Countercurrent Chromatographic Separation of Proteins Using an Eccentric Coiled Column with Synchronous and Nonsynchronous Type-J Planetary Motions

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Protein separation was performed using the high-speed countercurrent chromatograph (HSCCC) at both synchronous and nonsynchronous type-J planetary motions. The partition efficiency was evaluated with two different column configurations, eccentric coil and toroidal coil, on the separation of a set of stable protein samples including cytochrome C, myoglobin and lysozyme with a polymer phase system composed of 12.5% (w/w) polyethylene glycol 1000 and 12.5% (w/w) dibasic potassium phosphate. Better peak resolution was obtained by the eccentric coil than by the toroidal coil using either lower or upper phase as the mobile phase. The peak resolution was further improved using the eccentric coil by the nonsynchronous type-J planetary motion with the combination of 1066 rpm of column rotation and 1000 rpm of revolution.

Keywords Countercurrent chromatography, universal high-speed countercurrent chromatograph, eccentric coil, toroidal coil, partition efficiency, protein separation with polymer phase system

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Introduction

Countercurrent chromatography (CCC) is a type of liquid-liquid partition chromatography, but it has no solid support matrix which is commonly used in conventional column chromatography. In the past, various types of CCC instruments have been developed to achieve better peak resolution with short retention times.¹⁻⁴ Among them, high-speed CCC (HSCCC) instruments including the type-J multilayer coil planet centrifuge (CPC) and the cross-axis CPC have proven most useful for effective separation of chemical and biological compounds. The type-J multilayer CPC is mainly used for separation with organic-aqueous two-phase solvent systems, whereas the cross-axis CPC is used for separation with aqueous-aqueous polymer phase systems. In our laboratory, the improved small-scale model of cross-axis CPC has been developed and applied to the separation of standard stable protein samples⁵ and enzymes, such as several types of ribonucleases⁶ and collagenases⁷ with aqueous-aqueous polymer phase systems. In the type-J multilayer CPC, the separation column rotates about its axis synchronously in the same direction as the revolution whereas in the cross-axis CPC, the column revolves around the vertical central axis of the centrifuge while rotating about its

horizontal axis at the same angular velocity. Because of its highly complex mechanical design, the cross-axis CPC has not been successfully commercialized yet, whereas the simple commercial model of the type-J multilayer CPC has been widely used for separation of natural products. Sutherland *et al.* have reported that the toroidal coil was useful for separation of proteins using type-J CPC with aqueous-aqueous two-phase solvent systems.⁸⁻¹⁰ Their studies revealed that the type-J CPC can be used for the low interfacial tension solvent system such as aqueous-aqueous polymer phase system by selecting the column configuration. Recently, we have fabricated a universal HSCCC centrifuge which can perform both type-J and type-I planetary motions each by a pair of diagonally mounted columns.¹¹ Our previous studies revealed that with an eccentric coil, type-J planetary motion yielded better separation of two different sugar derivatives, 4-methylumbelliferyl sugar derivatives and 5-bromo-4-chloro-3-indoxyl sugar derivatives, than type-I planetary motion. When we tried to separate protein samples with an aqueous-aqueous polymer phase system composed of 12.5% (w/w) PEG 1000 - 12.5% (w/w) dibasic potassium phosphate, type-I planetary motion failed to separate proteins due to loss of the stationary phase from the column, whereas the type-J planetary motion successfully separated proteins by retaining a satisfactory amount of the stationary phase in the eccentric coil separation column. This paper describes protein separation with an aqueous-aqueous polymer phase system using both eccentric and toroidal coil separation

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columns under the synchronous and nonsynchronous type-J planetary motions.

Experimental

Apparatus

The HSCCC instrument employed in the present studies was constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The design principle of the original apparatus has been described in detail elsewhere.¹¹

Fig. 1 Schematic drawing of the nonsynchronous type-J rotation mode of the universal HSCCC centrifuge.

Synchronous type-J planetary motion

In the present HSCCC instrument, one set of diagonally located two columns undergoes the synchronous type-J planetary motion and the other set of two columns undergoes the synchronous type-I planetary motion. In our present study, all experiments for protein separation were performed by the type-J planetary motion because no stationary phase of aqueous-aqueous polymer phase systems can be retained in the column at the type-I planetary motion as described earlier.

Nonsynchronous type-J planetary motion

Hawes at Brunel University has designed and fabricated a new type of rotary-seal-free nonsynchronous coil planet centrifuge.¹² The instrument presents the possibility of achieving further improvement of partition efficiency for the separation and purification of various compounds.

In our present studies, in order to establish the flow-through system for the nonsynchronous type-J planetary centrifuge, a set of newly designed rotary seal units was placed under two columns, one column receiving the feed flow tube and the other column receiving the return flow tube as shown in Fig. 1. The flow tube distribution in Fig. 1 enables each flow tube to rotate without twisting. The flow tubes connecting neighboring columns do not twist, because the columns rotate at the same speed and each in the opposite direction. Figure 2A shows a photograph of the nonsynchronous type-J planetary centrifuge. The different column rotation speed is set by changing the planetary gear on the holder shaft. In the present study, two different column rotation speeds of 1066 and 1142 rpm were applied under the constant revolution speed at 1000 rpm by setting each special planetary gear on the holder.

Figure 2B illustrates the cross-sectional view of the rotary seal unit. When the upper part of the rotary seal unit connected to the column holder rotates at the angular velocity of ω_x , the lower part of the rotary seal unit set under the rotary frame rotates at the different angular velocity of ω . In order to protect the flow tube cleavage between the upper and the lower parts of the rotary seal unit from liquid leakage, two different O-rings 1

Fig. 2 Photograph of the nonsynchronous type-J rotation mode of the universal HSCCC centrifuge (A) and the rotary seal unit (B).

and 2 were set on the bottom of the upper part directly connected to the cylindrical column holder shaft and another O-ring 3 was set on the concave surface of the lower part of rotary seal unit. This centrifuge gives a relatively mild centrifugal force of around 100 g (at 1000 rpm). The compression of O-rings 1 and 2 can be controlled by adjusting the distance between the upper and the lower parts of the rotary seal unit. The three O-rings were successfully used for each CCC separation without any leaking of the mobile phase. Another separation was performed after exchanging the O-rings for new ones because the lower part is easily unfastened.

Figure 3A schematically illustrates the gear combination to perform the nonsynchronous type-J planetary motion for the present HSCCC instrument. The different gear ratio between the sun gear fixed in the central shaft and the planetary gear to rotate the column produces the different rotation speed at a

given revolution speed. The decreased toothed planetary gear (G5: the tooth number: $Z = 30$ for the rotation speed at 1066 rpm or G6: $Z = 28$ for the rotation speed at 1142 rpm) was exchanged from the synchronous toothed planetary gear (the tooth number: $Z = 32$). In order to adjust the gear combination, a small idle gear (G2 or G4) was newly set between the sun gear and the planetary gear.

Preparation of two different coiled assemblies

The eccentric coil and the toroidal coil used in the present study are schematically illustrated in Fig. 4.

Each eccentric coil assembly was prepared by winding 1 mm i.d. PTFE (polytetrafluoroethylene) tubing (Flon Kogyo Co., Tokyo, Japan) onto 5 cm long, 5 mm o.d. nylon pipes making a series of tight left-handed or right-handed coils (20 turns for 1 unit). These coil units were arranged symmetrically around

Fig. 3 Schematic illustration (A) and photograph (B) of the gear combination to perform the nonsynchronous type-J planetary motion for the universal HSCCC.

Fig. 4 Schematic drawing of the eccentric coil (A) and the toroidal coil (B) for the separation column.

Fig. 5 CCC separation of proteins obtained by the eccentric coil with the synchronous type-J rotation mode of the universal HSCCC centrifuge at counterclockwise (A) and clockwise (B) directions. Experimental conditions: apparatus, universal HSCCC (type-J synchronous rotation mode); column, eccentric coil (left-handed, total column capacity: 29.5 mL); sample, lower phase mobile = cytochrome C (2 mg) + myoglobin (8 mg) + lysozyme (10 mg), upper phase mobile = lysozyme (10 mg) + myoglobin (8 mg); solvent system, 12.5% (w/w) PEG 1000 – 12.5% (w/w) dibasic potassium phosphate; flow rate, 0.2 mL/min; fractionate, 0.4 mL/tube; rotation speed, 1000 rpm; revolution speed, 1000 rpm; rotation direction, counterclockwise for lower phase mobile, clockwise for upper phase mobile (rotation direction of the column was defined from the upper view of the apparatus). SF = solvent front.

the holder hub of 5 cm o.d. in such a way that the axis of each coil unit is parallel to the holder axis (12 units for the first layer and 18 units for the second layer). A pair of assemblies was mounted on the rotary frame on the diagonally located column holders and serially connected with the flow tube. The total column capacity was 29.5 mL.

The toroidal coil assembly was prepared by winding 1 mm i.d. PTFE tubing onto a 5 mm o.d. nylon pipe forming double layers of both left-handed coils: the first layer was 59 cm long with 266 turns and the second layer 89 cm long with 402 turns. After the first layer of the toroidal coil was wound around the holder to form a left-handed coil from the top to the bottom, the PTFE tubing of the first layer that reached the bottom was returned in a straight line to the top and then connected directly to the second layer of the toroidal coil. A pair of identical coil assemblies was connected in series to obtain a total column capacity of 29.8 mL.

Reagents

Polyethylene glycol (PEG) 1000 (M_w 1000), cytochrome C (horse heart) (M_w 12384), myoglobin (horse skeletal muscle) (M_w 17800), and lysozyme (chicken egg) (M_w 13680) 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 systems and sample solutions

An aqueous-aqueous polymer phase system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate 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 the two clear layers were formed.

The sample solutions were prepared by dissolving a set of standard proteins in 0.5 mL of each phase of the two-phase

Table 1 Analytical values obtained by the CCC separations of proteins using the eccentric coil with the type-J rotation at the CCW and the CW directions

A. Lower phase mobile

Rotating direction	Elution volume/mL (<i>K</i> value)			Peak resolution (<i>R_s</i>)		Theoretical plate number (<i>N</i>)	Theoretical plate number per column capacity (<i>N</i> /mL)	Stationary phase retention, %
	Cyt C	Myo	Lys	Cyt C/Myo	Myo/Lys			
CCW	21.6 (0.13)	26.2 (0.64)	37.3 (1.86)	1.2	1.6	446	15.1	30.8
CW	23.4 (0.23)	26.8 (0.66)	36.2 (1.85)	0.9	1.3	438	14.8	26.8

B. Upper phase mobile

Rotating direction	Elution volume/mL (<i>K</i> value)		Peak resolution (<i>R_s</i>)	Theoretical plate number (<i>N</i>)	Theoretical plate number per column capacity (<i>N</i> /mL)	Stationary phase retention, %
	Lys	Myo	Lys/Myo			
CCW	27.6 (0.78)	37.6 (1.93)	1.0	283	9.6	29.5
CW	27.5 (0.76)	37.3 (1.94)	1.1	376	12.7	28.1

CCW. Counterclockwise, CW. Clockwise, Cyt C. Cytochrome C, Myo. Myoglobin, Lys. Lysozyme. The theoretical plate number was calculated from the myoglobin peak for the lower phase mobile and the lysozyme peak for the upper phase mobile. The partition coefficient was expressed by K (C_U/C_L) for the lower phase mobile and K (C_L/C_U) for the upper phase mobile.

Table 2 Analytical values obtained by the CCC separations of proteins using the toroidal coil with the type-J rotation at the CCW and the CW directions

A. Lower phase mobile

Rotating direction	Elution volume/mL (<i>K</i> value)			Peak resolution (<i>R_s</i>)		Theoretical plate number (<i>N</i>)	Theoretical plate number per column capacity (<i>N</i> /mL)	Stationary phase retention, %
	Cyt C	Myo	Lys	Cyt C/Myo	Myo/Lys			
CCW	20.3 (0.11)	23.8 (0.44)	34.1 (1.40)	1.0	1.3	336	11.3	35.6
CW	20.4 (0.08)	24.7 (0.50)	34.0 (1.41)	1.2	1.3	454	15.2	34.2

B. Upper phase mobile

Rotating direction	Elution volume/mL (<i>K</i> value)		Peak resolution (<i>R_s</i>)	Theoretical plate number (<i>N</i>)	Theoretical plate number per column capacity (<i>N</i> /mL)	Stationary phase retention, %
	Lys	Myo	Lys/Myo			
CCW	26.8 (0.68)	40.5 (2.14)	1.0	243	8.2	31.5
CW	27.3 (0.73)	41.2 (2.21)	1.2	277	9.3	31.5

CCW. Counterclockwise, CW. Clockwise, Cyt C. Cytochrome C, Myo. Myoglobin, Lys. Lysozyme. The theoretical plate number was calculated from the myoglobin peak for the lower phase mobile and the lysozyme peak for the upper phase mobile. The partition coefficient was expressed by K (C_U/C_L) for the lower phase mobile and K (C_L/C_U) for the upper phase mobile.

solvent system used for separation.

Separation of protein samples

Each separation was initiated by completely filling the column with the stationary phase, followed by injection of the sample solution through the flow tube leading into the head of the coiled column with a syringe. Then, the mobile phase was pumped into the column using a reciprocating pump (Model LC-6A, Shimadzu Corp., Kyoto, Japan), while the column was rotated at a desired rotation speed under the revolution speed of

1000 rpm. The effluent from the column outlet was collected into test tubes using a fraction collector (Model CHF 100AA, Advantec, Tokyo, Japan).

Spectrophotometric analysis of fractions

Each collected protein fraction was diluted with an aliquot of distilled water and the absorbance was measured at 280 and 540 nm (for myoglobin) with a spectrophotometer (Model UV-1600, Shimadzu).

Fig. 6 CCC separation of proteins obtained by the toroidal coil with the synchronous type-J rotation mode of the universal HSCCC centrifuge at counterclockwise (A) and clockwise (B) directions. Experimental conditions: column, toroidal coil (left-handed, total column capacity: 29.8 mL). Other experimental conditions were the same as those described in Fig. 3.

Evaluation of partition efficiency

The efficiencies in protein separations were computed from the chromatogram and expressed in terms of theoretical plate number (N) and peak resolution (R_s) each according to the conventional formula.

Results and Discussion

Partition efficiency of the eccentric coil and the toroidal coil on protein separation with the synchronous type-J planetary motion

As mentioned earlier, the type-J multilayer CPC produces extremely low stationary phase retention from low interfacial tension two-phase solvent systems such as aqueous-aqueous polymer phase systems whereas the cross-axis CPC with a similar separation column shows sufficient retention of the stationary phase for protein separation. Compared to the complicated three-dimensional planetary motion of the cross-axis CPC, the type-J and type-I CPC provide the simple two-dimensional planetary motion. The universal HSCCC centrifuge simultaneously produces both the type-J and the type-I planetary motions. As mentioned earlier, our preliminary studies revealed that the synchronous type-I planetary motion

produced no stationary phase retention of aqueous-aqueous polymer phase systems, but the synchronous type-J planetary motion satisfactorily retained the stationary phase of aqueous two-phase solvent systems for protein separation with either eccentric coil or toroidal coil separation column.

In CCC separation, each sample component elutes according to its partition coefficient (K) in the two-phase solvent system used for separation, where the K value is defined as the concentration of the solute partitioned into the upper phase divided by the concentration of the solute partitioned into the lower phase. Using the 12.5% (w/w) PEG 1000 – 12.5% (w/w) dibasic potassium phosphate system, the K values of each protein used in the present study were determined as 0.02 for cytochrome C, 0.59 for myoglobin and 2.19 for lysozyme by the simple test tube measurement. With the lower phase mobile, the protein sample is eluted in the order of cytochrome C, myoglobin and lysozyme. With the upper phase mobile, the elution order is reversed, where cytochrome C that is almost partitioned into the lower phase may be predicted to take a long time for elution. CCC can select the upper or the lower phase of the two-phase solvent system for the stationary phase according to the K value of the target compound for separation.

Figure 5A illustrates protein separation obtained by the

Fig. 7 CCC separation of proteins obtained by the eccentric coil with the nonsynchronous type-J rotation mode of the universal HSCCC centrifuge at two different column rotation speeds. Experimental conditions: column, eccentric coil (right-handed, total column capacity: 29.5 mL); rotation speed, (A) 1066 rpm, (B) 1142 rpm; rotation direction, clockwise for lower phase mobile, counterclockwise for upper phase mobile. Other experimental conditions were the same as those described in Fig. 3. SF = solvent front.

eccentric coil at counterclockwise (CCW) rotation. With the lower phase mobile, stable protein samples including cytochrome C, myoglobin and lysozyme were satisfactorily separated from each other. With the upper phase mobile, the separation between lysozyme and myoglobin peaks was also achieved by the head to tail elution mode. Band broadening was observed in the myoglobin peak due to the high viscosity of the upper PEG mobile phase, which gives an enhanced axial flow pattern to produce sample band broadening, especially in later eluting peaks such as myoglobin. At clockwise (CW) rotation as illustrated in Fig. 5B, the lower phase mobile showed insufficient resolution between the cytochrome C and the myoglobin peaks compared with that obtained at CCW rotation. With the upper phase mobile, the lysozyme and the myoglobin peaks were well separated from each other. Table 1 summarizes the analytical values calculated by these chromatograms. The overall results indicated that the left-handed eccentric coil produced better resolution of proteins at CCW rotation for the lower phase mobile and at CW rotation for the upper phase mobile.

Figure 6A illustrates protein separation obtained by toroidal coil at CCW rotation. With the lower phase mobile, three stable protein samples including cytochrome C, myoglobin and lysozyme were satisfactorily separated from each other. With the upper phase mobile, lysozyme and myoglobin were well

separated from each other. Figure 6B illustrates protein separation obtained by the synchronous type-J planetary motion of toroidal coil at CW rotation. Table 2 summarizes the analytical values calculated by these chromatograms. Better resolution of proteins was obtained by either lower or upper phase used as the mobile phase at CW rotation. Our overall experiments revealed that the eccentric coil yielded substantially higher partition efficiencies than the toroidal coil.

Protein separation using an aqueous-aqueous polymer phase system with the nonsynchronous type-J planetary motion by the universal HSCCC instrument

In order to improve protein separation, two different rotation speeds of the nonsynchronous type-J planetary motion were applied using the universal HSCCC centrifuge. The different column rotation speeds under a given revolution speed were achieved by engaging the central stationary gear to the different planetary gear on the holder shaft through the idler gears.

Figure 7A illustrates protein separations obtained by the eccentric coil at a rotation speed of 1066 rpm under the revolution speed of 1000 rpm. With the lower phase mobile, the peak resolution of three proteins was improved compared with that obtained by the synchronous planetary motion at the rotation speed of 1000 rpm as shown in Fig. 5A (left side).

Table 3 Protein separation using the eccentric coil with the nonsynchronous mode of the universal HSCCC at the type-J rotation

A. Lower phase mobile					
Rotating speed/ rpm	Revolution speed/ rpm	Peak resolution (R_s)		Theoretical plate number (N)	Stationary phase retention, %
		Cyt C/ Myo	Myo/ Lys		
1066	1000	1.3	1.7	388	38.8
1142	1000	1.2	1.5	415	37.2

B. Upper phase mobile					
Rotating speed/ rpm	Revolution speed/ rpm	Peak resolution (R_s)		Theoretical plate number (N)	Stationary phase retention, %
		Lys/Myo			
1066	1000	1.3		283	37.2
1142	1000	1.3		270	36.1

With the upper phase mobile, the resolution between two protein peaks was similarly improved with the lower phase mobile. Figure 7B illustrates protein separations obtained by the eccentric coil at the column rotation speed of 1142 rpm under the revolution speed of 1000 rpm. With the lower phase mobile, the resolution between myoglobin and lysozyme peaks was decreased compared with that obtained by the synchronous planetary motion at the rotation speed of 1000 rpm as shown in Fig. 5A (left side). With the upper phase mobile, the peak resolution was similar to that obtained at the rotation speed of 1066 rpm. Table 3 summarizes the analytical values calculated from these chromatograms. In Table 3A, the peak resolution was enhanced although the theoretical plate number decreased, which was different from common column chromatography using the solid support. This may be caused by the increased mixing of two liquid phases in the rotating column, which produces the improved separation of analytes with band broadening for the decreased theoretical plate number. In general, better CCC separation requires a balance between the mixing and settling of two phases and a suitable flow rate of the mobile phase. A further increase in the rotation rate does not always produce an enhancement of separation performance.

The overall results indicate that the best separation of proteins was accomplished by the nonsynchronous planetary motion at the rotation speed of 1066 rpm under the revolution speed of 1000 rpm in the eccentric coil with an aqueous-aqueous polymer phase system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate.

Conclusions

Protein separation with an aqueous-aqueous polymer phase system was examined using the HSCCC centrifuge at both synchronous and nonsynchronous type-J planetary motions. The comparison between the eccentric coil and the toroidal coil at synchronous planetary motion revealed that better partition efficiency of proteins was obtained by the eccentric coil using an aqueous-aqueous polymer phase system composed of 12.5% (w/w) PEG 1000 - 12.5% (w/w) dibasic potassium phosphate. Separation of proteins was further improved by the nonsynchronous type-J planetary motion at the rotation speed of 1066 rpm under the revolution speed of 1000 rpm.

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