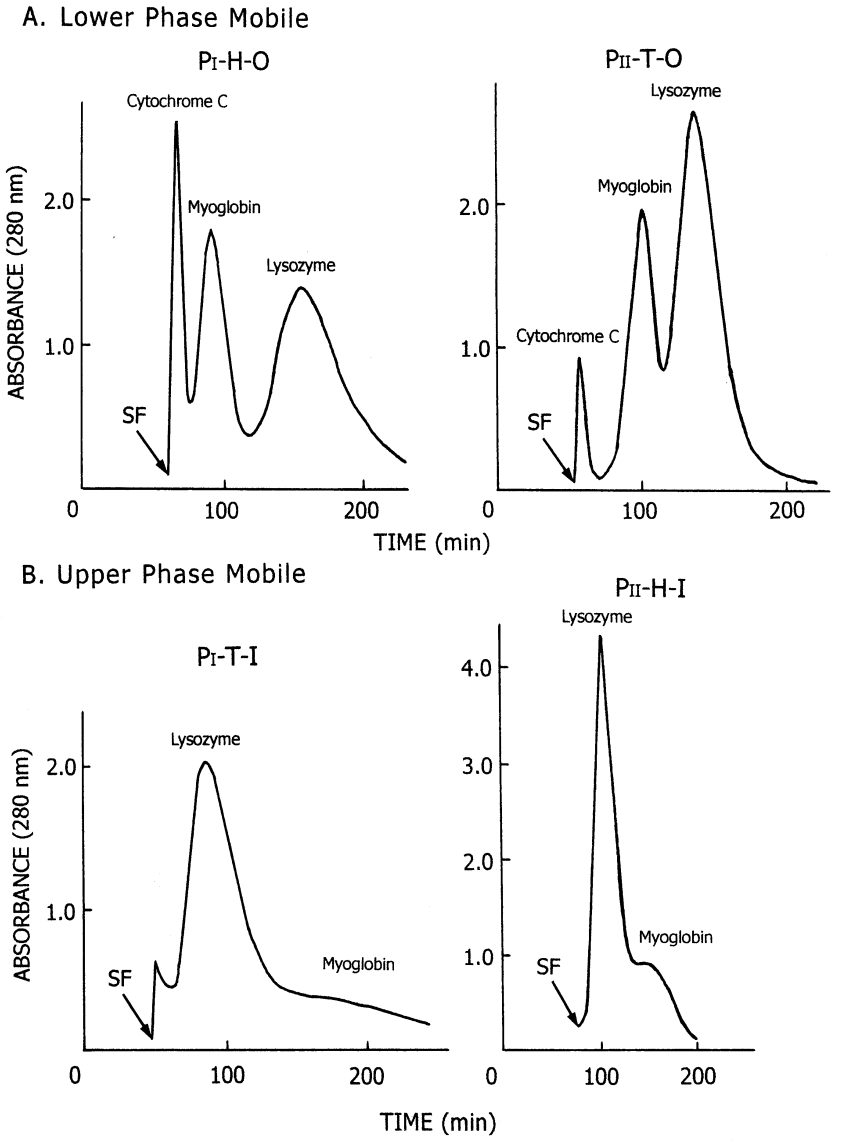

Figure 6. Countercurrent chromatography separations of proteins by the cross-axis CPC equipped with single-layer helical column assemblies at off-center position. Experimental condition: apparatus: cross-axis CPC equipped with single-layer helical column assemblies, 1.5 mm ID and 2.5 mm OD, and 26.0 mL capacity. Other conditions were same as those described in the Fig. 5 captions. SF = solvent front.

plates/column capacity (N/mL) is highest among those obtained by other spiral columns as summarized in Table 4.

In order to compare the partition efficiency between spiral column and helical column, the single-layer helical column (Column VI) was fabricated using the same size of 1.5 mm-ID tubing for Column II at equal capacity. Figure 6 illustrates the CCC chromatograms obtained by using this single-layer helical column mounted at off-center position ($\beta = 0.3$). As a result, each value of peak resolution between cytochrome C and myoglobin, myoglobin and lysozyme, was equal to that obtained by the single-layer spiral column in the P_I-H-O elution mode as shown in Table 4.

Table 3 summarizes the retention of the stationary phase in the coiled column measured experimentally after each separation was completed. As observed in the single-layer helical column commonly used in CCC as a portion of multilayer coil, the spiral column was also affected remarkably by the elution mode, especially, head–tail elution mode. With lower phase mobile, better stationary phase retention and peak resolution were obtained in the P_I-H-O elution mode than in the P_{II}-T-O elution mode. With upper phase mobile, the P_I-T-I elution mode gave better stationary phase retention than the P_{II}-H-I elution mode.

FUTURE DEVELOPMENT

The overall results described above indicated that spiral column assembly can be used for separation of proteins with a polymer phase system, at an efficiency as good as that obtained from the standard multilayer coiled column. The spiral column has an important advantage over the standard coiled column in that it has a potential to be fabricated from a solid disk with a spiral groove that can be molded for mass production. A set of these disks can be stuck up to form a multiple spiral disk assembly. This would ease manufacturing of the separation column for the cross-axis CPC for purification of biopolymers.

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