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Protein separation by nonsynchronous coil planet centrifuge with aqueous–aqueous polymer phase systems

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Abstract

Counter-current chromatographic separation of proteins was performed 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 rate 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 two different types of aqueous–aqueous polymer phase systems, i.e., PEG (polyethylene glycol) 1000–dibasic potassium phosphate, and PEG 8000–dextran T500 in 5 m*M* potassium phosphate buffer. Using a set of multilayer coiled columns prepared from 0.8 mm I.D. PTFE tubing with different volumes (11, 24, 39 ml), the effect of the column capacity on the partition efficiency was investigated under a given set of experimental conditions. Among these experiments, the best separation of proteins was attained using the 39 ml capacity column with a 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate system at 10 rpm of coil rotation under 800 rpm. With lower phase mobile at 0.2 ml/min in the head-to-tail elution, the resolution between cytochrome *c* and myoglobin was 1.6 and that between myoglobin and lysozyme, 1.9. With upper phase mobile in the head-to-tail elution, the resolution between lysozyme and myoglobin peaks was 1.5. In these two separations, the stationary phase retention was 35.0 and 33.3%, respectively. Further studies were carried out using a pair of eccentric coil assemblies with 0.8 mm I.D. PTFE tubing at a total capacity of 20 ml. A comparable resolution was obtained using both lower and upper phases as a mobile phase in a head-to-tail elution. The results of our studies demonstrate that the nonsynchronous CPC is useful for protein separation with aqueous–aqueous polymer phase systems.

Keywords: Counter-current chromatography; Instrumentation; Proteins

1. Introduction

Counter-current chromatography (CCC) has a unique feature that it eliminates the use of a solid

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support [1] as in the classical counter-current distribution (CCD) system. In a Craig CCD instrument the solute partitioning is carried out in three discontinuous steps of mixing, settling, and transfer of the mobile phase hence requiring long separation times. In contrast, in the CCC system the solute partition process continuously takes place between the two phases under a strong centrifugal force field combined with the particular geometry and/or a planetary motion of the separation column. Consequently, separations can be completed in a much shorter elution time.

Since the advent of CCC in 1970, a variety of CCC instruments have been developed for the separation and purification of natural and synthetic products [2–6]. Among those instruments, the nonsynchronous coil planet centrifuge (CPC) is considered most versatile in that it provides desirable combination between rotation (about its own axis) and revolution (around the centrifuge axis) of the coil holder [7–11]. Previous studies demonstrated that this apparatus was useful for partition of cells [7,9,11] and plasmid DNA [9] using aqueous–aqueous polymer phase systems as well as elutriation of cells according to their size and density [7–10,12].

Our previous studies have demonstrated that protein separation can be successfully performed using the cross-axis CPC [13–20]. The present paper describes the protein separation with aqueous–aqueous polymer phase systems using our rotary-seal-free nonsynchronous CPC.

2. Experimental

2.1. 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 [9,10] and a brief description is given here. The apparatus has a distinctive feature which allows a freely adjustable rotational rate of the coiled separation column (between 0 and ±10 rpm) at any given revolution speed, while the effluent is eluted through the rotating column without the use of conventional rotary seal device.

Fig. 1 illustrates the schematic drawing of the nonsynchronous CPC fabricated in our laboratory. Fig. 2 also shows the revolution mechanism of the apparatus which eliminates the need for the rotary seal.

The main motor drives the rotary frame 1 at an angular velocity, ω_a, around the central axis of the centrifuge. Rotary frame 1 carries a pair of countershafts which convey the motion to rotary frame 2: the lower countershaft rotates frame 2 via pulleys 3, 4, 5 and gears 3 and 4 whereas the upper countershaft rotates pulley 6 on a center piece via pulley 1 (stationary) and 2 and gears 1 and 2. When the side motor is at rest, these two countershafts synchronously rotate at $-\omega_a$ on rotary frame 1, and therefore both rotary frame 2 and pulley 6 (on the center piece) rotate at the same doubled speed at $2\omega_a$. In this case the column holder simply revolves with rotary frame 2 without rotating about its own axis.

When the side motor rotates at ω_b, this motion is conveyed through the upper countershaft changing the rotation speed of rotary frame 2 at $2\omega_a - \omega_b$. The difference in rotation rate between rotary frame 2 and pulley 6 on the center piece is then conveyed to the coil holder shaft through pulleys 7, 8 and 9, causing the column holder to rotate at ω_b about its own axis. Thus, the rotation–revolution ratio of the column holder is expressed as $\omega_b/(2\omega_a - \omega_b)$. The relationships in angular velocity between the motors and rotary frames are summarized in Table 1. More detailed description on this planetary mechanism is given elsewhere [9,10].

2.2. Preparation of two types of coiled column

Two types of coiled column were used: one is coaxial multilayer coil and the other eccentric coil assembly.

The multilayer coil was prepared by tightly winding a piece of 0.8 mm I.D.×1.59 mm O.D. PTFE tubing (Flon Kogyo, Tokyo, Japan) around the holder hub of 2.2 cm in diameter forming tight coiled layers between a pair of flanges spaced 23 cm apart. Three columns with different volumes 11 ml (two layers), 24 ml (four layers) and 39 ml (six layers) were employed to investigate the effect of column capacity on partition efficiency.

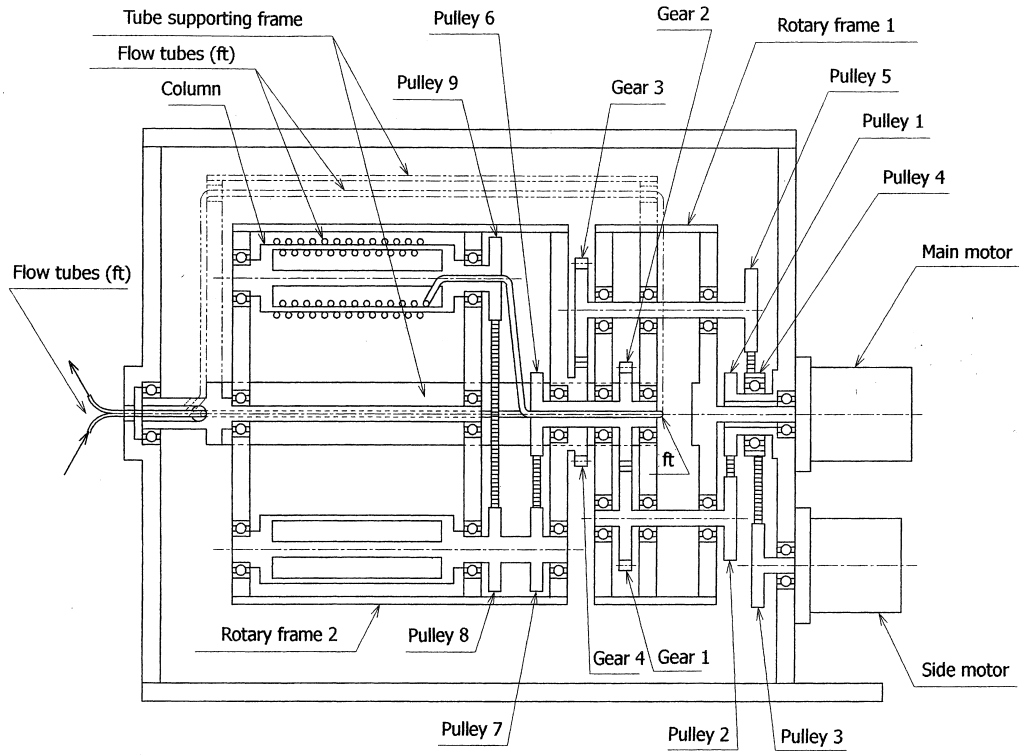

Fig. 1. Schematic drawing of the cross-sectional view of the nonsynchronous CPC fabricated in our laboratory.

The eccentric coil assembly was prepared by winding 0.8 mm I.D. 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.

2.3. Reagents

PEG (polyethylene glycol) 1000 (M_r: 1000), PEG

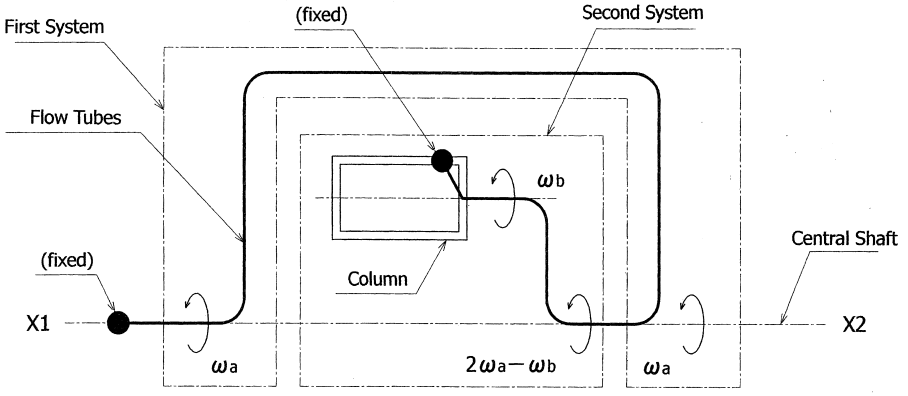

Fig. 2. Schematic illustration of the revolution mechanism of the nonsynchronous CPC without using rotary seals.

Table 1
Angular velocities of motors and rotary frames on nonsynchronous coil planet centrifuge

Main motor	Side motor	Rotary frame 1	Rotary frame 2	Column		
				Rotation	Revolution	Total
$+\omega_a$	0	$+\omega_a$	$+2\omega_a$	0	$+2\omega_a$	$+2\omega_a$
0	$+\omega_b$	0	$-\omega_b$	$+\omega_b$	$-\omega_b$	0
$+\omega_a$	$+\omega_b$	$+\omega_a$	$+2\omega_a-\omega_b$	$+\omega_b$	$+2\omega_a-\omega_b$	$+2\omega_a$

8000 (M_r: 8000), cytochrome *c* (horse heart), myoglobin (horse skeletal muscle), and lysozyme (chicken egg) were purchased from Sigma (St. Louis, MO, USA). Dextran T500 (M_r: 500 000) was purchased from Pharmacia (Sollentuna, Sweden). Monobasic and dibasic potassium phosphates, and sodium chloride were obtained from Wako (Osaka, Japan). All other chemicals were of reagent grade.

2.4. Preparation of aqueous–aqueous polymer phase systems and sample solutions

Three polymer phase systems were prepared [21]: 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; 4.4% (w/w) PEG 8000–7.0% (w/w) dextran T500 in 5 m*M* potassium phosphate buffer (pH 7.0) containing 2 *M* sodium chloride [19]; and 4.0% (w/w) PEG 8000–5.0% (w/w) dextran T500 in 5 m*M* potassium phosphate buffer (pH 7.0) containing 3 *M* sodium chloride [19], which performed the best separation of proteins when using the PEG 8000–dextran T500 systems in our previous studies. 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.

2.5. CCC separation procedure

Each separation was initiated by completely filling the column with the stationary 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, Kyoto, Japan), while the column was rotated at a given combination of the rotation (0–10 rpm) and revolution (800–1000 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, Tokyo, Japan).

2.6. Analysis of CCC fractions

Each collected protein 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).

3. Results and discussion

The nonsynchronous CPC offers a unique separation method for cells and macromolecules by freely adjustable coil rotation under a given centrifugal force field. In general, the retention of the stationary phase becomes less stable in the conventional CCC column when the density difference between the two solvent phases decreased. For example, the conventional high-speed CCC centrifuge with a multilayer coil separation column fails to retain the aqueous–aqueous polymer phase system which has a low interfacial tension and a small density difference between the two phases. This problem is largely alleviated in the present system by applying a low coil rotation rate which gives the two phases enough time to settle under a strong centrifugal force field. In the present studies, a series of experiments were performed to evaluate the capability of the apparatus for protein separation using aqueous–aqueous polymer phase systems.

The acceleration produced by the nonsynchronous planetary motion fluctuates in a plane perpendicular to the axis of the holder. The present system using the combination of low speed coil rotation (0–10 rpm) and high speed revolution quite resembles the

basic hydrodynamic equilibrium system (slowly rotation coil in unit gravity) [22,23] except that the unit gravity is replaced by a strong centrifugal force field. Clearly, in the present system 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, the head–tail orientation of the coil is defined by the Archimedean screw

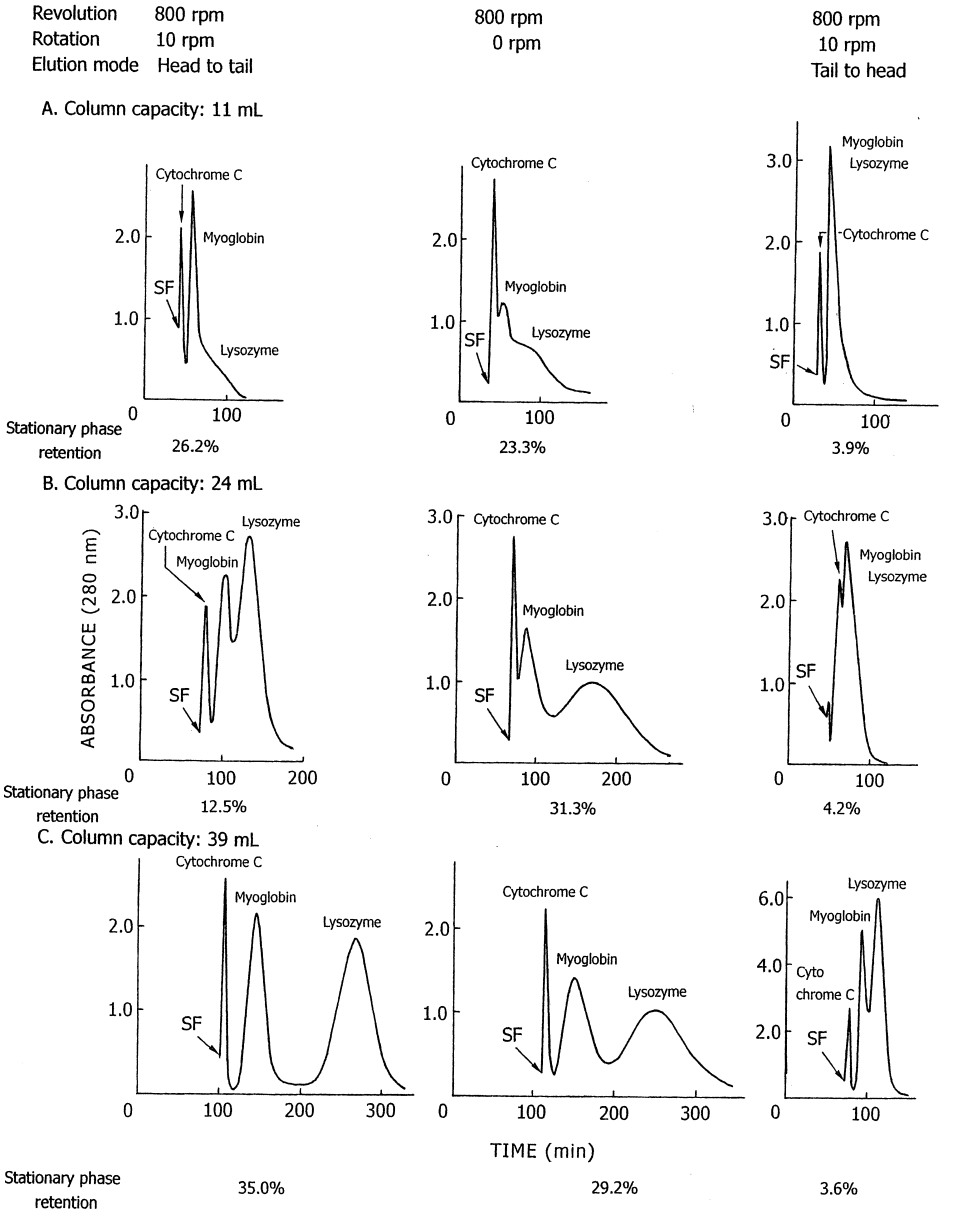

Fig. 3. CCC chromatograms of proteins obtained by three different capacities of coaxial multilayer coils. Experimental conditions: apparatus: nonsynchronous CPC equipped with coaxial multilayer coils, (A) 0.8 mm I.D.×1.59 mm O.D., and 11 ml capacity; (B) 24 ml capacity; (C) 39 ml capacity; sample: cytochrome *c* (2 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. Other conditions are described in the figure. SF=Solvent front.

force where all objects of different densities in a rotating coil are driven toward the head of the coil. Consequently, the satisfactory retention of the stationary phase of near 50% is attainable only by pumping either phase from the head end of the coil at a low flow-rate, whereas the introduction of the mobile phase from the tail end would lead to continuous carryover of the stationary phase resulting in detrimental loss of peak resolution.

Fig. 3 illustrates a set of CCC chromatograms obtained by three different capacities of coaxial multilayer coils (0.8 mm I.D.) with a polymer phase system composed of 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate. Using the lower phase as the mobile phase, the best separation was attained from the 39 ml capacity coil at 10 rpm rotation in head-to-tail elution mode where the resolution between cytochrome *c* and myoglobin peaks was 1.6 and that between myoglobin and lysozyme peaks, 1.9 with the stationary phase retention at 35.0%. As expected, the reversed rotation (tail to head elution mode) under otherwise identical experimental conditions resulted in severe loss of the stationary phase (retention 3.6%) with poor peak resolution.

With the upper phase mobile (Fig. 4), the best separation was obtained 10 rpm in the head to tail elution mode. Elution in the tail to head direction again resulted in low retention at 2.6%.

In order to improve partition efficiencies with lower phase mobile, the effect of revolution speed on peak resolution was examined using the 39 ml capacity multilayer coil. As shown in Fig. 5A and B, the peak resolution was effectively improved as the revolution speed is increased. At doubled flow-rate of 0.4 ml/min and revolution speed of 1000 rpm, the complete peak resolution of proteins was attained at a shorter separation time within 2.7 h as illustrated in Fig. 5C.

When using a different aqueous biphasic polymer phase system of 4.4% (w/w) PEG 8000–7.0% (w/w) dextran T500 in 5 m*M* potassium phosphate buffer containing 2 *M* sodium chloride, the separation of lysozyme and myoglobin was achieved with the upper phase mobile in the head to tail elution mode. Fig. 6 illustrates the CCC chromatogram obtained with the above polymer phase system. The resolution between lysozyme and myoglobin peaks was 1.5 while the stationary phase retention was decreased to 19.7%. Another polymer phase system composed of

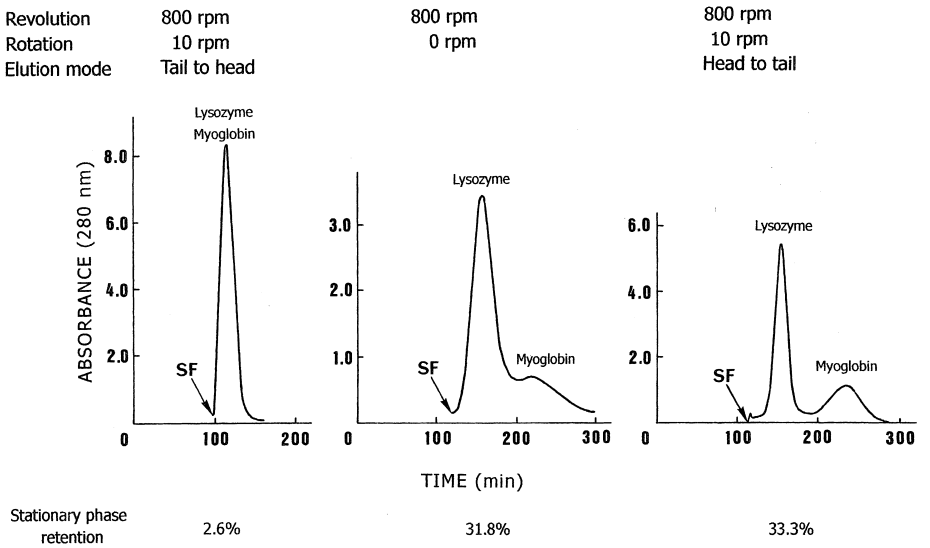

Fig. 4. CCC chromatograms of proteins obtained by the nonsynchronous CPC with coaxial multilayer coils. Experimental conditions: apparatus: nonsynchronous CPC equipped with coaxial multilayer coils, 0.8 mm I.D.×1.59 mm O.D., and 39 ml capacity; sample: myoglobin (8 mg) and lysozyme (10 mg); solvent system: 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase: upper phase; flow-rate: 0.2 ml/min. Other conditions are described in the figure. SF=Solvent front.

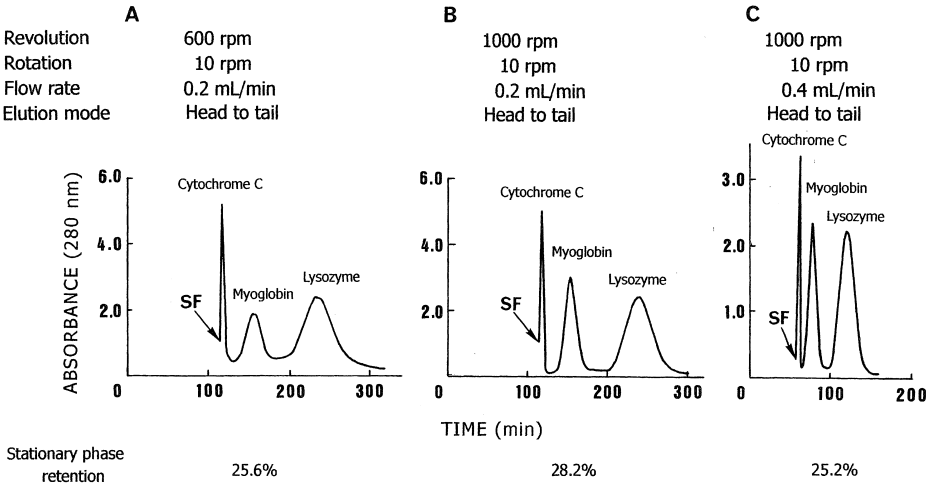

Fig. 5. CCC chromatograms of proteins obtained by the nonsynchronous CPC with coaxial multilayer coils. Experimental conditions: apparatus: nonsynchronous CPC equipped with coaxial multilayer coils, 0.8 mm I.D.×1.59 mm O.D., and 39 ml capacity. Other conditions as described in Fig. 3 caption and in the figure. SF=Solvent front.

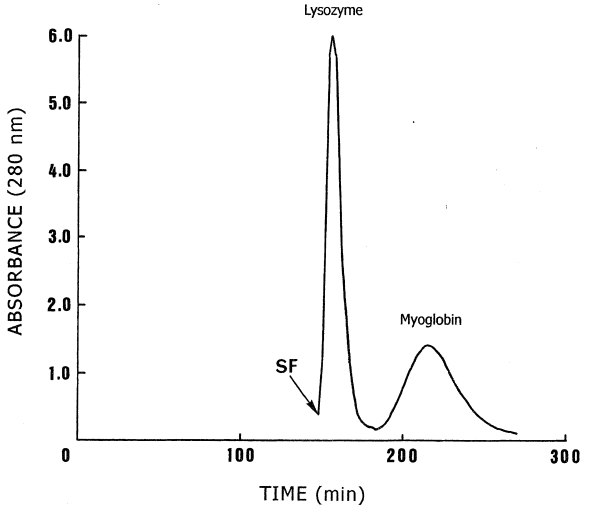

Fig. 6. CCC separation of lysozyme and myoglobin by the nonsynchronous CPC with aqueous–aqueous polymer phase systems. Experimental conditions: apparatus: nonsynchronous CPC equipped with coaxial multilayer coils, 0.8 mm I.D.×1.59 mm O.D., and 39 ml capacity; sample: myoglobin (8 mg) and lysozyme (10 mg); solvent system: 4.4% (w/w) PEG 8000–7.0% (w/w) dextran T500 in 5 m*M* potassium phosphate buffer (pH 7.0) containing 2 *M* sodium chloride; mobile phase: upper phase; flow-rate: 0.2 ml/min; revolution: 800 rpm; rotation: 10 rpm. SF=Solvent front.

4.0% (w/w) PEG 8000–5.0% (w/w) dextran T500 in 5 m*M* potassium phosphate buffer containing 3 *M* sodium chloride produced no stationary phase retention regardless of the elution mode.

Further experiments on partition efficiency of protein separation were performed with the eccentric coil assembly (0.8 mm I.D.) at the total capacity of 20 ml. As illustrated in Fig. 8, a good separation was obtained by eluting the lower phase at the head to tail elution mode. Table 2 summarizes the analytical data calculated from the chromatograms of Figs. 3, 4, 7 and 8.

4. Conclusion

A series of experiments in the present studies revealed that the nonsynchronous CPC can be effectively used for partition of proteins with aqueous–aqueous polymer phase systems. The best results are attained using the head to tail elution mode at 10 rpm of coil rotation and at 800–1000 rpm of revolution speed. The partition efficiency would be further increased by mounting a longer multilayer coil.

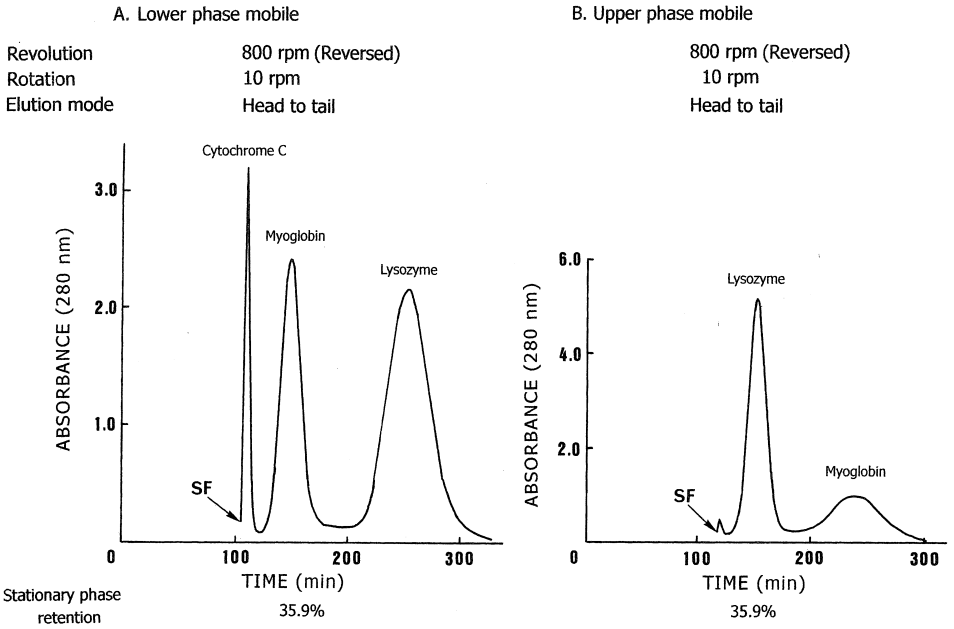

Fig. 7. CCC chromatograms of proteins obtained by the nonsynchronous CPC with coaxial multilayer coils at reversed revolution. Experimental conditions: apparatus: nonsynchronous CPC equipped with coaxial multilayer coils, 0.8 mm I.D.×1.59 mm O.D., and 39 ml capacity; sample: cytochrome *c* (2 mg), myoglobin (8 mg) and lysozyme (10 mg); solvent system: 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate; mobile phase: (A) lower phase, (B) upper phase; flow-rate: 0.2 ml/min; revolution: 800 rpm (reversed); rotation: 10 rpm. SF=Solvent front.

Table 2
Analytical values obtained from separations of proteins by a nonsychronous coil planet centrifuge with two different types of coiled column

Column	Mobile phase	Theoretical plates (*N*)	Resolution		Theoretical plates/column capacity (*N*/ml)	Ref.
			Cyt C/Myo	Myo/Lys (Lys/Myo)		
Coaxial multilayer coils (39 ml capacity)	LP	214	1.6	1.9	5.5	Fig. 3
	LP	281	1.7	2.0	7.2	Fig. 7A (reversed revolution)
	UP	378		(1.5)	9.7	Fig. 4
	UP	380		(1.4)	9.7	Fig. 7B (reversed revolution)
Eccentric coils (20 ml capacity)	LP	83	0.6	1.1	4.2	Fig. 8A
	LP	97	0.7	1.1	4.9	Fig. 8A (reversed revolution)
	UP	269		(0.9)	13.5	Fig. 8B
	UP	197		(0.9)	9.9	Fig. 8B (reversed revolution)

Abbreviations: LP=lower phase; UP=upper phase; Cyt C=cytochrome *c*; Myo=myoglobin; Lys=lysozyme. The value of the theoretical plate number was calculated from the myoglobin peak with the lower phase mobile and the lysozyme peak with the upper phase mobile in the chromatogram.

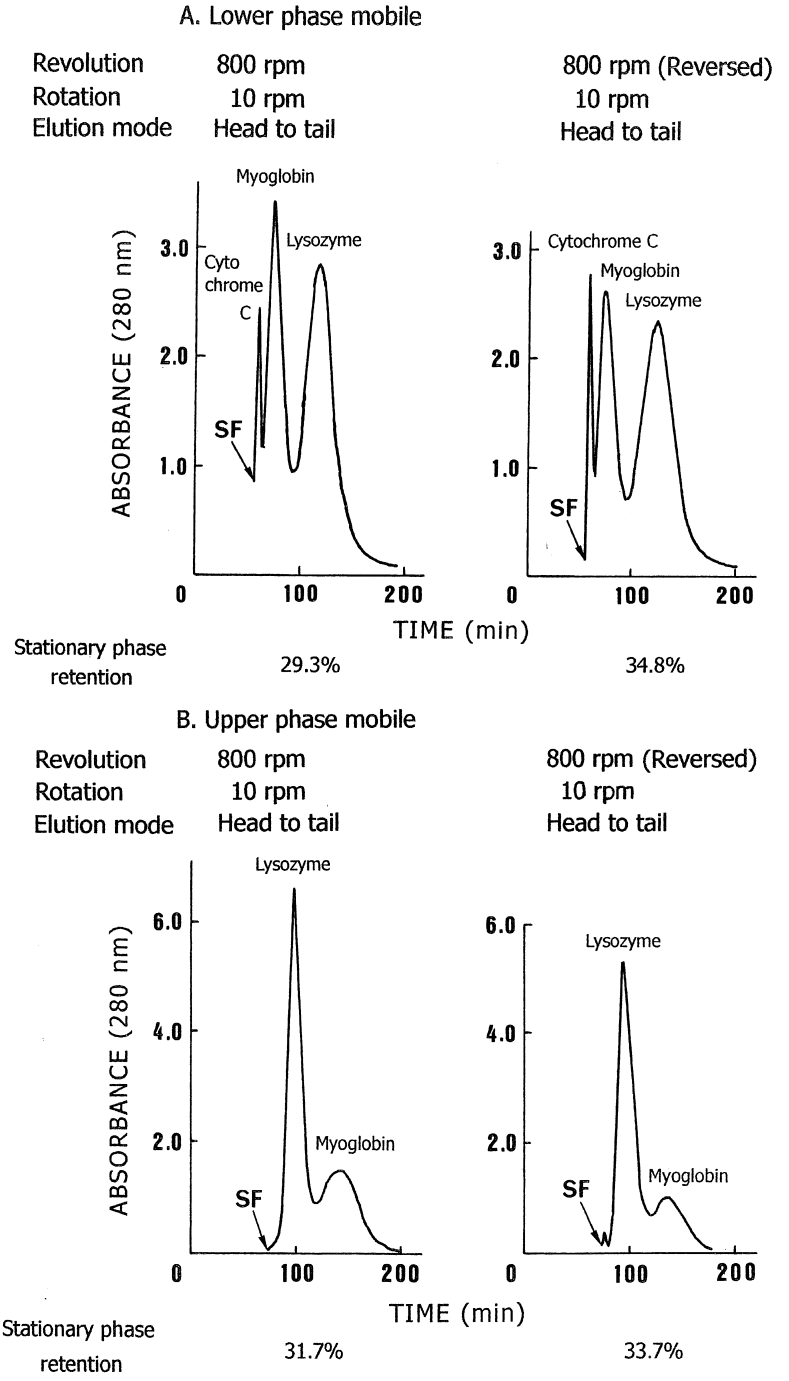

Fig. 8. CCC chromatograms of proteins obtained by the nonsynchronous CPC with eccentric coil assemblies. Experimental conditions: apparatus: nonsynchronous CPC equipped with eccentric coil assemblies, 0.8 mm I.D.×1.59 mm O.D., left-handed coils, 11 units, and 20 ml capacity; sample: cytochrome *c* (2 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: (A) lower phase, (B) upper phase; flow-rate: 0.2 ml/min; revolution: 800 rpm; rotation: 10 rpm. SF=Solvent front.

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