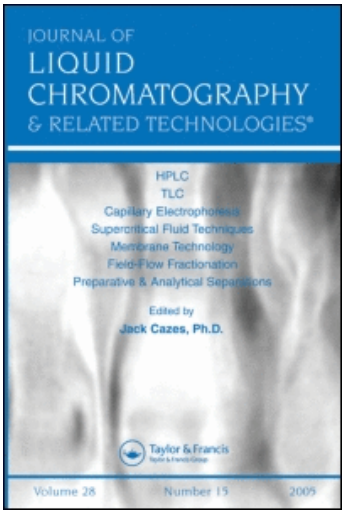

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Effects of the Planetary Motion of a Coiled Column on Protein Separation by the Nonsynchronous Coil Planet Centrifuge

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ABSTRACT

The effects of rotational speed and direction of revolution of the coiled separation column on protein separation were examined using a rotary-seal-free nonsynchronous coil planet centrifuge (CPC) fabricated in our laboratory. This apparatus has a unique feature that allows a freely adjustable rotational speed of the coiled separation column at a given revolution speed. The separation was performed using a set of stable proteins including cytochrome C, myoglobin, and lysozyme with an aqueous–aqueous polymer phase system composed of 12.5% (w/w) polyethylene glycol (PEG) 1000 and 12.5% (w/w) dibasic potassium phosphate. A series of experiments revealed that the head to tail elution mode produced better stationary phase retention and higher peak

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resolution regardless of the choice of the mobile phase. The best result was obtained in the head to tail elution mode by the clockwise (CW) coil rotation for the lower mobile phase or by the counterclockwise (CCW) coil rotation for the upper mobile phase, both under CCW revolution of the rotor.

Key Words: Nonsynchronous coil planet centrifuge; Planetary motion; Protein separation; Countercurrent chromatography; Polymer phase system; Retention of stationary phase; Cytochrome C; Myoglobin; Lysozyme.

INTRODUCTION

Countercurrent chromatography (CCC) has been widely used for separation and purification of various natural and synthetic products.[1–4] Among various CCC instruments developed in the past, the type-J multilayer coil planet centrifuge (CPC) and the cross-axis CPC have proven most useful and effective among all existing CCC instruments. The type-J multilayer CPC produces a synchronous planetary motion of the separation column, which revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity in the same direction. The cross-axis CPC, on the other hand, produces a synchronous planetary motion of the column in such a way that it revolves around the vertical axis of the centrifuge, while rotating about its horizontal axis at the same angular velocity. The difference in the planetary motion between these two instruments provides distinctive use of the two-phase solvent systems, where the type-J multilayer CPC performs excellent separation with organic–aqueous two-phase solvent systems, whereas the cross-axis CPC is used for highly polar two-phase solvent systems, such as aqueous–aqueous polymer phase systems. Our previous studies have demonstrated that protein separation can be successfully performed using the cross-axis CPC.[5–12]

The nonsynchronous CPC introduced first in 1979[13] has been considered most versatile because it provides a desirable combination between rotation (about its own axis) and revolution (around the centrifuge axis) of the coil holder.[13–17] Our previous studies revealed that this apparatus can be effectively used for partition of proteins with aqueous–aqueous polymer phase systems, where the best results are attained in the head to tail elution mode at 10 rpm of coil rotation and at 800–1000 rpm of revolution.[18] The present paper describes the effects of planetary motion of the coiled separation column on protein separation using the nonsynchronous CPC.

EXPERIMENTAL

Apparatus

The nonsynchronous 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.[15,16,18] The apparatus has a distinctive feature which allows a freely adjustable rotational rate (0–60 rpm) at any given revolution speed, while the effluent is eluted through the rotating column without the use of a conventional rotary-seal device.

Preparation of Coiled Column

The eccentric coil assembly used in the present study was prepared by winding 0.8 mm I.D. polytetrafluoroethylene (PTFE) tubing onto a set of 20 cm × 6 mm O.D. aluminum pipes making a series of tight left-handed coils. Eleven coil units were arranged symmetrically around the holder hub of 6 cm O.D. in such a way that the axis of each coil unit was parallel to the holder axis. The total column capacity was 20 mL.

Reagents

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

Preparation of Aqueous–Aqueous Polymer Phase System and Sample Solutions

The polymer phase system was prepared by dissolving 125 g of PEG 1000 and 125 g of dibasic potassium phosphate (anhydrous) 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.

Sample solutions were prepared by dissolving each sample mixture in 1 mL of the solvent consisting of equal volumes of each phase used for separation.

CCC Separation Procedure

Each separation was initiated by first completely filling the column with the stationary phase (either the upper or the lower phase), followed by the injection of 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 given combination of the rotation (0–60 rpm) and revolution (800 rpm) rates. The effluent from the column outlet 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 fraction was diluted with 2.5 mL of distilled water and the absorbance was measured at 280 nm with a spectrophotometer (Model UV-1600, Shimadzu).

RESULTS AND DISCUSSION

The nonsynchronous CPC has a unique mode of planetary motion, such that it provides freely adjustable coil rotation under a given centrifugal force field, where the acceleration produced by this motion fluctuates in a plane perpendicular to the axis of the holder. In the present system, using the combination of high speed revolution (800 rpm) and low speed coil rotation (0–60 rpm) both in either direction, the two phases are distributed in a rotating coil at nearly equal volumes from the head end, while any excess of either phase remains at the tail end. Here, head–tail orientation of the rotating coil is defined according to the Archimedean screw effect, where all objects of different densities present in the coil are driven toward the head of the coil. The hydrodynamic condition produces the stationary phase retention of 50% maximum of the total column capacity by pumping either phase from the head end of the coil, whereas the elution of the mobile phase from the tail toward the head results in no retention of the stationary phase. It has been found that the mode of the planetary motion

(direction of the coil rotation and the revolution) produces substantially different results in protein separation. For convenience, these two modes of planetary motion may be expressed as $P_{forward}$ (coil rotation and revolution in the same direction) (revolution/rotation = CW/CW or CCW/CCW) and $P_{backward}$ (coil rotation and revolution in the opposite direction) (revolution/rotation = CW/CCW or CCW/CW).

Figure 1 illustrates a set of CCC chromatograms obtained by the nonsynchronous CPC with a polymer phase system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate. These separations were all performed by the head to tail elution mode of lower phase mobile at various rotation speeds (0–60 rpm), at a constant high speed revolution of 800 rpm. Table 1 summarizes the analytical data computed from the chromatograms. $P_{forward}$ planetary motion (revolution/rotation = CW/CW) produces less retention of the stationary phase at higher rotation speeds, while $P_{backward}$ (revolution/rotation = CCW/CW) always gives high retention of stationary

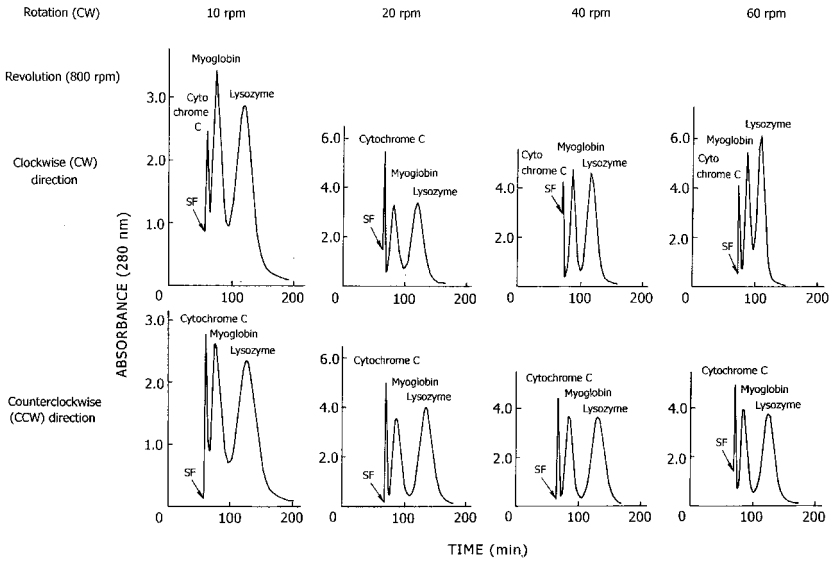

Figure 1. CCC chromatograms of proteins obtained by nonsynchronous CPC using the lower mobile phase eluted from the head toward the tail in two different modes of planetary motions. Experimental conditions: apparatus: nonsynchronous CPC equipped with an eccentric coil assembly, 0.8 mm I.D. × 1.59 mm O.D. and 20 mL capacity; sample: cytochrome C (2 mg), myoglobin (8 mg) and lysozyme (10 mg); solvent system: 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate; flow rate: 0.2 mL/min. Other conditions are described in the figure. SF, solvent front.

Table 1. Analytical values computed from CCC chromatograms of proteins obtained by the lower phase mobile at the head to tail elution mode using the nonsynchronous CPC.

Revolution (rpm)	Rotation (rpm)	Retention time (min)			Resolution factor (R_s)		Theoretical plates (N)	Stationary phase retention (%)
		Cyt C	Myo	Lys	Cyt C/Myo	Myo/Lys		
800 (CW)	10 (CW)	62	76	120	0.6	1.1	83	29.3
800 (CW)	20 (CW)	66	82	120	1.3	1.3	225	19.0
800 (CW)	40 (CW)	70	86	117	1.3	1.2	350	12.4
800 (CW)	60 (CW)	74	88	109	1.0	1.2	418	0
800 (CCW)	10 (CW)	60	75	124	0.7	1.1	97	34.8
800 (CCW)	20 (CW)	70	88	134	1.2	1.4	213	31.0
800 (CCW)	40 (CW)	66	84	130	1.2	1.5	210	33.3
800 (CCW)	60 (CW)	72	86	124	1.1	1.3	263	28.6

Note: CW, clockwise direction; CCW, counterclockwise direction; Cyt C, cytochrome C; Myo, myoglobin; Lys, lysozyme. The theoretical plates were computed from the myoglobin peak of each chromatogram.

phase at around 30%, regardless of the rotation speed. The best separation in these experiments was obtained at the coil rotation speed of 40 rpm in $P_{backward}$ (CCW/CW) planetary motion and at the revolution speed of 800 rpm. These results suggest that the eccentric coil assembly used in the present studies does not display the bilateral hydrodynamic distribution as observed in the type-J multilayer CPC with organic–aqueous solvent system. It is most likely that the difference in the stationary phase retention between the above two planetary motions may be caused by the effects of the Coriolis force, as observed in the toroidal coil CCC system,[19] and more recently, in the centrifugal partition chromatography.[20]

Figure 2 illustrates a set of CCC chromatograms obtained by the head to tail elution of the upper phase in $P_{forward}$ planetary motion (CW/CW) in the upper half, and $P_{backward}$ planetary motion (CCW/CW) in the lower half. As summarized in Table 2 computed from the chromatograms, both groups

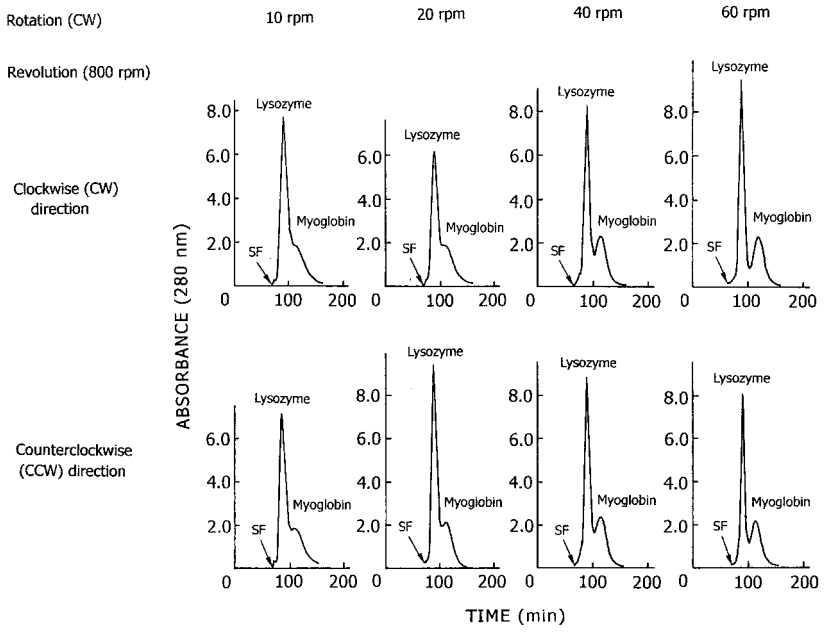

Figure 2. CCC chromatograms of proteins obtained by nonsynchronous CPC using the upper mobile phase eluted from the head toward the tail in two different modes of planetary motions. Experimental conditions: sample: myoglobin (8 mg) and lysozyme (10 mg); mobile phase: upper phase. Other conditions are described in Fig. 1. SF, solvent front.

Table 2. Analytical values computed from CCC chromatograms of proteins obtained by the upper phase mobile at the head to tail elution mode with the same flow direction of the lower phase mobile.

Revolution (rpm)	Rotation (rpm)	Retention time (min)		Resolution factor Lys/ Myo (R_s)	Theoretical plates (N)	Stationary phase retention (%)
		Lys	Myo			
800 (CW)	10 (CW)	90	113	0.5	198	17.5
800 (CW)	20 (CW)	88	108	0.5	247	24.4
800 (CW)	40 (CW)	88	112	0.8	364	20.0
800 (CW)	60 (CW)	86	119	1.3	440	24.0
800 (CCW)	10 (CW)	88	111	0.7	254	17.5
800 (CCW)	20 (CW)	88	110	0.7	366	17.5
800 (CCW)	40 (CW)	88	112	0.8	336	19.2
800 (CCW)	60 (CW)	88	114	1.0	603	17.5

Note: CW, clockwise direction; CCW, counterclockwise direction; Myo, myoglobin; Lys, lysozyme. The theoretical plates were computed from the lysozyme peak of each chromatogram.

show similar stationary phase retention at around 20%, regardless of the rate of coil rotation.

Figure 3 similarly illustrates a set of CCC chromatograms obtained by eluting with the upper phase from the head toward the tail in $P_{backward}$ planetary motion (CW/CCW) in the upper half, and in $P_{forward}$ (CCW/CCW) in the lower half. Table 3 summarizes the analytical data computed from these chromatograms. Good stationary phase retention of over 30% was obtained in $P_{forward}$ planetary motion (CCW/CCW), and only slightly less retention of 20–30% in $P_{backward}$ planetary motion (CW/CCW). Because of higher retention of the stationary phase, the peak resolution in these groups is better than those illustrated in Fig. 2. As mentioned earlier, the differences in retention level of the stationary phase in these four planetary

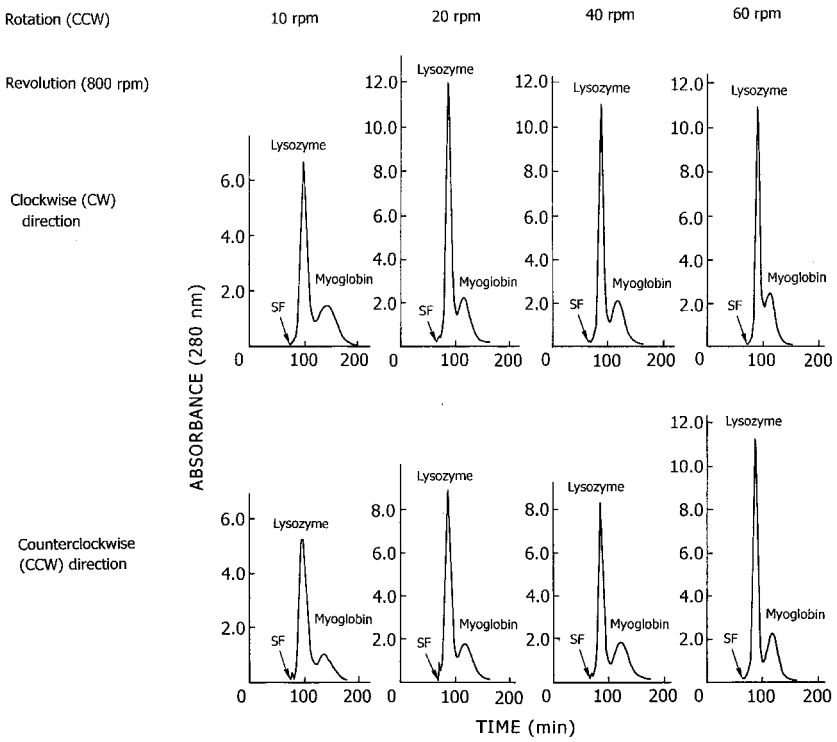

Figure 3. CCC chromatograms of proteins obtained by the upper mobile phase eluted from the head toward the tail in other modes of planetary motion. Experimental conditions are same as those described in Fig. 2. SF, solvent front.

Table 3. Analytical values computed from CCC chromatograms of proteins obtained by the upper phase mobile at the head to tail elution mode with the reversed flow direction of the lower phase mobile.

Revolution (rpm)	Rotation (rpm)	Retention time (min)		Resolution factor Lys/Myo (R_s)	Theoretical plates (N)	Stationary phase retention (%)
		Lys	Myo			
800 (CW)	10 (CCW)	100	141	0.9	269	31.7
800 (CW)	20 (CCW)	88	115	0.9	347	30.0
800 (CW)	40 (CCW)	86	118	1.0	386	21.5
800 (CW)	60 (CCW)	89	112	0.8	430	25.0
800 (CCW)	10 (CCW)	97	136	0.9	197	33.7
800 (CCW)	20 (CCW)	87	116	0.8	327	35.0
800 (CCW)	40 (CCW)	85	122	1.1	340	34.0
800 (CCW)	60 (CCW)	87	118	1.0	376	34.0

Note: CW, clockwise direction; CCW, counterclockwise direction; Myo, myoglobin; Lys, lysozyme. The theoretical plates were computed from the lysozyme peak of each chromatogram.

motions are most likely due to the effects of Coriolis force,[19,20] which will be investigated in the future.

One clear-cut finding is that partition efficiency expressed in terms of theoretical plate number (N) is strongly correlated with the coil rotation rates, i.e., the higher the coil rotation rate, the greater N value is obtained. This is apparently due to more efficient mixing of viscous polymer phases, which tend to form a laminar flow under gentle coil rotation.

CONCLUSION

The overall results of our present studies indicate that the partition efficiency on protein separation with aqueous–aqueous polymer phase systems, using the nonsynchronous CPC, is remarkably affected by the mode of planetary motion of the coiled separation column. The protein separation was optimized at $P_{backward}$ (CCW/CW) of the coil rotation for the lower phase mobile and at $P_{forward}$ (CCW/CCW) for the upper phase mobile, both in the head to tail elution mode.

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